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Biomolecular Cytokine Therapy

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As for the chronicity of inflammatory-immune diseases, the medication of them needs to be longterm and thus, quite safe with respect to side effects due to drug actions. Therapy of these diseases includes steroid and non steroid anti-inflammatories given in monotherapy or in combination with cytotoxic antimetabolites. Longterm administration of these active substances cumulate in side effects, not to speak of the probability of developing unresponsiveness to the drug in use. In principle, the earlier the intervention, the better the outcome of medication in therapy. In harmony with this principle, biopharmacology focuses on specific targets in early (acute) phase of inflammatory-immune diseases. One of these targets is the proinflammatory cascade of cytokines (IL1β, IL6, IL8, IL12, TNFα). Among them, the overproduction of tumor necrosis factor (TNFα) is suggested to orchestrate and escalate the disease phenotype. Hence, targeting of TNFα may restrict or stop the propagation of pathological reactions. TNFα in its excess can be captured at transcription, translation, secretion levels as well as in the extracellular soluble form. This latter approach is supported by clinical records emphasizing the use of recombinant antibodies and soluble receptors in trapping extra amounts of TNFα. This review serves as an illustration for the efficacy and safety of infliximab (antibody) and etanercept (soluble receptor) in the example of rheumatoid arthritis (RA). (Pathology Oncology Research Vol 9, No 1, 24–29, 2003)

Keywords: chronic inflammation, therapy, cytokines, infliximab, etanercept

Early phase RA: a target for cytokine therapy

Like other chronic inflammatory diseases, RA is of multifactorial (genetic + environmental) etiology. A self-perpetuating series of inflammatory reactions in concert with the predisposing genetic background of the touched person ultimately lead to degenerative and proliferative responses in target cell populations responsible for the disease phenotype. Should it be the activation-proliferation of synoviocytes (macrophage like and fibroblast like) or the process of angiogenesis or synovial swelling, the propagation of the disease mainly depends on the sequence and dominance of cytokines in cascade.[6,9,12,13,18] The origo for the liberation of these intercellular messages is the immune synapse (*Figure 1*). Activating each other, cells of the immune synapse (antigen presenting cells like macrophages, dendritic cells, B lymphocytes and the MHC restricted CD4+ T cells) promote the expression of first line cytokines (IL1β, IL2, IL12, TNFα, IFNγ) which, in response by inducing transcription of adhesive membrane components (CD11-CD54, CD40-CD154, CD95-CD95L, CD86 - CD28), give stimuli for the expression of second line cytokines and chemokines (IL1β, IL4, IL6, IL8, TNFα, GM-CSF, TGFβ). If imbalanced, the overproduction of TNFα liberates bioactive mediators - prostanoids, metalloproteinases, glycosidases - along with time in an amount leading to escalation (destruction of bone, cartilage) of the disease.[6,9,12,13,18] Thus, at least two levels of pharmacological interventions can be defined: the first at the immune synapse, the second at remote cells like osteoclasts, fibroblasts, vascular endothelial cells, cells in haematopoiesis (*Figure 1*). Even if not specific for the cytokines in action, the remote level is targeted by nonsteroid antiinflammatories, while both levels are involved in the actions of steroids.[8] Regarding the benefits of early intervention specific for cytokines, IL1β–TNFα–IFNγ seem to be the choice of bio-drug targeting. As for its regulatory role in cell survival and defense against infections, capturing the excess amount of TNFα seems to be the primary endpoint in cytokine therapy of RA.

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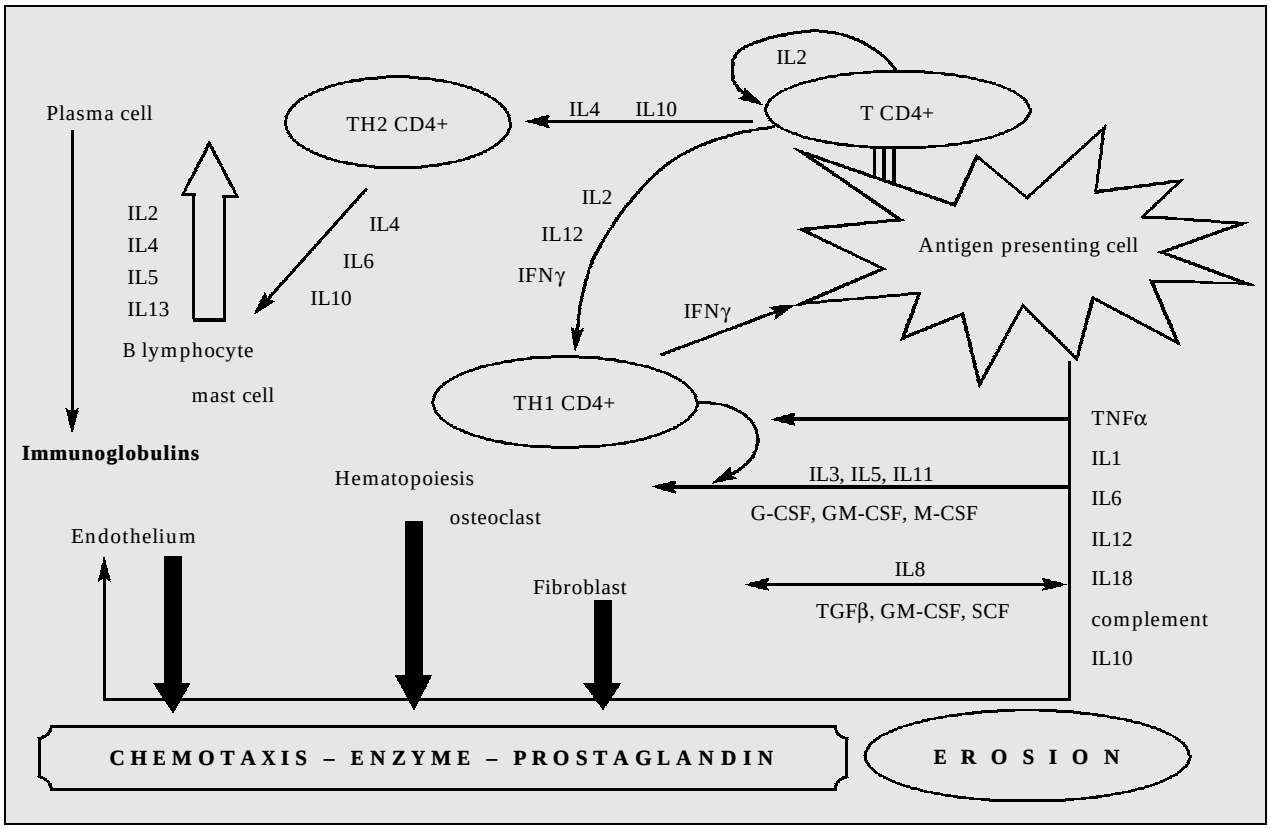

Figure 1. Cytokine cocktail for maintaining the inflammatory state

Trapping of cytokines

The standard therapy in RA[8] is based on steroids (glucocorticoids)/non steroids (gold, penicillamine, inhibitors of metalloproteases)/antimetabolites (methotrexate, leflunomide) with supplementary symptomatic medication (acetylsalycilic acid, inhibitors of cyclooxygenases). According to their mechanism of action, cytokine expression too, can be inhibited in a nonspecific manner with limitations in longterm use due to multiple toxic effects.[8,12,13] The latter to be avoided, the new concept of biomolecular therapy in trapping cytokines was introduced.

In a biopharmacological approach, TNFα can be captured by several ways:

1. structural-functional modulation of adhesive membrane determinants (MHC, integrins, selectins) to interfere with immune synapse for restricting the ongoing activation process;

2. a change in Th1/Th2 ratio of cytokines to diminish transcription/translation of TNFα (and other proinflammatory cytokines);

3. a blockade on angiogenesis to prevent immigration of inflammatory cells;

4. antibodies to TNFα capturing it either in its membrane bound or soluble, freed from the cell forms;

5. decoy receptors for TNFα competing with the natural ones in binding the ligand.

The first item is complicated by the dynamic replenishment of antigen presenting pockets in MHC.[10] Since the process of antigen recognition is flexible, it varies with extracellular and intracellular metabolic changes along with disease progression. This cannot be overcome by CD4+ T cells in the periphery with reduced variability in receptor β chains characteristic to RA.[23] Further, peripheral CD4+ T cells (resting and memory) in RA have shortened chromosome telomeres,[23] hence the targeting of immune synapse raises also the question of discrepancies in regeneration and maturation of the immune system.

The second and the third items are in part under preclinical and clinical phases.[4,16,17]

The following report deals with the safety and efficacy of infliximab and etanercept having already been authorized to enter clinical practice (1999-2001).

(A) Trapping TNFα in excess: recombinant antibodies

An immunocomplex containing TNFα bound to its specific antibody can be eliminated either by antibody-dependent cellular cytotoxicity and/or by recruiting the complements into action. Any of them should appear, both

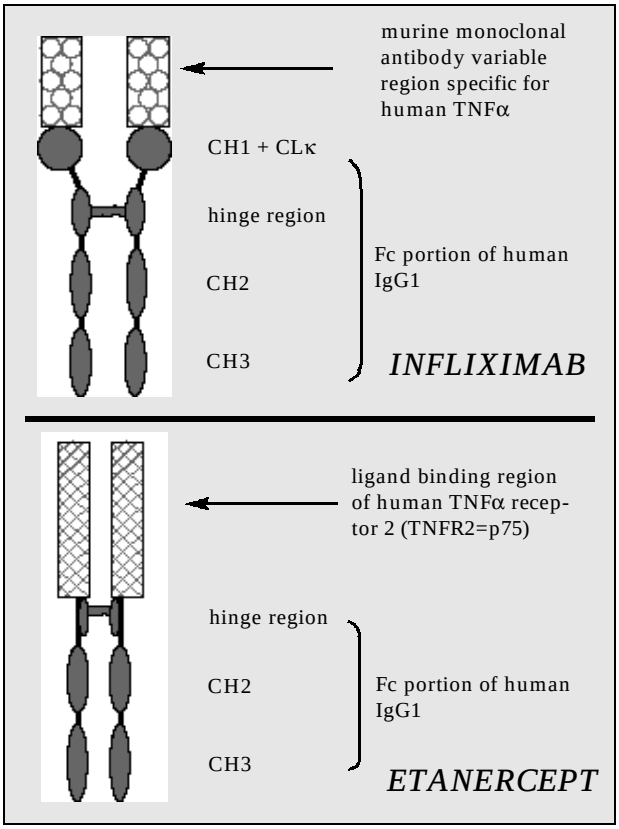

Figure 2. TNF-α modulating recombinant antibody and soluble receptor

processes require the lifetime of antibody be long enough to capture the ligand as well as to surrender the complex to elimination. To meet these requirements, the Fab portion of the antibody is either joined to an Fc region or, in advanced technology, to a neutral polymer (PEG) supporting the residence of biomolecule in the circulation. Contradictory to this, targeting of cytokines requires good penetration into tissues, too, with small antibody fragments (Fab) acting better than larger ones. Small molecules, however, are cleared from the circulation more easily, thus limiting their bioavailability. An illustration to this is *anakinra,* the recombinant human IL1 receptor antagonist interfering with signaling pathways of TNF receptor family members, exerting thus an indirect inhibition on the expression of TNFα. Given in subcutaneous daily doses, this small molecule has the highest absorption as compared to *infliximab* and *etanercept.* However, the short residential time in circulation makes *anakinra* less bioavailable (*Table 1,2*).

Infliximab, the recombinant chimeric antibody is produced by mouse myeloma cells.[6,13] The molecule contains sequences from human IgG1 constant and mouse variable regions (*Figure 2*). *Infliximab* is specific for TNFα of humans and chimpanzees either membrane bound or secreted in the extracellular space. As expected, *infliximab* prevents TNF from binding to its membraneous and soluble receptors. By initial parenteral administration the antibody has a serum half life approx. 9.8 days that is maintained by every 8 weeks dosing thereafter. Due to its intravenous administration, the maximum serum concentration of infliximab is reached within an hour (*Table 1*). Multiple clinical studies[6,12,13] confirmed that by 30 weeks of treatment, *infliximab* had a 20% improvement in ACR (parameters of the American College of Rheumatology) score in more than 50% of patients with active RA. During the same period of time this score was 20% in the placebo group (*Table 2*). By 54 weeks mainly the same figures were maintained. Besides antibody titers in serum characteristic to autoimmune diseases (anti-nuclear antibody and antibody to double stranded DNA, see *Table 2*), serial infusions of *infliximab* provoked the development of neutralizing (anti-chimeric) antibodies, too,[13] restraining the bioavailability of the molecule. A further improvement in biotechnology will provide fully human monoclonal antibodies (e.g. *adalimubab)* to counteract this effect.

To overcome the interference of anti-chimeric antibodies with the bioavailibility of *infliximab,* a combination therapy with methotrexate is accepted in clinical practice. Adverse events like infusion reaction-fever, hypotension, chills, are infrequent and it is only hypersensitivity to the biomolecule that has caused cessation of *infliximab* medication. Serious adverse events, like lymphomas[3] and more often the increased sensitivity to infections (e.g. Mycobacterium tuberculosis) are rare (0.1-1%) but worth to consider (*Figure 3*) because of their impact on public epidemy.[11]

(B) Trapping TNFα in excess: recombinant soluble receptors

Etanercept is a fusion protein (decoy receptor) secreted by CHO (chinese ovary) cells.[19] It combines the ligand binding portion of human TNF receptor 2 (TNFR2=p75= CD120b) with sequences of human IgG1 (*Figure 2*). In contrast to TNFR1 (p55=CD120a) being a constitutive membrane receptor, TNFR2 is inducible upon stimuli and can be found on almost every cell surfaces, except red blood cells and resting lymphocytes.[1] Having a higher affinity to its ligand (e.g. TNFα), TNFR2 is capable to capture the cytokine more easily than TNFR1. Since active TNFR2 is a trimer, the recombinant decoy receptor should not be a monomer to reach the required bioactivity in competing with natural receptors.[1,4]

Etanercept is administered twice weekly in 25 mg subcutaneous injections each.[6,13] The median half life in serum is 4.8 days and the maximum concentration is reached within 3 days (*Table 1*). Compared to *infliximab, etanercept* has higher affinity to binding the ligand, while

Table 1. **Pharmacological characteristics of TNF-α inhibitors**

Pharmac. character	**♦ nonspecific**	**♦ indirect**	**♦ specific**	**♦ specific**
	Leflunomide	*Anakinra*	*Infliximab*	*Etanerecept*
t 1/2	≈ 14 days	≈ 7–8 hours	≈ 9,8 days	≈ 4,8 days
Tmax	5–24 hours	3–9 hours	within an hour	2,5 days
Ka (M^{-1})	/////////	2,3 x 10^{10}	1,8 x 10^{9}	10^{10}
Target	de novo pyrimidine synthesis	IL1R Type I	mTNFα sTNFα	mTNFα sTNFα
Structure	isoxazol „ prodrug“	methyl. human IL1Ra 17,3 kD	murine-human chimera ≈150 kD	human–human fusion protein ≈150 kD
Complement	/////////	–	+	–
Cell lysis	cytotoxicity	–	+	–
Administration	p. o. 1 x / day 100 → 10–20 mg	s. c. 1 x / day 1–2 mg / kgbw	i. v. 4–8 x / wek 1–3 mg / kgbw	s. c. 2 x / week 25 mg

its residential time in circulation is shorter, even if longer than that of *anakinra*. *Leflunomide*, the inhibitor of de novo pyrimidine synthesis, has a shorter than *etanercept*, but longer than *infliximab* Tmax after an intensive first pass metabolism in the liver and the gut. The elimination half life of *leflunomide* (about 14 days) makes it more potent in availability and comparable in efficacy (*Table 1,2*), however, the indirect targeting of cytokines makes this antimetabolite more probable to result multiple toxic side effects along with its longterm administration.

A 20% improvement in ACR was achieved in 60-75% of RA patients on 12 weeks etanercept therapy versus the placebo 14-33 % effects.[6,12,13] Reports suggest that the benefit of *etanercept* treatment is also maintained over years. Besides the antibodies characteristic to autoimmune diseases, temporary appearance of antibodies of non-neutralizing nature were to be observed in sera of patients under treatment. Pharmakokinetic and pharmacodynamic parameters described above suggest a relative superiority in efficacy and safety of *etanercept* as compared to those of

Table 2. **Efficacy of TNF-α inhibitors**

Efficacy	**♦ nonspecific**	**♦ indirect**	**♦ specific**	**♦ specific**
	Leflunomide 24 weeks	*Anakinra 12 weeks*	*Infliximab 30 weeks*	*Etanerecept 12 weeks*
ACR 20 in pts%	≈ 52% Plac. 26%	≈ 34% Plac. 19%	≈ 53% Plac. 20%	≈ 60–75% Plac.14–33%
Antibody	/////////	Low titer if at all	• anti-chimeric neutralizing • ANA • a-dsDNS	• temporary, non neutralizing • ANA • a-dsDNS
Availability	82–95%	≈95%	↑	76%
Response	15 months (MTX 14 months)	???	more than 102 weeks?	more than 5 years
Administration	p. o. 1 x / day 100 → 10–20 mg	s. c. 1 x / day 1–2 mg / kgbw	i. v. 4–8 x / wek 1–3 mg / kgbw	s. c. 2 x / week 25 mg

infliximab or anakinra or leflunomide. Longterm administration of this fusion protein still has to face shortages in data of preclinical studies on stoichiometry, toxicology, carcinogenesis, and mutagenesis.

Trapping TNFα: a beauty or a monster?

Serious adverse events in anti-TNFα medications are mainly the consequences of imbalance in cell death and functional activation, conditions naturally regulated by the cytokine. Serious but rare in frequency adverse events so far were (myco)bacterial and fungal infections, lupus-like and demyelinating syndromes, blood dyscrasias and very rarely lymphomas (*Figure 3*). Considering the immune synapse as an activation site (the origo) in disease progression, tempting explanations to these adverse events are the intracellular signaling pathways shared by TNFR family members, IL1 receptors, Toll receptors (*Figure 4*). In balanced situations, TNFR-p55 initiates programmed cell death and the p75 receptor initiates activation of kinase cascades with transcription factors (NFκB, AP-1) at the end acting for gene expression promoting cell cycle and/or cellular functions.[4]

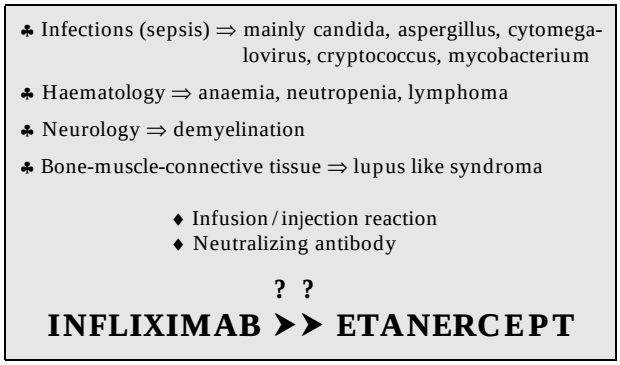

Figure 3. Serious adverse events in frequency of 1/1000 to 1/100 (1999–2002)

However, as for the shared signalizations, the caspase chain leading to apoptosis can be reached by p75 mediation as well[5,15] and the activation of NFκB and AP-1 can be performed by IL1 and Toll receptor signaling, too.[2,14,20] For p75 receptors, the key elements in these common signalizations are the RIP (mediating kinase receptor signals) and the FLIP (mediating Fas receptor signals) balancing proteins.[5,15] Ele-

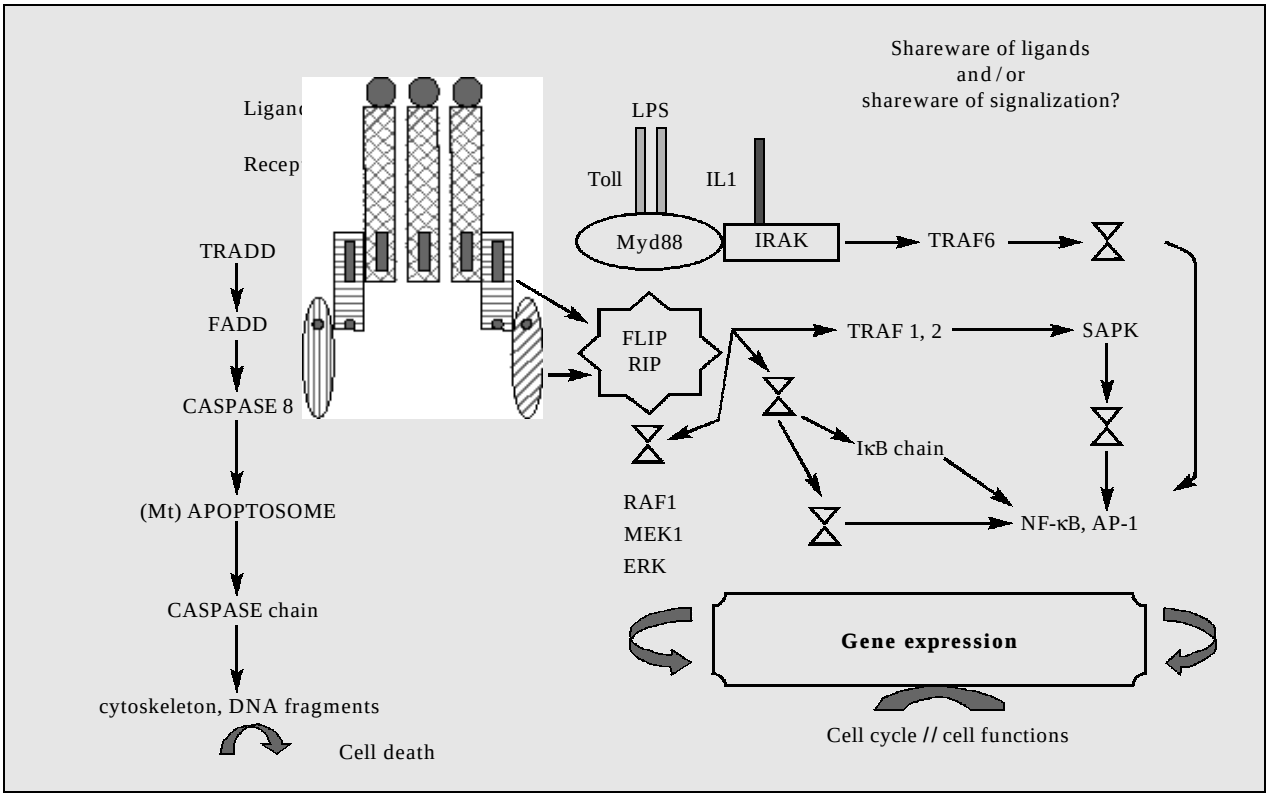

Figure 4. Signaling pathways of the TNFR family

AP-1: transcription factor, ERK: extracelllular signal regulated kinase, FADD: FAS associated death domain, FLIP: FLICE (caspase8) inhibitory protein, iB: inhibitory to NF-κB, IRAK: interleukin-1 receptor associated kinase, LPS: endotoxin, MEK: MAP / ERK kinase, (mt) mitochondrial, Myd88: myeloid differentiation primary response gene 88, NF-κB: transcription factor, RIP: receptor interacting protein, SAPK: stress activated protein kinase, TNFR: tumor necrosis factor receptor, TRADD: NF receptor associated death domain, TRAF: TNF receptor associated factor.

vated RIP concentrations in activated T lymphocytes lead to cell death (*Figure 4*), and the same applies for low concentrations of FLIP in cycling cells. The occupancy of crossing signaling pathways is determined by the presence or absence of ligands such as TNFα, the dominance of which, depending on the phase of RA can be influenced by the activation of non-MHC genes, too.[22]

For IL1 and Toll receptors the adapters IRAK and Myd88 respectively, both concluding in TRAF6 activated kinase cascade, mediate the activation of transcription factors NFκB and AP-1 *(Figure 4)*, critical for first line and second line cytokine expressions. By withdrawing a dominant cytokine (missing the ligand to TNFRs), anti-TNFα therapy in RA creates a change in the regulatory chain of cytokines. Consequently, signaling pathways for infections (e.g. mycobacterial and fungal) are less counteracted when leading to either cell cycle changes or to perturbations in differentiated functions of target cells. Multi-component cytokine traps perhaps, will give a further improvement in balancing the adverse effects of anti-cytokine therapy.[7]

In conclusion, it should be emphasized that even if rare in frequency, adverse events in *infliximab* and *etanercept* therapy reported so far, underline the necessity of stoichiometry and the close follow up in medication.

References

1. *Barclay AN, Brown MH, Law SKA, et al:* The Leukocyte Antigen Facts Book. Academic Press, 2nd edition, 1997.
2. *Baud V, Liu Z-G, Bennett B, et al:* Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. Genes Develop 13: 1297-1308, 1999.
3. *Brown SL, Greene MH, Gershon SK, et al:* Tumor necrosis factor antagonist therapy and lymphoma development : twenty-six cases reported to the Food and Drug Administration. Arthritis Rheum 46: 3151-3158, 2002.
4. *Beyaert R, Fiers W:* Tumor necrosis factor and lymphotoxin. In: Cytokines (Eds: Mire-Sluis A, Thorpe R) Academic Press, 1998, pp. 335–360.
5. *Budd RC*: Death receptors couple to both cell proliferation and apoptosis. J Clin Invest 109: 437–442, 2002.
6. *Choy EHS, Panayi GS:* Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med 344: 907–916, 2001.
7. *Economides AN,Carpenter LR,Rudge JS, et al:* Cytokine traps: multi-component, high-affinity blockers of cytokine action. Nat Med 9: 47-52, 2003.
8. *Goodman Gilman A, Rall TW, Nies AS, Taylor P:* The pharmacological basis of therapeutics. Pergamon Press – 8th edition, 1990.
9. *Hayashida K, Nanki T, Girschick H, et al:* Synovial stromal cells from rheumatoid arthritis patients attract monocytes by producing MCP and IL8. Arthritis Res 3: 118–126, 2001.
10. *Jensen PE, Weber DA, Thayer WP, et al:* Peptide exchange in MHC molecules. Immunol Rev 172: 229–238, 1999.
11. *Keane J, Gershon S, Wise RP, et al:* Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med 345: 1098-1104, 2001.
12. *Kvien TK, Uhlig T, Kristiansen IS:* Criteria for TNF-targeted therapy in rheumatoid arthritis. Drugs 61: 1711-1720, 2001.
13. *Lee DM, Weinblatt ME:* Rheumatoid arthritis – Review. Lancet 358: 903–911, 2001.
14. *Li Q, Verma IM:* NFB regulation in the immune system. Nat Rev Immunol 2: 725-734, 2002.
15. *Liu ZG, Hsu H, Goeddel DV, Karin M*: Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell 87: 565–576, 1996.
16. *Morita Y, Yang J, Gupta R, et al:* Dendritic cells genetically engineered to express IL-4 inhibit murine collagen-induced arthritis. J Clin Invest 107: 1275–1284, 2001.
17. *Nakajima A, Seroogy CM, Sandora MR, et al*: Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. J Clin Invest 107: 1293–1301, 2001.
18. *Nanki T, Lipsky PE:* Cytokine, activation marker, and chemokine receptor expression by individual CD4+ memory T cells in rheumatoid arthritis synovium. Arthritis Res 2: 415–423, 2000.
19. PubMed/Swissprot Accession No P20333.
20. *Reed JC:* Apoptosis-based therapies. Nat Rev Drug Disc 1: 111-121, 2002.
21. *Yung RL:* Etanercept Immunex. Curr Opin Investig Drugs 2: 216–221, 2001.
22. *Vingsbo-Lundberg C, Nordquist N, Olofsson P, et al:* Genetic control of arthritis onset, severity and chronicity in a model for rheumatoid arthritis in rats. Nat Genet 4: 401-404, 1998.
23. *Wagner UG, Koetz K, Weyand CM, Goronzy JJ:* Perturbation of the T cell repertoire in rheumatoid arthritis. Proc Natl Acad.Sci USA 95: 14447–14452, 1998.