PhD
Program in Translational
and Molecular Medicine
DIMET

(XXII cycle, academic year 2008-2009)

University of Milano-Bicocca
School of Medicine and Faculty of Science

Effect of anti-TNF therapy on T cell activation and effector functions in patients with chronic inflammatory diseases

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The research presented in this thesis was performed at INGM-National Institute of Molecular Genetics, Milan, headed by Prof. Sergio Abrignani, in collaboration with the Department of Dermatology-IRCCS Istituto Ortopedico Galeazzi, Milan and the Unit of Gastroenterology II IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan.

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Chapter 1

General Introduction

1. History of TNF: discovering its role in Immune System

Tumor Necrosis Factor (TNF) was identified in 1975 by Old and coworkers, as a macrophages cell product responsible for LPS-induced apoptosis of different cell types, including tumor cells (1). It was independently described as "cachectin", a factor that caused fever and death in mouse models during parasitic infection (2). When TNF cDNA was cloned (3) and the TNF protein was purified (4) it became clear that it was similar to lymphotoxin (LT-α). TNF and LT-α were the first-identified members of a large and growing gene superfamily (5) collectively known as TNF- and TNFR-related superfamily. The evidences of the effect of TNF-α on tumor cells (6) led to the hypothesis that it could be used as an anti-tumor agent, but its high systemic toxicity unattended these expectations. In parallel, further studies enlightened the crucial role of TNF on inflammatory processes and demonstrated that TNF was implicated in the pathogenesis of human chronic inflammatory diseases, including psoriasis, rheumatoid arthritis and Crohn's disease (7).

2. TNF/TNFR superfamily

Currently more than 40 members of the TNF/TNFR superfamily have been identified.

Most of the TNF/TNFR family members are expressed by immune cells and are directly coupled to signaling pathways, crucial for

proliferation, survival, differentiation and other protective functions of immunocompetent cells.

2.1 TNF

TNF-α is a type II transmembrane proteins biologically active as a self assembling, non covalent bound, trimer (8).

Several immunocompetent cells and to a lower level other cell types, produce TNF protein. The main source of TNF are monocytes and macrophages, Th1 and Th17 T cells, cytotoxic T cells, NK, mast cells and neutrophils. Among non-immune cells, TNF is produced by astrocytes, microglia cells, keratinocytes, smooth muscle and endothelial cells.

TNF-α is a 233 amino acid and 23 kDa protein (Figure 1) synthesized in a transmembrane form that is biologically active and can bind and cross-link both the TNFR-I and –II receptors. The trimeric tertiary structure is assembled by the interaction of aromatic residues at hydrophobic interfaces between the 3 individual compact "jellyroll" chains (9). The soluble form of TNF-α is a 157 amino acid protein derived from the transmembrane precursor through proteolytic cleavage that keeps untouched the transmembrane and intracellular portions. The catalytic enzyme responsible of this cleavage is a membrane-bound metalloproteinase called TNF-α converting enzyme (TACE, ADAM17) (10).

The human TNF-α gene is located within the class III region of the major histocompatibility complex (MHC) on chromosome 6 (position 6p21.3) (11-13).

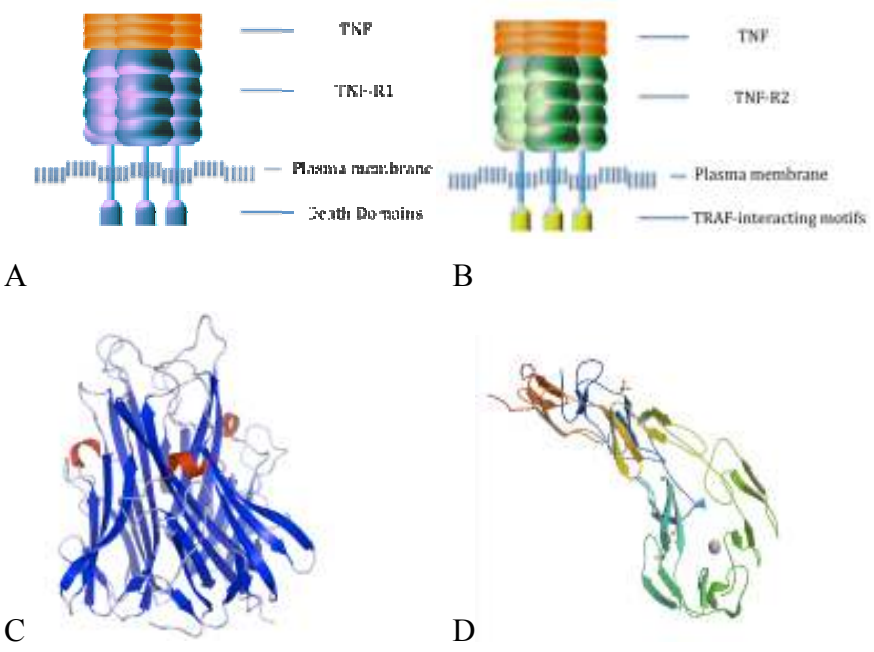

Figure 1: A) TNF bound to TNFR1 ; B) TNF bound to TNFR2; C) TNF crystal structure; D) TNFR crystal structure.

TNF-α gene is flanked by the genes encoding LT-α and LT-β and they closely resemble each other in genomic organization, consisting on 4 exones and 3 introns arranged over approximately 3 kb of DNA, probably deriving from a common ancestral gene (Figure 2A and 2B). Analysis of the transcriptional regulatory elements within the 5'-flanking region of the TNF-α gene has led to the identification of several elements that are important in the regulation of TNF-α gene expression, including a conserved kappa B element located downstream of the gene or the AU-reach elements (ARE) responsible for the stability of *TNF* mRNA (14-16).

A

B

Figure 2: A) TNF-α gene sequence; B) TNF-α genomic context.

In addition to the regulation at transcriptional level, TNF-α synthesis is also controlled at the level of mRNA elongation, mRNA processing, and at the level of translation (17).

Another regulatory site is at the level of proteolytic cleavage of transmembrane TNF-α protein that results in the release of the soluble TNF-α. The location of the TNF-α gene within the highly polymorphic MHC gene cluster arise the possibility that polymorphisms within the locus, including microsatellites and single nucleotide polymorphisms may play a role as genetic determinants of susceptibility to autoimmune and infectious diseases that are known to be MHC-linked (18). Both transmembrane and soluble form of TNF-α aggregate as homotrimers to efficiently cross-link the receptors (19). Even if with some differences, it has been shown that TNF membrane-bound form has more affinity for TNFR-II, compared with the soluble form (20).

2.2 TNFR

TNF-α can bind to two homotrimeric receptors: TNFR-I (p55) and TNFR-II (p75). These two receptors are type I transmembrane proteins characterized by cysteine-rich domains (CRD) that are the hallmark of the TNFR superfamily. 40 amino acid pseudorepeats are responsible for the formation of typical three intrachain disulphide bonds (21) (Figure 1D). Both TNF receptors are broadly expressed on different tissues, and most cell types, but in particular TNFR-I is the most frequently expressed in non-hematopietic cells, whereas TNFR-II is mainly expressed on T and B lymphocytes, NK cells, dendritic cells, monocytes and macrophages (22). Early studies using agonist antibodies have demonstrated that the two receptors signal distinct TNF activities (23). The majority of inflammatory responses classically attributed to TNF are mediated by the p55 TNFR.

Indeed, p55 was shown to be responsible for mediating cytotoxic signals and plays a critical role in mediating endotoxic shock, whereas p75 was shown to be capable of mediating proliferation signals in primary thymocytes and cytotoxic T cell lines (24). As compared to p55, p75 was found to be functionally predominant on activated T cells (25).

TNFR-I signaling is required for the host survival to infections with intracellular bacteria or parasites, such as *Mycobacterium tuberculosis* or *Leishmania major* (26-28), whereas TNFRII signaling is required for efficient response to extracellular fungal pathogen and is implicated in the development of experimental cerebral malaria (29). The members of the TNFR superfamily can be classified into three major groups based on their intracellular sequences.

TNFR-I belongs to the first group, including FAS, TRAIL-R1, -R2, -R4, containing a region, called death domain (DD), of 80 amino acids in its cytoplasmic domain. Activation of these receptors leads to recruitment of intracellular death domain containing adaptors, such as TNFR-associated death domain (TRADD) (30, 31). These molecules activate the caspase cascade and subsequently induce apoptosis.

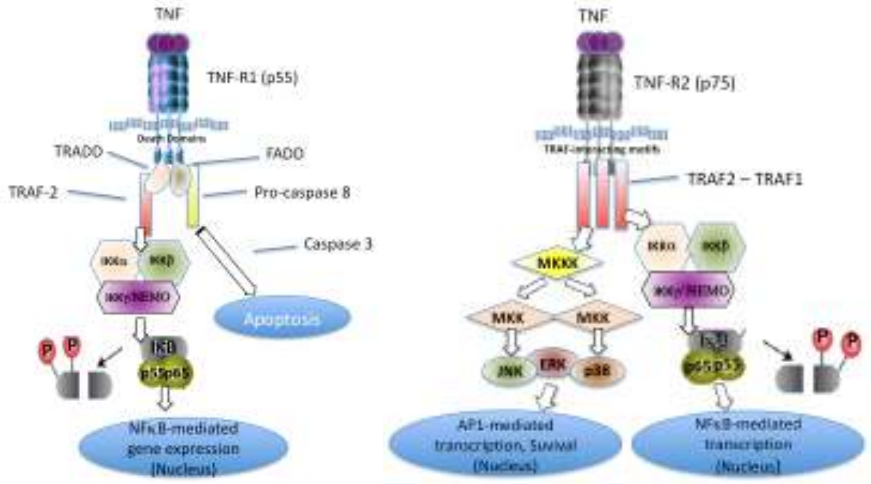

Figure 3: TNFR-I (left panel) and TNFR-II (right panel) signaling pathways.

Moreover, the activation of TNFR-I induces the activation and transcription of inflammatory genes through TRADD association with TNFR-associated factors (TRAF1 and TRAF2) and with receptor interacting protein (RIP), which leads to the activation of the nuclear factor-kB (NF-kB) and Jun N-terminal kinase (JNK) pathways (32-35) (Figure 3).

TNFR-II belongs to the second group of TNF-related receptors, that also includes CD27, CD40, LTβR, OX40, 4-1BB, RANK and that

contains TNF-receptor associated factor (TRAF)-interacting motifs (TIMs) in their cytoplasmic domain. Activation of TIM containing TNFR family members leads to the recruitment of TRAF and the subsequent activation of signal transduction pathways like those of NF-κB, PI3K, JNK, p38 and ERK (36). Upon stimulation TNFR-II can be cleaved from the cell surface or directly expressed as soluble isoform (Figure 3).

The third group of TNFR superfamily members including TRAIL-R3, and osteoprotegerin (OPG) do not contain signaling motifs but instead compete with the other two groups of receptors for their corresponding ligands.

3. Role of TNF in inflammation

TNF is one of the most important pro-inflammatory mediators with a central role in the inflammatory reactions mediated by the innate immune system. It is involved in the coordination of innate and adaptive immunity, as well as in the pathogenesis of the septic shock syndrome.

TNF production is triggered by different biological and physical inflammatory stimuli, including phorbol esters, ultraviolet light and bacterial or viral products. However, the major inducers of TNF are other cytokines including TNF itself, as well as IL-1, IL-2, IL-17, IFN-γ, TGF-β, GM-CSF. During the inflammatory processes, TNF signaling cascades leads to a range of cellular responses, which include cell death, survival, differentiation, proliferation and migration (37).

It is to note that the inflammatory effects of TNF are mediated mainly by the activation of the transcription factors NF-κB, AP-1 and mitogen-activated protein kinases (MAPKs), through the ligation of TNFR-I and/or TNFR-II receptors.

During inflammation, vascular endothelial cells respond to TNF by undergoing a number of pro-inflammatory changes which increase leukocyte adhesion, diapedesis and transendothelial migrantion (37). These include expression of P-selectins, ICAM-1 and VCAM-1 integrins. Mice lacking TNFR-I or TNF gene succumbed to very small doses of pathogens, such as *L. monocytogenes*, mycobacteria, *T. gondii* (38). Indeed, T cell-derived TNF induces the efficient NO production by activated macrophages and enhances their microbial killing. TNF is also a key factor for triggering the cross-talk between NK cells and dendritic cells (DC), a process that links innate and adaptive immunity: Conversely, TNF inhibition is mainly mediated by endogenous signals, such as IL-4, IL-6, anti-inflammatory cytokines, corticosteroids and prostaglandins. However, cellular responses to TNF vary depending on the cell types and microenvironment and are controlled by the balance among the activation of various pathways (39). Indeed, the protective effect of TNF in the course of infections is due to the action of strictly regulated small amounts of cytokine. On the contrary, when TNF is present in the systemic circulation, it produces toxicity in susceptible organs and could lead to several adverse effects. In the case of sepsis, the presence of infection in the blood is accompanied by release of TNF by macrophages in the liver, spleen and other sites leading to increased vascular permeability, loss

of plasma volume, increased blood pressure and intravascular coagulation (40).

4. TNF: role in the pathogenesis of chronic inflammatory diseases and anti-TNF immunotherapy.

The central role of TNF-α in the pathogenesis of several immuno-mediated and inflammatory disorders has been described. The pathogenetic role of TNF in chronic inflammatory diseases has been initially suggested by the finding that high levels of TNF were present in the synovium of joints in rheumatoid arthritis (41), where TNF-α mediates both inflammatory synovitis and articular matrix degradation (42). The level of TNF-α has also been found to be enhanced in psoriatic lesions as compared to the normal skin of psoriatic patients and it has multiple potential effects in the pathogenesis of this disease. TNF-α-directed biologic immunotherapy is a successful tool for the treatment of many inflammatory diseases (43). In particular, among the best studied are rheumatoid arthritis, Crohn's disease, and psoriasis/psoriatic arthritis.

4.1 Psoriasis

Psoriasis is a chronic inflammatory disease that affects the skin and joints of approximately 2% of the world's population. It commonly causes red, scaly patches to appear in the skin. The scaly patches caused by psoriasis, called psoriatic plaques, are areas of inflammation and keratinocyte hyperproliferation. Keratinocytes

rapidly accumulates at these sites and takes on a silvery-white appearance. Plaques frequently occur in the skin of the elbows and knees, but can affect any area including the scalp and genitals. In contrast to eczema, psoriasis is more likely to be found on the extensor aspect of the joint. The disorder is a chronic recurring condition that varies in severity from minor localized patches to complete body coverage. Psoriasis can also cause inflammation of the joints, which is known as psoriatic arthritis. 10-15% of people with psoriasis develop psoriatic arthritis. The genetic basis of psoriasis are known to be complex, with ten or more susceptibility loci, that are likely to be influenced by various environmental factors that act in the skin and/or immune system (44). Homozygous twins did not develop psoriasis together and factors that may augment the risk for disease development include stress, excessive alcohol consumption, and smoking (45). So far, 10-20 chromosome regions have been proposed to contain psoriasis genes, but very few genes have been identified (46). For example, the identity of the psoriasis susceptibility 1 locus *(PSORS1)* remains controversial One *PSORS1*-harbouring locus identified is the class I region of the major histocompatibility locus antigen cluster (MHC) HLA-Cw6, but whether the *PSORS1* locus is a classical MHC allele or a regulatory region is still unclear (47). Moreover, its low penetrance (about 10%) indicates that other important genetic and environmental factors are involved.

Gene expression studies revealed a molecular circuitry of inflammation in this disease and indicated that several (about 1300) genes are differentially expressed in psoriatic lesions as compared to non-psoriatic skin. The most of them are known to be regulated by

STAT and NF-κB transcription factors (44). In turn, key factors that can activate these transcription factors include TNF, LT, IL-1, IL-17, IL-20, IL-22 and IFNγ□cytokines□ (Figure 4).

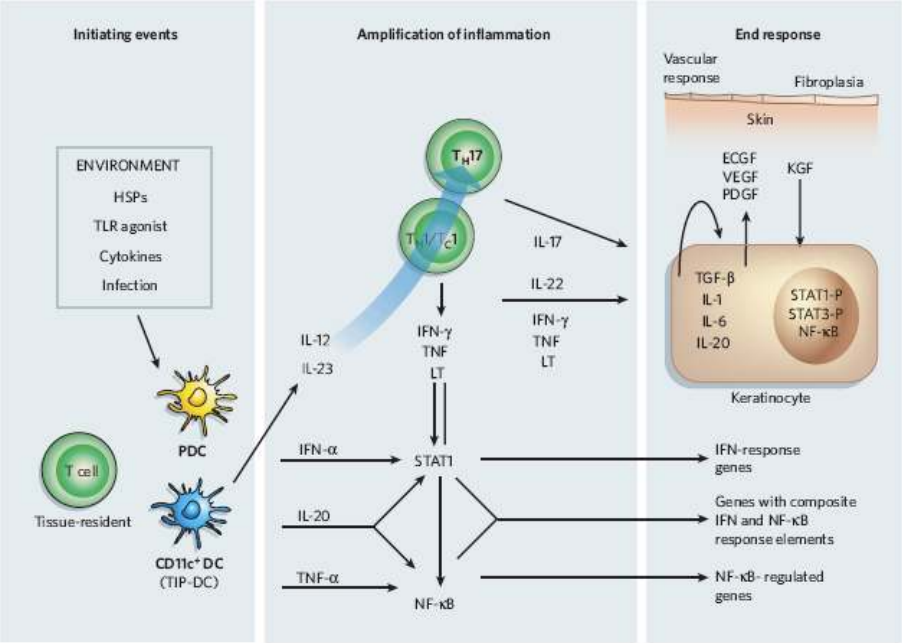

Figure 4: Cytokine network in psoriasis (Modified from Lowes et al., Nat. Rev. Immunol. 2007).

Activated DCs may contribute to the inflammation producing IFN-α, IL-12 and IL-23 and leading to the activation of T cells (blue arrow, Figure 4) that, in turn, synthesized T-cell-derived inflammatory cytokines (TNF, IFN-γ, IL-17). This cross-talk between DC and T cells develop in a self-sufficient circle that does not need any other stimuli to maintain the inflammation. Other cytokines synthesized by keratinocytes or stromal cells probably cross-regulate the epithelial–

stromal hyperplasia and fibroplasias: TGF-β, IL-1, IL-6 may act as keratinocyte autocrine and/or paracrine growth factors. However, TNF is one of the major cytokines produced in the psoriasic inflamed skin and is made by multiple cell types, including keratinocytes, Langerhans' cells and dermal mast cells (43). It induces the maturation of Langerhans' cells and is capable of promoting Langerhans' cell migration from the skin to the lymph nodes, where the process of T cell activation is initiated by antigen presentation. TNF may also influence cellular infiltration in the skin by inducing the expression of adhesion molecules on endothelial cells and keratinocytes. Finally, it may have a direct positive effect on keratinocyte proliferation and maturation (48).

4.2 Crohn's disease

Crohn's disease (CD) and ulcerative colitis are Inflammatory Bowel Diseases (IBD) characterized by strong cytokine-driven inflammation of the gut (49). Crohn's disease may affect any part of the gastrointestinal tract, causing a wide variety of symptoms. It primarily causes abdominal pain, diarrhea, vomiting, or weight loss, but may also cause complications outside of the gastrointestinal tract such as skin rashes and arthritis (50). Males and females are equally affected. Smokers are three times more likely to develop Crohn's disease (51).

It has been recently reported the association with more than 30 susceptibility loci (52), including those containing *CCR6* chemokine receptor, *STAT3* and *JAK2* transcription factors genes. Similarly, it

has been found a large environmental component, evidenced by the higher number of cases in western industrialized nations.

Although this pathology involves multiple genes and environmental conditions, about 10-20 % of patients with Crohn's disease present a mutant form of gene encoding NOD2 (53). NOD (nucleotide-binding oligomerization domain) proteins are intracellular pattern-recognition molecules implicated in the detection of bacterial peptidoglycans. It is associated with receptor interacting proteins (RIP2) that leads to NF-κB activation, through which it is involved in the regulation of pro-inflammatory response. Mutations found are gene frameshift mutations that alters the ability of the protein to sense the natural ligand and if it produces an increased or impared signaling is still unclear.

TNF-α expression in human macrophages was discovered in the colonic tissue and macrophages in both patients with CD and UC and serum levels of TNF-α correlate with clinical and laboratory indexes of intestinal disease activity (55-57). It is known, by early *ex-vivo* studies, that TNF could drive the overproduction of metalloproteinases (MMP) by mesenchiamal cells subsequent to T cell activation in the intestinal mucosa (54). MMPs overexpression by both mesenchiamal and activated T cells led to tissue injury and mucosal distruction.

Crohn's disease is associated with high Th1 and Th17 cytokine production (58, 59) that causes discontinuous ulceration and full thickness bowel wall inflammation, often including granulomas, in the small bowel and colon. Crohn's disease defects arise from a mucosal

immune system that overreacts to normal constituents of the mucosal microflora (Figure 5) (60, 61).

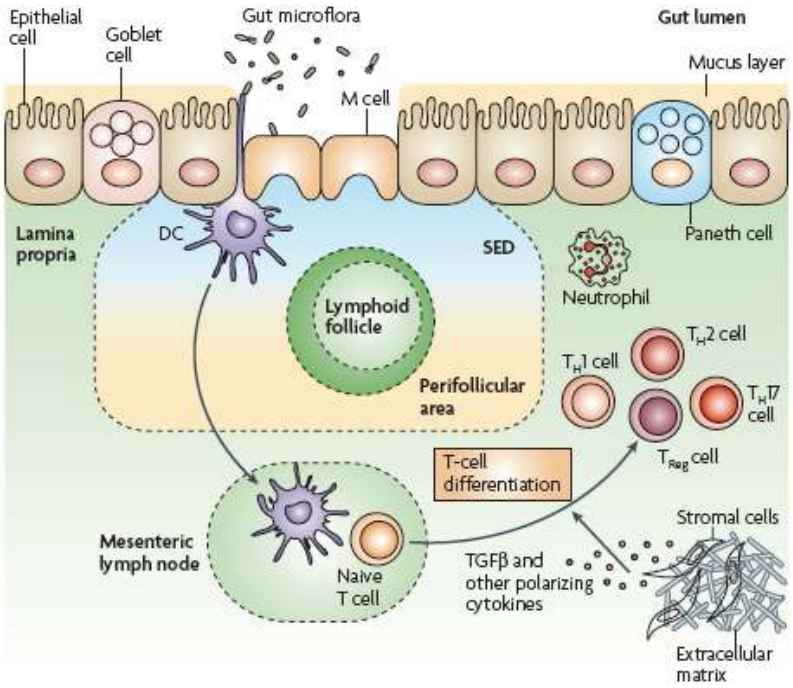

Figure 5: Key features of IBD. (Modified from Cho et al., Nat. Rev. Immunol. 2008).

Soluble TNFR-I and TNFR-II levels correlate with disease activity in IBD patients. More specifically, soluble TNFR-I is up-regulated in the serum of IBD patients as compared with healthy controls and could be used as a marker for disease activity (62). Soluble TNFR-II levels are significantly more elevated in serum from active CD patients as compared to UC and could be used as an additional parameter to discriminate the two diseases (63). Recently TNF receptor type 1-dependent activation of innate responses was shown to reduce intestinal damage-associated mortality (64).

Within this context, existing conventional treatments such as corticosteroids, and immunosuppressants aim broadly to block downstream inflammatory events such as the secretion of cytokines and the activation of neutrophils, regardless of the nature of the underlying T cell response that generated these events. These agents have sustained treatment of CD for many years despite their high toxicities. Conversely, antibodies against TNF-α have shown to target the mechanisms of inflammation more narrowly by eliminating a specific major inflammatory cytokine or by disrupting accumulation of cells at areas of inflammation (65).

4.3 Rationale for the use of TNF-blocking agents

TNF was not an obvious therapeutic target for the treatment of chronic inflammatory disorders, even if it was present in the inflamed tissues. Indeed, several other pro-inflammatory cytokines, such as IL-1, IL-6, GM-CSF, IL-8 and IL-17 were present as well.

The first indication that TNF could be a good target was provided by a study showing that pro-inflammatory cytokine production by dissociated rheumatoid synovial membrane cultures was dependent on the presence of TNF, but not on the presence of the closely related LTα (66). Other evidences in the same direction, were provided by the observation that mouse models developing erosive arthritis or strong colitis upregulated TNF-α and TNFR (67). Finally, the observation that the administration of TNF-α-specific mAb in these models was beneficial in vivo and could prevent experimental rheumatoid arthritis induced by type II collagen (68), (69)

conclusively defined the rationale for the use of TNF-blocking therapy.

Three anti-TNF-α agents are currently available for clinical use: etanercept, infliximab and adalimumab.

Chimeric monoclonal anti-TNF®, Infliximab (Remicade™) antibody comprises the mouse variable region Fv and the human constant Fc portion of the IgG1 immunoglobulins with high affinity and neutralizing capacity for TNF and poor toxicity (70).

Adalimumab is a recombinant fully human IgG1 monoclonal antibody specific for human TNF-α and shows very low toxicity.

Etanercept (Enbrel™) is a engineered p75 TNFR-II dimers linked to Fc portion of IgG immunoglobulin. Dimeric TNF-R-based TNF-blocking agents were found to be more effective in competing with the binding of TNF to the membrane receptors than a mononomer. Etanercept is an effective inhibitor, as demonstrated by studies in animal models (71) and subsequently by clinical trials.

There are evidences from studies on animal models and from clinical trials indicating that neutralization of TNF differentially modulates disease activity in chronic inflammatory diseases.

The effect of TNF-blockade is therapeutic in disorders in which TNF is overexpressed by monocytes and macrophages, and so it allows for the amelioration of the clinical features of the pathology (72). Indeed, the blockade of TNF activity in patients with rheumatoid arthritis (RA) by anti-TNF or soluble TNFR antagonists results in a dramatic decrease in disease activity and in some cases a complete remission although disease recurs after cessation of the therapy (73).

The blockade of TNF on RA patients showed to be able to inhibit other downstream cytokines. IL-6 is present at elevated levels in serum of RA patient and normalizes within few days after TNF-blocking. Reduced IL-8, MCP-1, IL-1, VEGF (and associated angiogenesis) serum levels, have been reported in RA patients undergoing anti-TNF therapy (74-76). In addition, a reduction of E-selectin, ICAM-1 and VCAM-1 adhesion molecules in the synovium of anti-TNF treated RA patients was also observed (77, 78).

In the treatment of psoriasis, the progressive changes in inflammatory cytokines and chemokines induced by TNF-blocking agents suggests that TNF strongly regulates some proximal cytokines, such as IL-1 and IL-8 and has more complex interactions to support inflammation driven by IFN-γ and STAT pathways (79). In addition, several DC products, such as iNOS and IL-23 are likely to be regulated by this cytokine and were found to be inhibited by anti-TNF therapy (80). However, the therapeutic actions of TNF inhibitors might not be as simple as blockade of the soluble cytokine, because Infliximab and Adalimumab could bind and block also the membrane-bound form of TNF, therefore modifying the biology of TNF expressing cells through ligation of surface complexes or even induction of apoptosis (81).

In Crohn's disease (CD), anti-TNF therapy results in a dramatic decrease in symptoms in up to 80% of the patients (82). Clinical studies have reported a dramatic improvement in CD patients treated with anti-TNF-α therapy such as infliximab and adalimumab (63). Reductions in the number of IFN-γ producing, lamina propria

mononuclear cells (LPMC) in colonic biopsies results from anti-TNF-α treated patients (83).

In the case of psoriasis, treatment of patients with anti-TNF leads to the clearing of skin lesions and a decrease in associated arthritis incidents. Patients receiving an anti-TNF-alpha agent as monotherapy experienced a high degree of clinical benefit and a rapid time of response to the treatment of moderate to severe plaque psoriasis compared with patients who received placebo (84).

In contrast, there are indications of increased of clinical severity of some disorders when treated with TNF-blocking agents. Multiple sclerosis (MS) and patients treated with anti-TNF mAb or soluble TNFR, had showed enhanced CNS lesions and disease activity in some cases (85-87). This was further supported by studies on mouse models, indicating that TNF-deficiency leads to exacerbation of autoimmune pathologies, such as experimental autoimmune encephalomyelitis (EAE) and murine lupus, with earlier disease onset and increased severity (88, 89).

This controversy in the effect of TNF therapy in different immune-mediated pathologies, points out the increasing need to clarify the impact of TNF blockade on systemic immunological homeostasis and on T cell mediated immune responses.

5. Introduction to T cell function

T lymphocytes can be distinguished in two compartments: the cytotoxic CD8+ T subset and the helper CD4+ T subset.

CD8+ T cells mediate effector functions and killing of viral infected cell, whereas CD4+ T cells play a central role in orchestrating a wide

range of immune responses. CD4+ T cells posses the ability to induce B cells to produce antibodies, macrophages to increase microbicidal activities and also the capacity to recruit cells of the adaptive and innate immunity to the sites of infection or inflammation.

Naïve CD4+ T cells can differentiate into at least 4 distinct groups determined by the pattern of signals they receive during their initial interaction with antigen: Th1, Th2, Th17 and iTreg (Figure 6).

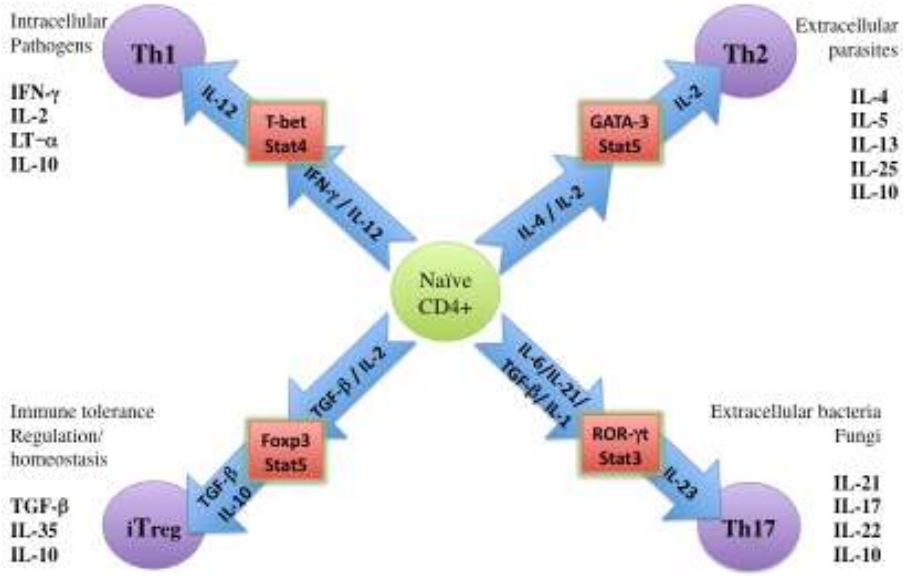

Figure 6: Summary of naïve-deriving CD4+ T lymphocyte subsets: cytokines and transcription factors critical for the differentiation and main functions are displayed.

Th1 cells mediate immune responses against intracellular pathogens (90, 91) and are particularly important for the resistance to mycobacterial infections. Pathogen-derived antigens induces the production of IL-12, mainly from dendritic cells, that plays the major inductive role for Th1 cells. Indeed, IL-12 appears to induce some IFN-γ production which then acts to up-regulate the key transcription

factor T-bet (92, 93) and leads to much more IFN-γ production, showing a positive feedback loop for Th1 cells as well.

Th1 principal cytokine products are IFN-γ, lymphotoxin α (LT-α), and IL-2. IFN-γ produced by Th1 cells is important in activating macrophages (94). LT-α has been identified as a marker for the disease progression in multiple sclerosis patients (95). IL-2 cytokine is essential for proliferation and long-term survival of lymphocytes (96).

Th2 cells mediate host defense against extracellular parasites including helminthes (90, 91). Naïve CD4 T cells could be induced to produce in vitro IL-4 if they were stimulated both with T-cell receptor ligands and IL-4 (97, 98). This in vitro differentiation requires a signaling pathway that includes the IL-4 receptor, the signal transducer and activator of transcription (Stat) 6 and the DNA-binding factor GATA-3 (99, 100). Th2 cells produce IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 (IL-17e). IL-4 is the positive feedback cytokine for Th2 cell differentiation and is the major mediator of IgE class switching in B cells (101), It leads to the production of several other cytokines, including IL-5, IL-13 and TNF-α. IL-5 and IL-9 play a critical role in recruiting eosinophils (102) and induce mucin production in epithelial cells during allergic reactions (103). IL-13 is the effector cytokine in the defence against of helminths and in the induction of airway hypersensitivity (104, 105).

Th17 cells were very recently identified and showed to play an important role in mediating immune response against fungi and extracellular bacteria (106). Th17 differentiation from naïve CD4 T cells requires TcR stimulation in the presence of IL-6, IL-1 and TGF-

β (107, 108). They produce IL-17a, IL-17f, IL-21, and IL-22. RORγt transcription factor has been identified as the master regulator of Th17 differentiation, but it is to note that IL-6, IL-21 and IL-23 use Stat3 for signal transduction (109). IL-21 is a stimulatory factor for Th17 differentiation and serves as positive feedback amplifier (110, 111). IL-17a and IL-17f can induce many inflammatory cytokines including IL-6 and chemokines such as IL-8, therefore playing a key role in promoting inflammatory responses (112).

Regulatory T cells (Treg) play a critical role in maintaining self-tolerance as well as in regulating immune responses (113). They are distinguished in natural occurring nTreg that directly differentiates in the thymus and inducible iTreg that differentiate in the peripheral compartment through the action of specific signals. In 2003, Foxp3 was reported as the master transcriptional regulator for natural occurring Treg cells (114), but high doses of TGF-β may also result in the induction of Foxp3+ iTreg cells from naïve CD4+ Foxp3- T cells (115). The association of NF-AT transcription factor with Foxp3 is required for the differentiation of regulatory T cells (Treg) (116, 117). Both nTreg and iTreg cells exert their suppressive functions through mechanisms requiring cell-cell contact (118) and through their production of cytokines, including TGF-β, IL-10, and IL-35.
IL-10 is cytokine with a potent immunosuppressive and anti-inflammatory activity. Its key function is to inhibit the production of pro-inflammatory cytokines, such as TNF, IFN-γ, IL-6, IL-12 (119). Il-10 was initially described as Th2-produced cytokine with the capacity to suppress Th1 cell proliferation (120), but IL-10 is also

produced by Th1, NKT cells, B cells, macrophages, Treg cells and keratinocytes. IL-10 production is also critical to suppress dendritic cell function (119) and for Treg-mediated prevention and cure of inflammatory bowel disease (121). Overall, the effect of IL-10 is to limit inflammatory reactions, prevent host damage and maintain self-tolerance.

5.1 T cell activation and TcR signaling pathway

T cell receptor (TcR) recognizes antigen-derived peptides bound to major histocompatibility complex (MHC) proteins on antigen presenting cells (APC). The interaction between the specific peptide-MHC complex and the TcR lead to TcR stimulation, which, in turn, leads to T cell cytokine secretion and promote proliferation and differentiation. This processes is collectively referred as T cell activation (Figure 7).

Initial evidences for the comprehension of the crucial events of TcR signaling pathway came from the observation that TcR-deficient Jurkat T cells could be stimulated with phorbol esters, which activates protein kinase C (PKC), and with Ca^{++} ionophores (122). These observations suggested that paradigm that TcR ligation transduces signals through PLC-γ to produce IP_3 and DAG, so activating PKC and inducing Ca^{++} mobilization. Protein tyrosin kinases (PTKs) could activate PLC-γ and cytosolic PTKs of the Src family (in particular Lck and Fyn) were being described in T cells. The recruitment of cytosolic PTK is due to the presence of motifs, designated immunoreceptor tyrosine-based activation motifs (ITAMs), which are phosphorilated by the PTKs (123). They serve as docking sites for the

recruitment of other kineses, such as the 70-kDa phosphoprotein Syk kinase family member ZAP-70 (ζ-associated protein of 70 kDa) (124).

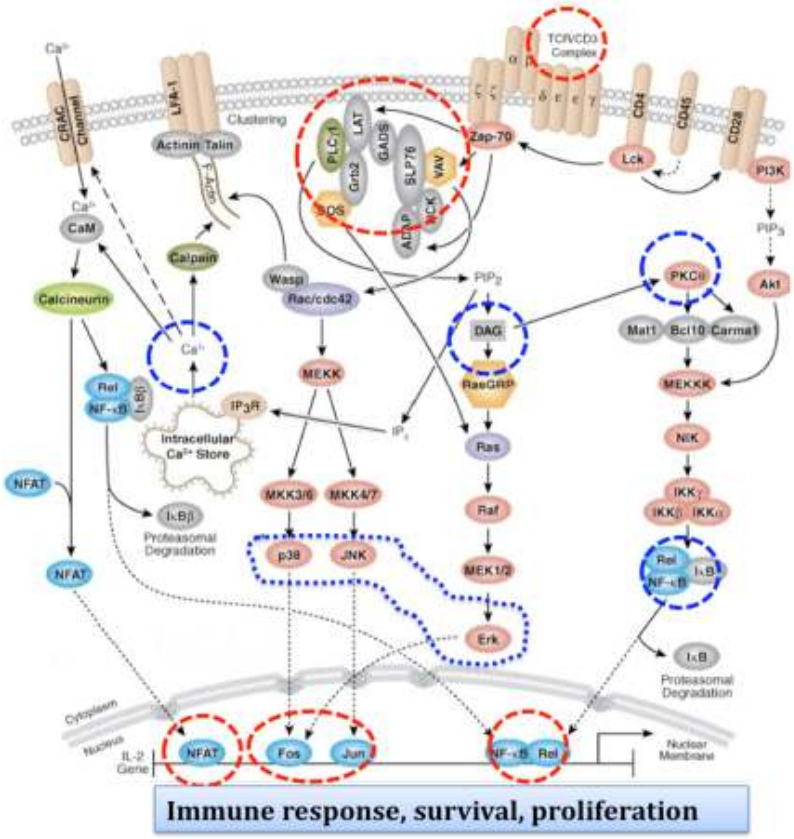

Figure 7: T cell activation and TcR signaling pathways. Modified from Cell Signaling technology® www.cellsignal.com.

Among the most important of the ZAP-70 targets are the transmembrane adapter protein LAT and the cytosolic adapter protein SLP-76 (124, 125). Both LAT and SLP-76 bind PLC-γ. Activated PLCγ1 hydrolyzes the membrane lipid $PI(4,5)P_2$, producing the second messengers IP_3 and DAG.

IP_3 stimulates IP_3R ion channel receptors on the endoplasmic

reticulum membrane, so inducing the release of Ca^{++} stores into the cytoplasm. This results in the activation of Ca^{++}-dependent transcription factors and to the dephosphorilation of the nuclear factor NFAT, thereby allowing its translocation to the nucleus. NFAT is a transcription factor family present in most cells of immune system and integrates several signaling pathways, including thymocyte development, T-cell differentiation, self-tolerance and tissue specific gene expression during development.

Production of DAG results in the activation of two major pathways involving Ras and PKCθ. Ras is a guanine nucleotide–binding protein required for the mitogen-associated protein kinase (MAPK) phosphorylation and activation cascade leading to the phosphorylation and activation of the extracellular signal-regulated kinase Erk1 and Erk2 (126). PKCθ is also recruited to plasma membrane by DAG and activated by Lck. PKCθ signals results in the activation of a very important transcription factor complex for T cell activation: NF-kB (127). PKCθ promotes the formation of a complex (128, 129) to induce the degradation of the regulatory subunit IKKγ of the IκB kinase (IKK) complex. Then, the catalytic IKK subunits phosphorylate the inhibitors of NF-κB, the IκB proteins (130, 131), which retain NF-κB dimers in the cytoplasm. Degradation of IκBα releases the NF-κB heterodimers, which then migrate to the nucleus and regulate gene expression. Activation of NF-κB signaling can be triggered also by TNF-α cytokine binding to TNFRs, especially to TNFR-I. Ligation of TNFR-I results in TRADD-dependent TRAF2 adapter recruitment (132-134) and TRAF2-mediated degradation IKKγ (132, 135, 136).

5.2 Positive and negative regulation of TcR signaling

T cell activation solely through the TcR results in a nonresponsive state (anergy) and in the enhanced apoptosis. Coligation of other cell surface receptors provides additional signals required for anergy avoidance and productive T cell activation. However, activation of these signaling pathways is regulated to ensure that T cells respond to appropriate ligands and for the proper duration. The most important costimulatory molecule of T cell is CD28, the binding of which provides the second signal necessary to promote T cell priming and to inhibit the activation-induced cell death (137). Following binding of CD28 to its ligands CD80 or CD86 on APCs, the PI3K kinase associates with the cytoplasmic tail of CD28 (138) and converts phosphatidylinositol PIP_2 into PIP_3. This latter is a docking site for PDK1 kinase and Akt. Through the activation of Akt, CD28 enhances the nuclear translocation of NF-κB, which has positive effects also on the expression of prosurvival genes, including Bcl-xl, and NFAT-regulated genes, such as IL-2.

CD28 engagement, as well as the engagement of other costimulatory molecules (CD2, CD5, CD30, 4-1BB, OX40, ICOS, LFA-1) results primarily in a quantitative rather than a qualitative change in T cell activation parameters (137).

As with positive regulation of T cell signaling, negative regulation is mediated through both TcR-generated signals and those induced by other cell surface receptors. Cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) are two examples of such receptors that limit the expansion and activation of TcR-triggered T cells and are important of for maintaining self-tolerance. CTLA-4

inhibitory receptor binds (like CD28) CD80 and CD86 on APC, so competing for the sequestration of ligands of the costimulatory pathways. Both CTLA-4 and PD-1 recruit SHP1 phosphatase that dephosphorylates and inhibits Lck and Zap-70 (139-143).

5.3 Current evidences of the role of TNF in T cell activation

Controversial findings have been reported about the effect of TNF on T cell activation. One study conducted on a hemagglutinin-specific TcR-transgenic mouse model has suggested that chronic exposure (3 weeks) to TNF attenuates broad range of T cell responses, including T cell proliferation and cytokine production *in vivo* (144). TNF long-term *in vitro* exposure also attenuates TcR signaling analyzed by measuring intracellular Ca^{++} mobilization, it suppress both Th1 and Th2 responses in a time and dose dependent manner and these effects could be reversed by neutralizing antibodies. Other studies have indicated TNF as a negative regulator of Th1 T cell responses. In a mouse model of mycobacterial infection where $TNF^{-/-}$ deficient mice succumbed to lung infection because of tissue destruction resulting from uncontrolled type 1 immune syndrome. This syndrome was characterized by expansion of activated CD4+ and CD8+ T cells and overproduction of Th1 cytokines (145). In support of this hypothesis, a very recent report has evidenced expanded Th1 and Th17 cell populations in a model of collagen-induced arthritis upon treatment with TNFR-Fc fusion protein or anti-TNF monoclonal antibody (146). Moreover, similar effect was found in collagen-immunized TNFR

p55-/- but not p75-/- mice, indicating a broader role of TNFR-I–mediated signaling in this model.

In contrast with this hypothesis, another group has demonstrated in a TNFR-II (p75) deficient mouse models, that signaling through TNFR-II lowers the T cell activation threshold since that $p75^{-/-}$ CD8+ requires about 5-fold TcR agonists for undergo to proliferation. Furthermore, the hypo-proliferative response displayed by $p75^{-/-}$ mice was associated with delayed kinetics of induction of acute activation markers and decreased production of IL-2 and IFN-γ cytokines. These effects could be only partially rescued by CD28 signaling, underlining the importance of TNF costimulatory activity (147, 148). An independent study on $TNF^{-/-}$ TcR-transgenic mice highlighted also the importance of TNF in the modulation of Ag-induced T cell apoptosis, showing the effect of TNF on the modulation of several aspects of homeostasis of peripheral CD8+ T cells. In this model, T cells developed in the absence of endogenous TNF exhibited an impaired response to TcR stimulation. In particular, $TNF^{-/-}$ CD8+ T cells showed a quantitatively reduced survival and homeostatic proliferation, impaired peptide-induced activation accompanied by decreased binding activity of NFκB and NF-AT transcription factors. Moreover, the recognition of self-antigens by these cells, in the absence of TNF, led to expansion of autoreactive T cells. Thus, from these evidences emerges that TNF is required for optimal survival and maximal antigenic responses as well as tolerance induction (149). Although these two latter studies indicate a positive regulatory effect of TNF on T cells activation, it cannot be excluded that a defect due to

the absence of TNF at early stages of T cell development is responsible for increased T cell responses.

The qualitative and quantitative analysis on T cell activation in humans upon blockade of TNF has not been provided so far.

Scope of the thesis

The aim of my PhD thesis project has been to study the T cell mediated immune responses in patients with chronic inflammatory diseases upon therapy with TNF-blocking agents.
In particular, we have studied the T cell responses in the peripheral circulation in parallel with the evaluation of T cell associated gene expression in the inflamed tissues, in order to find a correlation with clinical parameter of disease regression.
This would finally provide a thorough overview of the immunological changes occurring in T cell functions at level of both peripheral circulation and target organ upon TNF-blockade in correlation with the clinical outcome of the therapy. This information could also lead to the identification of key therapeutic events that associates with amelioration of chronic inflammatory diseases.

The project has developed as follows:

- In the first part of the project we wanted to characterize in psoriasis patients the impact of anti-TNF therapy on T cell responses and cytokine homeostasis in the peripheral circulation and in the in psoriatic plaques in correlation with the clinical amelioration of the disease (Chapter 1: submitted manuscript: *Anti-TNF therapy increases peripheral T cell responses and IL-10 expression in psoriasis patients*.)

- The second part is still ongoing and has the purpose to define the immunological and cellular mechanisms underlying the

enhancing effect of TNF-blockade on T cell responses to TcR stimulation. In particular the effector functions, the proliferative response and the activation of TcR-signaling molecules in T cells will be evaluated at baseline and after TNF-blockade, with the aim to investigate the possible negative regulatory role of TNF on T cell functions (Chapter 2, *Ongoing Work*).

- The last subproject wants to extend the analysis of differential gene expression to the intestinal mucosa of patients with inflammatory bowel diseases. The analysis will include cytokine, chemokine and adhesion molecule-related genes, as well as genes encoding transcription factors and lineage associated markers. This part of the study is in an early phase and will describe the modulation of the cellular and cytokine network in the inflamed intestinal mucosa during TNF-blocking therapy. The study will be completed by statistical analysis to evaluate the correlation between the modulation of single gene expression and the disease activity and by immunofluorescence microscopy on intestinal biopsies from individual patients. The final aim is to identify key immunological events that associated with the clinical response in different type of chronic inflammatory diseases (Chapter 3, *Ongoing work*).

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Chapter 2

Anti-TNF therapy increases peripheral T cell responses and IL-10 expression in psoriasis patients.

Submitted paper

Anti-TNF therapy increases peripheral T cell responses and IL-10 expression in psoriasis patients

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Abstract

TNF-blocking therapy is successfully used in the treatment of immune-mediated and inflammatory diseases. Although the inhibition of inflammatory pathways in target tissues by anti-TNF therapy has been clearly described, the impact of TNF-blockade on peripheral T cell responses in humans is still unclear. Here we studied T cell effector functions in psoriasis patients before and after treatment with anti-TNF by measuring a wide panel of cytokines as well as cell division upon in vitro stimulation. In parallel, the modulation of T cell cytokine gene expression was evaluated in psoriatic skin lesions. Results clearly evidenced that TNF-blockade increases T cell cytokine responses, mainly Th1 and Th17, in peripheral lymphocytes upon

stimulation. Importantly, TNF-blockade also induced a potent enhancement of IL-10 expression by different subsets of circulating leukocytes that was found to correlate with the clinical outcome of the therapy.

Despite the enhanced T cell cytokine responses in the peripheral circulation, in psoriatic skin lesions the overall effect of TNF-blockade was a diminished expression of Th1 and Th17 cytokine genes, paralleled by augmented expression of *Il10*.

These evidences indicate a negative regulatory role of TNF on T cell activation and effector functions and enlighten a new role for TNF-blockade in the up-regulation of IL-10 that may participate to the shut down of the inflammatory reaction.

Introduction

Anti-TNF therapy has been successfully applied to the treatment of several autoimmune and inflammatory diseases, such as rheumatoid arthritis, psoriasis and Crohn's disease (1, 2). Tumor necrosis factor is a pleiotropic cytokine critical for inflammation, neogenesis of lymphoid tissues, and host defence against various pathogens. As a pro-inflammatory molecule, TNFα possesses multiple activities including the induction of cytokines IL-1, IL-6 and IL-8, macrophage and neutrophil activation and is a key mediator of tissue damage in psoriasis and other types of inflammatory diseases (1, 3).

Psoriasis is chronic inflammatory skin disease, characterized by skin infiltration of activated T cells and myeloid and plasmacytoid DC (4, 5). At dermal level, T cells and dendritic cells aggregate in organized

lymphoid tissues that trigger and perpetuate T cell responses, which in turn mediate the immunological damage through the secretion of Th1 and Th17 type cytokines (4, 6). TNF-blockade has been shown to down regulate Th17 cytokine expression and other downstream pro-inflammatory molecules such as IL-8 and MCP-1 in psoriatic skin lesions (3, 6). Despite the evidences provided regarding the regulation of inflammatory pathways in inflamed skin, the impact of TNF-blockade on systemic immunological homeostasis and on T cell mediated immune responses in humans is still unclear.

Evidences on mouse models and in vitro studies have suggested a negative regulatory role of TNF on helper T cell responses, but the effect of a functional knockdown of TNF on T cell activation and effector functions in autoimmune patients has not been completely characterized. A previous study has indicated TNF as a negative regulator of Th1 T cell responses in a mouse model of mycobacterial infection (7). In support of this hypothesis, a very recent report has evidenced expanded Th1 and Th17 cell populations in a model of collagen-induced arthritis upon treatment with TNF-blocking agents (8).

More controversial findings have been reported on the effect of TNF on T cell activation. One study in a TcR-transgenic mouse model has suggested that chronic exposure to TNF attenuates TcR signaling and T cell proliferative responses (9, 10). Conversely, another group has demonstrated in a TNFR(p75)-deficient mouse models, that signalling through TNFRp75 lowers the T cell activation threshold (11-13).

In addition, TNF-deficiency leads to exacerbation of certain autoimmune pathologies such as experimental autoimmune

encephalomyelitis (EAE) and murine lupus (14, 15). This controversy points out the increasing need to clarify the impact of TNF depletion on immunological homeostasis in a clinical situation.

A more extensive characterization has been provided on regulatory T cell function in autoimmune patients upon TNF-blockade. Studies in patients with rheumatoid arthritis have reported that anti-TNF treatment was able to reverse the functional defect of regulatory T cells by increasing their suppressive capacity and upregulating FoxP3 mRNA and protein expression (16, 17). Increase of immune suppression in anti-TNF treated patients through the induction of regulatory T cells producing TGF-β and IL-10 has also been described (18). IL-10 is another key mechanism for maintaining immunological homeostasis and preventing tissue damage (19-22). It exerts its anti-inflammatory activity by inhibiting secretion of pro-inflammatory cytokines by T cells as well as by impairing dendritic cells functions (23, 24). Recent studies have demonstrated that IL-10 production can represent a normal stage in the Th1 differentiation programme (19, 20, 22). Moreover, a very recent report has described IL-10/IFNγ-producing T cells as an effector-like cell subpopulation with regulatory function (25). So far, there are no clear indications of the effect of TNF-blockade on IL-10 expression by different subsets of T cells and leukocytes.

Here we studied, in psoriasis patients treated with anti-TNF, the peripheral T cell responses in terms of phenotype, cytokine expression and T cell proliferation in parallel with the modulation of cytokine gene expression in psoriatic skin lesions.

We demonstrated that TNF-blockade induced an enhanced T cell responses in circulating lymphocytes and a potent enhancement of IL-10 expression that was found to correlate with the clinical outcome of the therapy. In psoriatic skin lesions the overall effect of TNF-blockade was a diminished expression of Th1 and Th17 cytokine genes and increased expression of *Il10*.

Material and methods

Patient population

26 healthy donors (HD) from IRCCS Ospedale Maggiore Foundation (Milan, Italy) and 29 patients from IRCCS Istituto Ortopedico Galeazzi (Milan, Italy) with a diagnosis of cutaneous psoriasis with PASI (Psoriasis Area and Severity Index) > 10, ranging in age from 18 to 70 were enrolled in this study. Criteria for patient selection included the absence of comorbidities such as diabetes mellitus, genetic diseases, HCV or HIV infection. Patients undergoing treatment with either CSA, methotrexate or systemic corticosteroids during anti-TNF therapy or within 3 weeks before the beginning of the therapy were excluded from the study. Patients were treated with TNF-blocking agents Infliximab (5 mg/kg i.v. at weeks 0, 2, 6 and every 8 weeks), Etanercept (25 mg s.c. twice a week) or Adalimumab (80 mg s.c. twice in the first infusion, 40 mg after 1 week and then 40 mg every 15 days). Blood samples were collected before therapy and 1, 3 and 6 months after treatment. Punch biopsies were collected from lesional skin under untreated conditions and after one month therapy in the same lesional area. The study was approved by the ethical

commission and written informed consent was obtained from all patients and donors before they entered the study.

Isolation and culture of cells

Peripheral blood mononuclear cells (PBMC) were prepared from buffy coats obtained from healthy volunteers or whole blood from patients by Ficoll gradient centrifugation (Lympholyte®, Cedarlane® Hornby, Ontario, Canada). Isolation kit for CD14+ monocytes, CD19+ B cells, CD8+, CD4+CD25– and CD4+CD25+ lymphocytes were isolated using Miltenyi Biotec isolation kit (Bergisch Gladbach, Germany) according to the manufacturer's instructions. PBMC and purified leukocyte subpopulations were cultured at a density of 1×10^6 cells/ml for 24-48 hours in RMPI 1640 + GlutaMAX™-I medium 10% FCS (Gibco®, Invitrogen™, Carlsbad, CA), Sodium Pyruvate 1mM and non essential amino acid 0.1mM (Gibco®) in 96-well flat-plates (Nunc, Roskilde, Denmark). Total PBMC, CD4+CD25- T cells were cultured in the presence of 0.05 - 0.5 μg/ml plastic-bound anti-CD3 antibody (eBioscience, San Diego, CA; OKT3). CD14+ monocytes were cultured in the presence of 1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO, USA). CD19+ cell population was cultured in the presence of 5 μg/ml CpG (Primm srl, Milan, Italy; TCGTCGTTTTGTCGTTTTGTCGTT). Controls were performed in the absence of stimulation. Supernatants were collected after 24-48h and tested for cytokine quantification. Purity of cells was detected by FACS analysis and percentage of dead cells was determined by adding 7AAD fluorescent dye (BD Biosciences).

For the intracellular detection of cytokines, PBMC at a density of 1 x 10^6 were cultured in flat-bottom plates in the presence or absence of 10 μg/ml TSST-1 superantigen, 1 μg/ml anti-CD28 and 1 μg/ml CD49d (BD Biosciences). After the first 2 hours, Brefeldin A (GolgiPlug, BD Biosciences) was added overnight. For stimulation with H1N1 influenza-derived protein, PBMC were cultured as described above in the presence of 10 μg/ml H1N1. After 12 hours, brefeldin A was added overnight.

Cytokine assay

For soluble IL-2, IL-4, IL-5, IL-10, IFN-γ, TNF-α detection in the culture supernatants, the human cytometric bead array (CBA) kit for Th1/Th2 cytokines (BD Biosciences) was used, according to the manufacturer's instructions. Data analysis was performed using FCAP software (Soft Flow Inc., New Brighton, MN). For soluble IL-17 detection, human IL-17 duo-set® ELISA kit was used (R&D Systems, Minneapolis, MN).

CFSE dilution assay.

To asses proliferation, 1 x 10^6 PBMC from patients, were labelled with 0,5 μM CFSE obtained from Invitrogen (Eugene, Oregon). Cells were cultured in the presence of 0.05 μg/ml plastic-bound anti-CD3 mAb (eBioscience) or in uncoated wells. After 3 to 6 days of culture 5 x 10^5 cells were collected and stained for flow cytometric analysis. The exclusion of dead cells was performed by adding 7AAD fluorescent dye (BD Biosciences).

Monoclonal antibodies and flow cytometric analysis

For immunostaining the following monoclonal antibodies (BD Biosciences): mouse anti-human CD3-FITC, CD4-PerCP, CD4-APC, CD8-APC, CD8-PE, CD25-PE, CD69-PE-Cy5, IFN-γ-APC; rat anti-human IL-2-APC, IL-10-PE were used.

Freshly isolated peripheral blood mononuclear cells (PBMC) were stained with conjugated monoclonal antibodies at 4 °C for 10 min and fixed using CytoFix or, for intracellular staining, CitoFix&Perm solution (BD Biosciences).

For intracellular IFNγ detection on CD4+ T lymphocytes, 2 x 10^6 PBMC were stained with anti-CD4-PerCP and Live/Dead fluorescent dye (Live/Dead Fixable dead cell stain kit, Invitrogen). Lymphocytes were then permeabilized using CitoFix&Perm solution (BD Biosciences) and stained for APC-conjugated anti-IFNγ. Samples were acquired using FACSCalibur (BD Biosciences) flow cytometer and data were analyzed with the FlowJo software (Tree Star, Ashland, OR).

RNA extraction and Real Time PCR

Total RNA from PBMC and lymphocyte subpopulations was isolated using RNeasy Mini kit (QIAGEN Gmbh, Hilden, Germany) under RNase-free conditions according to the instructions of the manufacturer. RNA from skin biopsies was extracted from tissues frozen in liquid nitrogen using the RNeasy fibrous tissue Mini Kit (QIAGEN).

cDNA was synthesized from a constant amount of total mRNA using Superscript III first-strand synthesis supermix (Invitrogen) as

described by the manufacturer. Gene expression was evaluated using TaqMan Gene Expression Mastermix and commercially available TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). For each sample, PCR reaction was performed in triplicate. Data were normalized to GAPDH housekeeping gene expression. RNA samples that were analyzed by TaqMan Low Density array Human Immune Panel (Applied Biosystems) were retrotranscribed by High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the instructions of the manufacturer.

Statistical analysis

For cytokine quantification, expression of surface marker and for real time PCR experiments, significance levels were calculated by Student's t-test and Wilcoxon rank test. Correlation between the cytokine increase and the decrease of PASI score was calculated by linear regression analysis and expressed as R-squared value. Data were analyzed by Prism 5 software (GraphPad Software Inc., La Jolla, CA). *P* values of less than 0.05 were considered statistically significant.

Results

Anti-TNF therapy up-regulates cytokine expression in αCD3 stimulated PBMC from psoriasis patients.

In order to study the effect of TNF-blocking therapies on T cell-mediated immune responses, we first measured Th1/Th2/Th17 and IL-10 cytokine secretion by PBMC from psoriasis patients in response to polyclonal stimulation with αCD3 antibody, before and during anti-TNF therapy. We analysed 29 patients treated either with soluble-TNFRII-Fc fusion protein (Etanercept, n=16) or with anti-TNF antibodies, such as the chimeric antibody Infliximab (n=8) and the recombinant humanized antibody Adalimumab (n=5).

Figure 1A shows the cytokine production by individual patients before therapy, after 1 month anti-TNF therapy and by healthy donors.

Enhancement of cytokine production was observed in PBMC from patients undergoing anti-TNF therapy as compared to the untreated group upon stimulation with αCD3 antibody. In particular IL-10 was enhanced to the highest level, but a significant enhancement was also observed for IL-2, IL-17 and IFNγ expression.

IL-4 and IL-5 Th2 cytokines were only moderately increased and significance levels were not reached. In contrast, no differences were observed in cytokine secretion by PBMC from untreated patients as compared to the healthy control group. The data are referred primarily to 1 month anti-TNF therapy, but for several patients experiments were performed also at 3 and 6 months after therapy with similar results (data not shown). At each time point clinical conditions were evaluated and disease severity was quantified by PASI (Psoriasis Area and Severity Index) score.

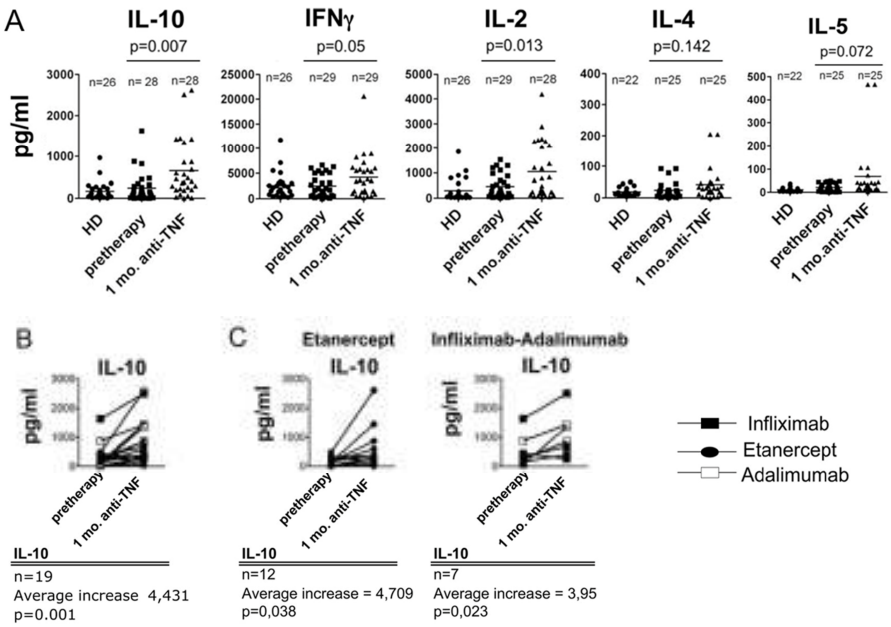


Figure 1: Increased cytokine expression in PBMC from anti-TNF-treated patients. PBMC from healthy donors and from psoriasis patients at baseline and after 1 month anti-TNF therapy (Infliximab, Etanercept, Adalimumab) were collected and activated with plastic-bound αCD3 antibody. (A) Th1, Th2, IL-10 and IL-17 cytokine production was measured in the supernatants after 24h. IL-10, IFN-g, IL-2, IL-4, IL-5, TNF-α and IL-17 cytokine levels secreted by PBMC from individual patients at baseline, after anti-TNF therapy (Infliximab, Etanercept or Adalimumab) and by healthy donor PBMC are shown in the figure. Significance levels were calculated by Student's t test and are indicated by horizontal bars. (B-C) IL-10 production in individual patients treated with different TNF-blocking agents. PBMC from patients before and after anti-TNF therapy were cultured in the presence of plastic-bound αCD3 for 24h and supernatants were collected for CBA analysis. IL-10 levels were measured in individual patients treated with Etanercept (n=12; filled circles) or monoclonal antibodies Infliximab and Adalimumab (n=7; filled square Infliximab, open square Adalimumab). Number of samples, cytokine average increase and significance levels, calculated by Student's t test, are reported below each diagram.

Similar data were obtained on PBMC from patients with Crohn's disease treated with TNF-blocking agents indicating that the phenomenon is attributable to the blockade of TNF function independently of the pathology (data not shown).

Longitudinal data analysis on PBMC from psoriasis patients evidenced that IL-10 enhancement occurred in 18 out of 19 individual patients with an average increase of 4.43 fold (Figure 1B). TNF-blockade significantly upregulated IL-10 production either with TNFR-based or with antibody-based treatment in individual patients as compared to their untreated counterparts (Etanercept, n=12 patients; Infliximab or Adalimumab, n=7 patients) (Figure 1C). Similarly to IL-10, other cytokines such as IL-2, IL-17, IFNγ□ □TNF-a and IL-4 were significantly upregulated to similar level in the two treatment groups (supplemental Figure 1).

Control experiments were performed by culturing in vitro αCD3-stimulated PBMC from patients before and after TNF-blockade, in the presence of soluble TNFR-Fc (Etanercept), showing no alteration in the increase of cytokine production induced by anti-TNF therapy (supplemental Figure 2).

Cytokine levels were also measured in the serum from patients before and after treatment, showing no detectable variations in IL-10, IL-2 or IFNγ induced by anti-TNF therapy (supplemental Figure 3).

The up-regulation of cytokine expression by anti-TNF was studied also by gene expression analysis on αCD3-stimulated PBMC by real-time RT PCR.

We analyzed the expression of a panel of T cell-derived cytokine genes as well as IL-10. Results in Figure 2 show that consistently

with cytokine secretion, mRNA for IL-10, IFNγ□and□ IL□2 was significantly enhanced after anti-TNF therapy.

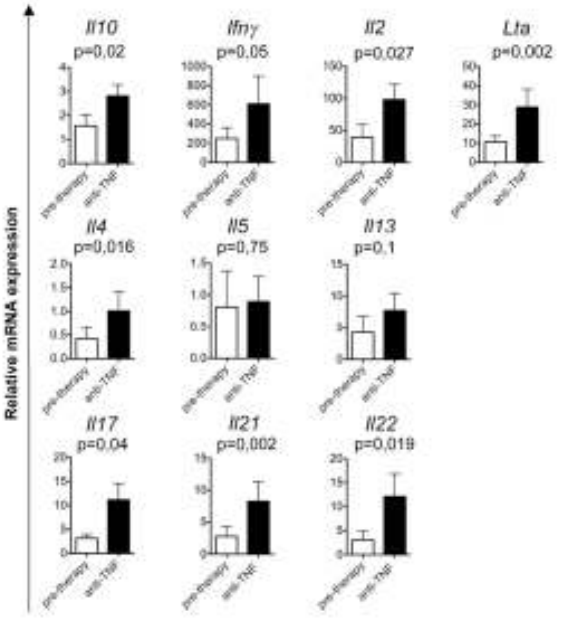


Figure 2: Multiple gene expression analysis in psoriasis patient PBMC. PBMC from psoriasis patients before (white bars) and after anti- TNF therapy (black bars) were cultured with plastic-bound αCD3 antibody for 5h and RNA was extracted and analyzed by quantitative real time PCR The mean ± SE of 10 experiments from different patients are presented relative to the expression of transcript encoding GAPDH. Each experiment was performed in triplicate. Significance levels, calculated by Wilcoxon Rank test, are shown in figure.

mRNA for Th2 cytokine IL-4 was also enhanced whereas *Il5* and *Il13* expression were not significantly altered.

Consistently, the expression of mRNA for IL-17, IL-21 and IL-22, Th17 cytokines was significantly increased after anti-TNF therapy. In parallel with the up-regulation of Th1, Th2 and Th17 cytokine

expression we also found in unstimulated PBMC an enhanced expression of *IL12B* gene encoding the common p40 subunit of IL-12 and IL-23 cytokines. A similar enhancement was observed in mRNA expression for the cytokine IL-27 which induces IL-10 and Th1 differentiation (22, 26) (supplemental Figure 4).

Increased IFNγ production by CD4 T cells in response to TSST-1 superantigen and antigen-specific stimulation in anti-TNF treated patients.

To determine whether upregulation of cytokine expression by anti-TNF corresponded to increased cytokine response to antigenic stimulation, we next evaluated the IFNγ response of CD4 T cells to in vitro activation with the staphylococcal superantigen TSST-1. Data in Figure 3A show that increased IFNγ production after TNF-blockade was observed in 3 out of 4 individual patients as determined by intracellular cytokine staining on CD4 T cells.

Similar results were obtained on CD4 T cells following in vitro stimulation with H1N1 influenza-derived protein (Figure 3B). This indicates that CD4 T cells have an increased cytokine response to antigen-specific stimulation following therapy with anti-TNF.

To define whether the effect of TNF-blockade on cytokine secretion corresponded to increased T cell activation in response to stimuli, we also evaluated the surface induction of the early T cell activation marker CD69, as well as CD4 and CD8 T cell proliferation, upon polyclonal activation of total PBMC. The surface induction of CD69 was evaluated in individual patients upon anti-TNF treatment and in untreated conditions. Data in Figure 3C show that CD3+ T

lymphocytes from treated patients displayed moderate but significantly increased expression of CD69 as compared to untreated counterparts.

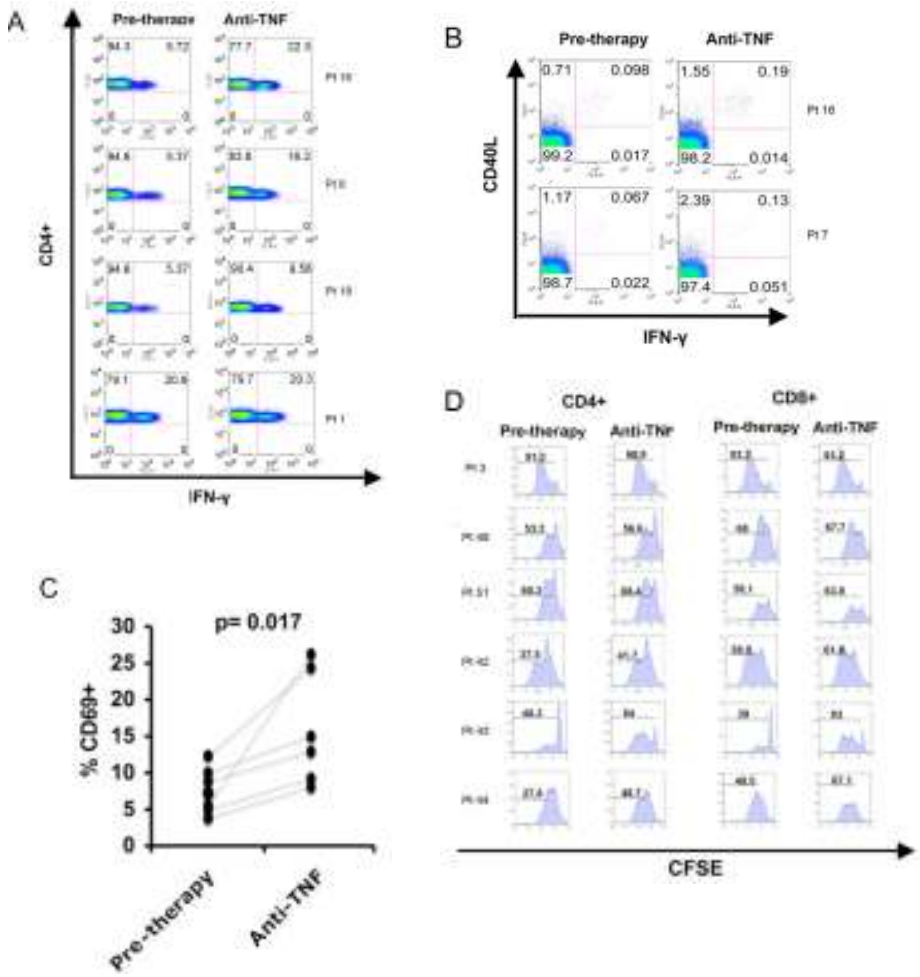


Figure 3: Increased IFN-g production by CD4+ T cells in response to TSST-1 and Influenza-derived antigen. (A) Intracellular cytokine staining for IFN-g on CD4+ gated T cells. PBMC were cultured for 12h in the presence of 10 µg/ml TSST-1 superantigen, 1 µg/ml aCD28 and 1 µg/ml aCD49d antibodies. Brefeldin A was added after the first 2h of stimulation. Shown are FACS analysis of CD4+ gated T cells from individual patients at baseline and after anti-TNF therapy (Pt1, Pt18=

Etanercept; Pt6, Pt16= Infliximab). (B) Costaining of IFN-g with CD40L on CD4+ gated cells. PBMC were cultured for 24h with 10 μg/ml H1N1 influenza-derived protein, 1 μg/ml anti-CD28 and 1 μg/ml CD49d. Brefeldin A was added in the last 12h. Shown are FACS analysis of CD4+ gated T cells from 2 individual patients before and during anti-TNF therapy (pt7= Etanercept; pt16= Infliximab). (C) Evaluation of the surface induction of CD69. PBMC were cultured for 6h in the presence of plastic-bound αCD3 antibody and CD69 surface expression was detected by flow cytometry. Percentages of CD69+ on CD3+ T cells in individual patients (n=6) analyzed before and after anti-TNF treatment are represented in the figure. (D) CD4+ and CD8+ T cell proliferation. PBMC from patients before and after 1 mo. anti-TNF therapy were labeled with 0.5 μM CFSE and cultured in presence of 0.05 μg/ml plastic-bound αCD3 for 6 days. Shown are FACS data on 7AAD gated cells. Numbers indicate the percentage of dividing CD4+ (left panels) and CD8+ (right panels) lymphocytes.

Proliferation of CD4 and CD8 T cells in response to αCD3 stimulation of total PBMC was not considerably altered in 4 out of 6 patients, after anti-TNF treatment as compared to baseline. Indeed no variation in the percentage of proliferating cells or in the number of cell divisions was observed (Figure 3D). However, in two patients that strongly up-regulated cytokine expression following anti-TNF therapy, both CD4+ and CD8+ T cell proliferation was augmented.

Finally, phenotype analysis on peripheral lymphocytes isolated from patients before and after anti-TNF therapy indicated that no increase either in the percentage of individual T and B cell lineage markers or in the percentage of peripheral T and B cells expressing activation markers (CD69, CD25, CD71 and HLA-DR) were induced by TNF-blockade (supplemental Table I). Together these data suggest that, in most of the patients, anti-TNF did not induce substantial increase in

the T cell activation state of circulating lymphocytes but rather increased the reactivity of T cells upon stimulation.

TNF-blockade upregulates gene expression of lineage-specific transcription factors T-bet and RORγt in CD4+CD25- T cells.

To further investigate the upregulation of cytokine expression in the T helper cell compartment, T cell derived cytokine analysis was performed on purified CD4+CD25- T cells stimulated with αCD3 showing increased production of IL-10, IFN-g, IL-2 and IL-17 cytokines (Figure 4A). Th2 cytokine IL-4 was also increased with a similar trend, however differences were not significant. Conversely, a moderate but significant increase was observed for IL-5. Data were confirmed by gene expression analysis on purified CD4+CD25- T cells (data not shown).

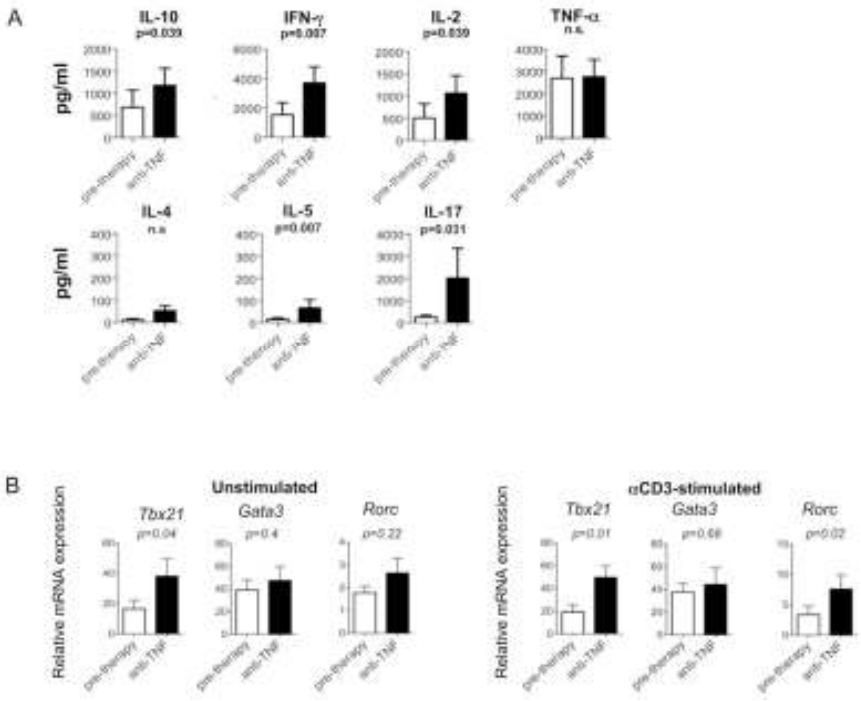


Figure 4: Increased cytokine secretion and *Tbx21, Gata3, Rorc* gene expression on CD4+CD25- T cells. (A) Cytokine secretion by αCD3-stimulated CD4+CD25- T cells from patients at baseline (white bars) and after therapy (black bars). Data represent the mean ± SE of 8 separate experiments performed on cells from different patients. Significance levels were calculated by Wilcoxon rank data analysis. (B) Gene expression analysis of lineage-specific transcription factors on CD4+25- cells. CD4+25- T cells isolated from individual patients before (white bars) and after TNF- blockade (black bars) were cultured for 5h in unstimulated or αCD3-stimulated conditions. RNA was extracted and analyzed by quantitative real time PCR for the expression of *Tbx21*, *Gata3* and *RORC* genes, encoding the lineage-specific transcription factors T-bet, GATA3 and RORγt respectively. Data represent the mean ± SE of 5 separated experiments performed on different patients relative to expression of GAPDH housekeeping gene. Each experiment was performed in triplicate.

To further investigate the Th1, Th2 and Th17 cytokine expression, we analyzed the level of mRNA for genes encoding the lineage-specific transcription factors T-bet, GATA3 and RORγt, in both unstimulated and αCD3 stimulated CD4+CD25- T cells from individual patients before and after anti-TNF therapy (Figure 4B). Consistently with cytokine production, we found that in CD4+CD25- T cells, *tbx21* was enhanced to significant levels in both unstimulated and αCD3-stimulated conditions (Figure 4B), whereas *RORC* expression was significantly enhanced only in αCD3-stimulated CD4+25- cells. *GATA3* expression was not considerably altered upon anti-TNF treatment (Figure 4 B).

Upregulation of IL-10 expression occurs in all IL-10-producing cell subsets and positively correlates with the clinical outcome of the therapy.

To understand whether the modulation of pro-inflammatory and anti-inflammatory cytokines induced by anti-TNF could correlate with the clinical response to the treatment, a linear regression analysis was performed to calculate the relationship between the percentage of decrease of the psoriasis score (PASI) and the fold increase of cytokine secreted by PBMC after treatment.

Results in Figure 5A demonstrate that the enhancement of IL-10 production induced by anti-TNF therapy positively correlated (R^2= 0.33) with the clinical amelioration of psoriasis disease (significance level p=0.009). In contrast, no significant correlation was found for the up-regulation of other cytokines, (IFNγ IL-17 or IL-2) and the disease response to the therapy, suggesting that among the cytokines up-regulated by anti-TNF, IL-10 may play a role in the clinical amelioration of the disease.

This was further confirmed by the analysis of cytokine up-regulation in patients divided on the basis of their response after 1 month treatment: highly responsive to anti-TNF therapy (>50% PASI decrease, n=8) and normally-low responding to the therapy (<50% PASI decrease, n=10). The analysis shows that the average fold-increase of IL-10 was significantly higher in the highly responding group as compared to the normally-low responders (Figure 5B). A similar trend was observed for IL-2 and IL-17 increase but differences were not significant.

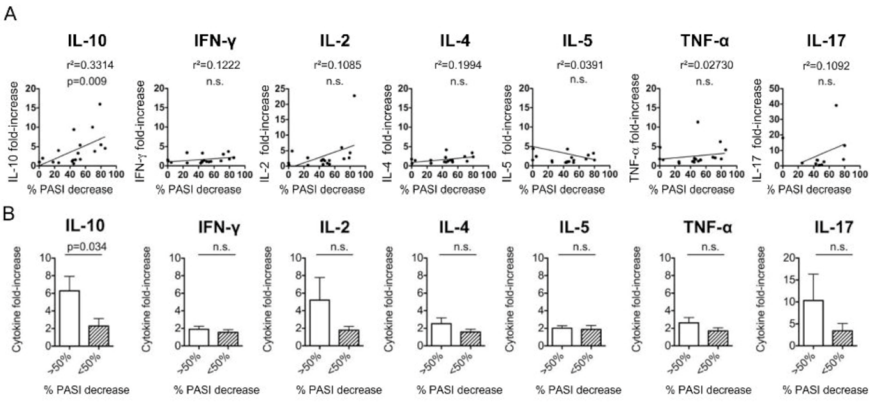


Figure 5: Up-regulation of IL-10 correlates with the clinical outcome of the therapy. (A) Correlation between the fold increase of cytokine production by αCD3-stimulated PBMC and the percentage of decrease of the PASI score was calculated by linear regression analysis in 19 individual patients after 1 month anti-TNF therapy. The correlation for single cytokine was expressed as R-squared values. (B) Fold-increase of cytokine secretion was compared in patients divided in two groups: highly responding (>50% of PASI decrease within 1 month anti-TNF therapy, n=8) and normally-low responding (<50% PASI decrease within 1 month anti-TNF therapy, n=10). Significance levels were calculated by Student's t test.

To further characterize the up-regulation of IL-10 expression by anti-TNF, we evaluated IL-10 protein secretion and mRNA expression in different immune cell subsets under both stimulated and unstimulated conditions.

CD4+CD25- and CD4+CD25+ cells were isolated from individual patient PBMC at different time-points during anti-TNF therapy and IL-10 modulation was investigated. Data in Figure 6A shows that in individual patients, the expression of IL-10 is up-regulated to higher level in CD4+CD25- T cells as compared to αCD3-stimulated

CD4+CD25+ cells, as evidenced by cytokine quantification in the supernatant.

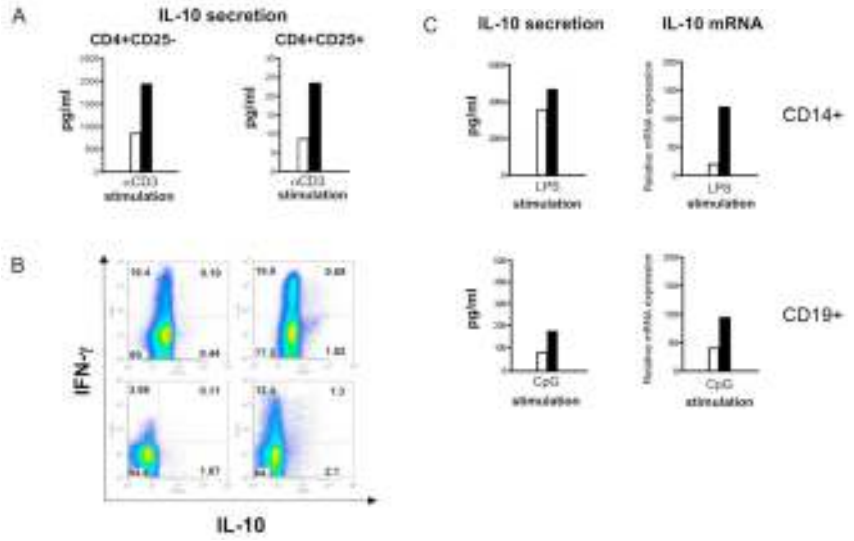


Figure 6

Expression of IL-10 is up-regulated in different IL-10-producing cell subsets.

(A and C) Different leukocyte cell subsets (CD4+25-, CD4+25+, CD14+ and CD19+) were separated from individual patient PBMC at baseline (white bars) and after therapy (black bars) and cultured in stimulated conditions (0.1 μg/ml plastic-bound αCD3 stimulation for T cell subsets, 1 μg/ml LPS stimulation for CD14+ and 5 μg/ml CpG for CD19+ cells). Supernatants were collected after 48h for cytokine detection and RNA was extracted after 5h for real time PCR experiments. IL-10 secretion and gene expression by different leukocyte cell subsets are shown in the figure (A. Pt 51; B. Pt 49) and are representative of at least 4 experiments. For real time PCR experiments, data were normalized to GAPDH housekeeping gene. (B) Intracellular cytokine staining for IL-10 and IFN-g on CD4+CD25- T cells. Cells were stimulated with αCD3/aCD28-coated beads for 30 h and Brefeldin A was added in the last 12 h. Shown are FACS profile of CD25+-gated T cells of 2 representative experiments out of 4 performed on different patients undergoing treatment with TNF-blocking agents.

CD4+CD25+ cells secreted lower amount of IL-10 that was moderately increased by anti-TNF, indicating that regulatory T cells are not the only source of IL-10 after anti-TNF therapy.

To investigate whether IL-10 increase occurs in activated Th1 cells producing IFNγ, we performed intracellular cytokine staining experiments for IL-10 and IFNγ□ □detection on CD4+CD25- T cells isolated from patients before and after TNF-blockade. From this experiments it was shown that upon anti-TNF treatment, both IL-10+ cells and IFNγ+IL-10+ double positive cells were augmented to similar levels (Figure 6B). This observation evidences that increased production of IL-10 occurs also in the potentially pathogenic subset of IFNγ□producing Th1 cells and this may have implications in the therapeutic effect of the treatment.

Results also exclude that a single expanded cell subset may be responsible for enhanced expression of IL-10. Indeed, up-regulation of IL-10 was observed also in the other IL-10-producing cell subsets, such as CD14+ cells and CD19+ B cell fraction (Figure 6C). Analogously, IL-10 enhancement was also observed in CD8+ T cell fraction (supplemental Figure 5). Up-regulation of IL-10 was evident on the constitutive mRNA expression in the absence of activating stimuli in all cell subsets (data not shown).

Cytokine gene expression in skin lesion after anti-TNF therapy

To evaluate the modulation of cytokine expression in the target tissue as compared to circulating lymphocytes we analyzed, in individual patients, cytokine expression in inflamed lesional skin before and after anti-TNF therapy. Gene expression analysis was performed for T cell

derived cytokines, inflammatory cytokines, chemokines and lineage associated markers. Figure 7A and B show two representative experiments of gene expression on regressing skin lesions from individual patients. The results clearly evidenced that the expression of T cell derived proinflammatory cytokine genes such as *Il17* and *Ifnγ* was strongly downregulated during remission of psoriatic plaque, despite in the same patients, TNF-blockade induced the up-regulation of Th1 and Th17 cytokines in peripheral lymphocytes (data not shown). Noticeably, the expression of chemokine genes such as *ccl3*, *cxcl10* and *cxcl11* was considerably downregulated in lesional skin upon TNF- blockade (Figure 7), suggesting that the inhibition of T cell migration in the target organ may plays a crucial role in the regression of inflammation.

Confirming previous observation from other groups, the downstream inflammatory cytokines *Il1b* and *Il8* were potently downregulated in psoriatic plaques upon anti-TNF treatment (3, 27).

The shut down of the inflammatory pathways in the skin corresponded to a decrease in the expression of T cell lineage marker genes such as *cd3*, *cd4*, *cd8* in 2 out of 4 biopsies (Figure 7A and data not shown).

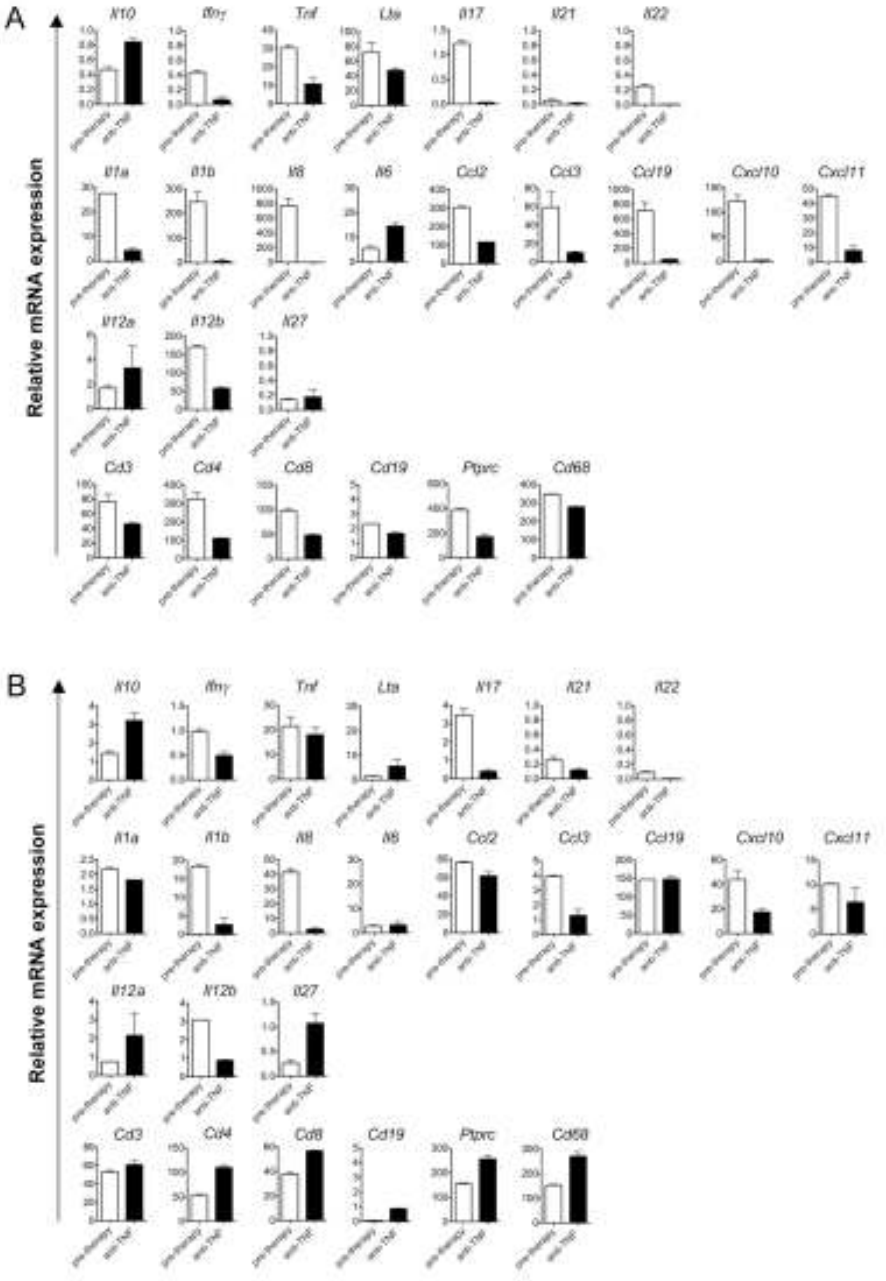


Figure 7: Down-regulation of pro-inflammatory gene expression and up-regulation of *Il-10* in skin lesions after anti-TNF therapy. Punch-biopsies from 4 individual patients were collected before (white bars) and after TNF- blockade (black bars) in the same lesional area. RNA was extracted and gene expression was analyzed by

real time PCR and by TaqMan Low Density array Human Immune Panel (Applied Biosystem). Values shown are the mean ± SD of triplicate samples. Data are normalized to GAPDH housekeeping gene. Figure shows two representative experiments (A. Pt 38; B. Pt 36) out of 4.

In the other 2 biopsies decreased expression of proinflammatory genes occurred without decrease of T cell lineage marker gene expression after 1 mo. therapy (Figure 7B and data not shown).
Despite the reduction of other cytokines and T cell associated genes, *Il10* mRNA was enhanced in psoriatic skin lesions after anti-TNF therapy (Figure 7), suggesting that other cell sources than T cells may account for this phenomenon.

Discussion

This study provides new insights on the impact of TNF-blockade on T cell responses and immunological homeostasis in patients with chronic inflammatory disease. In particular, it highlights the role of TNF-blockade in enhancing T cell effector functions in response to activating stimuli without altering the activation state of circulating lymphocytes. Moreover, it is enlightened a new role for TNF-blockade in the upregulation of IL-10 expression that was found to positively correlate with the clinical outcome of the therapy. Finally, it is clearly demonstrated the divergent effect of anti-TNF in up-regulating Th1 and Th17 pro-inflammatory cytokine responses in peripheral blood lymphocytes while inhibiting pro-inflammatory pathways in inflamed lesional skin. The up-regulation of Th1 and Th17 cytokines and, with a similar trend, also Th2 cytokines was

observed in PBMC and purified CD4+CD25- cells at both level of protein secretion and mRNA expression and was paralleled with the up-regulation of the expression of *Tbx21* and *RORC* encoding the Th1 and Th17 lineage-specific transcription factors T-bet and RORγt respectively.

These findings importantly demonstrate in humans a very recent evidence obtained in a mouse experimental model of collagen-induced autoimmune arthritis. The results of this study showed that anti-TNF therapy expanded Th1 and Th17 T cell populations, suggesting that TNFα, through TNFRp55, may be part of a negative feedback loop that limits Th17 and Th1 responses (8). A similar hypothesis came from a previous study on a mouse model of infection with Mycobacterium, showing that TNF-/- mice succumbed to lung infection because of tissue destruction caused by hyperactivation of Th1 responses. These evidences together with earlier studies indicating that TNF could act by attenuating TcR signaling, have lead to the hypothesis of a negative regulatory role of TNF on T cell responses (7, 9, 10).

Our data obtained on patients with a functional knock-down of TNF strongly support the role of TNF as a negative regulator of T cell effector functions. Nevertheless, we found that the proliferation of CD4+ and CD8+ T cells in response to αCD3 stimulation of total PBMC was not altered in 4 out of 6 patients. This could be due to the concomitant induction of immunosuppressor mechanisms such as IL-10 and the suppressor function of regulatory T cells that may limit T cell activation and expansion (17, 18).

The enhancing effect of TNF-blockade on T cell cytokine responses was observed also upon stimulation with superantigen and influenza-derived antigen. This may indicate an increased T cell reactivity to recall antigens or microbial pathogens that may account for the lack of evidence of increased susceptibility to infection that was expected to occur in patients after TNF depletion (28).

Increased reactivity of T cells, mostly Th1/Th17, could explain some of the controversial effect of TNF-blockade in autoimmune conditions (29). In fact, TNF-blockade was shown to increase both the rate and the frequency of relapse in patients with existing multiple sclerosis (30). Similarly, in murine models of lupus TNF-deficiency has been linked to exacerbation of the disease and increased anti-nuclear antibody production (15). In addition, a number of cases of development of psoriatic lesions has been reported in patients with different inflammatory diseases after anti-TNF treatment (31, 32).

As concerns the down regulation of T cell pro-inflammatory cytokines in the inflamed tissues, it has been hypothesized in the murine model of collagen-induced arthritis, that the mechanisms by which TNF-blockade is able to reduce disease activity while expanding population of Th1 and Th17 cells is preventing the migration of pathogenic T cells to the inflamed tissues (8). Consistently with this hypothesis, we observed decreased expression of chemokine genes including *cxcl10* and *cxcl11* mediating the recruitment of activated T lymphocytes and DC to the inflamed tissues. These results are in agreement with previous observation by other groups on the modulation of chemokine expression by anti-TNF in different types of pathologies (3, 27) and

support the hypothesis that inhibition of T cell migration to inflamed site may represents a key event in disease remission (8).

Reduced expression of T cell marker genes such as *Cd3, Cd4 and Cd8* was observed in two out of 4 patients after one month TNF-blockade. It has been reported that suppression of inflammatory genes in psoriatic plaques during TNF-blocking therapy occurs more quickly and to a larger extent than overall reductions in associated leukocyte subsets leading to the hypothesis that reduced T cell inflammatory genes may be due to a reduction of DC mediated T cell activation (3). In addition, it was postulated that the early decrease in chemokine mRNA including CXCL-10 and CXCL-11 could account for subsequent decrease in the infiltration of activated T cells. Our data strongly support this hypothesis and also suggest that other mechanisms may contribute to the early inhibition of T cell inflammatory cytokine expression in psoriatic plaques. This includes the upregulation of IL-10 expression.

On the basis of our data on circulating leukocytes, possible source of IL-10 could be the potentially pathogenic IFNγ-producing Th1 cells as well as other cell sources, including CD14+ cells.

In fact we demonstrated that up-regulation of IL-10 expression by anti-TNF occurred in all IL-10-producing cell subsets and the highest level of IL-10 up-regulation was observed in CD14+ cells. Collectively these data demonstrate a dual effect of TNF-blockade in increasing pro-inflammatory T cell reactivity in the peripheral circulation while blocking the downstream inflammatory cascade in psoriatic lesions.

At the same time, TNF-blockade up-regulates *Il10* expression both in peripheral lymphocytes and in the inflamed tissue suggesting a role for IL-10 in regulating the cytokine network during the shut down of the inflammatory reaction.

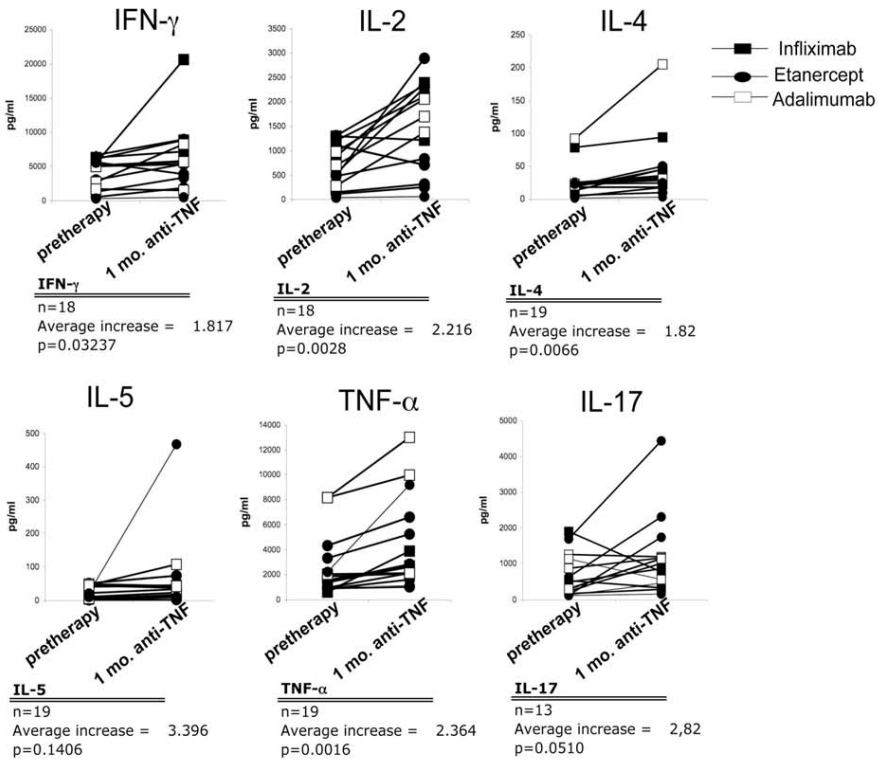
The dichotomy in the effect of TNF-blockade will help to distinguish the immunological mechanisms that are beneficial for clinical regression of autoimmune pathologies from thus that are potentially harmful, and may open new perspectives for the designing of selective immonotherapeutic approaches.

Acknowledgements

We thank Stefano Volinia (University of Ferrara, Italy) and Jens Geginat (German

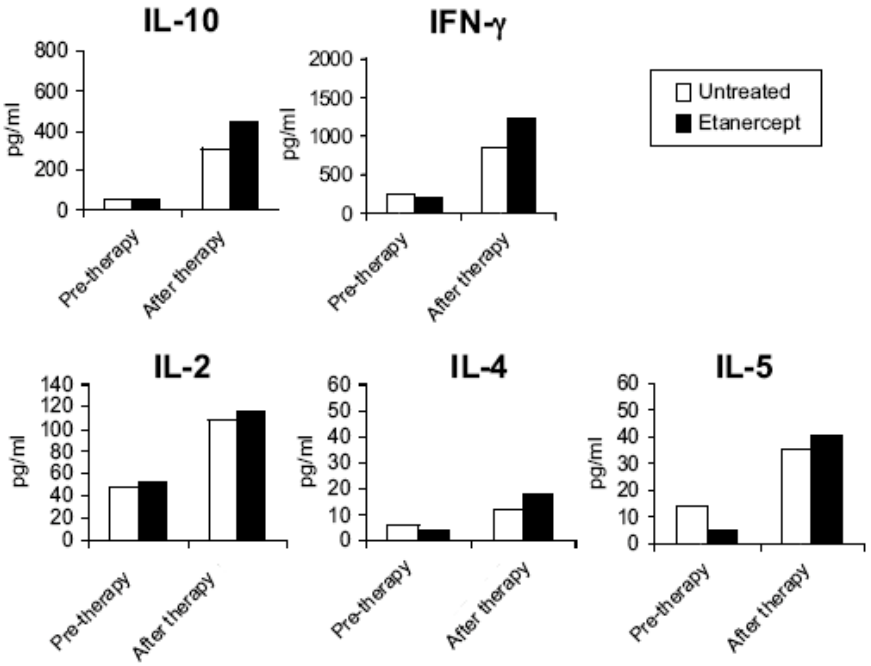
Rheumatism Research Center, Berlin, Germany) for the helpful discussion and suggestions. We also thank John W. Greiner (LTIB, NCI), Marta Catalfamo (CMRS, NIAID, National Institute of Health Bethesda, MD, USA), Daniela Talarico (DIBIT-HSR, Milan, Italy) and Giuseppe Banfi (Istituto Ortopedico Galeazzi, Milan, Italy) for critical reading of the manuscript.

Supplemental Figures

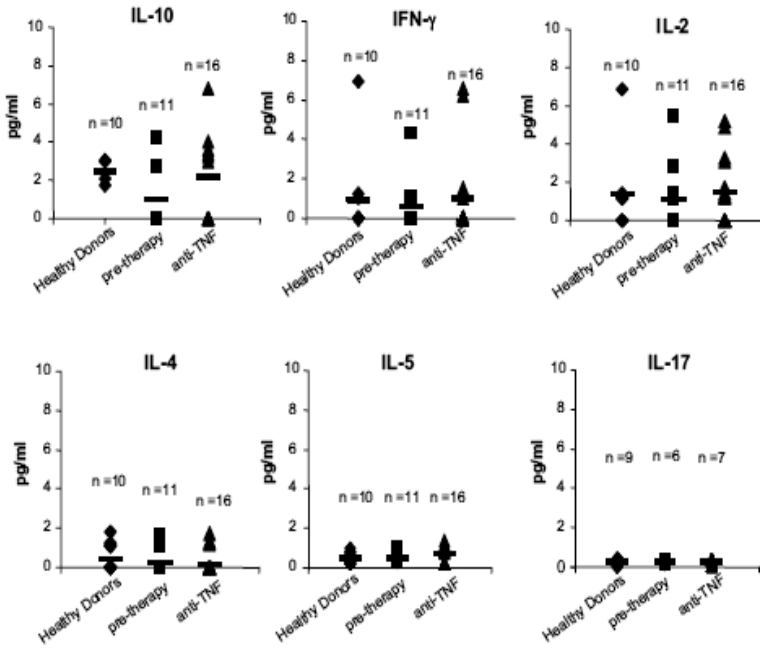


Supplemental Figure 1: Enhancement of cytokine production in individual patients treated with different TNF-blocking agents. PBMC from patients at baseline and after TNF-blocking therapy were stimulated with plastic-bound αCD3 antibody and supernatants were

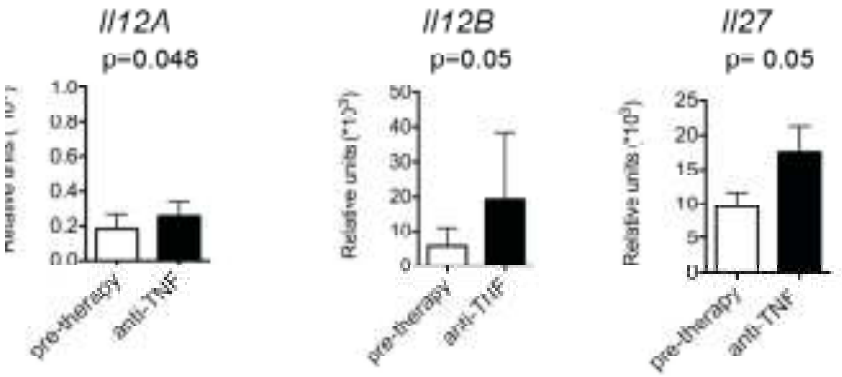
collected after 24h. Cytokine levels were evaluated by CBA analysis and ELISA assay. Cytokines were measured in individual patients before and after treatment with Etanercept (n=12; filled circle) and monoclonal antibodies Infliximab (n=3; filled square) and Adalimumab (n=4; open square). Number of samples, cytokine average increase and significance levels, calculated by Student's t test, are reported below each diagram.



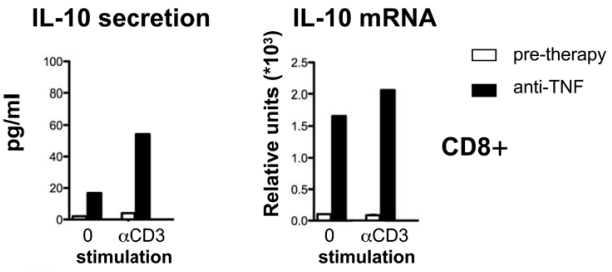
Supplemental Figure 2: Cytokine production by αCD3-stimulated PBMC in the presence of soluble Etanercept. PBMC from patients at baseline and after TNF-blocking therapy stimulated with 0.1 µg/ml plastic-bound αCD3 antibody were cultured in the presence or absence of 0.5 µg/ml Etanercept. Cytokines were measured in the 24 h supernatants by CBA analysis.



Supplemental Figure 3: Cytokine levels in serum of psoriasis patients. Serum from psoriasis patients at baseline and after anti-TNF therapy, and serum from healthy donors were collected. Cytokine levels were measured by ELISA (IL-17) and CBA analysis.



Supplemental Figure 4: Cytokine gene expression analysis in PBMC from anti-TNF treated patients. PBMC from psoriasis patients before and after anti- TNF therapy were cultered in the presence or absence of αCD3 for 5 hours. RNA was extracted and analyzed by quantitative real time PCR and by TaqMan Low Density array Human Immune Panel (Applied Biosystem). Il12A and Il12B expression was measured on unstimulated PBMC, Il27 expression was measured on stimulated PBMC. The mean ± SE of n=5 (Il27) and n=4 (Il12A and Il12B) patients are presented relative to the expression of transcript encoding GAPDH. Each experiment was performed in triplicate. Significance levels, calculated by Wilcoxon Rank test, are shown in figure.



Supplemental Figure 5: IL-10 expression in CD8+ T cell fraction. CD8+ cell subset was separated from individual patient PBMC before and after anti-TNF

therapy and cultured in the presence of 0.1 μg/ml plastic-bound αCD3. Supernatants were collected after 48h for IL-10 detection. For real time PCR experiment, mRNA was extracted after 5h of αCD3 stimulation and data were normalized to GAPDH housekeeping gene. One representative experiment (pt 43) out of 4 performed on different patients is shown in the figure.

	T Cells				
	CD3	CD3-CD69	CD3-CD25	CD3-HLA-DR	CD3-CD71
Healthy Control Donors (n=31)	72,13 ± 6,78	0,40 ± 0,50	2,64 ± 2,20	3,15 ± 1,99	0,32 ± 0,37
Psoriasis Patients (n= 11)	72,81 ± 12,25	0,68 ± 0,47 *	3,95 ± 2,54 *	3,27 ± 2,50	1,31 ± 1,61*
Psoriasis Patients 1 month after TNF therapy (n=10)	77,28 ± 6,97	0,66 ± 0,55	3,2 ± 1,40	4,84 ± 4,58	0,79 ± 1,16 *
Psoriasis Patients 3 months after TNF therapy (n=5)	77,20 ± 6,28	0,41 ± 0,43	2,08 ± 0,95	1,49 ± 0,84	0,14 ± 0,12 **

	On total lymphocytes	
	CD4	CD8
Healthy Control Donors (n=31)	35,51 ± 10,16	26,89 ± 7,24
Psoriasis Patients (n= 11)	35,63 ± 9,14	29,66 ± 8,23
Psoriasis Patients 1 month after TNF therapy (n=10)	37,54 ± 7,74	30,50 ± 7,71
Psoriasis Patients 3 months after TNF therapy (n=5)	42,44 ± 8,87	28,36 ± 4,37

	NK cells	B cells	
	CD56	CD19	CD19-CD71
Healthy Control Donors (n=31)	11,18 ± 4,51	11,94 ± 4,82	4,23 ± 2,67
Psoriasis Patients (n= 11)	13,98 ± 7,37	10,65 ± 4,02	4,17 ± 1,72
Psoriasis Patients 1 month after TNF therapy (n=10)	11,16 ± 6,82	11,45 ± 5,24	4,02 ± 2,15
Psoriasis Patients 3 months after TNF therapy (n=5)	14,62 ± 6,15	9,08 ± 2,69	n.d.

	% CD4+CD25hi	% FoxP3+	
Healthy Control Donors (n=9)	0,67 ± 0,37	83,85 ± 4,85	
Psoriasis Patients (n= 9)	1,08 ± 0,48	88,60 ± 6,70	* significant vs HD
Psoriasis Patients after anti-TNF therapy (n=18)	1,22 ± 0,59	88,44 ± 6,01	** significant vs untreated psoriasis patients

Table I: Lineage and activation marker expression by circulating PBMC. Freshly isolated PBMC from healthy volunteers (n=31), untreated psoriasis patients (n=11) and patients undergoing anti-TNF therapy (1 mo.-treated n=10; 3 mo.-treated n=5) were analyzed by flow cytometry for the expression of T, B and NK cell lineage (CD3, CD4, CD8, CD19, CD56) and activation markers (CD69, CD25, HLA-DR and CD71). Numbers represent the percentage of positive cells on total lymphocyte gate. Significance level was calculated by Student's t-test.

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CHAPTER 3

TNF-blockade enhances T cell response to TcR stimulation

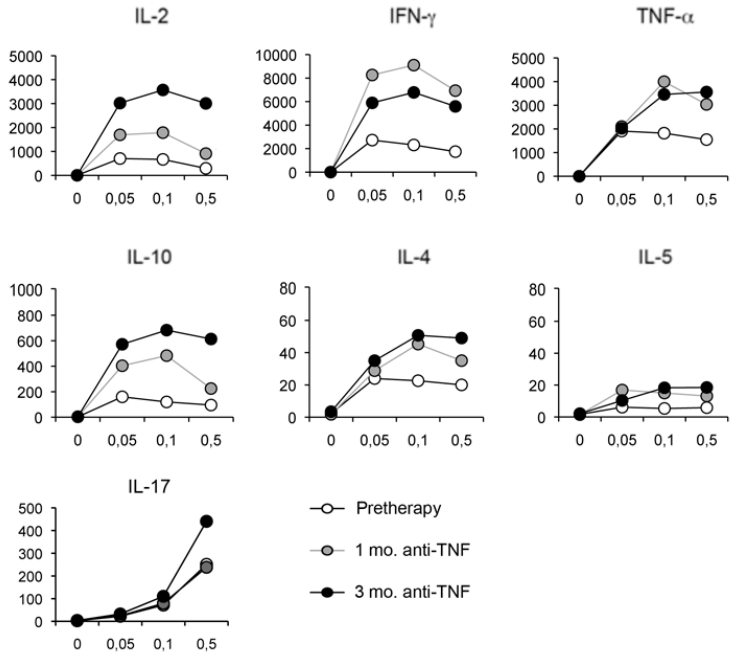
Ongoing work

TNF-blockade enhances T cell response to TcR stimulation

Our previous studies have evidenced an increased cytokine production in αCD3-stimulated PBMC as well as in CD4+ CD25- T cells from anti-TNF treated patients. However, from these data we could not establish whether the increased responses in the peripheral circulation was due to expanded T helper cell subsets, as hypothesized by recent studies on a mouse model of rheumatoid arthritis (1), or whether it was due to a hyper-responsiveness of T cells to TcR stimulation, according to the negative regulatory role of TNF suggested by Cope et coworkers (2, 3).

In order to specifically investigate these two possibilities, we evaluated the following parameters:

- the cytokine profile in single patients before and after anti-TNF treatment
- the circulating level of polarized T helper subsets during TNF-blockade
- the proliferative response of CD4+CD25- T cells in patients following TNF-blocking therapy
- the induction of T cell activation markers before and after TNF-depletion
- the phosphorylation of signaling molecules of the TcR signal transduction pathway



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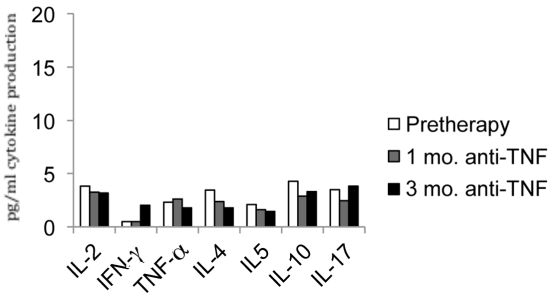

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Figure 1: A) αCD3 dose-dependent cytokine profile in PBMC from individual patient after TNF-blockade. B) Basal level of cytokine production by PBMC before and after anti-TNF treatment. PBMC were collected and activated with different doses of plastic-bound αCD3 antibody. CBA analysis was performed for IL-10, IFN-γ, IL-2, IL-4, IL-5, TNF-α cytokine levels secreted in the supernatants after 24h. IL-17 cytokine production was evaluated by ELISA test. Data from one representative patient (Pt 2) out of 19, at baseline and after anti-TNF therapy are shown in figure 1.

Figure 1 refers to the evaluation of cytokine production in individual patients before and after 1 - 3 months of anti-TNF therapy in response to different doses of αCD3 antibody.
The data showed that enhanced cytokine production by TNF-blocking could be observed for all cytokines tested, showing that no substantial variation in the cytokine profile was induced.
Figure 1B represents the basal level of cytokine production by PBMC in the absence of stimuli, indicating that no variation in the cytokine secretion was induced and that the enhancement was strictly dependent on TcR stimulation.

We next extended the analysis to the IFN-γ production in response to αCD3 stimulation by intracellular cytokine staining on both CD4 and CD8 T cell subsets, before and after TNF-blocking therapy.
Figure 2 shows the FACS analysis of CD4+ gated and CD8+ gated T cells from a psoriasis patient (Pt 23) at baseline and after 1 month and 3 months of anti-TNF therapy (etanercept).
As shown in figure 2, an increase of IFN-γ-producing cells was observed both in CD4+ and CD8+ T cells. Notably, data also evidenced a moderate increase of the Mean Fluorescence Intensity (MFI), suggesting an increased production of IFN-γ by single cells.
We next analyzed the expression of Th1-associated trafficking receptor CXCR3 and of Th2-associated trafficking receptor CCR4 in circulating CD4 T cells to evaluate whether variation of these T helper subsets was induced by TNF-blockade.

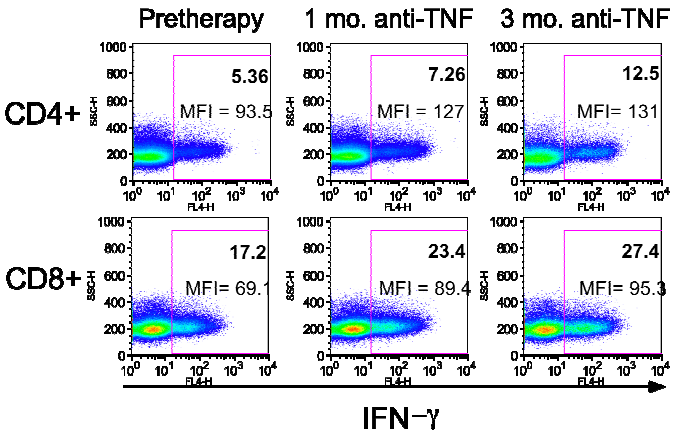

Figure 2: TNF-blockade enhances IFN-γ-production by both CD4+ and CD8+ T lymphocytes in response to αCD3 stimulation. PBMC from an individual patient were cultured for 12h in the presence of αCD3/αCD28-coated beads. Brefeldin A was added after the first 2 hours of stimulation. Numbers refer to the frequency (bold) of IFN-γ+ cells and to Mean Fluorescence Intensity (MFI) value in the indicated gates.

The data shown in Figure 3 referred to preliminary results indicating no substantial variation in the CXCR3+ or CCR4+ expression upon TNF depletion.

This first set of data does not support the hypothesis of a selective expansion of Th1 cells in the circulating compartment.

However, experiments are in progress to evaluate the frequency of circulating T cells expressing the Th17-associated markers CCR6.

We also wanted to determine whether variation occurs in the circulating memory T cell subsets, including central memory CCR7+CD45RA-, effector memory CCR7-CD45RA- and effector T_{EMRA} CCR7-CD45RA+.

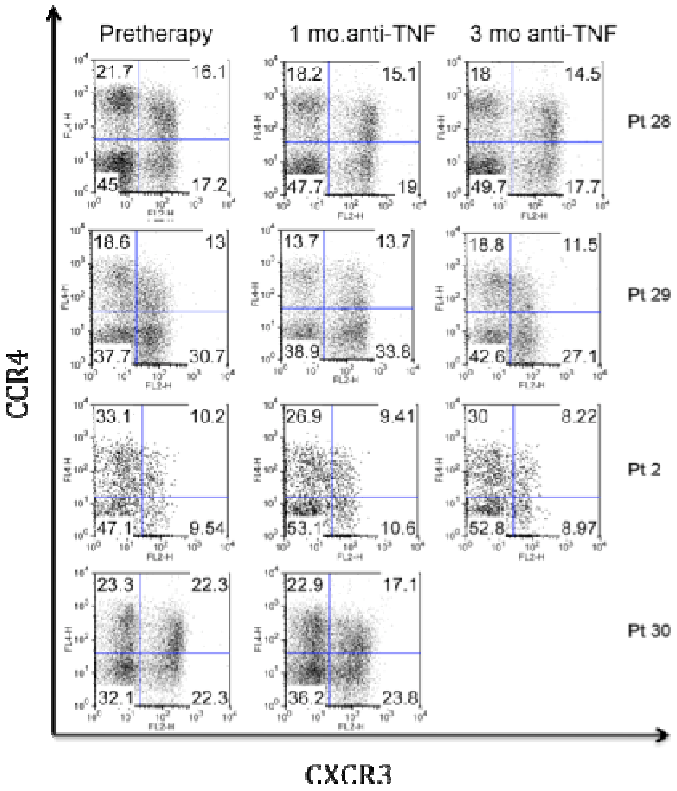

Figure 3: CXCR3 and CCR4 expression evaluation on CD4+ T cell from anti-TNF treated patients. FACS analysis of the expression of CXCR3 and CCR4 on CD4+ T cells from 4 patients at baseline and after 1 - 3 months of anti-TNF treatment.

The percentage of these subsets on CD4+ and CD8+ gated PBMC before and after anti-TNF treatment are represented in Figure 4.

No variation in the frequency of these subsets was observed in CD4+ or in CD8+ T cell compartment during anti-TNF treatment.

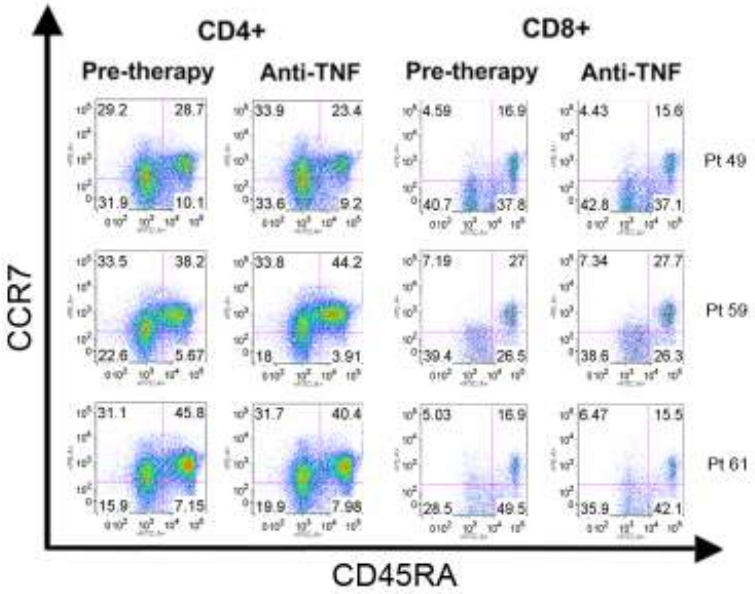

Figure 4: Central and Effector memory T cell subset analysis. PBMC from individual patients (n=3) was analyzed by flow cytometry to discriminate the frequency of CCR7+CD45RA+ naïve, CCR7+CD45RA- central memory, CCR7-CD45RA- effector memory, and CD45RA+CCR7- effector memory expressing CD45RA (T_{EMRA}) cell subsets before and after anti-TNF treatment.

TNFRs expression on PBMC during TNF-blockade. To further investigate the effect of TNF-blockade on T cells, we evaluated the expression of TNFR-I (p55) and/or TNFR-II (p75).

In particular, we analyzed by flow cytometry the percentage of TNFRs+ cells on CD3+ T cells and CD14+ monocytes from patients before and during anti-TNF therapy.

As shown in figure 5, CD3+ T cells expressed mainly TNFR-II, whereas CD14+ monocytes were mainly double positive for TNFR-I and TNFR-II.

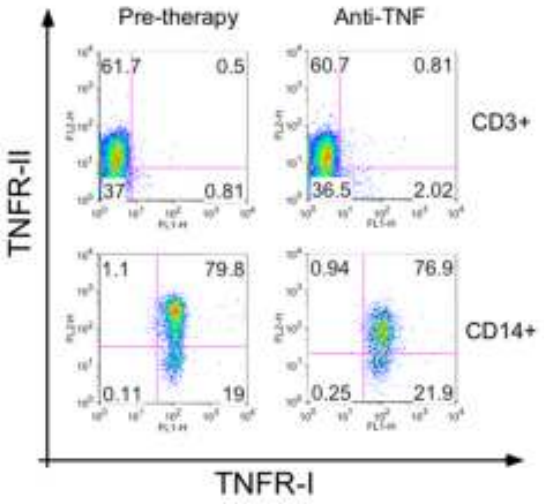

Figure 5: TNFR-I (p55) and TNFR-II (p75) detection on CD3+ T lymphocytes, CD19+ B cell and CD14+ monocytes from anti-TNF treated patients. FACS analysis of the percentage of TNFRs+ cells on CD3+ T cells and CD14+ monocytes from total PBMC from one (Pt 43) representative patient out of 3, at baseline and after 1 month of TNF-blockade.

The results from these experiments did not evidence any variation in the expression of TNFRs on circulating T cells and monocytes after anti-TNF treatment as compared with the untreated controls.

TNF-blockade enhances CD4+CD25- T cell proliferation and induction of activation markers in response to αCD3 stimulation.

In order to investigate the modulation of T cell activation by TNF-blockade, we evaluated whether T cells from anti-TNF-treated patients also increased their proliferative capacity in response to TcR stimulation. Our previous data on total PBMC indicated that the cell proliferation was altered by anti-TNF treatment only in 2 out of 6 patients. However, the effect of TNF-blockade on proliferation of

responder CD4 T helper cells might be masked by the increased suppressive activity of regulatory T cells that has been reported to occur during TNF-blockade. Thus, we evaluated the effect of TNF-blockade on proliferative response of purified CD4+CD25- T cells to αCD3 stimulation.

As shown in the Figure 6, our preliminary data indicated an increased proliferation of activated T cells after TNF-blocking treatment as compared with the untreated counterpart.

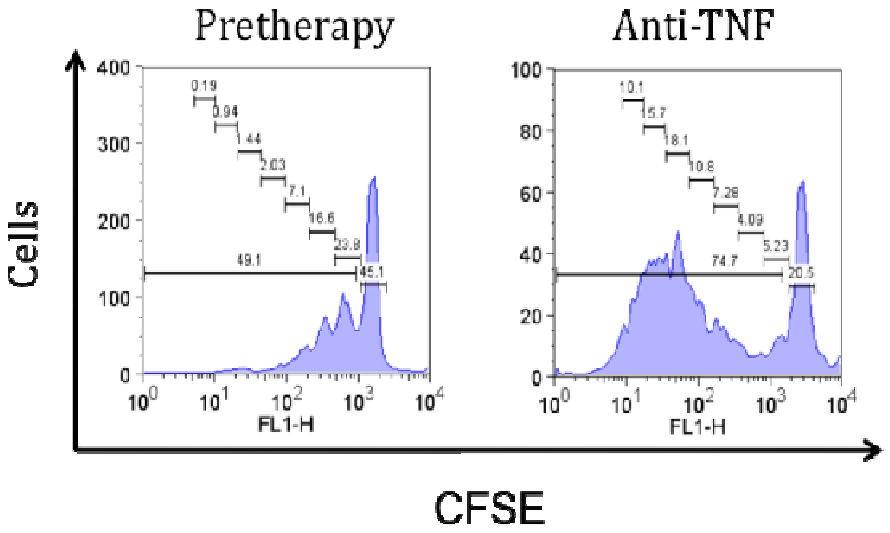

Figure 6: CD4+CD25- purified cell proliferation. CFSE-labeled CD4+CD25- responder T cells freshly purified from a representative individual patient out of 3, before and after TNF-blocking therapy, were cultured in the presence of 0,05 μg/ml αCD3 for 8 days. Shown are FACS data on activated and living CD25+7AAD-gated T cells. Numbers indicate the percentage of proliferating lymphocytes for all cell divisions.

The average number of cell divisions (proliferative index) changed from a value of 1,42 at the baseline to 2,63 upon TNF-blockade,

indicating increased number of cell divisions. This was also evidenced by a higher percentage of cells in the last generations.

Then, in order to investigate T cell activation in response to TcR stimulation upon TNF-blockade, we analyzed the surface induction of CD69 early activation marker, CD25 intermediate and CD71 late activation markers in individual patients.
As shown in Figure 7, our results evidenced a considerable enhancement of the induction of CD25 (IL-2Rα) and CD71 (transferrin receptor) upon TNF-blockade after 24h of TcR stimulation. Similar results were obtained, at lower level, for CD69 induction after 3h (data not shown) and 24h of αCD3 stimulation.
We found an enhanced level of expression of these markers on both naïve (CD45RA+) and memory (CD45RA-) T cells in individual patients after treatment with anti-TNF.
The evidence that TNF-blocking treatment increased the surface induction of activation markers also in the naïve compartment supports the hypothesis that TNF-blockade induces an increased T cell response to TcR stimulation, rather than a selective expansion of polarized T helper cells.
This will be confirmed by the future evaluation of induction of surface activation markers on sorted naïve T cells.

Experiments are in progress to investigate the phosphorylation state of signaling molecules and transcription factors of the TcR signaling pathway.

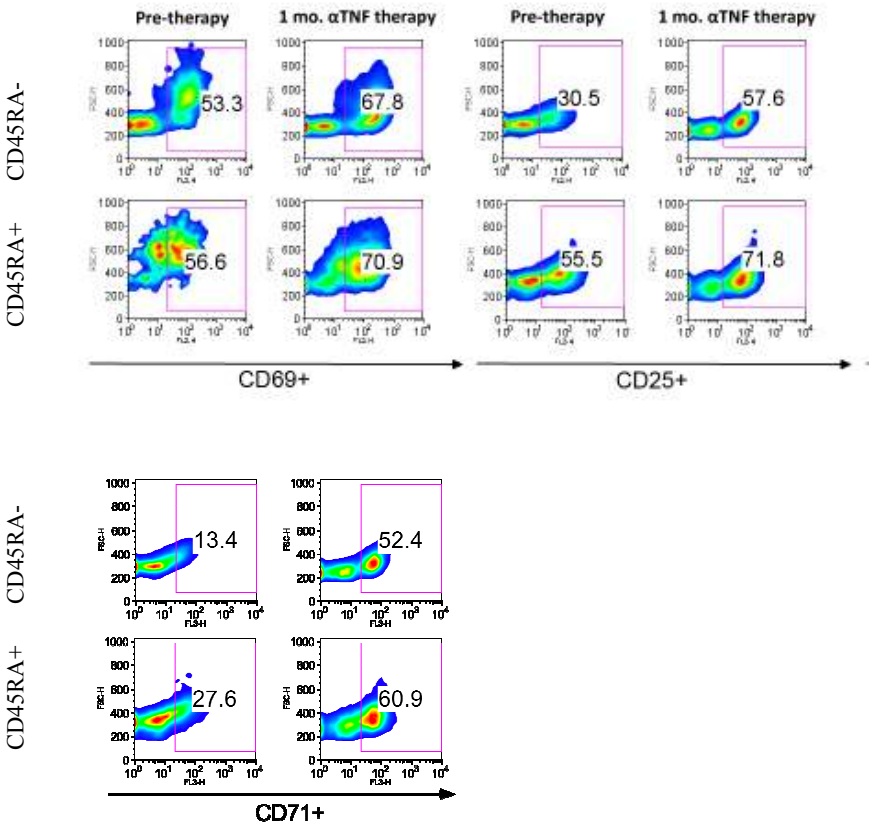

Figure 7: Activation marker induction on CD4+CD25- T cells. Purified CD4+CD25- T lymphocytes were cultured in the presence of plastic-bound αCD3 for 24h and stained for CD69+, CD25 and CD71. Shown in the figure are the expressions of CD69, CD25, CD71, in the memory and in the naïve T cell compartments in one representative patient out of 4, before and after TNF-blockade.

In a preliminary experiment we measured phospho-SLP-76 and phospho-c-Jun on protein extracts obtained from CD4+CD25- T cell from one individual patient before and after TNF-blocking treatment and stimulated at different time point with αCD3.

The results from this preliminary experiment suggested that TNF-blockade induced an enhancement of the activation state of these molecules and, in particular for c-Jun a possible longer kinetic.

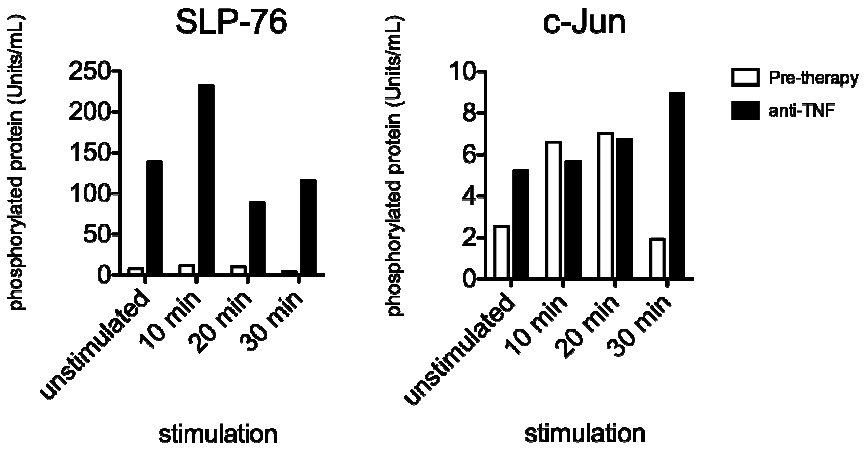

Figure 8: Modulation of phosphorylation state of molecules of TcR signaling pathway by TNF-blockade. CD4+CD25- T cells isolated from patient before and after TNF-blockade were stimulated with 0,05 μg/ml plastic-bound αCD3 for 10, 20 and 30 min. and the proteins were extracted. The extracts were processed and quantified for the subsequent CBA flex set analysis of different phosphoproteins, according to manufacturer's instructions. Data refers to one representative patient (Pt 61).

Other samples are required to establish the effect of TNF-blocking treatment on the phosphorylation of ERK1, ERK2 and p38 MAP kinases.

These observations, if has confirmed by higher number of samples, could provide a molecular explanation for the increased CD4+CD25- T cell response to TcR stimulation induced by TNF-blockade.

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Chapter 4

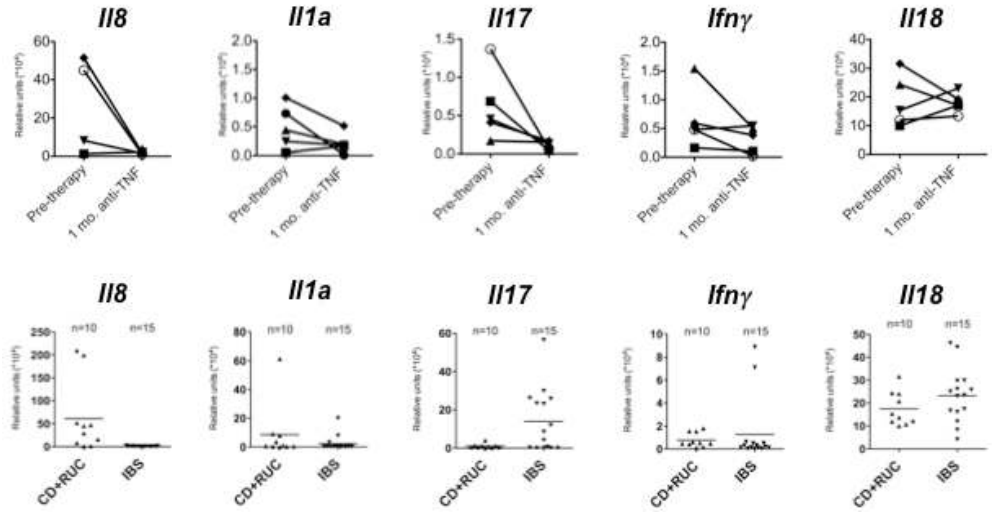
Modulation of cytokine gene expression by TNF-therapy in intestinal mucosa of patients with Inflammatory Bowel Disease

Ongoing work

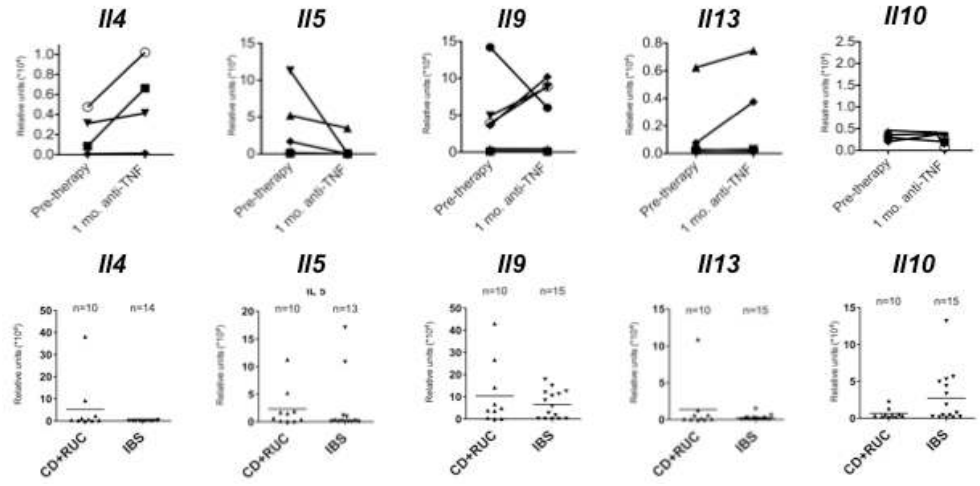
Modulation of cytokine gene expression by TNF-blocking therapy in intestinal mucosa of patients with Inflammatory Bowel Disease.

In this part of the study we extended the gene expression analysis, which we initially performed on psoriasis plaques, to the inflamed intestinal mucosa of patients with inflammatory bowel diseases (IBD). While modulation of pro-inflammatory gene expression by TNF-blockade in psoriasis and rheumatoid arthritis has been described by other studies (1-3), no information about the modulation of cytokine and chemokine network have been reported on intestine of IBD patients upon anti-TNF immunotherapy. Therefore, we analyzed the expression of 96 genes, including a wide panel of cytokine, chemokine and adhesion molecule-related genes, as well as transcription factor, lineage associated markers and activation markers.

The analysis was performed by real-time PCR on RNA extracted from intestinal biopsies from patients with Crohn's disease and ulcerative colitis at baseline and after 6 weeks of infliximab treatment, as well as biopsies from patients with irritable bowel syndrome (IBS) that represented the control group.


Il8
Il1a
Il17
Ifnγ
Il18
Pre-therapy
1 mo. anti-TNF
CD+RUC
IBS
n=10
n=15

A


Il4
Il5
Il9
Il13
Il10
Pre-therapy
1 mo. anti-TNF
CD+RUC
IBS
n=10
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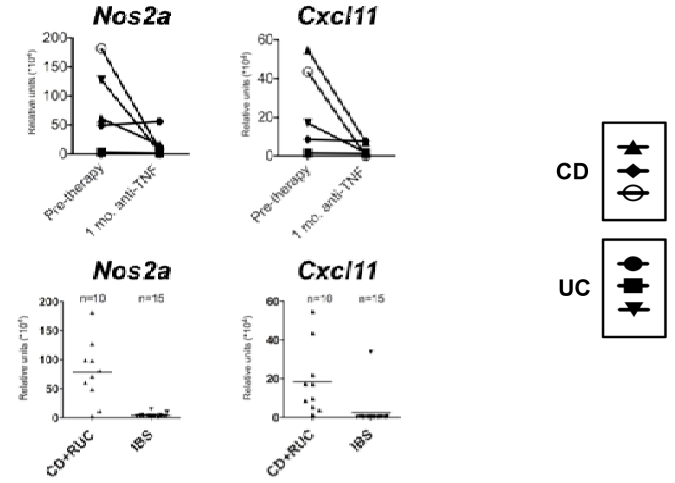
n=13
n=15

B



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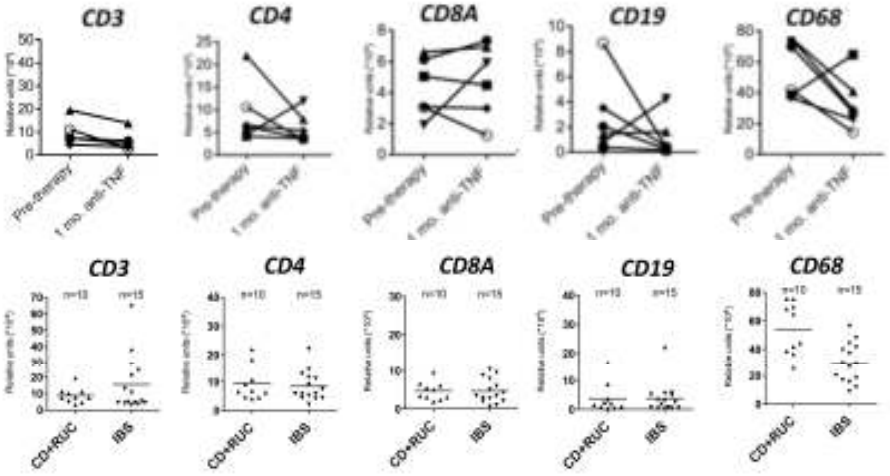

D

Figure 1: Modulation of cytokine and chemokine gene expression in intestinal mucosa of IBD patients following anti-TNF therapy. Analysis was performed by real-time PCR on RNA extracted from intestinal biopsies from patients with Crohn's disease and ulcerative colitis at baseline and after 6 weeks of infliximab treatment, as well as from patients with irritable bowel syndrome (IBS) that represented the control group. Data were normalized with a mean of three different housekeeping genes. The upper part of each panel of the figure shows the expression of these

genes in individual patients from Crohn's disease (CD) or ulcerative colitis (UC) before and during anti-TNF therapy. The lower part of each panel shows the expression of the same genes in intestinal mucosa of IBD patients in comparison with patients with irritable bowel syndrome (IBS).

We focused our attention on genes encoding relevant pro-inflammatory and anti-inflammatory cytokines, as well as lineage associated marker genes, and chemokine genes (Figure 1).

Figure 1 A refers to genes involved in Th1 and Th17 cell mediated responses and innate immunity. The results showed that *Il17* pro-inflammatory cytokine is strongly downregulated in IBD patients after therapy. Similarly, *Il8* expression was considerably decreased in IBD patients after anti-TNF therapy. In this case, increased level of *Il8* expression was also found in IBD patient as compared with IBS controls. These data are in agreement with our previous results on skin lesion. Higher number of samples is required to reach statistical significance.

Figure 1 B refers to genes involved in the Th2 cell mediated response and to the anti-inflammatory cytokine IL-10 gene. We observed also a decreased expression of *Il5* that could correlate with disease amelioration. If confirmed on a larger number of samples from Crohn's disease and ulcerative colitis patients, these evidence would be in accordance with the involvement of Th-2 cytokine mediated responses in the pathogenesis of ulcerative colitis (4, 5).

Figure 1 C showed the gene expression of the inducible nitric oxide synthase (iNOS) *Nos2a*, and of the *Cxcl11* chemokine.

Nos2a gene expression is activated by inflammatory stimuli in a NFκB-dependent manner. It is involved in dendritic cell function and

is part of anti-microbial mechanisms of activated macrophages. A decrease of iNOS expressing TipDC has been described in regressing skin lesions of psoriasis patient undergoing anti-TNF therapy (1-2). The results in figure 1 D indicated *Nos2a* expression downregulation also in IBD patients treated with anti-TNF. This, in turn, could be associated with a decreased DC-mediated activation of T cells.

Very importantly, we found that *Cxcl11* expression was considerably downregulated by TNF-blocking therapy. Moreover, higher level of *Cxcl11* mRNA was observed in IBD patients in comparison with the IBS control group.

Cxcl11 is a chemokine induced by IFN-γ and represents the cognate ligand of CXCR3 chemokine receptor. Through the high affinity binding to CXCR3 it can recruit activated memory T cells to the inflammation sites. One study on a mouse model of collagen-induced arthritis (CIA) has hypothesized that inhibition of cell migration was responsible for the downregulation of T cell mediated response in the inflamed tissue induced by TNF-blockade (6). Our data of gene expression on psoriatic plaques strongly support this idea (Chapter 1) that is further confirmed by these data o IBD patients. In parallel to *Cxcl11* downregulation, we detect a decrease in the gene expression of T cell lineage associated markers, in particular CD3 and CD4 (Figure 1 D).

Collectively, our data on skin lesions from psoriasis patients and intestinal mucosa from IBD patients could definitively demonstrate that the downregulation of chemokine expression by TNF-blockade and the consequent diminished T cell recruitment to inflamed tissue represents the key events in the inhibition of T cell-mediated pro-

inflammatory response in patients with chronic inflammatory diseases upon TNF-blocking treatment, despite the enhancement of the peripheral T cell reactivity induced by TNF-blockade itself.

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Chapter 5

Summary, conclusions and future perspectives

Summary

TNF blocking agents have been applied to the treatment of different autoimmune and inflammatory disorders, including rheumatoid arthritis, psoriasis and Crohn's disease. Although the inhibition of inflammatory pathways in target tissues by anti-TNF therapy has been described in psoriasis and rheumatoid arthritis, the impact of TNF-blockade on peripheral T cell responses in humans is still unclear. Studies on patients and on mouse models have reported very conflicting findings on the effect of anti-TNF treatment on peripheral T cells.

This controversy points out the increasing need to clarify in a clinical situation the role of TNF-blockade on T cell activation and effector functions.

In this study, we wanted to investigate:

- Effect of anti-TNF immunotherapy on cytokine homeostasis and T cell response and, in parallel, the modulation of cytokine gene expression in the target organ.
- Effect of TNF-blockade on peripheral T cell activation in response to TcR stimulation.
- Modulation of cytokine and chemokine gene expression in the intestinal mucosa of patients with inflammatory bowel diseases undergoing anti-TNF immunotherapy.

The first part of the study clearly demonstrated in psoriasis patients that anti-TNF therapy enhanced, in the peripheral circulation, the T cell cytokine production in response to activating stimuli. Increased expression of Th1, Th17, IL-10 and with similar trend also Th2 cytokines was observed both at level of protein secretion and mRNA expression and in response to different stimuli. Consistently with cytokine production, we found that the expression of Th1 and Th17 master regulator transcription factors, *Tbx21* and *RORC* genes, was enhanced to significant level in αCD3-stimulated CD4+25- cells. Similar data were obtained on T cells from patients with Crohn's disease and ulcerative colitis, indicating that this effect was dependent on the anti-TNF therapy, rather than on the disease.

IL-10 expression was also enhanced on different circulating leukocytes subsets and in skin biopsies. Importantly, increased production of IL-10 by stimulated PBMC from patients treated with anti-TNF was also found to positively correlate with the clinical outcome of the therapy.

Despite the enhanced T cell cytokine responses in the peripheral circulation, in psoriatic skin lesions the overall effect of TNF-blockade was a diminished expression of pro-inflammatory Th1 and Th17 cytokine genes, paralleled by the downregulation of *ccl3, cxcl10* and *cxcl11* chemokine genes, involved in the recruitment of activated leukocytes to the inflamed tissue.

Data from this first part of the project clearly evidence a dual role of TNF-blockade that increases the T cell response to stimulation in the

circulating compartment, while downregulates T cell cytokine expression in the inflamed tissue. It also enlightens a new role of TNF-blockade in enhancing IL-10 expression that may have implications in the shut down of the inflammatory reaction in regressing skin lesions.
It also suggests that the inhibition of cell migration in the target organ caused by downregulation of the chemokine expression may represent the crucial event in inhibition of T cell mediated inflammatory response in psoriatic plaques upon TNF-blocking therapy.

These evidences opened the question of whether the increased response of T cells to stimulation that was induced by TNF-blockade, was due to an increased responsiveness of T cells to TcR stimulation, as suggested by Cope and coworkers in studies on transgenic mice (1, 2), or whether it could be due to a selective expansion of polarized T cells in the peripheral circulation, as suggested by other studies (3). Thus, to specifically investigate these two possibilities, in the second part of the study we have examined both cellular and molecular events of T cell activation in individual patients upon TNF-blockade.
In parallel, we evaluated in patients before and after TNF-blockade: the cytokine profile of activated T cells, the circulating percentage of T cells expressing the Th1-associated receptor CXCR3 and the Th2-associated receptor CCR4, as well as the proliferative response and surface induction of activation markers in aCD3-stimulated CD4+CD25- T cells. Preliminary experiments to evaluate the phosphorylation of signaling molecules of TcR pathaway are in progress.

The results showed no substantial variation in the Th1, Th17 and Th2 cytokine profile during 1-3 months of TNF-blocking treatment. Lymphocytes from individual patients exhibited enhanced cytokine production in response to stimulation and unchanged basal level in unstimulated conditions, indicating that the increase of cytokine expression is strictly dependent on TcR stimulation.

The expression of CXCR3 and CCR4 in circulating CD4+ T cells was found unaltered upon TNF-blockade and experiments are in progress to assess the variation of Th17-associated receptor CCR6 expression.

These results did not provide evidences supporting the hypothesis of a selective expansion of polarized T cell subpopulations after TNF depletion.

Conversely, we found an increased proliferation of purified CD4+CD25- T cells after TNF-blockade, evidenced by a higher frequency of dividing cells and by an enhanced average number of cell divisions. In parallel, we observed a considerably augmented surface induction of intermediate and late activation markers, such as IL-2 receptor alpha (CD25) and transferrin receptor (CD71), in response to aCD3 stimulation. This effect was evidenced on both naïve (CD45RA+) and memory (CD45RA-) CD4+CD25- T cells. Additional comprehension of the mechanisms underlying this phenomenon could derive from the analysis of the phosphorylation state of molecules of the TcR signaling pathway. A pilot experiment has indicated an increased level of phosphorylated SLP-76 adapter protein and phosphorylated c-Jun transcription factor in aCD3-stimulated CD4+CD25- T cell from patients after TNF-blockade.

These data suggest that TNF-blockade increases T cell activation in response to TcR stimulation and support the hypothesis of a negative regulatory role of TNF on T cell function.

The last part of the project had focused on the analysis of the modulation, induced by anti-TNF therapy, of cytokine and chemokine gene expression in intestinal mucosa of patients with inflammatory bowel diseases.

We will confirm the gene expression data by confocal microscopy imaging of specific molecule in the inflamed tissue and by statistical correlation of differential expression of single genes with the clinical response to the treatment.

Our data indicate that *Il8* cytokine as well as *Il17* cytokine gene expression was considerably downregulated upon TNF-blockade. *Il8* gene expression was also found upregulated in the IBD patient as compared with irritable bowel disease (IBS) controls.

Very importantly, we found also that *Cxcl11* chemokine expression was strongly downregulated in intestinal mucosa of IBD patients upon anti-TNF treatment. This was paralleled by a decrease of the T cell lineage-associated markers genes *Cd3* and *Cd4*.

These results strongly support the hypothesis that decreased migration of T cells to the inflamed tissue, as consequence of the downregulation of chemokine molecules, is the key event of the inhibition of T cell mediated inflammatory reaction during anti-TNF treatment. This may explain the clinical benefit of anti-TNF therapy in chronic inflammatory diseases, despite the enhancement of T cell response to stimulation induced in the peripheral circulation.

Together, these data definitively demonstrate in humans the dual role of TNF-blockade on T cell mediated response and will help to distinguish the immunological mechanisms that are beneficial for clinical regression of chronic inflammatory diseases from thus that are potentially harmful, and may open new perspectives for the designing of selective immonotherapeutic approaches.

Conclusions and future perspectives

In this study we definitively demonstrate in patients, a dual role of TNF-blockade in modulating T cell-mediated cytokine responses. Indeed, anti-TNF therapy enhances the pro-inflammatory T cell cytokine responses to stimuli in the peripheral circulation, while it inhibits T cell-mediated inflammatory reaction in the inflamed tissue. In particular, we demonstrated that TNF-blocking therapy considerably increased the Th1, Th17 and, with the same trend, also Th2 cytokine response to stimulation in both total PBMC and purified CD4+CD24- T cells. Increased T cell response in the peripheral circulation induced by anti-TNF therapy was observed in both psoriasis and inflammatory bowel disease patients.

Upregulation of Th1 and Th17 cells was observed both as protein secretion and mRNA expression and was further confirmed by the upregulation of expression of the master regulator transcription factor genes responsible for the Th1 and Th17 cell differentiation, *Tbx21* and *RORC*. A recent report has evidenced expanded Th1 and Th17 cell populations in a mouse model of collagen-induced arthritis upon treatment with TNFR-Fc fusion protein or anti-TNF monoclonal antibody (3). Notley and coworkers also described a Th1/Th17 increased T cell response in TNFR p55-/-, but not p75-/- mouse, so indicating a broader role of TNFR-I–mediated signaling in this effect (3). Another study reported that TNF negatively regulates Th1 T cell responses in a mouse model of mycobacterial infection. $TNF^{-/-}$ deficient mice, in this case, succumbed to lung infection

because of tissue destruction resulting from uncontrolled type 1 immune syndrome. This syndrome was characterized by expansion of activated T cells and overproduction of Th1 cytokines (4). These data were in accordance with results of studies on hemagglutinin-specific TcR-transgenic mice, suggesting that chronic exposure to TNF attenuates broad range of T cell responses, including T cell proliferation and cytokine production *in vivo* (1). Our data demonstrate in humans the hypothesis emerged from these describe them in a clinical situation.

The enhancement of T cell response to stimulatory signals, that we observed in the peripheral circulation, could also explain some of the controversial clinical effect of TNF-blockade in the treatment of autoimmune diseases (5). Indeed, TNF-blockade was shown to increase both the rate and the frequency of relapse in patients with existing multiple sclerosis (6). Similarly, in murine models of lupus TNF-deficiency has been linked to exacerbation of the disease and increased anti-nuclear antibody production (7). In addition, a number of cases of development of psoriatic lesions has been reported in patients with different inflammatory diseases after anti-TNF treatment (8, 9).

The enhancing effect of TNF-blockade on T cell responses was observed also upon stimulation with TSST-1 superantigen and influenza-derived antigen. This may indicate an increased T cell reactivity to recall antigens or microbial pathogens that may

justify the lack of evidence of increased susceptibility to infection that was expected to occur in patients after TNF depletion (10).

Noticeably, our data show for the first time that TNF-blockade induces upregulation of IL-10 production in peripheral leukocytes upon stimulation and this positively correlates with clinical response. Moreover, increased IL-10 mRNA expression was also found in skin lesions after treatment.

In contrast to the increase of cytokine production in response to stimulation, the proliferation of CD4+ and CD8+ T cells in αCD3-stimulated PBMC was not altered in 4 out of 6 patients. This could be due to the concomitant induction of immunosuppressive mechanisms, such as regulatory T cell activity that has been shown to be increased in patients upon treatment with TNF antagonists (11, 12).

In skin lesions, we found a marked decrease of Th1 and Th17 cytokine gene expression, as well as in the expression of genes encoding chemokines, such as *Cxcl10* and *Cxcl11* that mediate the recruitment of activated T cells to the inflamed tissue. In parallel, we also found a reduced expression of T cell lineage-associated marker genes, including *Cd3*, *Cd4* and *Cd8*. Thus, our data suggest that TNF blockade inhibits proinflammatory T cell mediated responses in psoriatic plaques, despite of the increased responsiveness of proinflammatory T cells in the peripheral circulation, because of the inhibition of chemokine molecules that prevents the recruitment of T cells to the inflamed tissue.

These findings also opened the question of whether the increased response of T cells to stimulation that was induced by TNF-blockade, was due to an increased responsiveness of T cells to TcR stimulation, or whether it could be due to a selective expansion of polarized T cells in the peripheral circulation. In favor of the first hypothesis, Cope and coworkers showed that TNF long-term *in vitro* exposure also attenuates TcR signaling analyzed by measuring intracellular Ca^{++} mobilization and it suppresses both Th1 and Th2 responses in a time and dose dependent manner and that these effects could be reversed by neutralizing antibodies (1, 2).

Our data showed in patients with chronic inflammatory diseases undergoing anti-TNF therapy, an enhanced Th1, Th17 and also Th2 cytokines production, without qualitative variations in the cytokine profile or in the basal level of cytokine production in unstimulated conditions. This indicated that the effect is strictly dependent on TcR stimulation.

Phenotypic analysis in the circulating CD4 T cell compartment indicated no variation in the percentage of T helper cells expressing the CXCR3 or CCR4 receptors was induced by TNF blockade. These data are not in favor of the hypothesis of the selective expansion of Th1 and Th17 cell subsets.

In addition, the surface expression of TNFR-I and –II on T cells and monocytes during anti-TNF therapy was not altered. Similarly, we did not find any variation in the circulating

percentage of CD4 and CD8 T cell memory subsets. Conversely, a recent study indicated that anti-TNF therapy causes a reduction of $CD8^+$ T_{EMRA} cells with antimicrobial activity against *M. tuberculosis* (13). However, in this study the decreased frequency of circulating $CD8^+$ T_{EMRA} was evidenced only after 2 weeks of anti-TNF treatment and returned to normal level after 1 month.

This first set of data indicates that no variation in circulating level of polarized T cell is induced by TNF-blockade, but rather an increase in the response of T cells to TcR stimulation. Therefore, we focused on the evaluation of the modulation of T cell activation induced by TNF-blockade.

Preliminary results indicated in patients an enhanced proliferative response to stimulation of the activated CD4+CD25- T cells after TNF-blocking. Interestingly, we also found a strong increase of surface induction of intermediate and late T cell activation markers CD25 and CD71 in naïve and memory CD4+CD25- T cells subsets in response to polyclonal stimulation.

The evidence that TNF-blockade increased the activation markers induction also in the naïve compartment supports the hypothesis that TNF-blockade qualitatively modulates T cell activation in response to TcR triggering, possibly by regulating TcR and/or TNFR signaling molecules.

Preliminary experiments showed enhanced phosphorylation of SLP-76 adapter protein and c-Jun transcription factor on αCD3.stimulated CD4+CD25- T cell from one individual patient

before and after TNF-blockade. Future investigation of the mechanisms underlying the effect of TNF on T cell activation will be focused on the analysis of signaling molecules of TcR and TNFR pathways, with particular attention to common signals that represent the intersection between these two pathways. Experiments are in progress to evaluate the phosphorylation of Erk and p38 MAP kinases. In addition, we will evaluate by immunostaining the kinetics of apoptosis induced by anti-CD3 (AICD, activation induced cell death), distinguishing between the early (AnnexinV+ cells), intermediate (AnnexinV+7AAD+ double positive cells) and late apoptosis (7AAD+ cells).

In the last part of the study, we evaluated the modulation induced by TNF-blocking therapy on cytokine and chemokine network by gene expression analysis, in the intestinal mucosa of patients with inflammatory bowel diseases. A considerable downregulation of the *Il17*, *Il8* and, with similar trend, also *Ifng* gene expression was found. Interestingly, we observed a decrease in the *Il5* gene expression in the intestinal mucosa of patients after anti TNF therapy. This is in accordance with an involvement of Th2 cytokines in the pathogenesis of ulcerative colitis that has been shown by Strober and coworkers (14, 15).

Remarkably, we found *Cxcl11* chemokine expression strongly downregulated in intestinal mucosa of IBD patients upon anti-TNF treatment and it was associated with a decrease of the T cell lineage-associated markers genes *Cd3* and *Cd4*. Thus, we found

that anti-TNF therapy induced downregulation of T cell recruitment signals, and that this may represents the crucial events to the inhibition of T cell mediated inflammatory response in the intestinal mucosa of IBD patients.

Conclusively, this study provides an overview of the immunological changes in psoriasis and IBD patients undergoing anti-TNF therapy. It highlights the dual role of TNF-blockade that induces an upregulation of T cell activation and effector functions in response to TcR stimulation in the peripheral compartment, while it inhibits proinflammatory T cell-mediated responses in the inflamed tissue.
These data suggest a negative regulatory role of TNF on the T cell activation and importantly indicate that the downregulation of chemokine molecules by TNF-blocking therapy represents the essential event for the inhibition of proinflammatory T cell-mediated processes in the target tissue.

In future, we will investigate more specifically the modulation of signaling pathways underlying the effect of TNF-blockade on T cell functions. We will confirm by imaging in confocal microscopy the variation of chemokine and cytokine molecules in psoriatic skin lesions and inflamed intestinal mucosa of patients undergoing ant-TNF therapy, also distinguishing the specific role of cells responsible of their production. Moreover, we are

planning to explore the molecular mechanisms underlying the regulation of IL-10 cytokine expression by TNF.

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Publications:

Abstract: Gallo B., Raeli L., Crosti M.C., Parazzoli D., Altomare G., Abrignani S. and Reali E. "T cell phenotype and effector function in patient undergoing anti-TNFα therapy". SIICA, Trieste, June 4-7 2007

Abstract: Bosè F., Gallo B., Raeli L., Frigerio E., Scavelli R., Crimi M., Altomare G, Abrignani S. and Reali E. "Immunoregulatory function in psoriasis patients undergoing anti-TNF therapy". SIICA, Rome June 11-13, 2008

Article: Bosè F., Raeli L., Garutti C. , Frigerio E., Cozzi A., Crimi M., Caprioli F., Scavelli R., Altomare G., Abrignani S. and Reali E. **"**Anti-TNF therapy increases peripheral T cell responses and IL-10 expression in psoriasis patients". 2009 Submitted.

Ringraziamenti

Voglio rivolgere un sentito ringraziamento a tutta la fondazione INGM per avermi concesso l'opportunità di svolgere questo PhD program in un ambiente nuovo e in continua espansione. Ringrazio INGM soprattutto perché in essa ho potuto essere accompagnato nella mia crescita professionale da figure eccellenti, quali il Direttore Scientifico Prof. Sergio Abrignani e, molto più direttamente, dalla mia Tutor Dr.ssa Eva Reali con la quale ho condiviso (oltre al cognome, accidentalmente e innumerevoli volte) una infinità di esperienze formative e dalla quale ho imparato molto.

In questa avventura, durata tre anni, posso dire di essere cresciuto insieme all'istituto, che ho visto essere inizialmente composto solo da 5 o 6 persone e divenire pian piano una sorta di grande e numerosa famiglia. Tra quelle 5 o 6 persone c'era già e c'è tutt'ora una persona che ho scoperto anche essere un buon amico, Pasquale Creo, che ringrazio anche per le cene post-esperimento in Duomo a mezzanotte. Ringrazio tutti coloro che in hanno lavorato con me o mi hanno aiutato e dato consiglio nelle molteplici attività svolte nel progetto, in particolar modo Monica Moro, Mariacristina Crosti, Paola Gruarin, Rossana Scavelli, Dario Parazzoli, Alessio Giavazzi, Manila Boca, tutti i dottorandi DIMET in INGM e, *dulcis in fundo*, Barbara Gallo e soprattutto Francesca Bosè per avermi aiutato, se era necessario sostituito e anche sopportato tutti i giorni e tutto il giorno in laboratorio.

Un ringraziamento a parte va ad amici e familiari per non avermi fatto mai mancare il loro sostegno in questi anni.