Distinct Effects of Anti-Tumor Necrosis Factor Combined Therapy on TH1/TH2 Balance in Rheumatoid Arthritis Patients

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The immune balance in patients with rheumatoid arthritis (RA), a disease characterized by TH1 dominance, treated by the preferred combined anti-tumor necrosis factor (anti-TNF) and methotrexate (MTX) therapy was evaluated by assessing the chemokine and cytokine receptors as well as apoptosis induction. A meta-analysis of combined therapy by TNF blockers and MTX in 15 RA patients, MTX monotherapy in 20 RA patients, and 11 diagnosed but untreated RA patients was performed by assessing several immune markers in the whole lymphocyte population, as well as in specific CD4 cells, by both flow cytometry and image analysis. A significant downregulation of CXCR3 and IL-12 receptors (both TH1 markers) and a significant increase in the chemokine receptor CCR4 and, to a lesser extent, IL-4R (both TH2 markers) were found; a particularly marked increase was found in patients treated by combined therapy. This phenomenon was pronounced in CD4 cells and was accompanied by a high proportion of apoptotic cells. The therapeutic effect of MTX and TNF blockers may be due to apoptosis induction in lymphocytes infiltrating from the inflammation site and restoring the TH1/TH2 balance.

Several clinical trials of the anti-tumor necrosis factor (anti-TNF) agents etanercept, infliximab, and adalimumab, used alone or in combination with methotrexate (MTX), in patients with early or established rheumatoid arthritis (RA) have produced consistent improvements in symptoms as well as in function, with a dramatic slowing of radiographic damage compared to that with MTX treatment alone (15, 20, 22). The anti-TNF agents combined with optimal doses of MTX currently constitutes the best therapeutic regimen for the management of symptoms in RA patients that fail to adequately respond to traditional disease-modifying antirheumatic drugs (DMARDs). Previous trials demonstrated that the gold-standard treatment with MTX alone selectively modulates the TH1/TH2 balance in active rheumatoid arthritis patients (11). In the present research, the contribution of the combined treatment to the immune status in these patients, especially in CD4+ T cells, was assessed.

Pathologically, RA is characterized by the proliferation and activation of lymphocytes that have a predominant CD4+/T-helper phenotype, and the synovial tissue in RA (the pannus) becomes infiltrated with lymphocytes of this type (5, 14, 17). In RA, large quantities of proinflammatory cytokines, especially of the TH1 profile, were found in the synovial fluid and membrane (4, 21). These cytokines are produced by activated macrophages or T cells and strongly contribute to synovial cell proliferation and cartilage destruction in RA (14, 28).

A variety of anti-TNF agents are used in RA treatment. Etanercept is a TNF receptor-IgG fusion protein (16). Infliximab is a mouse-human chimeric antibody (7, 22), and adalimumab (Humira) is the third TNF antagonist; it was constructed from a fully human monoclonal antibody (MAb) (3, 25). All of these agents block TNF-α activation and have proven to be effective for downregulating the inflammatory reactions associated with autoimmune diseases, including RA (6, 23, 27, 29). Several recent studies have indicated that the neutralization of TNF-α is sufficient to induce a clinical improvement in RA (15, 20, 22, 27, 29).

The question of whether the anti-inflammatory effect of the currently preferred combined therapy of MTX together with TNF blockers is due to an induction of both TH1/TH2 balance and apoptosis in lymphocytes infiltrating from the site of inflammation was assessed. A single report (26) recently demonstrated that infliximab is an inducer of apoptosis in mononuclear cells (MNC) derived from RA patients, as was established in Crohn’s disease with the induction of apoptosis in the gut. However, no extensive research has been conducted regarding the differential apoptotic effects of TNF blockers compared to MTX alone and regarding the possible reactive oxygen species involvement. In addition, the ability of combined therapy to modulate the immune response toward TH1/TH2 balance has been scarcely reported (11, 27). As the anti-TNF and MTX mechanisms of action still are debated, the aim was to establish and distinguish alternative or additive anti-TNF immune effects. It is unclear, however, whether a local decrease in inflammatory cells results from an increased rate of apoptosis or from altered cell trafficking and peripheral cell pooling. We hypothesized that TNF-α inhibition is associated with an induction of apoptosis, resulting in a shift in the TH1/TH2 balance.

MATERIALS AND METHODS

The present study was conducted in collaboration with the Sheba Medical Center, Tel-Hashomer, Israel, in accordance with the Declaration of Helsinki.
Blood samples were obtained from populations of RA patients diagnosed according to the American College of Rheumatology (ACR) criteria. Subjects were requested to grant their informed consent to donate 5 ml blood for examination. Blood samples were analyzed at a cell population level using fluorescence-activated cell sorter (FACS) flow cytometry.

MNC were isolated from peripheral blood leukocytes of 46 RA patients (average age, 59 years) and 9 healthy controls (average age, 50 years) using the Ficoll-Paque procedure. The RA patients were classified according to the ACR criteria (2). The six criteria for disease activity assessment (duration of morning stiffness, fatigue, joint pain by history, joint tenderness or pain in motion, soft-tissue swelling in joints or tendon sheaths, and erythrocyte sedimentation rate) were monitored for each patient. Of the participating RA patients, 35 were carefully selected, all with stable, long-standing disease (median disease duration, 12 years) and classified as active using the recently developed and validated disease activity score based on 28 joints (DAS-28) (24). The remaining 11 RA patients were diagnosed but classified as inactive (with signs of remission). Folate supplementation was routinely administered (up to 5 mg, one to two times per week). Blood was collected within 2 to 3 days after the administration of drugs.

All treated patients took MTX or combined therapy for at least 6 months prior to in vitro examinations. For logistical reasons, it was not possible to monitor patients before and after the initiation of therapy. Therefore, all patients initially examined at least 6 months after the administration of therapy while being classified as having active disease and were evaluated by the same physician throughout the study.

All patients taking MTX (7.5 to 15 mg/kg of body weight/week) and the anti-TNF blockers infliximab (200 mg intravenously every 6 to 8 weeks), adalimumab (40 mg subcutaneously every 14 days), and etanercept (25 mg subcutaneously twice a week) were included in this study, unless they were taking other drugs known to affect the immune system.

Following separation, cells were washed twice in saline and once in phosphate-buffered saline (PBS) and then suspended in enriched RPMI medium at a concentration of 1 x 10^6 cells/ml. Before any cytometric analysis, viability was checked by trypan blue exclusion. Each type of experiment was performed in triplicate.

Cytokine receptor and chemokine receptor evaluation. MNC were evaluated under serum-free conditions for CD4, CCR3, CXCR4, interleukin-12R (IL-12R), and IL-4R expression. Flow-cytometric staining and analysis of the receptors were performed as previously described (19). Briefly, 1 x 10^6 MNC were fixed with 4% paraformaldehyde and then stained in PBS (Ca and Mg free) supplemented with 5% bovine calf serum (HyClone, Logan, UT). Primary MAbs were detected with secondary phycoerythrin, antigen-presenting cells (APC), or fluorescein isothiocyanate (FITC)-conjugated goat antimouse MAbs (Sigma) (1:100). After the final wash, cells were analyzed using FACScan (Becton-Dickinson, Mountainview, CA).

Double staining with FITC-annexin V and PI. For the analysis of apoptosis, phosphatidylserine (PI) exposure on the outer lealet of the plasma membrane was detected using the annexin V assay. Early and late apoptosis was measured simultaneously by double staining with PI and FITC-annexin V. 1 x 10^6 cells were washed with PBS and re-suspended in the binding buffer (Gentzime, Cambridge, MA). FITC-annexin V was added at a final concentration of 1 µg/ml, and PI was added simultaneously. After 10 min of incubation in the dark at room temperature, cells were analyzed either by using the Optical LiveCell Array (LCA; Molecular Cytomics, Boston, MA) for individual cell measurements or by FACS flow cytometry for population measurements (FACScan; Becton-Dickinson, Mountainview, CA).

Optical LiveCell Array. Cells were introduced into the Optical LiveCell Array (Molecular Cytomics, Boston, MA), a densely packed hexagonal transparent array of micron-sized wells which hold individual cells in suspension. The Optical LiveCell Array enables real-time visualization, manipulation, and sequential measurement of cellular activities in living individual cells within a population. Cells within the Optical LiveCell Array were manipulated and measured using an imaging system. For each assay, at least three areas were imaged and analyzed (about 100 individual cells).

Imaging system. An Olympus CellR system was mounted on an inverted ix81 microscope (Tokyo, Japan) that was equipped with a submicron Marzhauser-Wetzlar motorized stage type SCAN-IM with an Lstep controller (Wetzlar-Steindorf, Germany), 14-bit ORCA II C4742-98 cam (Hamamatsu, Japan), and a filter wheel. Fluorescence cubes with the following excitation filters, dichroic mirrors, and emission filters (all obtained from Chroma Technology Corporation [Brattleboro, VT]) were used: for FITC, 470 to 490 nm, 505-nm long pass, and 510 to 530 nm, respectively; for phycoerythrin, 536 to 555 nm, 560-nm long pass, and 570 to 610 nm, respectively; for APC, 620 to 650 nm, 660-nm long pass, and 680 to 690 nm, respectively. Olympus CellR software was used for image analysis. An in-house software package was developed to utilize the highly ordered LiveCell Array honeycomb structure for automating cell recognition.

Data analysis. Data were expressed as means ± standard deviations (SD) and were analyzed by the two-tailed paired Student t test. Differences were considered significant at P < 0.05. Correlations were assessed using linear correlation coefficients, and where data were not normally distributed, nonparametric correlation coefficients (Kendall’s tau-b and Spearman’s correlation coefficients) were used.

RESULTS

To explore the regulation of immune markers of CD4 cells (TH1/TH2) derived from RA patients undergoing combined treatment with MTX and TNF-α inhibitors, the chemokines CXCR3 and CCR4, which correlate with the TH1 and TH2 subtypes, were examined. Two groups of long-standing RA patients, 20 patients treated by low-dose MTX (our reference group for the combined treatment group) and 15 patients treated by combined therapy (MTX and TNF blocker) were examined. The number of patients treated with stand-alone anti-TNF agents was minimal, so they were not included in the study. For logistic reasons, it was not possible to monitor patients before and after the initiation of therapy. Therefore, all treated patients were classified as active according to their DAS 28 score and were examined in vitro at a static phase at least 6 months after the initiation of drug therapy. The last group to be examined consisted of 11 diagnosed but untreated RA patients. Table 1 summarizes the clinical characteristic phenotype of the patients examined.

All experiments examined the chemokine expression by both FACS and image analyses. All lymphocytic cells were gated by forward scatter (FSC) and side scatter (SSC) in the FACS analysis, as were the specific CD4 subtypes, were examined. Two groups of long-standing RA patients, 20 patients treated by low-dose MTX (our reference group for the combined treatment group) and 15 patients treated by combined therapy (MTX and TNF blocker) were examined. The number of patients treated with stand-alone anti-TNF agents was minimal, so they were not included in the study. For logistic reasons, it was not possible to monitor patients before and after the initiation of therapy. Therefore, all treated patients were classified as active according to their DAS 28 score and were examined in vitro at a static phase at least 6 months after the initiation of drug therapy. The last group to be examined consisted of 11 diagnosed but untreated RA patients. Table 1 summarizes the clinical characteristic phenotype of the patients examined.

Figure 1A summarizes the FACS results and indicates a decrease in the level of the CXCR3 marker in both lymphocytic populations (all lymphocytic cells and CD4-marked cells) in the combined treatment group compared to the levels for the untreated and MTX-treated groups. The CCR4 receptor was upregulated in the CD4 subpopulation in patients undergoing combined treatment only (Fig. 1A). Two notable phenomena were observed regarding the TH1/TH2 profile in the combined treatment group only: a significant decrease in the CXCR3 receptor and a significant increase in the CCR4 receptor, identifying a distinct immune status in these patients. Moreover, while the untreated and MTX-treated patients exhibited a TH1 profile with CXCR3
dominance and the highest TH1/TH2 ratio (0.64), the cells of the combined treatment group showed a TH2 dominance with CCR4 profile and the smallest TH1/TH2 ratio (0.34) (Fig. 1B). Within the CD4 cell populations, the differences in TH1/TH2 ratios were more substantial: the ratio in the combined treatment group (0.25) was about 10 times lower than that in the untreated group (2.1) (*, P ≤ 0.05, indicating a significant difference compared to the untreated RA group. The distribution pattern of these receptors using the Live-Cell Array (LCA), which enables live measurements of individual cells, was analyzed further. As expected, similar results were observed when imaging the different cells on the LCA and by flow analysis. In Fig. 1C, the whole lymphocytic population shows a significant decrease in the average percentage of CXCR3 expression in the combined treatment group compared to that of the untreated group, while CCR4 receptor expression was slightly changed. Figure 1D and E demonstrate the appearance of these markers, specifically in the CD4 subpopulation. There is a decrease in the CXCR3 and an increase in the CCR4 expression, a phenomenon also observed in the FACS analysis. The LCA permits the correlation between the cell area and ligand expression. In lymphocytes derived from patients treated by combined therapy, two cell populations are clearly distinguishable with reference to cell size, blast lymphocytes and small lymphocytes, especially among the CD4 cells expressing the CXCR3 receptor (Fig. 1E). In the combined treatment group, the CD4 cells expressing the TH1-type CXCR3 receptor (CD4+ CXCR3+) are significantly larger (average cell area, 0.726 ± 0.64 mm²) than the lymphocytes that do not express this receptor (CD4+ CXCR3+) (cell area, 0.636 ± 0.47 mm²; P ≤ 0.01). This finding implicates blast lymphocytes as a primary target for combined therapy, as RA is characterized by a hyperproliferation of TH1 cells (13, 14). Subsequent experiments examined two other markers of the TH1/TH2 subsets, IL-12R and IL-4R. As in prior experiments, the whole lymphocyte population as well as the specific CD4 population were examined (Fig. 2). As shown in Fig. 2A, the most significant changes occurred only in the CD4 subpopulation. In the combined treatment group, significant changes were observed, a decrease in IL-12R and an increase in IL-4R, again demonstrating the pattern shift in the TH1/TH2 balance. Significant changes also were observed in the immune repertoire of CD4 cells in MTX-treated patients (Fig. 2A). Inter-
Remarkably, a decrease in IL-4R expression was observed in the entire lymphocytic population in the combined treatment group, in contrast to an increase in IL-4R in the CD4 sub-group. 

The smallest ratio between the two markers IL-12R/IL-4R (ratio, 3) was observed in the CD4/H11001 subpopulation of the combined treatment group, indicating a reestablishment of the immune T-helper lymphocytic balance in these patients (Fig. 2B). The nine healthy controls exhibited 16% CD4/H11001 IL-12R and 10% CD4/H11002 IL-4R, yielding a TH1/TH2 ratio of 1.6, indicating that untreated RA patients have a significant TH1 profile compared to that of healthy controls, and although both MTX treatment and combined treatment groups exhibited a much lower TH1/TH2 ratio than the non-treated groups, they did not reach the level of healthy controls (Fig. 2B).

The LCA was used to determine the cytokine receptor appearance at an individual cell level with reference to cell size. Among the CD4 cells under combined treatment, two cell populations are clearly distinguishable according to cell size, namely, blast and small lymphocytes (Fig. 2D); this is not the case for lymphocytes derived from the untreated patients (Fig. 2C). Under combined treatment, the average cell size in the subpopulation expressing the TH1-type IL-12R receptor (CD4+ IL-12R+; average cell area, 0.735 × 10^{-4} ± 2.138 × 10^{-5} mm²) is significantly larger than that of the subpopulation that does not express this phenotype (CD4+ IL-12R-; average cell area, 0.59 × 10^{-4} ± 1.834 × 10^{-5} mm²; P < 0.01). This finding has particular significance, considering that RA is characterized by TH1 hyperproliferation. Importantly, both types of analyses (FACS and imaging) showed similar results regarding the percentage of the two subpopulations (whole and specific CD4 lymphocytes) and their chemokine expression. The high correlation between the two types of measurements strongly corroborates the results.

Figure 3 demonstrates the positioning of individual cells within the LCA, labeled with both anti-CD4 and anti-IL-12R antibodies. Two out of eight cells are marked by both CD4 and IL-12 antibodies in various degrees. Notably, other cells were marked by either the CD4 or the IL-12 antibody. Further experiments will take advantage of the LCA’s ability to distinguish and correlate receptor expression at an individual cell resolution.

The final set of experiments concentrated on the apoptotic effect of the different treatments in RA patients. As Fig. 4 demonstrates, all TNF inhibitors, as well as MTX, slightly
increase the proportion of apoptotic cells compared to that of untreated RA patients. Notably, a significant increase occurs only in the combined treatment group. As a matter of interest, healthy controls exhibited 7.5% annexin V−PI− cells, a low rate of spontaneous apoptosis. Future research will further link apoptotic induction to the immune repertoire changes observed in patients.

**DISCUSSION**

Little is known about the role of chemokines and the interaction of their receptors, which are essential for the recruitment of selective lymphocyte subsets during inflammation in the pathogenesis of RA. Recent studies have revealed that TH1 and TH2 cells preferentially employ the chemokine receptors CXCR3 and CCR4, respectively, in the process of accumulation into inflammatory sites (18). The CXCR3 and CCR4 expression and the IL-12R and IL-4R expression on lymphocytes derived from untreated, MTX-treated, and MTX-plus-anti-TNF-treated RA patients were evaluated. Studies have indicated that the combined MTX plus anti-TNF agents are the best choice for RA patients (15, 20), but their interaction and mechanism of action are still debated. It has been demonstrated in our previous study that in active RA patients receiving MTX treatment only, MTX selectively modulates the TH1/TH2 balance (11). In the present study, we suggest that the therapeutic effect of anti-TNF-plus-MTX combined therapy is achieved by correcting a TH1/TH2 imbalance (producing a shift from the TH1 to TH2 type) that may be involved in RA pathogenesis.

In the present study, the image analysis of individual cells using the LiveCell Array revealed results similar to those of FACS analysis, thus corroborating the cell population results as well as pinpointing different CD4 subpopulations according to cell size and other distinguishing parameters of individual cells. All experiments were established on a certain static active phase (at least 6 months after therapy initiation) and were not monitored before or thereafter, a point intended for future investigation. The further determination of these significant results will assess their correlation to variations in therapy or stage or severity of disease. Two cell subpopulations are clearly distinguishable according to cell size: blast and small lymphocytes. Under combined therapy, the size of cells expressing the TH1-type receptors CXCR3 and IL-12 was significantly larger than the size of cells that did not express these markers. Previously, we showed that an increase in the CD4+ lymphoblast population was evident upon in vitro exposure to MTX (13) and that in nonactive RA patients, the major CD4+ CD28− lymphocyte subpopulations appeared to be activated by MTX, subsequently transforming them into a major hyperblast population. The current findings may implicate TH1 blast cells as a primary target of MTX and anti-TNF therapy and also may support previous results in which the apoptotic effect of MTX was pronounced in activated blast lymphocytes and not in small resting lymphocytes (13). Future experiments will further focus on the relation between cell population and individual cell measurements with reference to size, activation status, and phenotype, as well as cytokine secretion and regulation by these cells. In previous research, others have presented a high correlation between elevated serum IL-10 and the percentage of CD4+/CCR4+ T cells in RA patients (31). Future experiments will examine such correlations. When measuring the receptor expression in the whole lymphocytic population, or specifically in CD4 cells, the most pronounced change was noted in the CD4+/CCR4+ T cells in RA patients (31). Future experiments will examine such correlations. When measuring the receptor expression in the whole lymphocytic population, or specifically in CD4 cells, the most pronounced change was noted in the CD4 cells, indicating a major role of this subpopulation in RA and confirming previous observations (5, 9, 30).

Finally, results demonstrate the induction of apoptosis, especially in patients undergoing combined treatment. The evaluation of apoptosis induction by anti-TNF agents plus MTX might be useful to assess the clinical improvements in RA.
patients after the administration of therapy. MTX alone previously has been demonstrated to induce apoptosis (8, 10, 12). Here, we show that the apoptotic effect may be strengthened by combined anti-TNF and MTX therapy. Future studies will focus on the evaluation of apoptosis in different CD4 subpopulations, i.e., CXCR3-positive and IL-12R-positive cells (TH1 markers) and CCR4-positive and IL-4R-positive cells (TH2 markers), to determine the mechanism of immune balance in patients. LCA may aid in analyzing this mechanism in individual cells. We assume that the local decrease in inflammatory cells and the treatment efficiency in patients receiving combined therapy are due to an increased induction of apoptosis, especially of the TH1 subset, which previously was reported to be more sensitive to apoptotic triggers (1, 11, 12), resulting in a shift in TH1/TH2 balance.

REFERENCES
1. Alvarez-Lara, M. A., et al. 2004. The imbalance in the ratio of TH1 and TH2 helper lymphocytes in uromelia is mediated by an increased apoptosis of TH1 subset. Nephrol. Dial. Transplant. 19:3084–3090.
2. Arnett, F. C., et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 31:315–324.
3. Bang, L. M., and G. M. Keating. 2004. Adalimumab: a review of its use in rheumatoid arthritis. BioDrugs 18:1221–1239.
4. Cañete, J. D., et al. 2000. Differential TH1/TH2 cytokine patterns in chronic arthritis: interferon gamma is highly expressed in synovium of rheumatoid arthritis compared with seronegative spondyloarthropathies. Ann. Rheum. Dis. 59:263–268.
5. Chen, D. Y., J. L. Lan, F. J. Lin, and T. Y. Hsieh. 2004. Proinflammatory cytokine profiles in sera and pathological tissues of patients with active untreated adult onset Still’s disease. J. Rheumatol. 31:2189–2198.
6. Cronstein, B. N. 2005. Low-dose methotrexate: a mainstay in the treatment of rheumatoid arthritis. Pharmacol. Rev. 57:163–172.
7. Ebert, E. C. 2009. Infliximab and TNF alpha system. Am. J. Physiol. Gas-trointest. Liver Physiol. 306:G612–G620.
8. Fairbanks, L. D., et al. 1999. Methotrexate inhibits the first committed step of purine biosynthesis in mitogen-stimulated human T-lymphocytes: a metabolic basis for efficacy in rheumatoid arthritis. Biochem. J. 342:143–152.
9. Firestein, G. S. 2005. Immunologic mechanisms in the pathogenesis of rheumatoid arthritis. J. Clin. Rheumatol. 11(Suppl. 3):39–44.
10. Genestier, L., et al. 1998. Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of activated peripheral T cells. J. Clin. Invest. 102:322–328.
11. Herman, S., N. Zurgil, P. Langevitz, M. Ehrenfeld, and M. Deutsch. 2008. Methotrexate selectively modulates TH1/TH2 balance in active rheumatoid arthritis patients. Clin. Exp. Rheumatol. 26:317–323.
12. Herman, S., N. Zurgil, P. Langevitz, M. Ehrenfeld, and M. Deutsch. 2004. The immunosuppressive effect of methotrexate in active rheumatoid arthritis patients vs. its stimulatory effect in nonactive patients, as indicated by cyto- metric measurements of CD4+ T cell subpopulations. Immunol. Invest. 33:351–362.
13. Herman, S., N. Zurgil, P. Langevitz, M. Ehrenfeld, and M. Deutsch. 2003. The induction of apoptosis by methotrexate in activated lymphocytes as indicated by fluorescence hyperpolarization: a possible model for predicting methotrexate therapy for rheumatoid arthritis patients. Cell Struct. Funct. 28:122.
14. Hildner, K., et al. 1999. Tumour necrosis factor (TNF) production by T cell receptor-primed T lymphocytes is a target for low dose methotrexate in rheumatoid arthritis. Clin. Exp. Immunol. 118:137–146.
15. Klæreskog, L., et al. 2004. Therapeutic effect of the combination of etanercept and methotrexate compared with each treatment alone in patients with rheumatoid arthritis: double-blind randomised controlled trial. Lancet 363:675–681.
16. Klinkhoff, A. 2004. Biological agents for rheumatoid arthritis: targeting both physical function and structural damage. Drugs 64:1267–1283.
17. Kremer, J. M. 2005. Selective costimulation modulators: a novel approach for the treatment of rheumatoid arthritis. J. Clin. Rheumatol. 11(Suppl. 3):S55–62.
18. Kunkel, E. J. 2002. Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. Am. J. Pathol. 160:347–355.
19. Lee, B., J. Ratajczak, R. W. Doms, A. M. Gewirtz, and M. Z. Ratajczak. 1999. Coreceptor/chemokine receptor expression on human hematopoietic cells: biological implications for HIV-1 infection. Blood 93:1145–1156.
20. Lee, Y. H., et al. 2008. Meta-analysis of the combination of TNF inhibitors plus MTX compared to MTX monotherapy, and the adjusted indirect comparison of TNF inhibitors in patients suffering from active rheumatoid arthritis. Rheumatol. Int. 28:553–559.
21. Linsen, L., et al. 2005. Peripheral blood but not synovial fluid natural killer T cells are biased towards a TH1-like phenotype in rheumatoid arthritis. Arthritis Res. Ther. 7(R493–R502).
22. Lipsky, P. E., et al. 2000. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-tumor necrosis factor trial in rheumatoid arthritis with concomitant therapy study group. N. Engl. J. Med. 343:1584–1602.
23. Maini, R. N., and P. C. Taylor. 2000. Anti-ctytokine therapy for rheumatoid arthritis. Annu. Rev. Med. 51:207–229.
24. Prevoo, M. L. L., et al. 1995. Modified disease activity scores that include twenty-eight joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum. 38:44–48.
25. Shen, C., et al. 2005. Adalimumab induces apoptosis of human monocytes: a comparative study with infliximab and etanercept. Aliment. Pharmacol. Ther. 21:251–258.
26. Tak, P. P. 2005. Effects of infliximab treatment on rheumatoid synovial tissue. J. Rheumatol. Suppl. 74:31–34.
27. Tamaka, Y. 2008. Paradigm shift in the treatment of rheumatoid arthritis by biologics. Rinsho Byori 58:309–315.
28. VanderBorgh, A., P. Geusens, J. Raus, and P. Stinissen. 2001. The auto-immune pathogenesis of rheumatoid arthritis: role of autoreactive T cells and new immunotherapies. Semin. Arthritis Rheum. 31:160–175.
29. Weaver, A. L. 2004. The impact of new biologicals in the treatment of rheumatoid arthritis. Rheumatology 43(Suppl. 3):i7–23.
30. Weyand, C. M., E. Bryl, and J. J. Goronzy. 2000. The role of T cells in rheumatoid arthritis. Arch. Immunol. Ther. Exp. 48:429–435.
31. Yang, P. T., et al. 2004. Increased CCR4 expression on circulating CD4+ T cells in ankylosing spondylitis, rheumatoid arthritis and systemic lupus erythematosus. Clin. Exp. Immunol. 138:342–347.