MONOCLONAL ANTIBODY THERAPY OF RHEUMATOID ARTHRITIS

By

Deepa Rajan Hammaker

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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DEDICATION

To my mom, Padma Rajan

and my husband Jeff Hammaker,

whose love and encouragement have helped me achieve this goal

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

OBJECTIVES: To (a) determine the immunological effects of a PRIMATIZED® anti-CD4 antibody alone or in combination with methotrexate in RA patients, (b) determine the immunological effects of a chimeric anti-CD25 antibody in RA patients who are partially refractive to methotrexate and (c) compare interleukin-15 levels in the serum of RA patients and healthy controls and determine if there is a correlation between this cytokine and serum TNF-α, CD122 expression, and disease activity.

PATIENTS AND METHODS: (a) Eight RA patients were selected, four received anti-CD4+placebo and the other four received anti-CD4+methotrexate for 4 weeks. The immunological effects were assessed on peripheral blood by flow cytometry and thymidine incorporation assays. (b) Six RA patients were given anti-CD25 antibody (0.02-60mg) along with methotrexate for 26 days. The immunological effects were assessed on peripheral blood by flow cytometry, thymidine assays, and ELISA. (c) Blood and disease activity from twenty-one RA patients were obtained and serum IL-15 and TNF-α levels were measured by ELISA. IL-15R β chain (CD122) expression was measured by flow cytometry.

RESULTS: (a) The anti-CD4 antibody caused a selective and significant decrease in the number of CD4+ T cells. No inhibition of PHA or mitogenic antibody stimulated proliferation was observed. (b) The anti-CD25 antibody caused a significant decrease in the percent CD25+ cells. Anti-CD25 antibody caused a significant decrease in PHA or mitogenic antibody stimulated proliferation. Clinically, the anti-CD25 antibody caused a

significant decrease in the number of tender and swollen joints (c) Elevated serum IL-15 was measured in 10 out of 21 RA patients but not in controls. No correlation was observed between IL-15 and TNF-α, CD122 expression or disease activity. CONCLUSIONS: (a) Methotrexate did not alter the effects of the PRIMATIZED® anti-CD4 antibody. Changes in antibody development processes have yielded two antibodies with different functions. (b) Anti-CD25 induced decrease in CD25+ T cells was associated with clinical benefit. It appears that the antibody bound CD25 and prevented interaction of IL-2 and IL-2R. The exact mechanisms of action are not clear. (c) Serum IL-15 levels in RA may be a more sensitive indicator of inflammation than TNF-α and may be a valuable tool in diagnosis.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease characterized by persistent intense immunologic activity, with the accumulation of activated local leukocytes within the inflamed synovial joint (1). The uncontrolled, inflammatory cascade initiated by these cells induces the destruction of bone and cartilage. Although the synovial joints are the primary targets of the immune response in RA, inflammatory lesions can manifest systemically in other organs (2).

The etiology of RA is unknown. Moreover, there is no cure or way of preventing the disease (3). Although the pathogenesis of RA is not completely understood, substantial progress has been made in defining some of the mechanisms involved in the initiation and propagation of the disease. It is thought that an arthrotropic agent elicits the immune response (4). Evidence suggests that one of the earliest events in RA is the intense infiltration of activated, memory CD4+ T cells into the synovium (5). The recruitment of the CD4+ T cells into the synovial tissue suggests an antigen specific response. These activated T cells express CD69, CD25, HLA-DR, as well as adhesion molecules such as LFA-1 and VLA-4 (6). The CD4+ T cells that extravasate into the synovial tissue in this manner are CD45RO+, suggesting prior activation. This migration is enhanced by cytokines such as IL-15 (7) and IL-16 (8), which act as chemoattractants for memory CD4+ T cells. Evidence has suggested that these activated CD4+ T cells orchestrate the local synovial inflammation and cellular infiltration that are followed by a number of subsequent uncontrolled inflammatory events. The synovial CD4+ T cells produce IFN-γ and augment activation of B cells, macrophages, and other antigen presenting cells (9). Furthermore, these T cells stimulate monocytes/macrophages in an uncontrolled manner to produce cytokines such as IL-1 and TNF-α (10). These cytokines in turn enhance the production of matrix metalloproteinases (MMPs), serine proteases and cathepsins that initiate joint destruction (11).

Based on the delineated events in the pathogenesis of RA, therapeutic agents that target the molecules of the inflammatory cascade have been developed. Biological agents such as monoclonal antibodies have been genetically engineered and studied for their ability to treat RA. The principle of monoclonal antibody therapy is to specifically target and inactivate the key players in the inflammatory cascade, thereby inducing immunosuppression. Thus far, monoclonal antibodies have been developed to (a) interfere with the activation of CD4+ T cells, (b) block the entry and accumulation of mononuclear cells in the inflamed synovial tissues by disrupting binding of adhesion molecules to endothelial cells and (c) to neutralize the pro-inflammatory cytokines such as TNF-α and IL-6 in the synovium.

In two chapters of the dissertation, we have studied the immunological effects of two monoclonal antibodies used clinically, in the treatment of RA. The first is a PRIMATIZED® anti-CD4 antibody that selectively binds CD4 molecule on T helper cells. The second is a chimeric anti-CD25 antibody that specifically binds CD25 and targets activated cells. The cells that express both CD4 and CD25 are thought to play an important role in the pathogenesis of RA. In the third chapter, we have evaluated the prevalence of a pro-inflammatory cytokine, IL-15, in the serum of RA patients. The potential role of IL-15 in the pathogenesis of RA has suggested its use in monoclonal antibody therapy.

I. PRIMATIZED® Anti-CD4 Antibody Therapy of Rheumatoid Arthritis

Evidence for the role of CD4+ T cells came from the observations of Jaffe (12) who noted that RA patients who developed AIDS and profound CD4+ cell depletion saw an improvement of their rheumatic symptoms, suggesting that CD4+ T cells might play an important role in the initiation and progression of RA. Second, synovial CD4+ T cells, in some patients, accumulated in lymphoid-tissue like structures that were similar in appearance to germinal centers found in lymph nodes (13). These structures supported the notion that the inflammation in rheumatoid arthritis is a result of an antigen-driven T cell response. This, along with the observation that certain MHC class II molecules, whose primary function is antigen presentation to CD4+ T cells, are associated with RA strengthened the role of CD4+ T cells in the development of the disease. Weyand and Goronzy (14) reasoned that if RA is T helper mediated, MHC class II molecules could present an unknown antigen to CD4+ T cells and trigger their activation and clonal proliferation. They found clones of CD4+CD28- T cells in the blood of RA patients but not healthy controls. The CD4+CD28- T cell clones are enriched in the joints and are autoreactive (15,16). They express perforin, granzyme B and are capable of lysing P815 target cells (17). They are also capable of producing high levels of IFN-γ, without the need for co-stimulatory signals from CD28 (18). These clones have a skewed TCR repertoire with dominance of certain V_β genes, suggesting clonal expansion (15). The extent of CD4+CD28- T expansion has been correlated with clinical manifestation of RA (19).

The association of MHC class II with RA has been known for nearly 20 years (20). Weyand et. al. (21) have found that some RA patients who are homozygous for HLA-DRB1 alleles have a greater risk for severe disease. This suggested that MHC class II might have a more complex role in the development of RA (21). The MHC class II molecules may play a greater role in thymic selection by determining the peripheral T cell repertoire. It has been hypothesized that defective positive and negative selection processes in RA patients might lead to the generation of auto-reactive T cells (22). The self-reactive clones then enter the periphery where they might encounter a self-antigen and mount an immune response. In addition, there may be defects in maintaining peripheral tolerance in RA patients, which could lead to contribute to autoimmunity.

The proposed role of CD4+ T cells has been studied in animal models of RA. In vivo treatment with anti-CD4 antibody prevented type II collagen induced arthritis when the murine antibody was administered before the onset of the disease (23). The clinical signs were reversed when the antibody was administered shortly after the first symptoms of arthritis appeared (24).

The success of anti-CD4 antibodies in animal models suggested its use in humans. Several investigators used murine anti-CD4 antibodies to treat patients with RA (25-27). These antibodies induced an immediate decrease in the number of circulating CD4+ T cells. In the majority of patients, murine anti-CD4 antibodies caused profound CD4+T cell depletion. It was thought that killing CD4+ T cells would prevent activation of other cells and production of pro-inflammatory cytokines, thus ameliorating RA. However, several problems were associated with these antibodies. The responders as well as the non-responders experienced prolonged lymphopenia, which posed risks of opportunistic infections. In addition to the short serum half-life, these antibodies were highly immunogenic. They induced an immune response that resulted in the production of human anti-mouse antibodies, which reduced the effectiveness of the treatment (28). Chimeric antibodies (murine variable and human constant region) have been designed to overcome these problems (29, 30). These antibodies had longer serum half-lives and were less immunogenic than their murine counterparts. However, Fc-receptor mediated functions such as ADCC and complement mediated lysis (CML) caused prolonged lymphopenia (31).

Non-depleting anti-CD4 antibodies were developed and used in clinical trials because studies showed that depletion of CD4+ cells was not necessary to observe beneficial clinical effects. More importantly, non-depleting anti-CD4 antibodies were shown to be more effective in animal studies than the depleting ones (32, 33). Two kinds of non-depleting antibodies have been tested in clinical trials in the treatment of RA. The PRIMATIZED® anti-CD4 antibody is an IgG1 antibody with primate complementarity determining regions (CDRs) and human constant region (34). Animal studies have shown that this antibody has low immunogenicity, as determined by human anti-immunoglobulins, and did not cause the depletion of CD4+ T cells (35). The humanized anti-CD4 antibodies have murine CDRs and human constant regions. Currently, two humanized anti-CD4 antibodies are being tested in clinical trials for the treatment of RA (36, 37).

Clinical trials with the PRIMATIZED® anti-CD4 antibody to treat RA have been conducted. The Phase I single-dose, dose-escalating trial demonstrated that the anti-CD4 antibody had a benign safety profile. The results indicated that the antibody was non-depleting and had very low immunogenicity. The antibody's mechanism of action included two elements. First, the antibody bound CD4 such that it blocked the interaction of CD4 with MHC, which resulted in decreased response to recall antigens (38). Second, the CD4 molecule was internalized and re-expressed (i.e. CD4 expression was modulated) within 72 hours of treatment. The Phase II/III multi-dose, dose-escalating trial confirmed these results (39).

The production protocols were then modified to optimize yield, giving a newly synthesized (modified) PRIMATIZED® anti-CD4 antibody. In this paper, we have shown the results of a phase II/III randomized, double-blinded, placebo-controlled trial in which methotrexate (MTX) was used concomitantly with this modified PRIMATIZED® anti-CD4 antibody.

II. Chimeric Anti-CD25 Antibody Therapy of Rheumatoid Arthritis

Evidence suggests that CD25+ (IL-2R_{α} chain positive) T cells play an important role in the initiation and propagation of the disease. As mentioned earlier, one of the earliest events in RA is an uncontrolled inflammatory response in the synovium, possibly induced by an arthrotropic agent (4). These infiltrating cells are CD4+, a fraction of which express CD25, suggesting that they may have been recently activated (40). Other evidence supports a central role for systemic T cell activation and CD25+ T cells in the progression of RA. Many RA patients have elevated numbers of CD25+ T cells in the peripheral blood compared to healthy controls (41). Moreover, in early RA, many patients have high levels of serum IL-2 and IFN-γ, which correlate with progression of early disease (42). RA patients have high levels of soluble CD25 (sCD25) in the peripheral blood, which also suggests the importance of CD25+ cells (43). sCD25 is produced by enzymatic cleavage of the transmembrane form of CD25 and is released into circulation by activated T cells (44). sCD25 may be produced by activated T cells in an attempt to regulate the activational state of the cell in that sCD25 prevents the formation of the high affinity IL-2R and further activation by IL-2 (45). sCD25 levels correlate positively with disease activity in RA suggesting a role for CD25+ T cells in RA (46).

Several immunosuppressive therapies have been designed to prevent T cell activation or deplete cells that express CD25. Cyclosporin, FK506 and Rapamycin are potent immunosuppressive agents that specifically inhibit T cell activation. Cyclosporin and FK506 interfere with the signal transduction processes involved in the activation of T helper cells as well as the production of cytokines such as IL-2 and IFN-γ. IL-2 induced cytokines such as IL-6, TNF-α, and IFN-γ, which have been implicated in the pathogenesis of RA are consequently inhibited. These pharmacologic agents bind to their intracellular receptors, immunophilins, and inhibit the activation of calcineurin. The inhibition of calcineurin prevents activation of a host of transcription factors such as NF-AT, NF-κB, AP-1, and CREB, which in turn inhibits IL-2 gene transcription (47). Cyclosporin and FK-506 also inhibit IL-3, IL-4, GM-CSF, TNF-α and IFN-γ expression (47). The blocking of these cytokines results in the inhibition of T-cell activation and ultimately the suppression of T-cell-dependent immune responses. Rapamycin functions by inhibiting cytokine and growth-factor dependent stimulation of T helper cells. It suppresses IFN-γ production and IL-4 dependent autocrine stimulation of T helper clones (48). DAB_{486}IL-2 fusion protein has been used successfully in clinical trials to treat patients with severe, refractory RA (49). DAB_{486}IL-2 binds to and depletes CD25+ cells and the beneficial effects were seen as early as 7 days and persisted 4-30 weeks beyond the treatment period. These studies suggest that immunosuppressive therapies that target CD25+ cells can be beneficial in treating RA.

In this study, we hypothesize that CD25+ T cells play an important role in the progression of RA and that treatment with an anti-CD25 antibody will reduce disease activity. The concept of using anti-CD25 antibodies as means of achieving selective immunosuppression or tolerance is well established in transplantation (50, 51). However, anti-CD25 therapy has not been used in the treatment of RA. This is a logical step in monoclonal antibody therapy of RA because anti-CD25 antibodies selectively target the high affinity IL-2R on recently activated T cells that have been implicated in the pathogenesis of the disease.

Simulect® (basiliximab) is a chimeric, IgG monoclonal antibody that recognizes the CD25 antigen that is expressed on activated but not resting or memory T cells. *In vitro* experiments have demonstrated that Simulect® inhibited mixed lymphocyte reaction (MLR) and induced antibody dependent cell cytotoxicity (ADCC) on a CD25+ cell line through its human FcR (52). Simulect® has been used successfully as a prophylactic immunosuppressive agent in combination with cyclosporine and corticosteroids in kidney transplantation (53). Simulect's® serum half-life was approximately 7 days (53). It is the first chimeric anti-CD25 antibody used in the treatment of RA. In a 33-day, open-label clinical trial, 6 patients with active RA, who were partially responsive to methotrexate, (MTX) were treated with Simulect®. In this paper, we report the immunological effects of this chimeric anti-CD25 antibody and discuss its mechanisms of action.

III. Interleukin-15

Interleukin-15 (IL-15) is a pleiotropic cytokine that belongs to the four-helix bundle family, of which IL-2 is a member (54). IL-15 and IL-2 do not share sequence homology but have structural and functional similarities (54). Like IL-2, IL-5 induces T, B and NK cell activation (55, 56), proliferation (57), and co-stimulation of immunoglobulin synthesis (58). However, unlike IL-2, IL-15 is produced by monocytes and macrophages, not by activated T cells (54). IL-15 is a chemoattractant for human peripheral blood (PB) T cells (59). IL-15 binds to a heterotrimeric receptor composed of a unique IL-15R_{α} chain, and the β and γ chains of the IL-2R (60). The similarities in function between IL-2 and IL-15 have been attributed to the usage of the same receptors.

Abnormalities in IL-15 production have been associated with the development and maintenance of chronic inflammatory diseases such as rheumatoid arthritis (RA) (61). High concentrations of IL-15 have been found in RA synovial fluid (SF) and IL-15 is expressed by monocytes and macrophages in the synovial lining (62). Evidence suggests that IL-15 may play an important role in recruiting PB T cells into the synovium. *In vitro* experiments by McInnes et. al. demonstrated that IL-15 induced specific polarization and migration of CD45RO+ PB T cells into RA SF, which were inhibited by anti-IL-15 antibodies (62). In addition, IL-15 stimulates activation and proliferation of RA PBMCs. IL-15 activates PB lymphocytes of RA patients by rapidly upregulating CD69 expression. RA PBMCs and SF T cells exhibited a significantly higher stimulation index in response to IL-15 than healthy PBMCs (62). These data suggest that IL-15 may continually recruit

T cells from RA PB into the synovium, and induce their activation and proliferation, contributing to the pathogenesis of RA. While these studies have shown the importance of IL-15 in the synovium of RA patients, very little is known about IL-15 levels in RA serum. In this paper, it is proposed that because RA is a systemic disease, serum IL-15 levels may be elevated in RA patients compared to healthy controls, contributing to the pathogenesis of the disease.

Evidence suggests that IL-15 may be an important regulator of TNF-$_{\alpha}$ production in the rheumatoid synovium. McInnes et. al. have shown that IL-15 activated CD45RO+ PB T cells from RA patients induced TNF-$_{\alpha}$ synthesis by blood or synovial derived macrophages in a contact-dependent mechanism (63). These results demonstrate the importance of IL-15 in regulating TNF-$_{\alpha}$ production in the RA synovium as well as PB. TNF-$_{\alpha}$ occupies a pivotal role in the regulation of synovial inflammation in RA because it regulates synthesis of other pro-inflammatory cytokines such as IL-1$_{\beta}$. These cytokines in turn enhance production of matrix metalloproteinases (MMPs) that initiate joint destruction (64). Many RA patients have elevated TNF-$_{\alpha}$ levels in the serum, which correlates with disease activity (65). Because IL-15 regulates TNF-$_{\alpha}$ production, the authors wanted to determine if there is a correlation between the two cytokines in RA serum.

IL-15 binds the high affinity IL-15R complex that is composed of the alpha, beta and gamma chains. IL-15R β chain (CD122) has been of particular interest because it is necessary for IL-15 internalization, signal transduction (65), IL-15 mediated activation and proliferation of healthy PBMCs (66). Studies on CD122 expression in RA PB are limited and contradictory (67, 68). In this study, the numbers of CD122+ cells and CD122 MFI have been measured in RA patients and healthy controls by flow cytometry. It is hypothesized that CD122 expression may be elevated in RA patients compared to healthy controls and that there may be a correlation between CD122 expression and serum IL-15 levels. The goals of this study were to (a) measure IL-15 levels in the serum of RA patients and healthy controls, (b) determine if there is a correlation between serum TNF-α and IL-15 levels in RA patients (c) measure CD122 expression in PB of RA patients and healthy controls and (d) determine if there is a correlation between CD122 expression and serum IL-15 levels.

These projects were conducted in the lab of Dr. David Yocum and represent on-going research in the area of rheumatoid arthritis therapy. The projects are continuing applied lab clinical adjuncts to evaluate the immunological effects of the therapies and to determine their mechanisms of action. The author of the dissertation has performed the majority of the experiments included in the three papers in Appendices A, B and C and is the first author of these papers.

PRESENT STUDY

The methods, results, and conclusions of this study are presented in the papers appended to this dissertation. The following is a summary of the most important findings.

I. PRIMATIZED® Anti-CD4 Antibody

The modified PRIMATIZED® anti-CD4 antibody used in this clinical trial has very different effects from that used in the Phase I single dose, dose-escalating trial. The flow cytometric results indicate that the modified antibody decreases the number of CD4+ T cells in both the anti-CD4+placebo treated group as well as the anti-CD4+MTX group. A simultaneous decrease was observed in CD2+, CD3+ and CD28+ T cells. However, the number of CD8+ T cells and the CD8 mean fluorescence intensity (MFI) remained stable throughout the trial. The CD4/CD8 MFI ratio dropped significantly at weeks 3 and 5, but returned to baseline levels by week 8 in both groups of patients. The effects of the modified PRIMATIZED® anti-CD4 antibody on mitogenic antibodies (anti-CD2, CD3, CD28, CD69) + PMA or mitogen (PHA, ConA or PWM) induced proliferation of PBMCs were measured. The results indicate that, unlike the original antibody, the modified anti-CD4 did not consistently inhibit proliferation through week 8 for either group. This indicated that a decrease in the number of circulating CD4+ T cells was not accompanied by a corresponding decrease in the PBMC proliferative response. Neither the decrease in CD4+ cell number nor the ability of PBMCs to proliferate in response to stimuli was altered by the concomitant administration of MTX.

II. Chimeric Anti-CD25 Antibody

The percent CD25+ cells in the peripheral blood at baseline was significantly higher in RA compared to healthy controls ($p=0.01$, Mann-Whitney). The chimeric anti-CD25 antibody induced a significant decrease in the percent CD25+ cells on day 0 +2h compared to baseline (17.6 ± 1.0 to $3.5 \pm 0.7\%$, $p<0.001$, paired t-test). The percent CD25+ cells on day 33 was 1.0 ± 0.3 %. The percentages of CD8, CD4, CD3, CD20 (B cells) and CD16 (NK cells) remained stable throughout the trial. The administration of the anti-CD25 antibody had variable effects on PHA as well as mitogenic anti-CD3 antibody induced proliferation through day 8 + 2h. Between day 12 +2h and day 33 there was a significant decrease in PHA stimulated proliferation ($p<0.05$). Proliferation by stimulation with anti-CD3 antibody decreased significantly at day 26 + 2h and day 33. Supernatant IL-2 levels in PBMCs stimulated with mitogenic anti-CD3+ anti-CD28 antibodies or PHA were low or undetectable through day 4 + 2h. The supernatant IL-2 levels increased significantly at day 12 +2h compared to baseline. Elevated supernatant IL-2 levels were observed through day 33. One patient, who had a low CD3 MFI at baseline, did not respond to the antibodies or PHA stimulation. The supernatant IL-2 levels in this patient were undetectable (< 7.0 pg/ml) between days 4 and 33. There were no treatment related changes in the plasma levels of IFN-γ, TNF-α, IL-2 and IL-6, indicating that the antibody did not cause cytokine release syndrome. Clinically, there was a significant decrease in the numbers of tender and swollen joints compared to baseline ($p<0.05$).

III. Interleukin-15

Interleukin-15 levels were elevated in the serum of 10 out of 21 RA patients compared to healthy controls. IL-15 was not detectable in the 8 healthy controls. Elevated serum TNF-α levels were observed in 6 out of 21 RA patients. The majority (4 out of 6) of these patients also had detectable IL-15 levels. There was no direct correlation between the two cytokines. Numbers of CD122+ cells and CD122 MFI in PB did not differ significantly in RA patients compared to healthy controls. The results indicate that not all CD8+ T cells express CD122. A significant inverse correlation exists between the numbers of CD122+ cells and CD122 MFI. No correlation was observed between serum IL-15 levels and CD122 expression or disease activity. These results indicate that serum IL-15 may be a more sensitive indicator of the inflammation in RA than TNF-α. However, since disease activity did not correlate with serum IL-15 levels, this cytokine may not accurately measure disease progression.

FUTURE STUDIES

Although there is no cure or method of preventing RA, new therapies, particularly genetically engineered monoclonal antibodies, have been shown to suppress inflammation and ameliorate symptoms. Monoclonal antibodies provide ways of achieving specific immunosuppression without adverse side effects. There are many therapeutic targets in the synovium as well as peripheral blood of RA patients. T cells, pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, and adhesion molecules have all been targets of therapy. An ideal antibody therapy will specifically target a molecule or cytokine that is involved in the earliest processes of inflammation that initiate RA and the suppression of which leads to sustained clinical improvement. In addition, an ideal antibody will have low immunogenicity to prevent the development of anti-immunoglobulins that reduce its effectiveness and have a long serum half-life to ensure minimal number of treatments.

Future studies involving the original and modified PRIMATIZED® anti-CD4 antibodies are needed to fully elucidate their mechanisms of action. In particular, studies are needed to understand the differences in the abilities of these antibodies to induce apoptosis. The mechanisms of action of the chimeric anti-CD25 antibody also need to be examined. Studies are needed to elucidate the processes involved in local accumulation of interleukin-2 and determine the clinical relevance of this effect. In addition, multiple dose trials are necessary and evaluating the immunological effects in these studies may be very important in determining optimal dosing of this antibody.

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APPENDIX A

Immunological Effects of a PRIMATIZED® anti-CD4 Antibody Used Concomitantly with Methotrexate in Active Rheumatoid Arthritis

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ABSTRACT

OBJECTIVE: To (a) determine the immunological effects of a PRIMATIZED® anti-CD4 antibody alone and in combination with methotrexate in a randomized, double-blind, placebo controlled trial and (b) elucidate its mechanisms of action.

MATERIALS AND METHODS: Eight patients with active RA were chosen: four (Group A) received the PRIMATIZED® antibody with placebo and four (Group B) were treated with the PRIMATIZED® antibody and MTX (10 mg, once weekly). The antibody was administered bi-weekly (at either 40 or 80 mg per injection) for 4 weeks and peripheral blood was obtained prior to, during and after treatment for analysis.

RESULTS: Flow cytometric analysis of groups A and B demonstrated significant decreases in CD4+ T cells by week 5 compared to baseline (33% and 39% for group A and group B, respectively) ($p<0.05$) that persisted until week 8. A simultaneous decrease was observed in CD2+ CD3+ and CD28+ T cells. The CD4/CD8 MFI ratio dropped significantly at week 3 compared to baseline and persisted through week 5 ($p<0.05$), returning to baseline levels at week 8. The prevalence and MFI of CD8+ T cells

remained stable throughout the study. PBMC's were tested for their ability to proliferate in response to mitogenic antibodies + PMA or mitogens. The results show no change in the PBMC proliferative response to mitogenic antibodies through week 8.

CONCLUSION: These results demonstrated significant CD4+ T cell depletion with this PRIMATIZED® anti-CD4 antibody. This effect was not altered by MTX. While CD4+ T cell depletion was significant, the PBMC's proliferative responses to mitogens and mitogenic antibodies were not affected.

KEYWORDS: Rheumatoid arthritis, anti-CD4, monoclonal antibody, immunological effects

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease that targets the synovium of the joints (1). While the etiology of RA is unknown, CD4+ T cells are thought to play an important role in the initiation, propagation and severity of the disease (2). One of the earliest events in RA is the intense infiltration of activated CD4+ CD45RO+ (memory) T cells into the synovium, suggesting an antigen specific T cell response (3). Evidence for the role of CD4+ T cells also came from the observations of Jaffe (4) who noted that RA patients who developed AIDS and profound CD4+ cell depletion saw an improvement of their rheumatic symptoms, suggesting that CD4+ T cells might play an important role in the initiation and progression of RA. Furthermore, the association of RA with certain MHC class II molecules, whose primary function is antigen presentation to CD4+ T cells, has strengthened the role of these cells in the development of the disease (5). Weyand et. al. found that auto-reactive CD4+CD28- T cell clones were enriched in the peripheral blood and synovium of RA patients, suggesting clonal expansion of T helper cells (6). Because class II MHC molecules determine T cell repertoire during thymic selection, auto-reactive T cells may be generated by defective selection in RA patients (7). These auto-reactive clones then enter the periphery where they may contribute to autoimmunity. The effectiveness of T cell directed therapies such as cyclosporin and FK506 further supports the role of CD4+ T cells in RA (8). Finally, the proposed role of CD4+ T cells in RA has also been studied in animal models of rheumatoid arthritis. Treatment with anti-CD4 antibody prevented type II collagen-induced arthritis (9). These suggest that CD4+ T cells might play an important role in the pathogenesis of RA and that anti-CD4 antibodies may be valuable in treatment of the disease.

The success of anti-CD4 antibodies in animal models suggested their use in humans with RA. Several investigators used murine and chimeric (mouse/human) anti-CD4 antibodies to treat patients with RA (10-12). These antibodies induced an immediate decrease in the number of circulating CD4+ T cells (13). However, depletion was not associated with clinical efficacy (13). In addition, these antibodies had a short serum half-life and were highly immunogenic. They induced the production of human anti-mouse antibodies, causing rapid clearance of the anti-CD4 antibodies, thereby reducing its effectiveness (13).

The PRIMATIZED® anti-CD4 antibody was one of the first non-depleting, hyperchimeric (CDR-grafted) antibodies to be used in clinical trials for the treatment of RA. In animals, the PRIMATIZED® anti-CD4 antibody was shown to be specific for CD4+ T cells, and have longer serum half-life (5 days) than previous antibody treatments (14). The PRIMATIZED® antibody had very low immunogenicity because of its primate complementarity determining regions (CDRs) and human IgG1 constant region (14). Moreover, these animal studies demonstrated that the PRIMATIZED® anti-CD4 antibody was non-depleting such that antibody binding to CD4 did not induce concurrent cell death (15). *In vitro* experiments with human PBLs demonstrated that this anti-CD4 antibody inhibited mixed lymphocyte reaction and IL-2 production but did not induce complement mediated lysis (CML) (15).

The phase I single-dose, open label, dose-escalating clinical trial using this PRIMATIZED® anti-CD4 antibody demonstrated CD4 modulation (i.e. internalization and re-expression) without cell depletion (16). CD4 expression decreased rapidly within 72 hours after infusion of the antibody in a dose-dependent manner. A decrease in mitogen and antigen induced proliferation 4-8 weeks post-treatment suggested that the anti-CD4 had immunosuppressive effects. The multi-dose, dose-escalating trial confirmed the immunological effects of the phase I trial (17). The present study combines the PRIMATIZED® anti-CD4 antibody with a placebo or MTX and determines the immunological effects of this combination therapy and proposes mechanisms to explain the anti-CD4 antibody's effects.

Materials and Methods

Patients

Eight patients taking methotrexate were recruited from the rheumatology clinics at the University of Arizona, Tucson, Arizona. All patients fulfilled the 1987 American College of Rheumatology (ACR) criteria for diagnosis of RA, with disease duration 6 months or longer (18). They had failed at least one, but no more than four DMARD due to lack of efficacy and had active disease as defined by the presence of 3 or more swollen or tender joints and the presence of the following criteria: erythrocyte sedimentation rate

(ESR) $\geq$ 28mm/h; morning stiffness $\geq$ 45 min; and rheumatoid factor (RF) positivity. DMARDs were stopped 4 weeks prior to treatment in all patients. During this period, the dose of nonsteroidal antiinflammatory drugs (NSAIDS) was not changed. Concurrent oral steroid usage was allowed if the dose was stable and less than or equal to 10 mg of prednisone per day. Written consent was obtained from all patients after they had been informed, both orally and in writing, of the possible risks of treatment.

Antibody dosing regimen

A detailed description of the PRIMATIZED® anti-CD4 monoclonal antibody is given elsewhere (14). Briefly, it is an IgG1 antibody (λ light chain) with macaque primate variable region and human constant regions. It was produced by a CHO transfectoma cultured in hollow fiber bioreactors. Eight patients with active RA were selected for the study. Four patients (Group A) received the PRIMATIZED® antibody (2 injections/week at either 40 mg or 80 mg per injection) with placebo. Four patients (Group B) were treated with the PRIMATIZED® antibody (2 injections/week at either 40 mg or 80 mg per injection) and methotrexate (10 mg, once weekly). Antibody treatments were administered for weekly for 4 weeks and peripheral blood was obtained prior to, during and after therapy for analysis.

Reagents and Antibodies

The following mitogenic antibodies were used in the proliferation experiments: mab 9.3, specific for CD28 (ascitic fluid, a gift from Dr. Jeffrey Ledbetter, Bristol-Meyers Squibb, Seattle, WA) was used at a final concentration of 1:100. Mabs A32 and G38, specific for CD3 and CD69, respectively, gifts from Dr. Shu Man Fu (University of Virginia, Charlottesville, VA) were used at a final concentration of 1:1000 and 1:400. Mabs $T11_2$ and $T11_3$, specific for CD2, gifts from Dr. Ellis Reinherz (Dana-Farber Cancer Institute, Boston, MA) were each used at a final concentration of 1:100. Phorbol myristate acetate (PMA, Sigma) was used at a final concentration of 1 ng/ml. The mitogens, phytohemagglutinin (PHA, Sigma), concanavalin A (Con A, Sigma) were each used at 0.5 mg/ml, 0.1 mg/ml and 0.01 mg/ml; pokeweed mitogen (PWM, Sigma) was used at 1 mg/ml, 0.5 mg/ml and 0.01 mg/ml.

Immunofluorescence staining and flow cytometric analysis

The following Mabs were used for indirect immunofluorescence staining: anti-CD2, anti-CD28 and anti-fas (Immunotech, Marseille, France); anti-CD3 and anti-CD8 (Coulter, Miami, FL); anti-CD4 (Leu 3a) and anti-CD69 (Leu 23) (Becton Dickinson, Mountain View, CA) and anti-CD4 (OKT4) (Ortho Diagnostics, Raritan, NJ). Whole blood (100μl) was incubated with combinations of the above antibodies for 30 minutes at 4°C. The RBCs were lysed and the WBCs were fixed by Coulter Multi-Q-Prep. The cells were analyzed by Coulter Epics XL-MCL (Coulter Corporation, Miami, Florida).

Culture conditions and proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by Ficoll-Hypaque (Sigma) centrifuge method (19). PBMCs were then washed, counted and plated in 96 well plates at 2×10^5 cells/well in complete media (RPMI-1640 supplemented with 10 mM HEPES, 10% heat inactivated FCS, Penicillin-Streptomycin (10000 U). The indicated antibodies and PMA or mitogens were added and the cells were incubated for 72 hours at 37°C in a humidified 5% CO_2 incubator after which ^{3}H-thymidine was added (1 μCi, New England Nucleotides, Boston, MA). The cells were harvested after 24 hours and analyzed on a Beckman scintillation counter. The results are expressed as mean cpm of the groups $\pm$ SE.

Statistical Analysis

Paired t-tests were performed in each group (A or B) to compare data obtained at weeks 3, 5 and 8 with baseline.

RESULTS

Effects of the PRIMATIZED® anti-CD4 antibody on T cell population

The effects of the PRIMATIZED® anti-CD4 antibody on the number of CD4+ T cells were determined by flow cytometry using antibodies to two different epitopes on the CD4 molecule, Leu 3a and OKT4. Leu 3a, which binds to the first domain (D1) of the CD4 molecule, competes with the PRIMATIZED® anti-CD4 antibody. The OKT4 antibody binds to the fourth domain (D4), which is proximal to the plasma membrane. A decrease in the number of CD4+ T cells was observed in group A (placebo+anti-CD4) from week 3 through week 8 compared with baseline (FIGURE 1.1). Decreases in numbers OKT4+ and Leu 3a+ cells were statistically significant at week 5 ($p<0.04$) and week 8, ($p<0.04$). Similarly, there was a significant decrease in the number of CD4+ T cells in group B (MTX+anti-CD4) at weeks 3 and 5 ($p<0.02$; $p<0.005$) but not at week 8. There was no significant difference between groups A and B.

To determine if the decrease in number of CD4+ T cells in the peripheral blood was due to depletion or specific endocytosis of the CD4 molecule, other T cell markers CD2, CD3, CD8 and CD28 by flow cytometry. Significant decreases were observed in the number of CD2+, CD3+ and CD28+ T cells in group A at weeks 3, 5 and 8 ($p<0.01$, $p<0.005$ and $p<0.002$, respectively) (FIGURE 1.2). The number of CD8+ T cells was stable throughout the trial. Similar results were seen with group B (FIGURE 1.2). Significant decreases were observed in the numbers of CD2+, CD3+ and CD28+ T cells at weeks 3, 5 and 8 ($p<0.02$, $p<0.003$, $p<0.005$). As with group A, the number of CD8+ T cells remained stable throughout the trial.

Effect of the PRIMATIZED® anti-CD4 antibody on CD4 mean fluorescence intensity (MFI)

To determine the effects of the anti-CD4 antibody on CD4 expression, the MFI of CD4 (OKT4) and CD8 were measured from week 0 through week 8 in both treatment groups. CD8 MFI remained stable throughout the trial in groups A and B. A significant decrease in the CD4/CD8 MFI ratio at week 3 was observed, which persisted until week 5 ($p<0.02$, $p<0.003$ respectively, t test) in group A (FIGURE 1.3). At week 8, the

CD4/CD8 MFI ratio returned to baseline levels. Similar results were seen in group B ($p<0.05$ (week 3), $p<0.005$ (week5), FIGURE 1.3).

Effects of PRIMATIZED® anti-CD4 antibody on proliferative response

To determine if the decrease in the number of CD4+ T cells was accompanied by a decrease in function, we measured the ability of the PBMCs to respond to various stimuli by ^{3}H thymidine incorporation assay. These stimuli included mitogenic antibodies (anti-CD2, CD3, CD28 and CD69) + PMA as well as mitogens (PHA, Con A, PWM). The mitogenic antibodies + PMA have been shown previously to activate PBMCs and cause proliferation (20-23). No consistent or significant changes in proliferation were noted in group A or B in response to mitogenic antibodies + PMA from week 0 through week 8 (FIGURE 1.4). A significant decrease in proliferation was observed in response to the mitogens at week 5 ($p<0.05$) compared to baseline. However, at week 8, no significant difference was observed compared to baseline. Furthermore, there was no significant difference in proliferative ability between the placebo and MTX treated groups.

Discussion

The proposed role of CD4+ T cells in the pathogenesis of RA made these cells targets for immunotherapy. Monoclonal antibodies directed at the CD4 molecule were developed to induce selective immunosuppression via CD4+ T cells. Initially, murine and chimeric anti-CD4 antibodies were used in clinical trials to treat RA. However, these antibodies were highly immunogenic, which limited their usage. They also caused specific depletion of CD4+ T cells (lymphopenia) in the majority of patients, which posed risks of opportunistic infections. The PRIMATIZED® anti-CD4 antibody was developed to circumvent these problems. This antibody was hyperchimeric (with primate CDRs and human constant regions) and had low immunogenicity. *In vivo* experiments in primates demonstrated that this antibody was non-depleting (15). *In vitro* experiments demonstrated that the PRIMATIZED® anti-CD4 antibody inhibited mixed lymphocyte reaction with human PBLs and inhibited IL-2 production, indicating its immunosuppressive properties (14). The phase I, single dose, open-label, dose-escalating trial demonstrated that the PRIMATIZED® anti-CD4 antibody induced CD4 modulation and a decrease in mitogen and antigen induced proliferation (16). These results were confirmed by the multi-dose trial (17). The goals of this study were to determine the immunological effects of this PRIMATIZED® anti-CD4 antibody in combination with MTX or placebo and to elucidate its mechanisms of action. The results suggest that the PRIMATIZED® anti-CD4 antibody used in this study has very different effects on the T helper cells than that used in the phase I trial. While the reasons for the altered function are not clear, it has been hypothesized that changes in antibody production and storage protocols may have modified its functions (24).

The results suggest that the PRIMATIZED® anti-CD4 antibody used in this study has very different effects on the T helper cells from the previous study. Yocum et. al. demonstrated that the original antibody caused specific modulation of the CD4 molecule without cell depletion (16). CD4 expression decreased rapidly at 72 hours post treatment and returned to baseline levels at 2 weeks post treatment. The modified antibody however, functions by killing CD4+ T cells. The significant decrease in the number of CD4+ T cells at week 5 in both MTX and placebo treated patients suggests that it is a depleting antibody.

The authors propose that the modified antibody induces complement mediated lysis or antibody dependent cell cytotoxicity. Alternatively, the modified antibody may be cross-linked through its Fc receptor triggering apoptosis of the T helper cell. Oyaizu et. al. have shown that CD4 cross-linking by anti-CD4 antibodies induce apoptosis of normal CD4+ T cells by up-regulating fas and down-regulating bcl-2 expression (25, 26). Choy et. al. have shown that cM-T412, a depleting chimeric anti-CD4 antibody cross-links Fc-receptors and induces apoptosis of antibody coated cells (27).

Yocum et. al. demonstrated that the original antibody decreased the proliferative response of PBMCs to mitogens and recall antigens such as *Candida, Trichophyton*, and tetanus toxoid at 4-8 weeks post treatment (17). *In vitro* experiments such as mixed lymphocyte reaction and tetanus toxoid proliferation assay confirmed that the original anti-CD4 antibody inhibited T cell activation and proliferation (14). The results of this study indicate that the modified antibody did not induce any consistent changes in the proliferative response of PBMCs to mitogenic antibodies or mitogens. This contradicted the hypothesis that depletion of CD4+ T cells from peripheral blood would be accompanied by a sustained decrease in proliferation. The reasons for this remain unclear. One explanation is that at baseline the majority of the proliferating cells were

CD4+ while at week 8, they may have been primarily CD8+ T cells. The mitogenic antibodies (anti-CD3, anti-CD2 etc.) do not distinguish proliferation between these cell populations. Using a CD4+ T cell specific mitogen such as jacalin may elucidate how the modified antibody affects T helper function.

While the original antibody's mechanism of action is unknown, evidence suggests that non-depleting anti-CD4 antibodies primarily function by modulating CD4 molecules on the cell surface. Bartholomew et. al. have demonstrated that the humanized anti-CD4 antibody, 4162W94, modulated CD4 molecules *in vivo* in a similar manner (28). Other mechanisms have been proposed to explain the effects of non-depleting anti-CD4 antibodies. They can block T cell/MHC interactions by inhibiting CD4-MHC binding. This abrogates the co-stimulatory signal needed for T cell activation and the T cell becomes unresponsive to further stimuli (29). Non-depleting antibodies have been shown to alter cytokine production by T helper cells. Treatment with the humanized anti-CD4 antibody, OKTcdr4a, showed a significant decrease in IL-2 and IFN-γ mRNA levels in mitogen stimulated PBMCs from antibody treated patients compared to placebo controls (30). Another humanized anti-CD4 antibody, YHB.46, inhibits production of IL-2, IL-4 and IL-10 by PBMCs from healthy controls in in-vitro experiments (31). Finally, non-depleting anti-CD4 antibodies can also inhibit intracellular interaction between $p56^{lck}$ and CD3 by a mechanism that is not completely understood. Data suggests that certain non-depleting anti-CD4 antibodies bind CD4 causing transmission of an inhibitory signal to the T cell (32, 33).

Further studies are needed to fully explain the mechanisms of action of both PRIMATIZED® antibodies. The objectives of these studies will be to (a) evaluate the effects of the original anti-CD4 antibody on cytokine production by CD4+ T cells stimulated with mitogenic antibodies and (b) determine if the original anti-CD4 antibody prevents signal transduction by inhibiting $p56^{lck}$ activation. Specifically, the authors propose to measure differences in $p56^{lck}$ activation in T helper cells treated with and without the PRIMATIZED® antibody. The results of this trial indicate that the modified antibody depletes T helper cells but the mechanism by which this occurs is not clear. One potential mechanism is that the modified antibody cross-links CD4 molecules and induces apoptosis of the CD4+ cell. Coating plates with either PRIMATIZED® anti-CD4 antibody and incubating with CD4+ T cells will mimic cross-linking through Fc receptor. Flow cytometric analysis of propidium iodide negative/ annexin V positive cells will determine if cross-linking induces apoptosis. It is expected that the modified, but not the original antibody, to induce apoptosis.

In summary, these results demonstrated significant CD4+ T cell depletion with the modified antibody. This effect was not altered by the combination with MTX. While CD4+ T cell depletion was significant, the PBMC's proliferative responses to mitogens and mitogenic antibodies were not affected.

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APPENDIX B

Immunological Effects of a Chimeric Anti-IL-2 Receptor Alpha Chain (CD25) Antibody in the Treatment of Active Rheumatoid Arthritis

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ABSTRACT

Objective: The goals of this phase I study were to (a) determine the immunological effects of Simulect®, a chimeric anti-IL-2 receptor α chain (CD25) antibody in active rheumatoid arthritis (RA) and (b) elucidate its mechanisms of action.

Methods: Six patients with active RA, who had a partial response to methotrexate, were chosen. Intravenous bolus injections were administered on day 0(0.02mg), day 4(0.2mg), day 8(2mg), day 12(20mg), day 19(40mg) and day 26(80mg). Blood was obtained for analysis at baseline, 2(+2h), 8(+8h) and 24 hours(+24h) post injection and on day 33 (follow up). Flow cytometric analysis, ^{3}H-thymidine incorporation assay, and cytokine ELISAs were performed.

Results: The percent CD25+ cells at baseline was significantly higher in RA patients than healthy controls ($p=0.01$, Mann-Whitney). Simulect® caused a significant decrease in %CD25+ cells on day 0+2h compared to baseline (17.6 ± 1.0 to $3.5\pm0.7\%$, $p<0.001$, paired t-test). The %CD25+ cells remained significantly decreased through day 33 ($1.0\pm0.3\%$, $p<0.001$). The percentages of CD8, CD4, CD3, CD20 and CD16+ cells

remained stable throughout the trial. Simulect® induced an initial increase in PHA stimulated PBMC proliferation between day 4+2h and day 8+2h and a significant decrease at day 12+2h, which persisted through day 33 ($p<0.05$). The anti-CD3 antibody induced proliferation decreased significantly at day 26+2h and day 33. Supernatant IL-2 levels in anti-CD3+anti-CD28 antibody or PHA stimulated cells were low or undetectable through day 4+2h. The IL-2 levels increased significantly at day 12+2h ($p<0.05$) compared to baseline. Elevated supernatant IL-2 levels were observed through day 33. There were no treatment related changes in the plasma levels of IFN-γ, TNF-α, IL-2 and IL-6. Clinically, there was a significant decrease in the numbers of tender and swollen joints compared to baseline ($p<0.05$).

Conclusions: The clinical and immunological effects of Simulect® supported our hypothesis that CD25+ T cells play an important role in the progression of RA. Simulect® effectively targeted the CD25 molecule in vivo, resulting in decreased proliferation without cell depletion. Decrease in number of CD25+ cells was associated with a positive clinical effect. We have proposed mechanisms to explain Simulect's® action.

Keywords: rheumatoid arthritis, chimeric, anti-CD25 antibody, immunological effects

Rheumatoid arthritis (RA) is a systemic inflammatory disease that primarily affects the synovial joints (1). There is no known cure or way of preventing the disease. While the etiology of RA is unknown, evidence suggests that CD25+ (IL-$2R_{\alpha}$ chain positive) T cells play an important role in the initiation and propagation of the disease. One of the earliest events in RA is an uncontrolled inflammatory response in the synovium, possibly induced by an arthrotropic agent (2). The infiltrating cells are CD4+, a fraction of which express CD25, suggesting that they may have been recently activated (3). Other evidence supports a central role for systemic T cell activation and CD25+ T cells in the progression of RA. Many RA patients have elevated numbers of CD25+ T cells in the peripheral blood compared to healthy controls (4). Moreover, in early RA, many patients have high levels of serum IL-2 and IFN-γ, which correlate with progression of early disease (5). In addition, RA patients have high levels of soluble CD25 (sCD25) in the peripheral blood (6). This sCD25 is produced by enzymatic cleavage of the transmembrane form of CD25 and is released into circulation by activated T cells (7, 8). It may be produced by activated T cells in an attempt to regulate the activational state of the cell in that sCD25 prevents the formation of the high affinity IL-2R and further activation by IL-2. The sCD25 levels correlate positively with disease activity in RA suggesting a role for CD25+ T cells in RA (9). T cell directed therapies that prevent the CD25 expression or deplete CD25+ cells have been effective in treating RA. Cyclosporine A and FK-506 inhibit T cell activation, production of IL-2 and IFN-γ, and T cell proliferation (10). DAB_{486}IL-2 toxin fusion protein has been used successfully in clinical trials to treat RA patients by depleting CD25+ cells (11). While IL-2 binds to its receptor on activated T cells, the DAB_{486} toxin kills the cell.

It is hypothesized that CD25+ T cells play an important role in the progression of RA. The authors propose that treatment with an anti-CD25 antibody will reduce disease activity. The concept of using anti-CD25 antibodies as means of achieving selective immunosuppression or tolerance is well established in transplantation (12, 13). However, anti-CD25 therapy has not been used in the treatment of RA. This is a logical step in monoclonal antibody therapy of RA because anti-CD25 antibodies selectively target the high affinity IL-2R on recently activated T cells that have been implicated in the pathogenesis of the disease.

Simulect® (basiliximab) is a chimeric, IgG monoclonal antibody that recognizes the CD25 antigen that is expressed on activated but not resting or memory T cells. *In vitro* experiments have demonstrated that Simulect® inhibited mixed lymphocyte reaction (MLR) and induced antibody dependent cell cytotoxicity (ADCC) on a CD25+ cell line through its human FcR (14). Simulect® has been used successfully as a prophylactic immunosuppressive agent in combination with cyclosporine and corticosteroids in kidney transplantation (15). Simulect's® serum half-life was approximately 7 days (15). It is the first chimeric anti-CD25 antibody used in the treatment of RA. In a 33 day, open-label clinical trial, 6 patients with active RA, who were partially responsive to methotrexate

(MTX) were treated with Simulect®. In this paper we report the immunological effects of this chimeric anti-CD25 antibody and discuss its mechanisms of action.

Patients and Methods

Patients

Six patients who had partial response to MTX therapy were recruited from the rheumatology clinics at the University of Arizona, Tucson, Arizona along with seven age-matched (mean age >35) healthy controls. All patients fulfilled the 1987 revised American College of Rheumatology (ACR) criteria for diagnosis of RA with disease duration of 6 months or longer (16). A partial response to methotrexate was defined as the presence of at least 6 swollen and 9 tender joints in patients who had been receiving a maximally tolerated MTX dose (not exceeding 20 mg/week) for at least 3 months prior to day 0. Concurrent oral steroid and NSAID usage was allowed if the dose was stable for at least 6 weeks prior to day 0 and oral steroid usage was less than or equal to 10 mg/day. All patients gave written consent for participation in the study at screening. Patients with known allergies to murine proteins were also excluded from the study.

Antibody Dosing Regimen

Simulect®(basiliximab) is a chimeric, IgG monoclonal antibody that recognizes the CD25 antigen expressed on activated T cells. It is composed of murine variable and human constant region and inhibits the binding of IL-2 to its receptor. In this open label, intrapatient dose-escalating trial, each patient was administered the following doses through an intravenous bolus injection:

Day 0 - 0.02 mg	Day 12 - 20 mg
Day 4 - 0.20 mg	Day 19 - 40 mg
Day 8 - 2 mg	Day 26 - 60 mg

Peripheral blood was drawn at baseline, on days 0-26 at 2 (+2h), 8 (+8h), and 24 hours (+24h) post injection and on day 33 (follow up).

Reagents and Antibodies

The following mitogenic antibodies for used in the proliferation and cytokine stimulation assays. Mab 9.3, specific for CD28 (ascitic fluid, a gift from Dr. Jeffrey Ledbetter, Bristol-Meyers Squibb, Seattle, Washington) was used at a final concentration of 1:100. Mab A32, specific for CD3 (a gift from Dr. Shu Man Fu, University of Virginia, Charlottesville, Virginia) was used at a final concentration of 1:1000. PHA (Sigma) was used at final concentrations of 80 ug/ml, 16 ug/ml, 1.6 ug/ml.

Immunofluorescence Staining and Flow Cytometric Analysis

The following Mabs were used for direct immunofluorescence staining: anti-CD2, anti-CD28, and HLA-DR (Immunotech, Marseille, France); anti-CD3, anti-CD4, anti-CD8, anti-CD16, anti-CD25 (Coulter, Miami, Florida); anti-CD20 (Becton Dickinson, Mountain View, California). Whole blood ($100_{\mu}l$) was incubated with combinations of the above antibodies for 30 minutes at 4°C. The RBCs were lysed and WBCs were fixed by Coulter Multi-Q-Prep (Hialeah, Florida). The cells were analyzed by Coulter Epics XL-MCL, Coulter Corporation, Hialeah, Florida.

^{3}H-Thymidine Incorporation Assay

Peripheral blood was drawn 2 hours post-injection on treatment days. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque (Sigma) method (17). The PBMCs were then washed, counted, and plated in triplicate in 96 well plates at $2x10^5$ cells/ well in RPMI-1640 supplemented with $NaHCO_3$. Anti-CD3 antibody or PHA were added and the cells were incubated for 72 hours at 37°C in a humidified 5% CO_2 incubator after which ^{3}H thymidine was added (1 μCi, New England Nucleotides, Boston, Massachusetts). The cells were harvested after 24 hours and analyzed on a Beckman scintillation counter. The results are expressed as percent change of baseline.

ELISA

Peripheral blood was drawn 2 hours post-injection on treatment days. The isolated PBMCs were incubated with PHA or anti-CD3+anti-CD28 antibodies in triplicates for 72 hours. Supernatants of the stimulated cells were pooled and assayed for IL-2 and IL-4 using commercial kits (OptEIA kits, Pharmingen, California). Determinations were performed in duplicate. Plasma levels of IL-2, TNF-α, and IFN-γ were measured in triplicate using commercial sandwich ELISA kits (R&D Systems, Minneapolis, MN). Plasma IL-6 levels were measured in duplicate by sandwich ELISA (R&D Systems, Minneapolis, MN).

Clinical Efficacy

RA disease activity was measured by swollen joint counts, tender joint counts (18). All disease activity measurements were carried out at the start of therapy (day 0) and at days 19, 26 and 33 (follow up).

Results

Percent CD25+ cells in the peripheral blood of RA patients

Prior to treatment with Simulect®, the percent CD25+ cells was measured by flow cytometry (FIGURE 2.1). The percent CD25+ cells in the peripheral blood of RA patients was significantly increased compared to 13 age-matched healthy controls (Mann Whitney, $p = 0.01$). The percent CD25+ cells decreased significantly at day 0 +2 h (17.6 $\pm$ 1.0% to 3.5 $\pm$ 0.7%, paired t-test, $p<0.0001$) (FIGURE 2.2). Prior to the injection on day 4, CD25 expression had increased to 10.4 $\pm$ 1.6% but was significantly lower than baseline ($p<0.0001$). Percent CD25+ cells remained at significantly lower levels compared to baseline through day 33 ($p<0.0001$). The decrease in CD25+ cells was not accompanied by a corresponding decrease in T cells (CD3, CD4 and CD8), B cells (CD20) and NK cells (CD16) (FIGURE 2.3).

Simulect's® effect on CD4+ T cells

To determine how Simulect® affects lymphocyte subsets, we determined percent CD25 on CD2, CD4, CD8, CD16 and CD20+ cells. The data show that CD25 is primarily expressed on CD4+ T cells (FIGURE 2.4) and therefore, Simulect's® effect is observed primarily on CD4+ T cells.

Effect of Simulect® on IL-2 dependent proliferation

^{3}H-thymidine incorporation assays were performed to determine anti-CD25 antibody's effects on mitogenic anti-CD3 or PHA induced proliferation. Stimulation with PHA induced a biphasic proliferative response (FIGURE 2.5). A significant decrease in proliferation was observed on day 0 +2h (paired t-test, $p<0.05$), followed by an increase on day 4 and 8. By day 12, proliferation significantly decreased compared to day 0 (paired t-test, $p<0.05$) and remained until day 33. Stimulation with anti-CD3 antibody resulted in a similar biphasic proliferative response (FIGURE 2.6). However, Simulect® induced decrease in anti-CD3 induced proliferation was not apparent until day 33 (paired t-test, $p<0.05$).

Simulect's® effects on IL-2 and IL-4 Production

Anti-CD25 antibody's effects on Th1 (IL-2) and Th2 (IL-4) cytokine production, in response to anti-CD3+anti-CD28 stimulation, were measured by ELISA (FIGURE 2.7). Supernatant IL-2 levels in anti-CD3+anti-CD28 stimulated cells were low or undetectable through day 4+2h. The IL-2 levels increased significantly at day 12+2h ($p<0.05$)

compared to baseline. Elevated supernatant IL-2 levels were observed through day 33. Supernatant IL-4 in anti-CD3+anti-CD28 stimulated cells was detectable at day 0 pre infusion through day 4+2h. Supernatant IL-4 levels decreased at day 8 compared to baseline and remained undetectable through day 26. Similar results were obtained with PHA stimulated cells. An inverse correlation was observed between IL-2 and IL-4 levels in 2 patients in PHA stimulated cells (SBF Spearman ρ= -0.80), BYS (ρ = -0.71)).

Simulect's® effects on Plasma IL-2, TNF-α, IFN-γ and IL-6 levels

To determine if Simulect® induces cytokine release syndrome in these RA patients, plasma cytokine levels of IL-2, IFN-γ and TNF-α were measured in triplicates. In the majority of patients, plasma levels of these cytokines were low or undetectable throughout the trial (FIGURE 2.8-2.12). Slight increases in plasma IL-2, IFN-γ and TNF-α were observed in one patient (AEV) but the fluctuations did not correlate with times of treatment. Simulect's® effects on plasma IL-6 are shown on Figure 6D. Plasma IL-6 levels were undetectable in 2 out of 6 patients. In the remaining 4, plasma IL-6 levels consistently decreased to 1.75 pg/ml (low limit of detection) at 2 hours post-injection at days 2, 4, 8, 12 and 19. This pattern does not appear in these patients on day 26.

Clinical effects of Simulect®

Simulect® induced a significant decrease in the number of tender and swollen joints on day 33 compared to pre-infusion in 5 out of 6 patients (paired t-test, $p<0.05$, TABLE

2.1). The treatment was discontinued in one patient (FIC) after day 12 due to aseptic meningitis. In the 5 patients who showed clinical improvement, RF and CRP levels did not show a corresponding decrease.

Discussion

CD25+ T cells have been implicated in the pathogenesis of RA. The goals of this study were to (a) evaluate the immunological effects of a chimeric anti-CD25 antibody in RA patients who are partially refractive to MTX and (b) elucidate its mechanisms of action. To our knowledge, this is the first chimeric anti-CD25 antibody used in the treatment of RA.

In this study, we have demonstrated that Simulect® did not cause depletion of CD25+ cells. While *In vitro* experiments have shown that Simulect® is capable of inducing ADCC in CD25+ T cell lines (14), our results indicate that it does not deplete T, B or NK cells *in vivo.* This contrasts data obtained with anti-CD25 antibodies used to cause regression of tumors in mice, which primarily depleted CD4+CD25+ T cells (19). Simulect® significantly inhibited IL-2 dependent proliferation induced by mitogenic antibodies or PHA. Anti-CD25 antibodies have been reported to inhibit T cell proliferation induced by cross-linking the TCR and CD2 (20).

Simulect® did not inhibit IL-2 (Th1) production by PBMCs stimulated by mitogenic antibodies or PHA. The results indicated that the anti-CD25 antibody may have prevented the formation of high-affinity IL-2 receptors, causing the accumulation of IL-2 in the supernatant. Alternatively, IL-2 production may have been upregulated to compensate for the presence of the anti-CD25 antibody. This contrasts with other monoclonal antibody therapies such as anti-CD4 antibody that inhibit production of Th1 (IL-2) and Th2 (IL-4) cytokines (21). Our results demonstrate a decrease in IL-4 in the supernatant of PBMCs stimulated with mitogenic anti-CD3 or PHA. We propose that the decrease in IL-4 is due to the formation of high-affinity IL-4 receptor. In preventing the formation of the high-affinity IL-2R, Simulect® allows for increased availability of the IL-2R$_{\gamma}$ chain, which is a component of the high-affinity IL-4R (22). The increased uptake of IL-4 may account for some of the beneficial effects of this antibody.

Our results demonstrated that Simulect® did not induce cytokine release syndrome. Levels of IFN-γ, TNF-α□□ and IL-2 in plasma were generally undetectable. However, in one patient, the levels of these cytokines fluctuated throughout the trial but did not appear to correlate with administration of the treatment. On the contrary, plasma levels of the pro-inflammatory cytokine, IL-6 appeared to be influenced by the treatment. Elevated levels of IL-6 have been observed in the plasma of RA patients and have correlated with disease activity (23). Simulect® induced a consistent decrease in plasma IL-6 levels at 2 hours post injection on days 0-19. This suggests that the major producers of IL-6 in the peripheral blood express the high-affinity IL-2R and by blocking the interaction of IL-2 with its receptor, Simulect® inhibits IL-6 production. This may also contribute to the beneficial effects of this antibody. Treatment with Simulect® resulted in a significant decrease in the number of tender and swollen joints on day 33 compared to baseline ($p<0.05$). Taken together, these results support our hypothesis that CD25+ T cells play an important role in the progression of RA.

In regard to Simulect's® mechanisms of action, we propose that the antibody primarily affects CD4+ T cells circulating in the peripheral blood. It binds to recently activated CD4+ T cells and prevents interaction of IL-2 with its receptor. Consequently, there is inhibition of T cell activation and proliferation. It appears that IL-2 production is not inhibited. On the contrary, it is possible that there is a local accumulation of IL-2. The clinical effects of this phenomenon are unclear. Simulect® might increase uptake of IL-4 due to increased formation of high-affinity IL-4 receptors and inhibit IL-6 production in the peripheral blood by preventing interaction of IL-2 with its receptor.

One question that these results raise is how does Simulect® induce peripheral tolerance in RA patients. Peripheral tolerance in healthy individuals is maintained by three mechanisms: deletion (activation induced cell death), anergy and immunoregulatory cells. In mice, immunoregulatory cells have been characterized as a subset of CD4+CD25+ cells that are thought to prevent the activation of autoreactive T cells (24, 25). Sakaguchi et. al. have shown that removal of these immunoregulatory cells from mice induces autoimmunity while amelioration of symptoms is observed when these cells are reconstituted (26). In addition, these cells have been shown to inhibit IL-2 production by a mechanism that is not cytokine mediated. The CD4+CD25+ cells require activation through the TCR in addition to cell contact between the immunoregulatory cells and the responder cells. Thornton et. al. proposed that these cells function by blocking the delivery of a co-stimulatory signal because the suppressor activity can be abrogated by addition of exogenous IL-2 or anti-CD28 antibody (27). Simulect's® effects on these CD4+CD25+ immunoregulatory cells are not known. We propose that Simulect® does not interfere with the function of these cells. It does not deplete these CD25+ suppressor cells, which might induce autoimmunity. Instead, it prevents the binding of CD25 and IL-2, and this may be beneficial since excess IL-2 has been shown to abrogate the suppressor function of these cells. Furthermore, these cells are thought to act as suppressors by cell-cell contact that does not involve CD25 (27). Our results suggest that the CD4+CD25+ suppressor cells bound Simulect® in peripheral blood but this did not result in an exacerbation of RA.

The second question raised by our results is what effects does Simulect® have on sCD25. We propose that Simulect® binds sCD25 and at saturation levels, it removes sCD25 from the peripheral blood by depositing it in the secondary lymphoid organs. We hypothesize since sCD25 levels have been positively correlated with disease activity, the removal of sCD25 may contribute to the beneficial effects of this treatment.

We have proposed several experiments to address how Simulect® influences IL-2, IFN-γ, and IL-4 production by stimulated CD4+ T cells. We want to distinguish between our hypotheses that the increased IL-2 in the supernatant of stimulated cells is due to (a) increased IL-2 production or (b) IL-2 accumulation due to lack of high-affinity IL-2R. Semi-quantitative measurements of IL-2 mRNA can be used to determine which of these hypotheses is true. To determine if (a) IL-4 production is inhibited or (b) there is increased uptake, we propose to incubate cells with anti-IL-4 antibodies that prevent uptake of IL-4. The levels of IL-4 in the supernatant of these cells can distinguish between the two possibilities.

In conclusion, Simulect® was effective at saturating the CD25 molecule in vivo, resulting in decreased proliferation without cell depletion. CD25 saturation was associated with a positive clinical effect.

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APPENDIX C

Elevated serum interleukin-15 levels in rheumatoid arthritis (RA): Correlation with serum TNF α, IL-15R β (CD122) expression and disease activity

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Abstract:

Objectives: The goals of this study were to (a) measure IL-15 levels in the serum of RA patients and healthy controls, (b) determine if there is a correlation between serum TNF-α and IL-15 levels in RA patients (c) measure CD122 expression in PB of RA patients and healthy controls and (d) determine if there is a correlation between CD122 expression and serum IL-15 levels.

Materials and Methods: Peripheral blood (PB) was obtained from 21 RA patients and 12 healthy controls. Disease activity was measured by ESR, tender joint count, and swollen joint count. Serum IL-15 and TNF-α levels were measured by sandwich ELISA. Number of CD122+ cells and CD122 MFI in PB was measured by flow cytometry.

Results: IL-15 levels were elevated in the serum of 10 out of 21 RA patients compared to healthy controls. Elevated serum TNF-α levels were observed in 6 out of 21 patients. The majority of these patients (4 out of 6) also had detectable IL-15 levels. Numbers of CD122+ cells and CD122 MFI in PB did not differ significantly in RA patients compared to healthy controls. A significant inverse correlation exists between numbers of CD122+

cells and CD122 MFI. No correlation was observed between serum IL-15 levels and CD122 expression or disease activity.

Conclusion: IL-15 is a pro-inflammatory cytokine that may play an important role in the pathogenesis of RA. This paper demonstrates that RA serum has elevated levels of IL-15 than that of healthy controls, supporting the hypothesis. The potential role of IL-15 and its receptor in systemic inflammatory response has suggested their use in treatment of RA.

Interleukin-15 (IL-15) is a pleiotropic cytokine that belongs to the four-helix bundle family, of which IL-2 is a member (1). IL-15 and IL-2 share similarities in biological effects including T cell activation (2), proliferation (3), and co-stimulation of immunoglobulin synthesis (4). However, unlike IL-2, IL-15 is produced by monocytes and macrophages, not by activated T cells (1). IL-15 is a chemoattractant for human peripheral blood (PB) T cells (5). IL-15 binds to a heterotrimeric receptor composed of a unique IL-15R_{α} chain, and the β and γ chains of the IL-2R (6). The similarities in function between IL-2 and IL-15 have been attributed to the usage of the same receptors.

Abnormalities in IL-15 production have been associated with the development and maintenance of chronic inflammatory diseases such as rheumatoid arthritis (RA) (7). High concentrations of IL-15 have been found in RA synovial fluid (SF) and IL-15 is expressed by monocytes and macrophages in the synovial lining (8). Evidence suggests that IL-15 may play an important role in recruiting PB T cells into the synovium. *In vitro* experiments by McInnes et. al. demonstrated that IL-15 induced specific polarization and migration of CD45RO+ PB T cells into RA SF, which were inhibited by anti-IL-15 antibodies (8). In addition, IL-15 stimulates activation and proliferation of RA PBMCs. IL-15 activates PB lymphocytes of RA patients by rapidly upregulating CD69 expression. RA PBMCs and SF T cells exhibited a significantly higher stimulation index in response to IL-15 than healthy PBMCs (8). These data suggest that IL-15 may continually recruit T cells from RA PB into the synovium, and induce their activation and proliferation, contributing to the pathogenesis of RA. While these studies have shown the importance of IL-15 in the synovium of RA patients, very little is known about IL-15 levels in RA serum. In this paper, it is proposed that because RA is a systemic disease, serum IL-15 levels may be elevated in RA patients compared to healthy controls, contributing to the pathogenesis of the disease.

Evidence suggests that IL-15 may be an important regulator of TNF-$_\alpha$ production in the rheumatoid synovium. McInnes et. al. have shown that IL-15 activated CD45RO+ PB T cells from RA patients induced TNF-$_\alpha$ synthesis by blood or synovial derived macrophages in a contact-dependent mechanism (9). These results demonstrate the importance of IL-15 in regulating TNF-$_\alpha$ production in the RA synovium as well as PB. TNF-$_\alpha$ occupies a pivotal role in the regulation of synovial inflammation in RA because it regulates synthesis of other pro-inflammatory cytokines such as IL-1$_\beta$. These cytokines in turn enhance production of matrix metalloproteinases (MMPs) that initiate joint destruction (10). Many RA patients have elevated TNF-$_\alpha$ levels in the serum, which correlates with disease activity. Because IL-15 regulates TNF-$_\alpha$ production, the authors wanted to determine if there is a correlation between the two cytokines in RA serum.

IL-15 binds the high affinity IL-15R complex that is composed of the alpha, beta and gamma chains. IL-15R β chain (CD122) has been of particular interest because it is necessary for IL-15 internalization, signal transduction (12), IL-15 mediated activation and proliferation of healthy PBMCs (2). Studies on CD122 expression in RA PB are limited and contradictory (13, 14). In this study, the numbers of CD122+ cells and CD122 MFI have been measured in RA patients and healthy controls by flow cytometry. It is hypothesized that CD122 expression may be elevated in RA patients compared to healthy controls and that there may be a correlation between CD122 expression and serum IL-15 levels.

The goals of this study were to (a) measure IL-15 levels in the serum of RA patients and healthy controls, (b) determine if there is a correlation between serum TNF-α and IL-15 levels in RA patients (c) measure CD122 expression in PB of RA patients and healthy controls and (d) determine if there is a correlation between CD122 expression and serum IL-15 levels.

Patients and Methods

Patients

Twenty-one patients were recruited from the rheumatology clinics at the University of Arizona, Tucson, Arizona. All patients fulfilled the 1987 American College of Rheumatology (ACR) criteria for diagnosis of RA, with disease duration 6 months or longer (15). Patients on oral steroid usage were included if the dose was stable and less than or equal to 15 mg of prednisone per day. Patients on methotrexate (MTX) were included in the study if the dose was less than or equal to 15 mg per week and blood was drawn at least 3 days after the last dose. Patients on Embrel and NSAIDS were included while those on Arava (lefluonomide) were excluded from the study. Twelve age matched healthy volunteers were chosen. Written consent was obtained from all participants prior to drawing blood.

Clinical Evaluations

RA disease activity was measured by swollen joint count and tender joint count (16). A complete blood count (CBC) with differential, and erythrocyte sedimentation rate (ESR) were performed.

ELISA

IL-15 and TNF-$_\alpha$ levels were measured in the serum of 21 RA patients and 8 healthy controls using a commercial sandwich ELISA kit (R&D, Minneapolis, MN). The results are expressed as a mean $\pm$ SE of triplicate culture for RA patients and as mean $\pm$ SE of duplicate culture for the healthy controls.

Immunofluorescence staining and flow cytometric analysis

The following Mabs were used for direct immunofluorescence staining: anti-CD2, CD28, HLA-DR (Immunotech, Marseille, France), anti-CD3, CD4, CD8, CD16 (Coulter, Miami, Florida), anti-CD20, CD122 (Becton Dickinson, Mountain View, CA). Whole blood (100μl) was incubated with combinations of the above antibodies for 30 minutes at 4°C. The RBCs were lysed and the WBCs were fixed by Coulter Multi-Q-Prep. The cells were analyzed by Coulter Epics XL-MCL (Coulter Corporation, Miami, Florida). The results are expressed as numbers of cells (% Cells x WBC).

Statistical analysis

Comparisons between RA and healthy control groups were determined by Mann-Whitney. Correlations between variables were determined by Spearman's correlation coefficient.

Results

Serum IL-15 and TNF-alpha levels in RA patients and healthy controls

ELISAs were performed to compare IL-15 levels in RA and healthy serum. Ten out of 21 RA patients had detectable IL-15 levels ($\geq$ 2.0 pg/ml) (Table 3.1). IL-15 was undetectable in the healthy control serum (< 2.0 pg/ml). Serum IL-15 levels in RA patients were significantly higher than healthy controls (p=0.047, Mann Whitney). Serum TNF-$_{\alpha}$ levels were measured to determine if there was a correlation with serum IL-15. Six out of 21 RA patients had detectable TNF-$_{\alpha}$ levels ($\geq$ 4.4 pg/ml) (Table 3.1). The majority of these patients (4/6) had detectable levels of IL-15. Serum TNF-$_{\alpha}$ levels in the healthy controls were undetectable (< 4.4 pg/ml).

Prevalence and MFI of CD122 in RA patients and healthy controls

The prevalence (numbers) of CD122+ cells and CD122 MFI were measured in the PB of 21 RA patients and 12 healthy controls by flow cytometry. No significant difference in prevalence of CD122+ cells was observed between RA patients and healthy controls (FIGURE 3.2). CD122+ cells were further characterized by flow cytometry (FIGURE

3.3). The results showed that CD122 was predominantly expressed on NK (CD16+) cells and CD8+ T cells. CD122 was not detectable on CD4+ T cells. Similar results were obtained with the healthy control group.

CD122 MFI was examined in RA and healthy controls and was not significantly different in the two groups. Within the RA group, CD122 MFI correlated inversely with numbers of CD122+ cells (ρ=-0.487, p<0.03) (FIGURE 3.4). This correlation was not observed in healthy controls. CD122 MFI correlated with absolute counts of CD25+ cells (ρ=0.806, p<0.001) but not with CD25 MFI.

Correlation of serum IL-15 and CD122 expression with disease activity and duration

Disease activity was measured by ESR, and the number of tender and swollen joints and duration was calculated from the year of diagnosis of RA. CD122 expression and serum IL-15 levels did not correlate with either disease activity or duration.

Discussion

Abnormalities in IL-15 have been observed in the serum of patients with chronic inflammatory diseases such as systemic lupus erythematosus (17), ulcerative colitis, hepatitis C (18) and multiple sclerosis (19). McInnes et. al. have demonstrated elevated levels of IL-15 in RA synovial fluid and tissue compared to OA patients (8). However, very little is known of IL-15 levels in the serum of RA patients. In this study, serum IL-

15 levels of 21 RA patients were measured by ELISA. The results demonstrated that 10 out of 21 RA patients had elevated serum IL-15 levels compared to healthy controls. Together with results of McInnes et. al., the data support the hypothesis that IL-15 may be important in the pathogenesis of RA. To determine if there is a correlation between IL-15 and TNF-α, the serum TNF-α levels of 21 RA patients were measured by ELISA. Elevated TNF-α levels were detected in 6 out of 21 RA patients. The majority of these patients (4 out of 6) also had detectable levels of serum IL-15. These results support *in vitro* experiments by McInnes et. al. who demonstrated that IL-15 stimulated TNF-α production by PB macrophages. However, because not all patients who had elevated serum TNF-α had detectable IL-15, the data suggest that regulation of TNF-α may be more complex *in vivo*. The data indicate that IL-15 may be a more sensitive indicator of inflammation in RA than TNF-α, which may be useful in diagnosis of the disease. However, the lack of correlation with disease activity suggests that IL-15 may not accurately represent disease progression.

Because RA is characterized by systemic lymphocyte activation, it was hypothesized that prevalence of CD122+ cells and CD122 MFI may be elevated in RA patients than healthy controls. The flow cytometric data indicated that prevalence of CD122+ cells and MFI were not significantly higher in RA patients compared to healthy controls. This contrasts results of Itoh et. al. who found that the prevalence of CD122+ cells was significantly lower in RA patients ($p<0.05$) than in healthy controls (13). The data are consistent with those of Creamer et. al. who found no significant difference in CD122 MFI between RA and healthy PBMCs (14). The results of this study demonstrated that RA and healthy naïve CD4+ T cells do not express CD122. Kanegane et. al. demonstrated similar results with healthy PBMCs (2). In this study, the data demonstrated that CD122 is expressed on the majority of NK cells, but on only a small percentage of CD8+ T cells. A significant inverse correlation was observed between prevalence of CD122+ cells and CD122 MFI in RA that was not present in the healthy controls. While this suggests that a small percentage of CD122+ cells are CD122^{bright} and the majority of cells are CD122^{dim}, the clinical relevance to RA is unclear.

In conclusion, IL-15 is a key regulatory cytokine that bridges macrophage and T cell derived processes that lead to joint destruction in RA. The inflammatory properties of IL-15 along with elevated levels of the cytokine in RA synovium and serum makes it a target for monoclonal antibody therapy. Antibodies to IL-15R components have been suggested for the treatment of RA. Anti-CD122 antibodies, in particular, have been used successfully in transplantation to prolong renal allograft survival in primates. Therapies targeting the IL-15R$_{\alpha}$ chain are also being developed and tested in mice for their immunosuppressive properties (12, 20). Studies characterizing IL-15R$_{\alpha}$ expression in RA are needed and will be critical in determining the effectiveness of this therapy.

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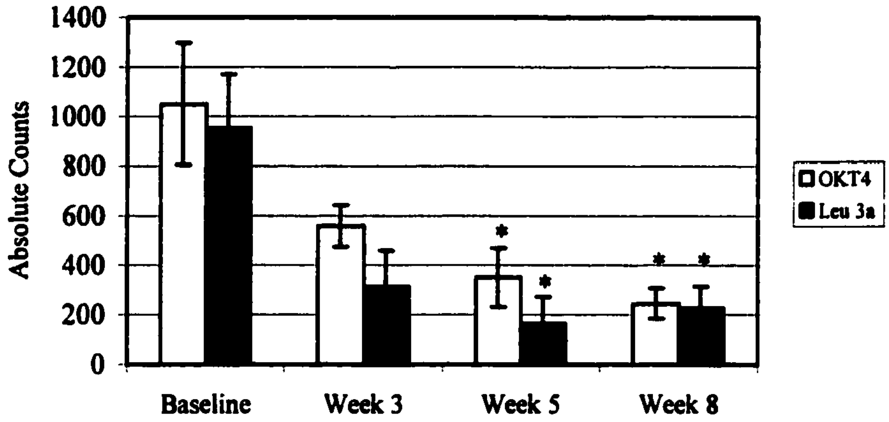
(a)



(b)

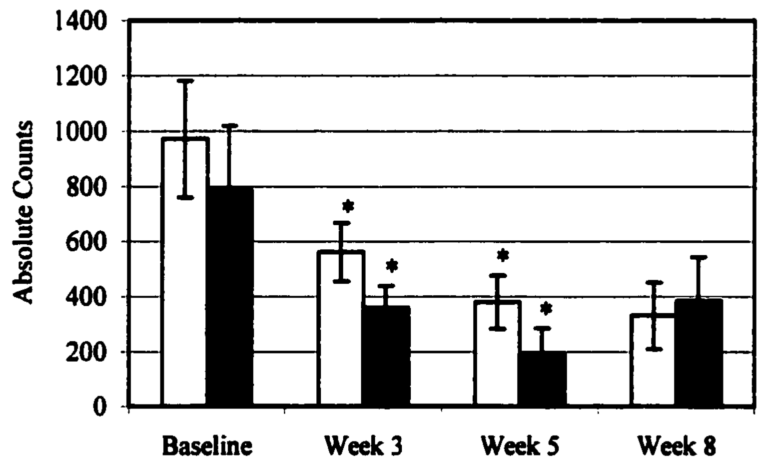
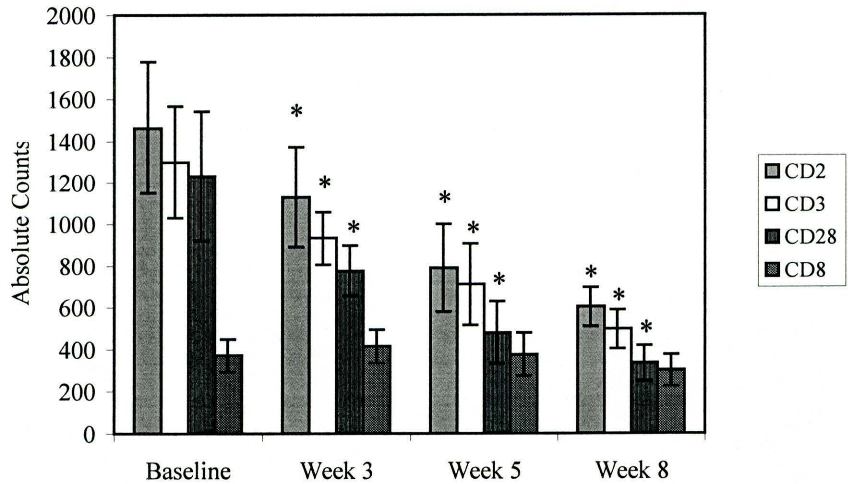


FIGURE 1.1 Effects of PRIMATIZED® anti-CD4 antibody on numbers of CD4+ T cells in group A and group B

(a)



(b)

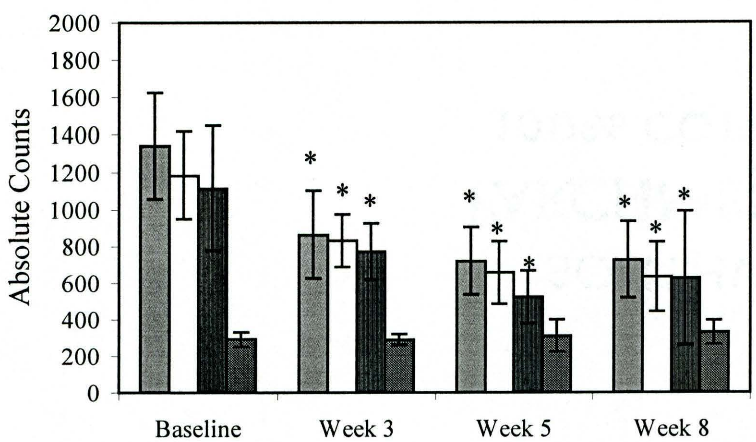
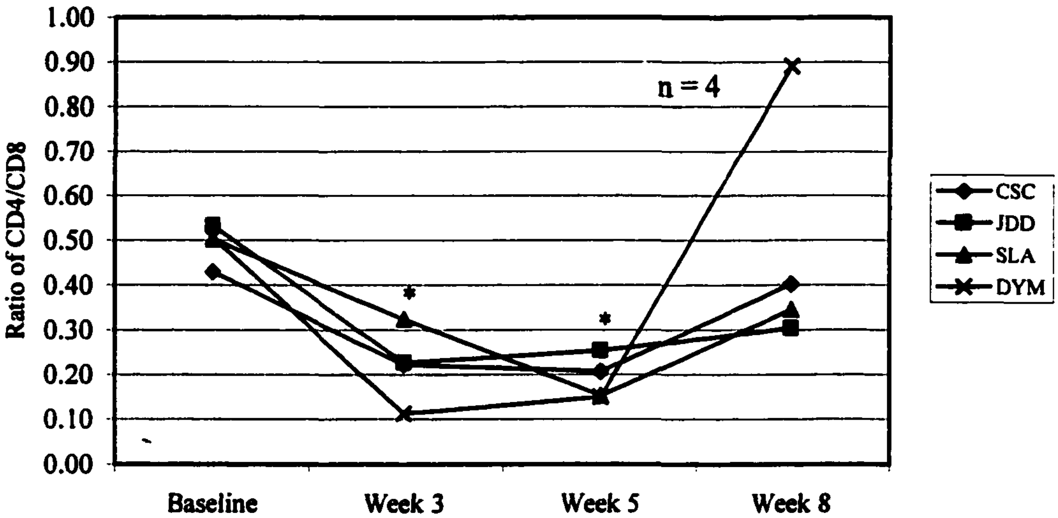


FIGURE 1.2. Effects of PRIMATIZED® anti-CD4 antibody on numbers of CD2+, CD3+ and CD28+ T cells

(a)



(b)

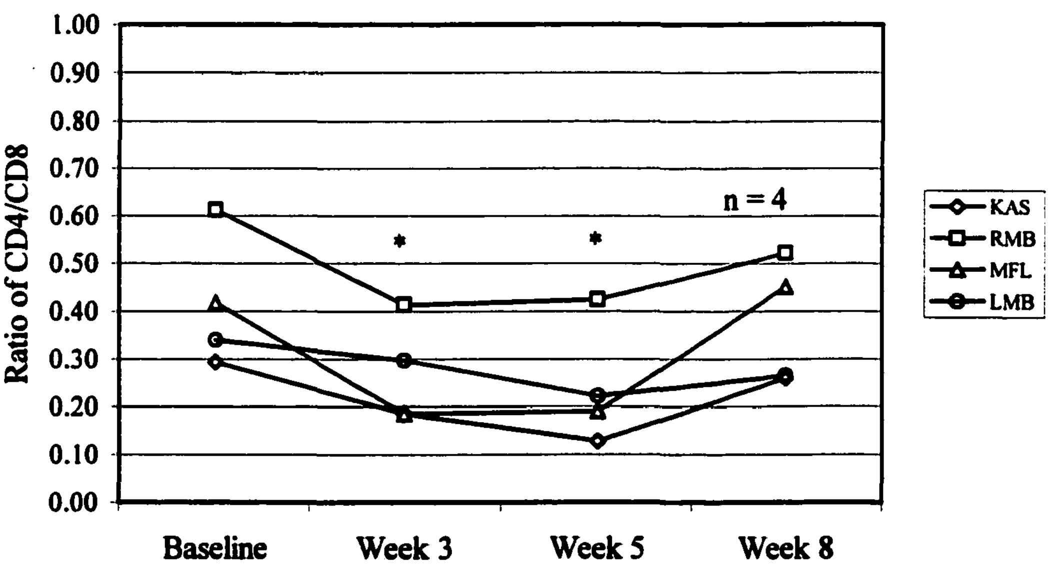
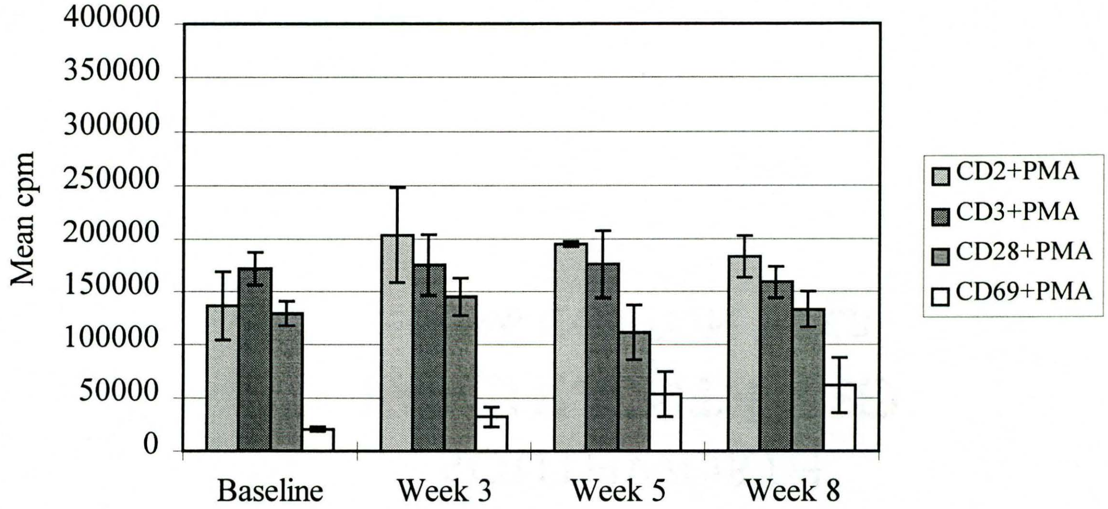


FIGURE 1.3 Effects of PRIMATIZED® anti-CD4 antibody on CD4/CD8 MFI Ratio

(a)



(b)

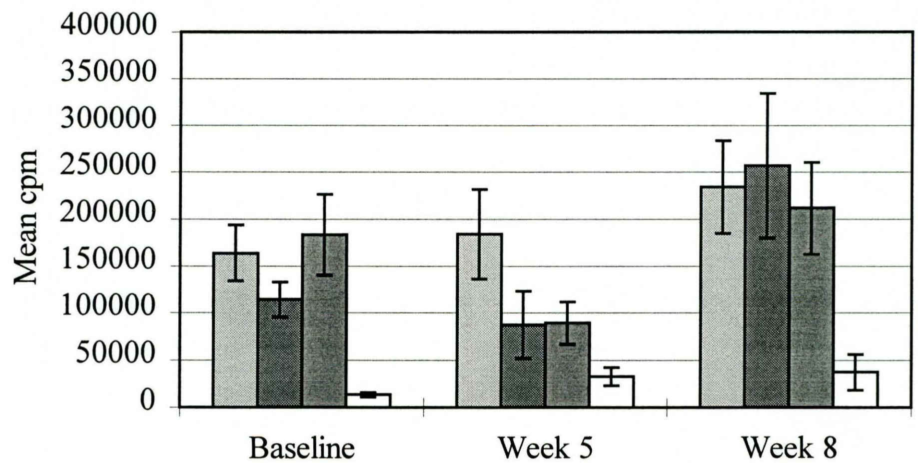
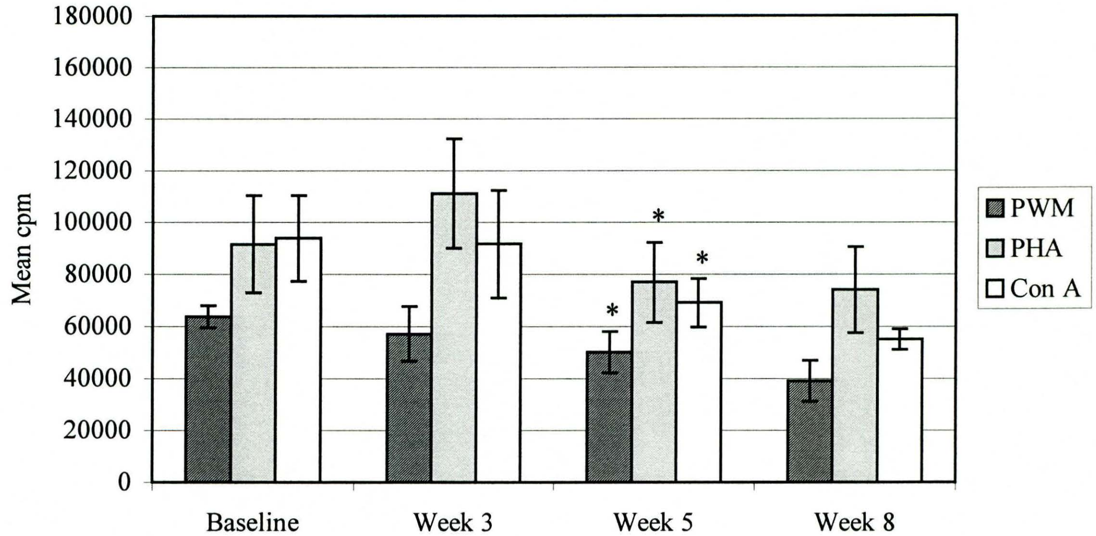


FIGURE 1.4 Effects of PRIMATIZED® anti-CD4 antibody on mitogenic antibody induced proliferation

(a)



(b)

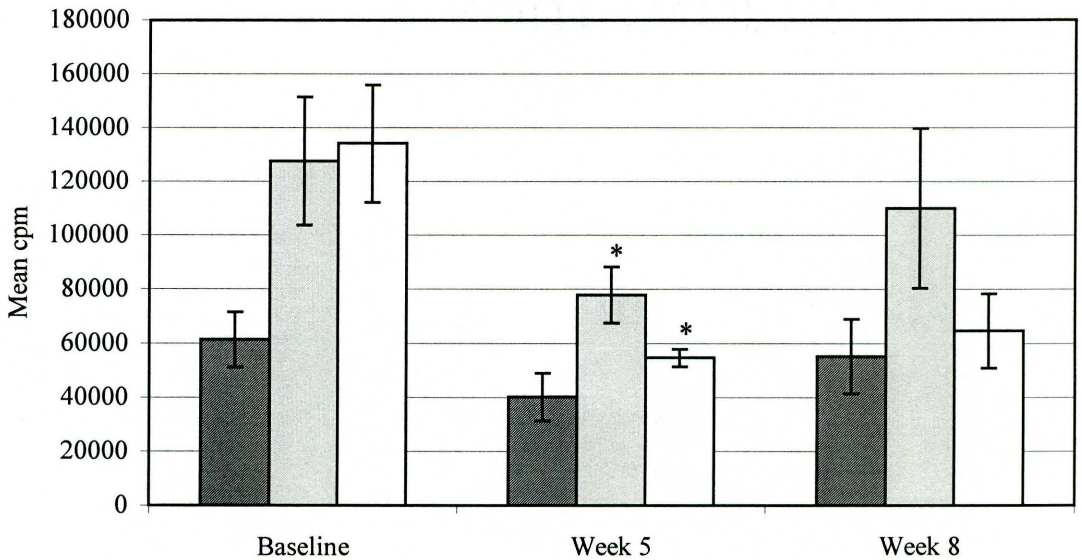


FIGURE 1.5 Effects of PRIMATIZED® anti-CD4 antibody on mitogen induced proliferation

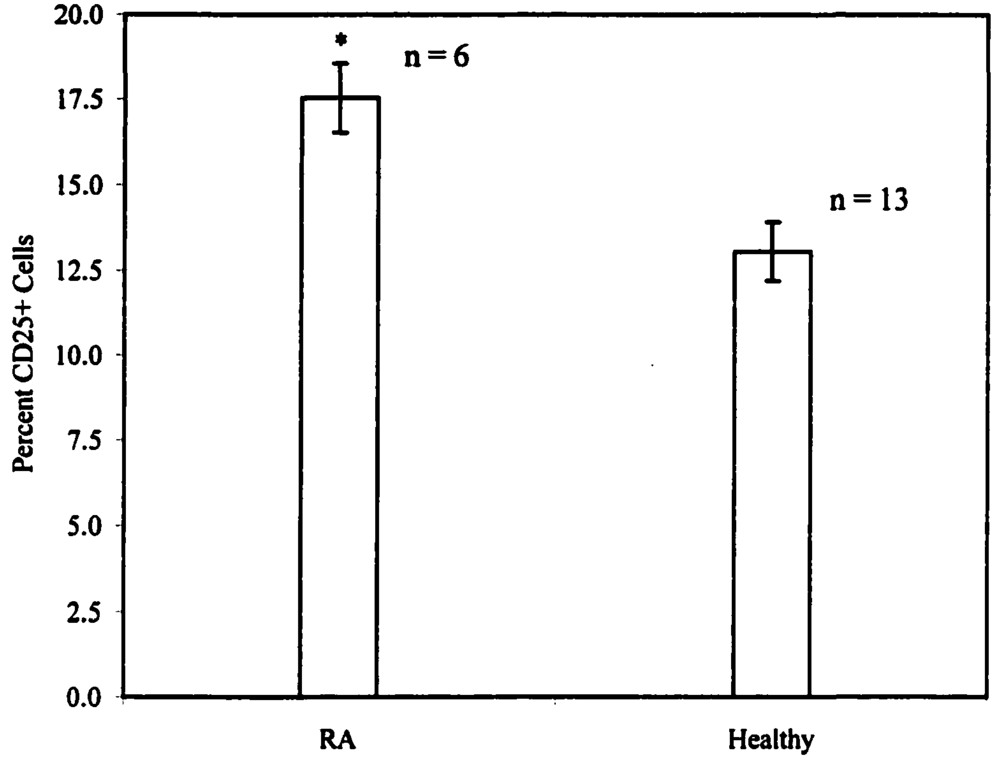


FIGURE 2.1 Percent CD25+ cells in RA and healthy controls

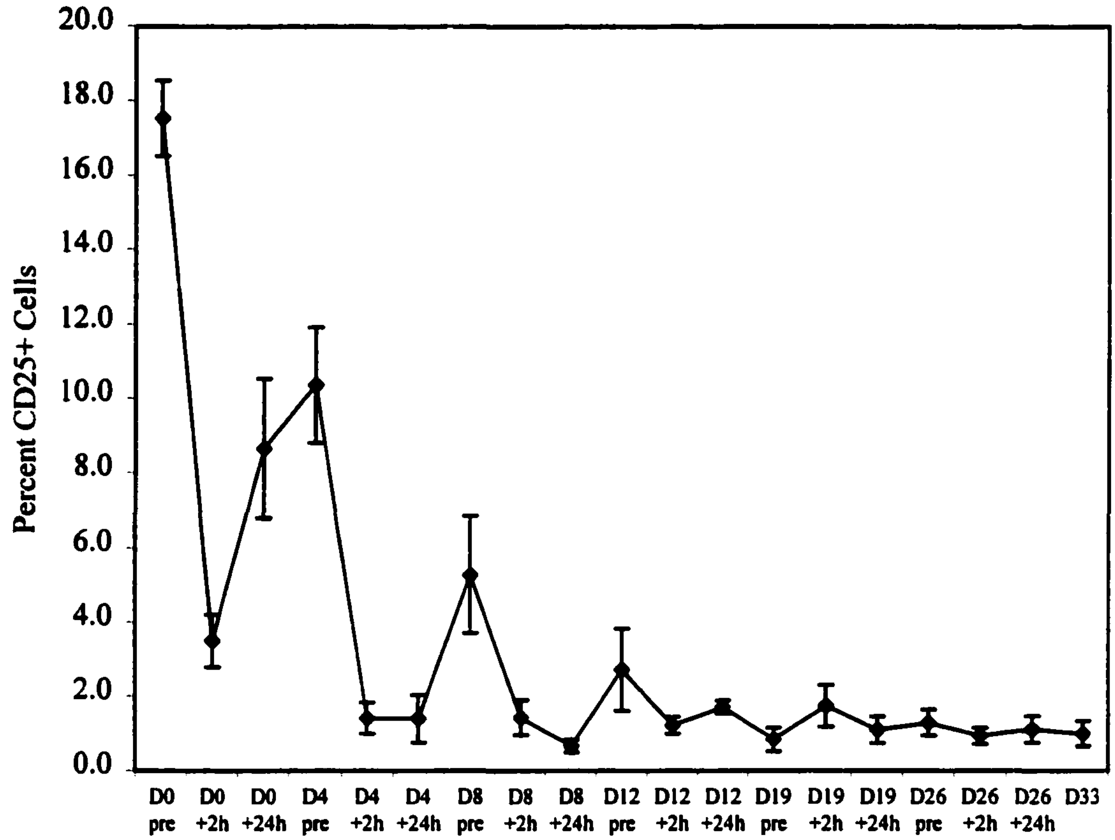


FIGURE 2.2 Effects of Simulect on percent CD25+ cells

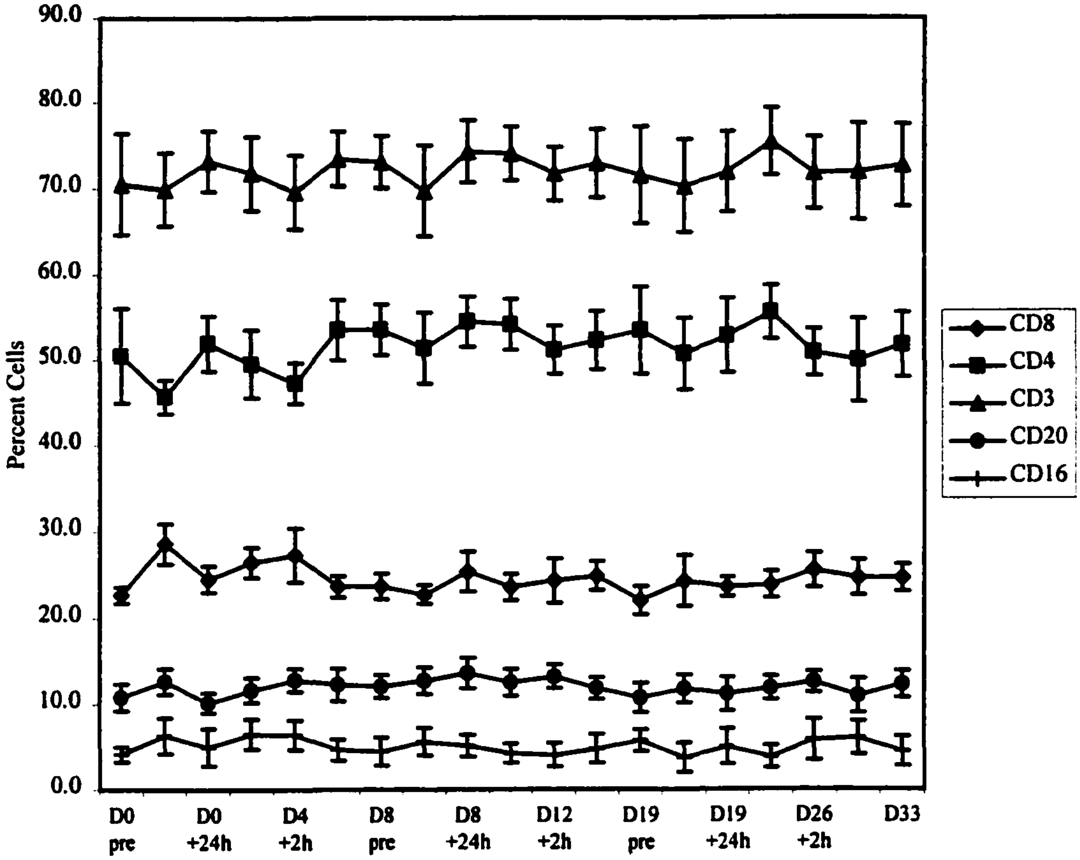


FIGURE 2.3. Simulect® does not affect T, B and NK cell numbers

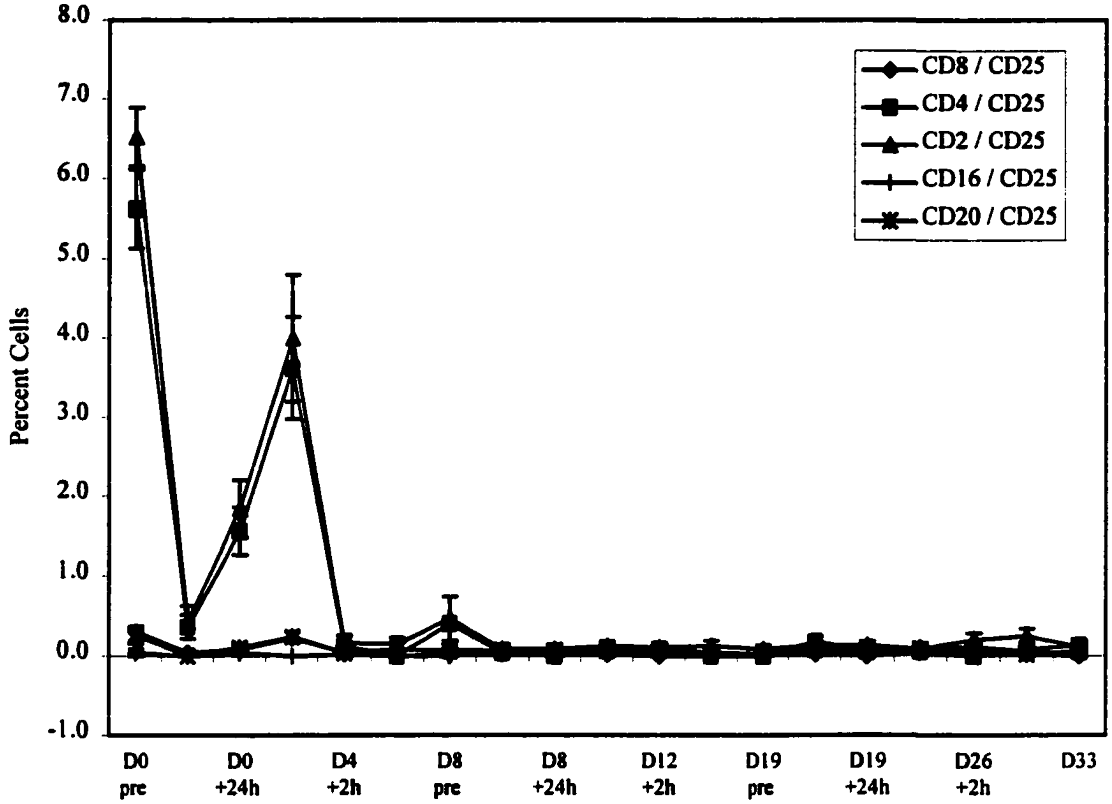


FIGURE 2.4. CD25 is expressed primarily on CD4+ T cells

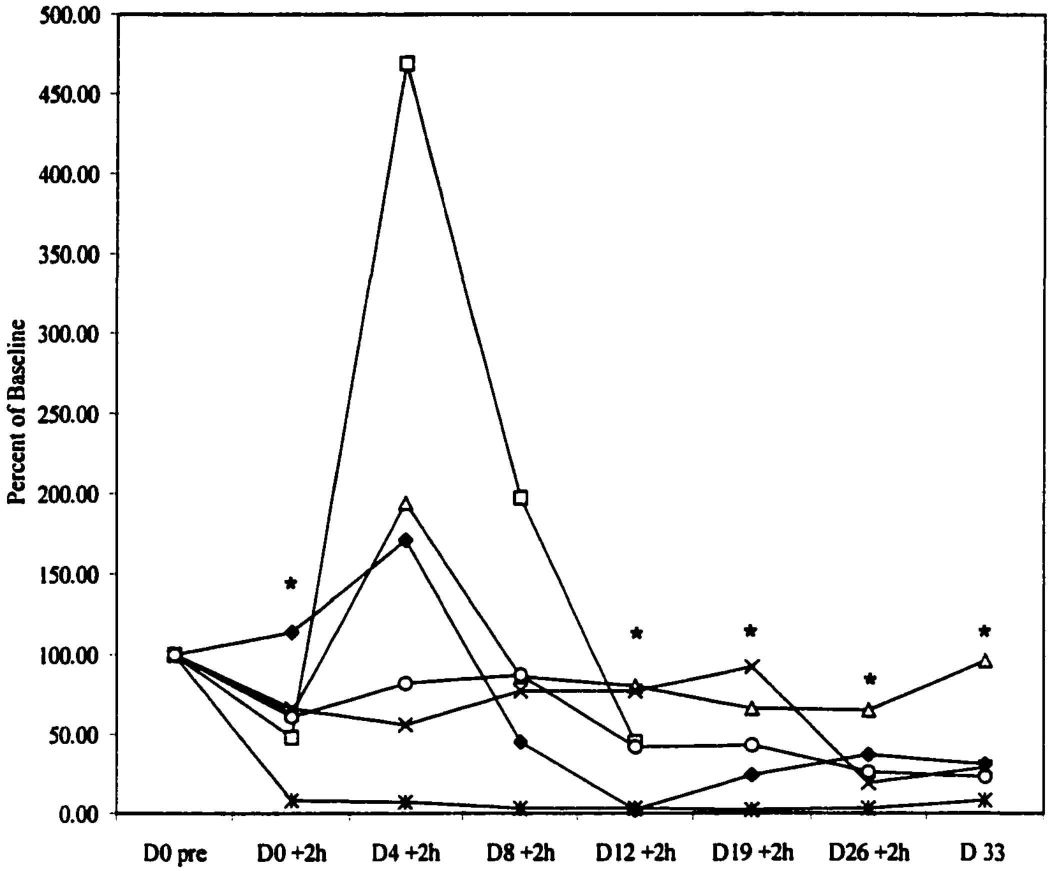


FIGURE 2.5. Effect of Simulect® on (PHA induced) IL-2 dependent proliferation

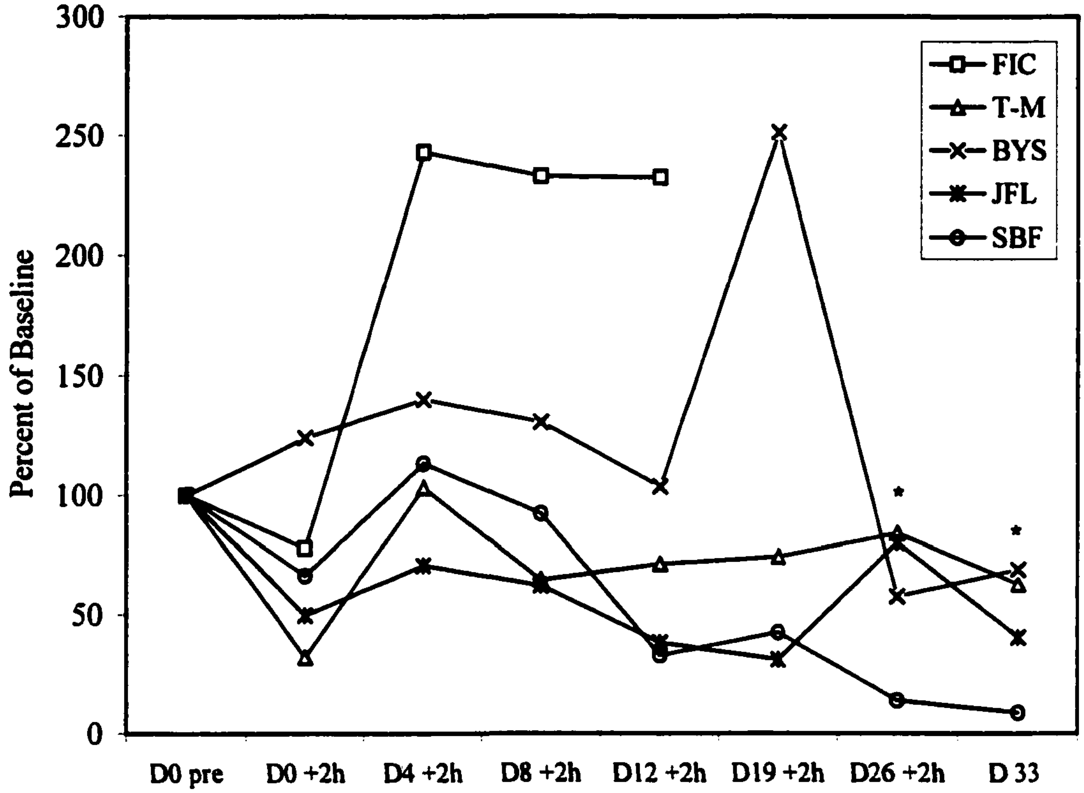


FIGURE 2.6. Effect of Simulect® on (anti-CD3+anti-CD28 induced) IL-2 dependent proliferation

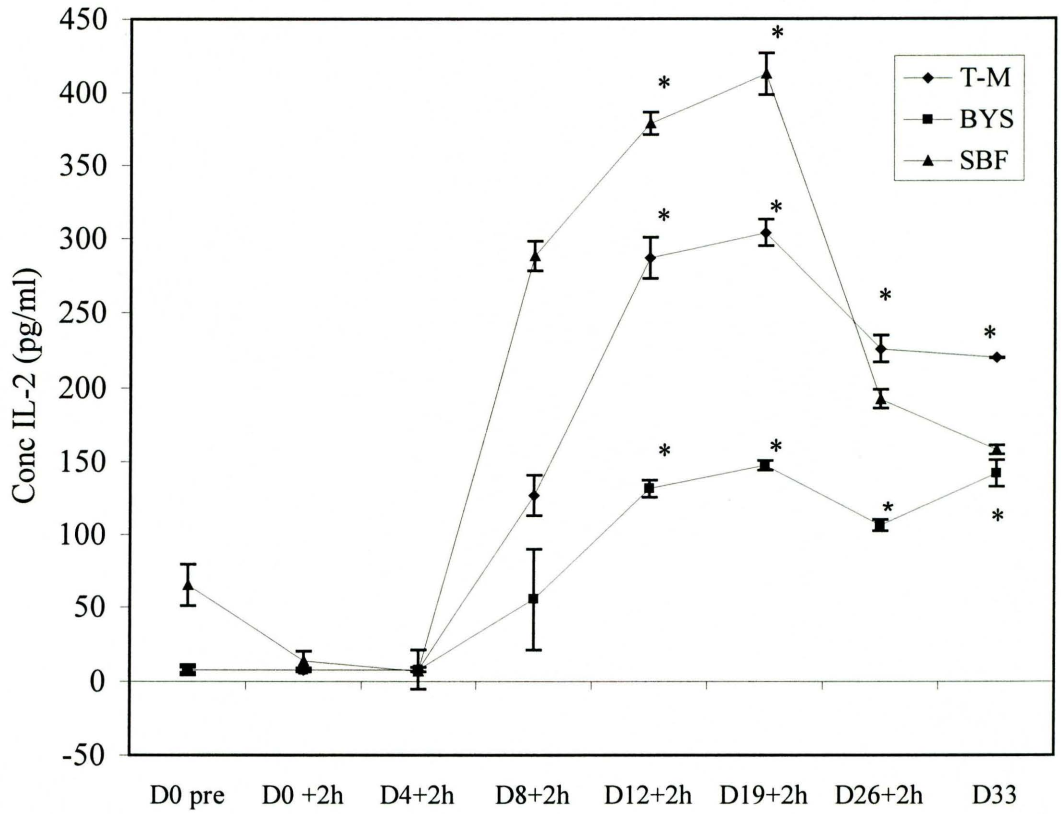


FIGURE 2.7. Simulect's® effects on IL-2 production by anti-CD3+anti-CD28 stimulated cells

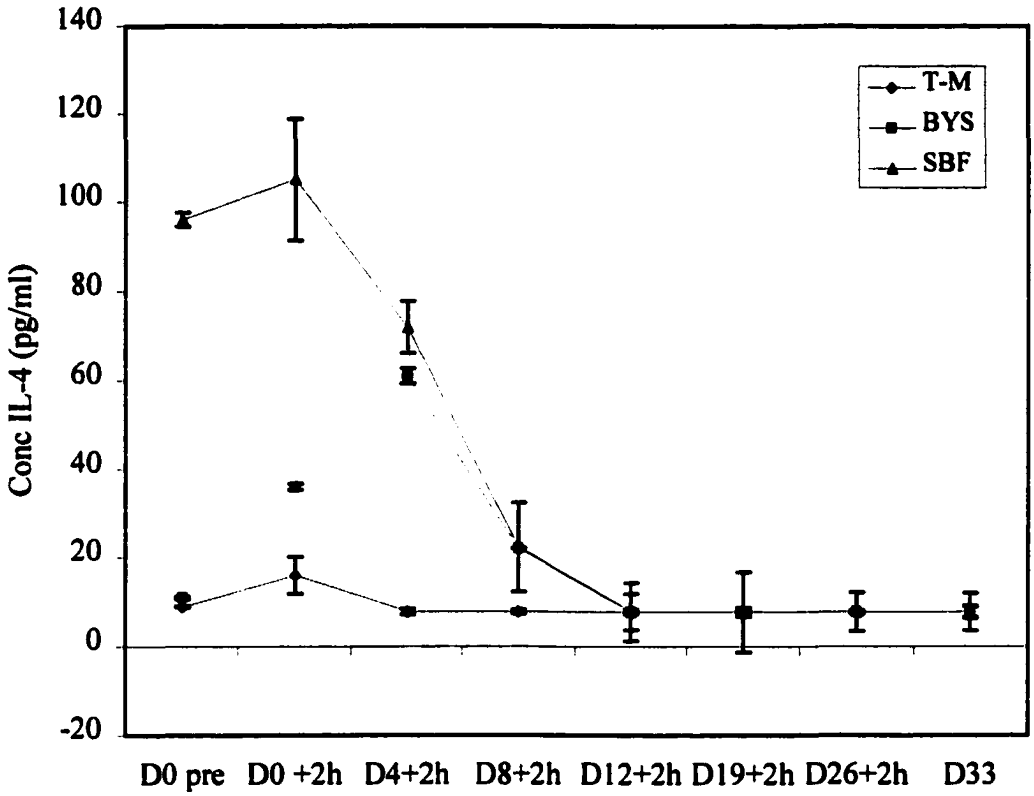


FIGURE 2.8. Simulect's® effects on IL-4 production by anti-CD3+anti-CD28 stimulated cells

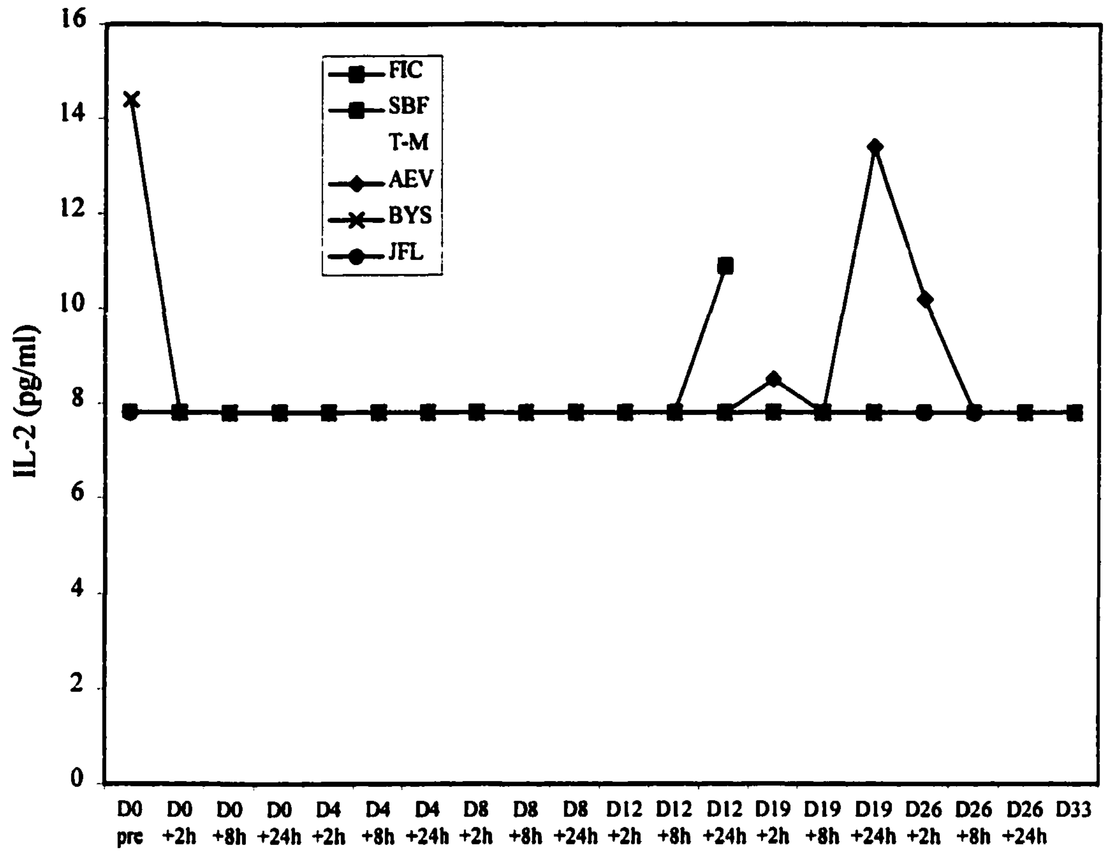


FIGURE 2.9. Simulect's® effects on plasma IL-2 levels

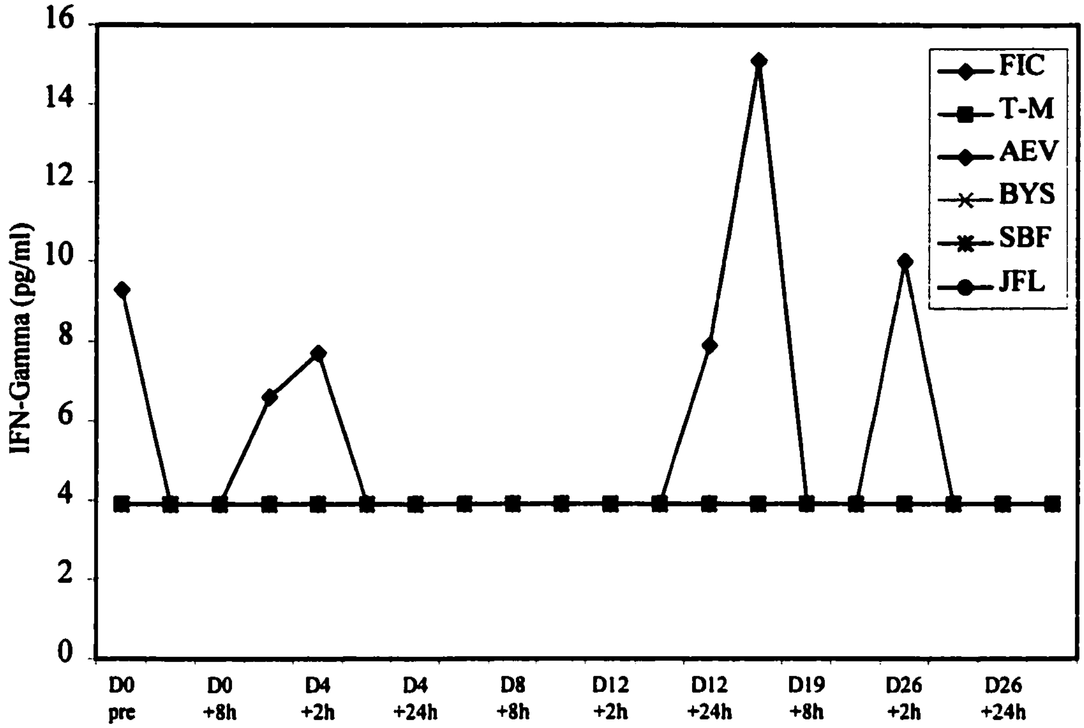


FIGURE 2.10. Simulect's® effects on plasma IFN-γ levels

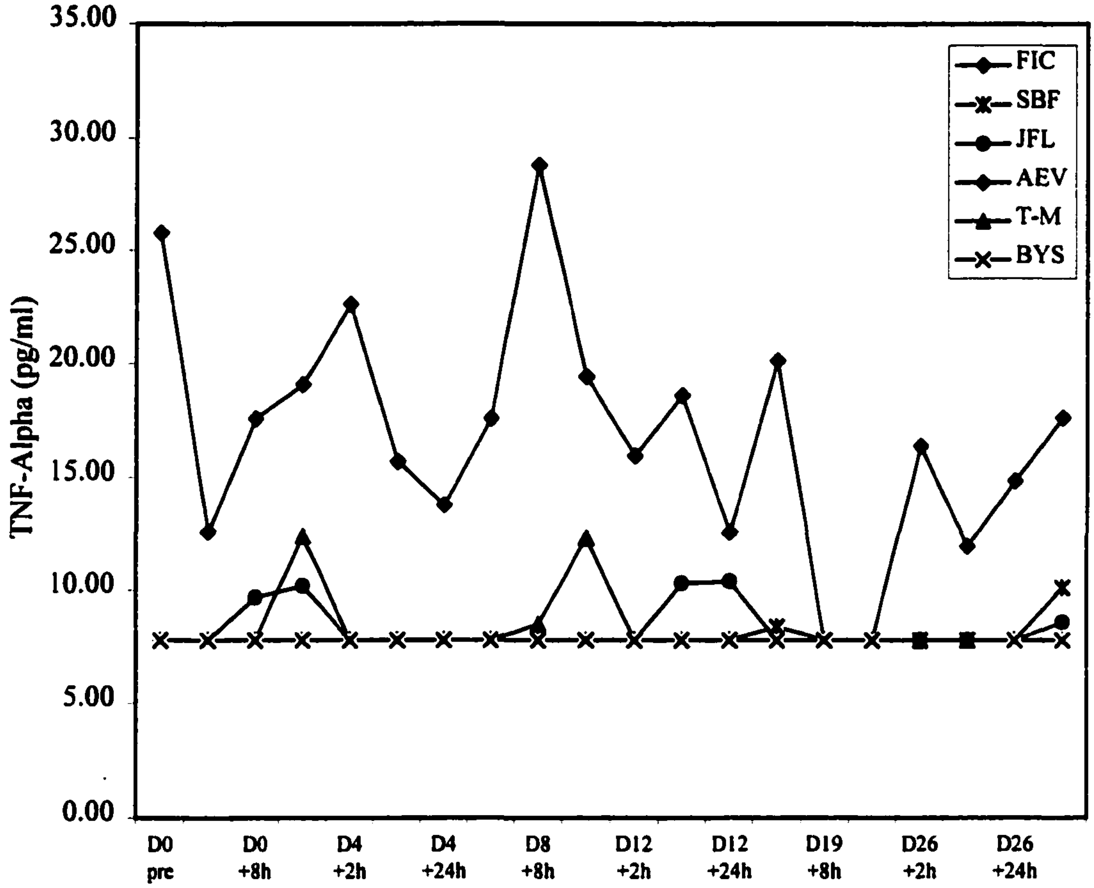


FIGURE 2.11. Simulect's® effects on plasma TNF-α levels

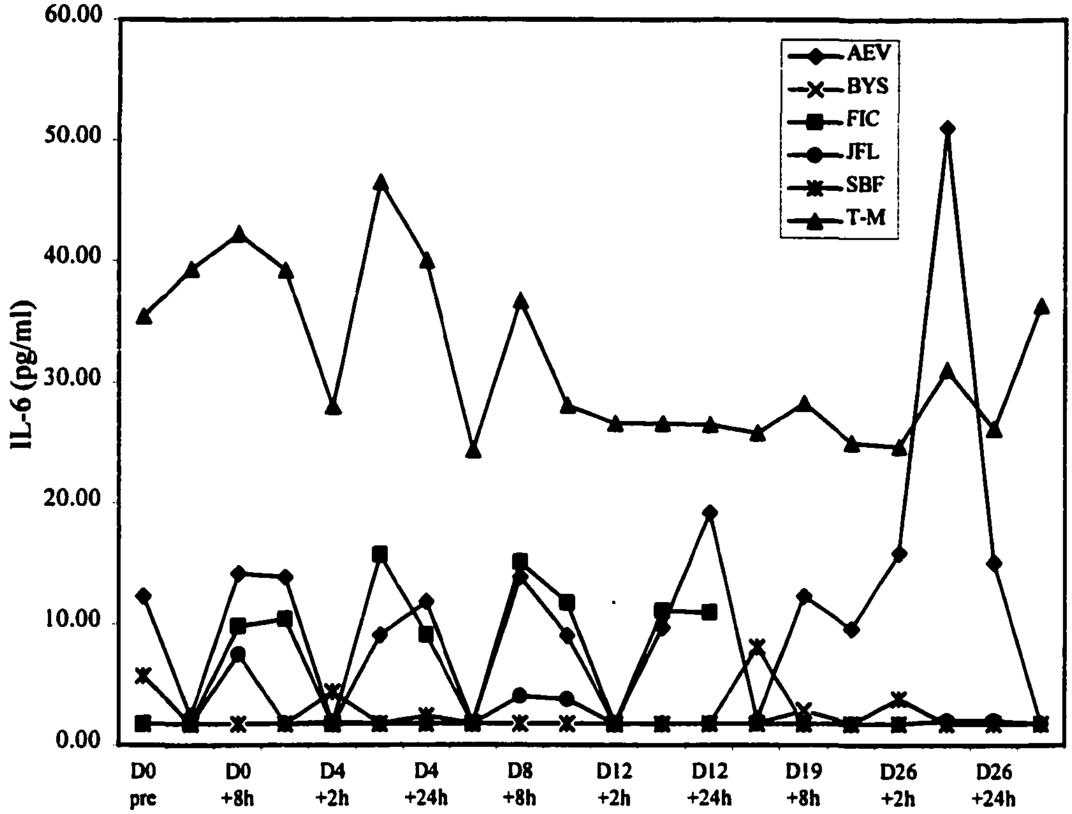


FIGURE 2.12. Simulect's® effects on plasma IL-6 levels

	Day 0	(Pre-infusion)	Day 33	(Follow up)
Pt.	Tender	Swollen	Tender*	Swollen*
1	14	16	5	3
2	32	31	24	34
3	37	20	11	8
4	36	25	26	8
5	17	17	3	3

TABLE 2.1. Effects of Simulect® on number of tender and swollen joints

Pt #	IL-15 (pg/ml)	TNF-α (pg/ml)
1	ND[a]	ND[b]
2	ND	ND
3	8.7 ± 0.3	ND
4	ND	ND
5	2.8 ± 0.0	ND
6	ND	294 ± 7.3
7	2.3 ± 0.2	ND
8	2.9 ± 0.2	317 ± 4.8
9	4.3 ± 0.9	190 ± 2.0
10	9.4 ± 0.2	ND
11	ND	ND
12	ND	ND
13	ND	117 ± 3.3
14	5.4 ± 0.2	37 ± 2.8
15	41.3 ± 0.7	ND
16	ND	ND
17	3.8 ± 0.6	ND
18	ND	ND
19	3.3 ± 0.5	19 ± 11.3
20	ND	ND
21	ND	ND

[a]ND = Non-detectable; low detection limit = 2.0 pg/ml
[b]ND = Non-detectable; low detection limit = 4.4 pg/ml

TABLE 3.1. Serum IL-15 and TNF-α levels of RA patients.

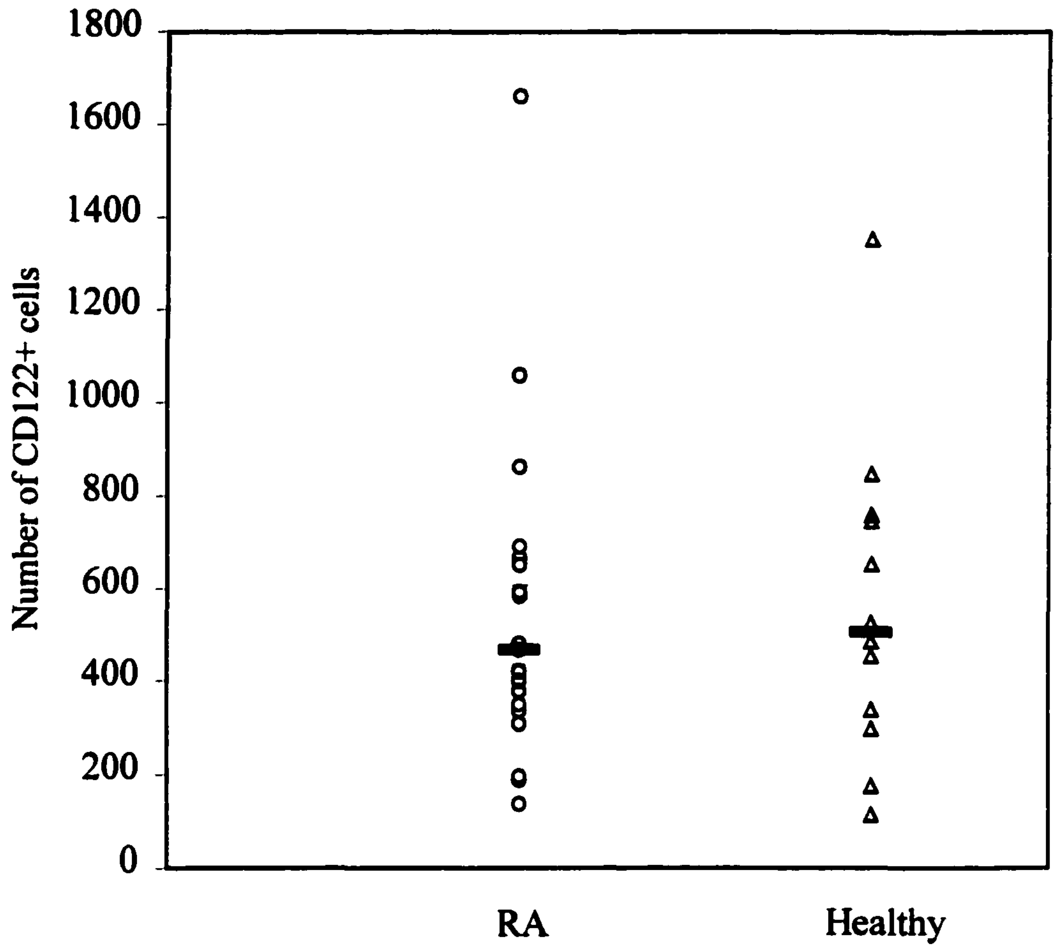


FIGURE 3.1. CD122 expression in RA and healthy peripheral blood

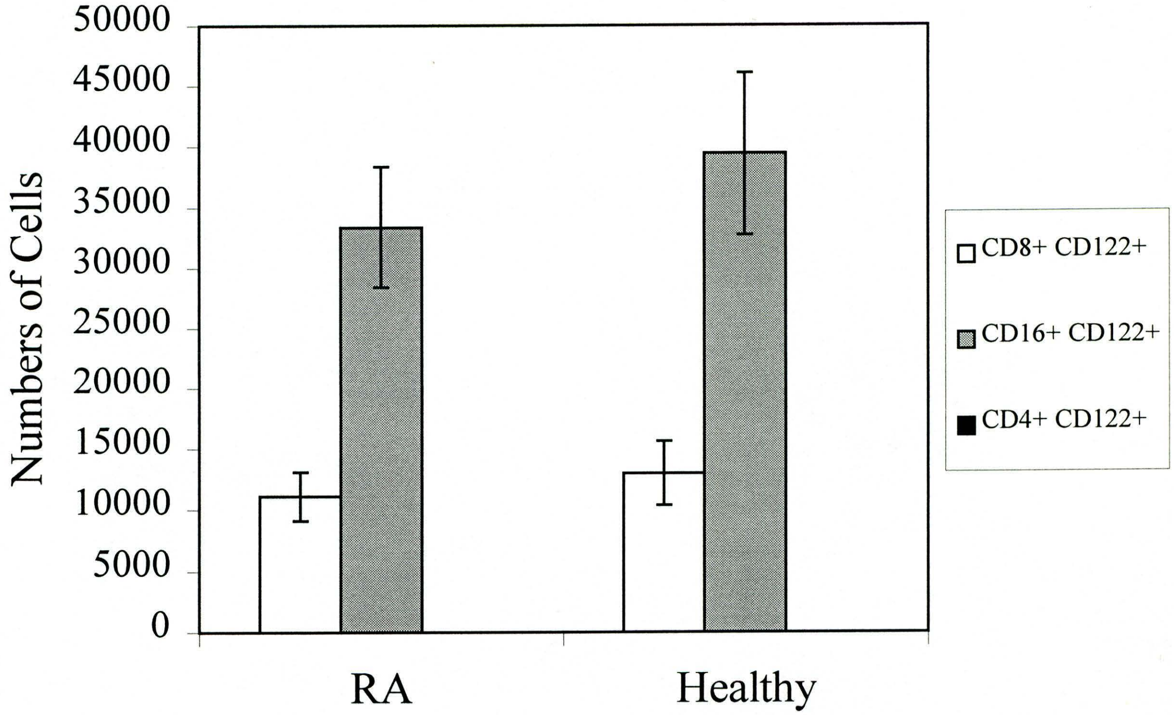


FIGURE 3.2. CD122 Expression on Cell Subsets

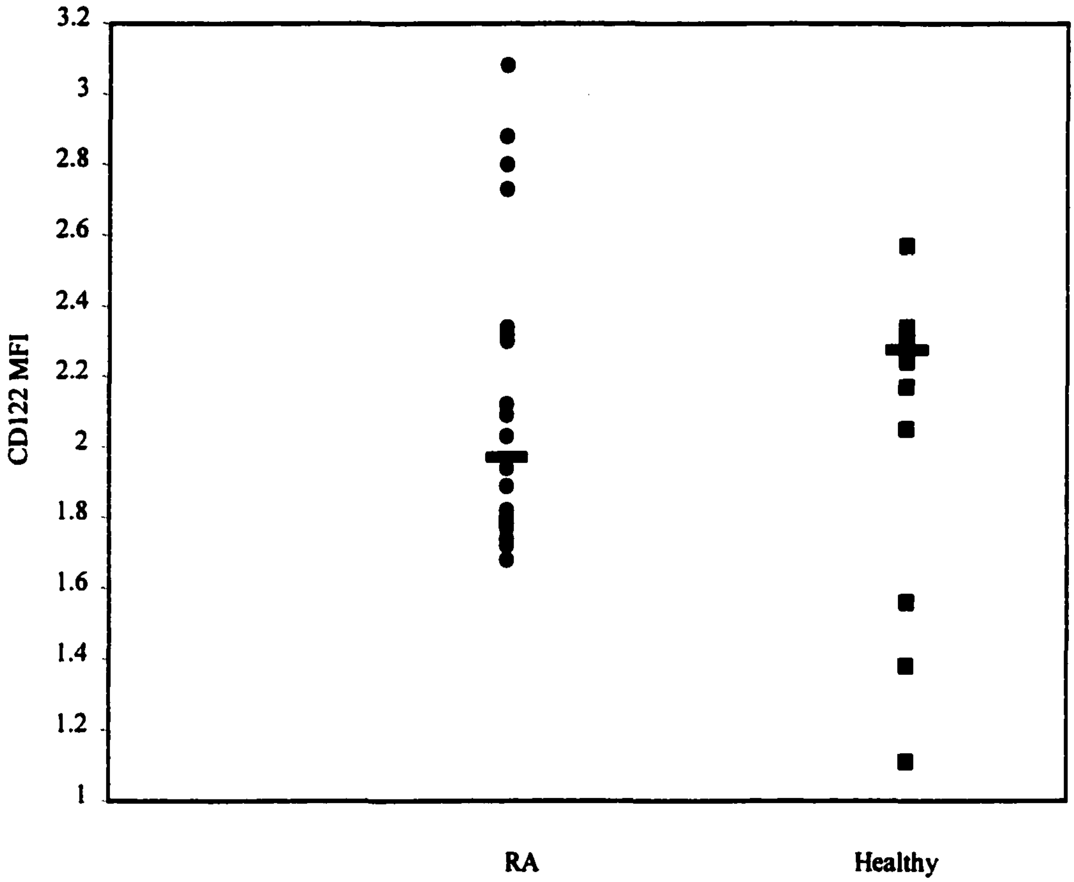


FIGURE 3.3. CD122 MFI in RA and healthy peripheral blood

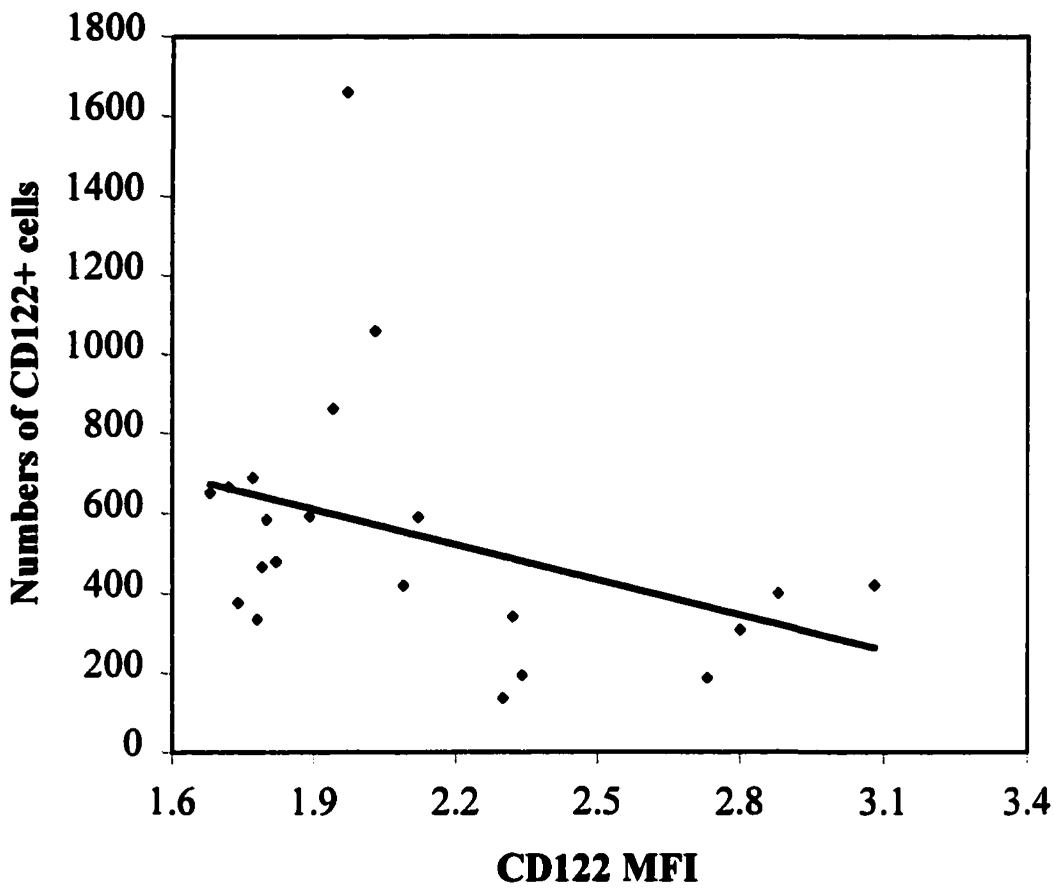


FIGURE 3.4. Inverse correlation between CD122 MFI and number of CD122+ cells