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Thèse préparée sous la direction du Dr Carlo Chizzolini, CC

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# TRANSFORMING GROWTH FACTOR BETA 1 (TGF-β1) et FIBROSE :

## Modulation du TGF-β1 messager dans les monocytes/macrophages par contact avec les lymphocytes T

Thèse

présentée à la Faculté de Médecine

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par

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Thèse de :

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Intitulée :

**TRANSFORMING GROWTH FACTOR BETA 1 (TGF-β1) ET FIBROSE :**
**Modulation du TGF-β1 messager dans les monocytes/macrophages par contact avec les lymphocytes T**

La Faculté de médecine, sur le préavis de Monsieur Jean-Michel DAYER, professeur honoraire au Département médecine interne, et de Monsieur Carlo CHIZZOLINI, chargé de cours au Département de médecine interne, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

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N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives à la présentation des thèses de doctorat à l'Université de Genève".

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**To my parents**

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# ACKNOWLEDGEMENTS

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# *ABBREVIATIONS*

# ABBREVIATIONS

| | |
|---|---|
| **AB** | Antibody |
| **BAL** | Bronchoalveolar lavage |
| **CD** | Cluster Differentiation |
| **C-SMAD** | Common-Smad |
| **CTGF** | Connective Tissue Growth Factor |
| **DNA** | Desoxyribonucleic Acid |
| **dcSSc** | diffuse cutaneous Systemic Sclerosis |
| **EC** | Endothélial Cell |
| **ECM** | Extracellular Matrix |
| **ET-1** | Endothelin-1 |
| **GAPDH** | Glyceraldehyde-3-Phosphate Dehydrogenase |
| **GM-CSF** | Granulocyte/Macrophage Colony-Stimulating Factor |
| **GVHD** | Graft-Versus-Host-Disease |
| **HLA** | Human Leukocyte Antigen |
| **HSC** | Hepatic Stellate Cell |
| **ICAM** | Inter Cellular Adhesion Molecule |
| **IFN** | Interferon |
| **IL** | Interleukine |
| **IPF** | Idiopathic Pulmonary Fibrosis |
| **I-SMAD** | Inhibitory-Smad |
| **LAP** | Latency Associated Peptide |
| **lcSSc** | Limited cutaneous Systemic Sclerosis |
| **LTBP** | Latent Transforming Growth Factor Binding Protein |
| **LTGF** | Latent Transforming Growth Factor |
| **MCP-1** | Monocyte Chemoattractant Protein-1 |
| **MNC** | Mononuclear Cell |
| **MHC** | Major Histocompatibility Complex |
| **mRNA** | messenger Ribonucleic Acid |
| **PCR** | Polymerase Chain Reaction |

| | |
|---|---|
| **PDGF** | Platelet Derived Growth Factor |
| **RP** | Raynaud Phenomenon |
| **RPA** | Ribonuclease Protection Assay |
| **R-SMAD** | Receptor activated Smad |
| **RT** | Reverse Transcriptase |
| **SMAD** | Small Mothers Against Decapentaplegic |
| **SSc** | Systemic Sclerosis |
| **TGF-β** | Transforming Growth Factor Beta |
| **TGF-βRI** | Transforming Growth Factor Beta Receptor I |
| **TGF-βRII** | Transforming Growth Factor Beta Receptor II |
| **Th1** | T helper type 1 cells |
| **Th2** | T helper type 2 cells |
| **TNF-α** | Tumor Necrosing Factor Alpha |
| **TIMP** | Tissue Inhibitor of Metalloproteinase |

# *INTRODUCTION (FRANÇAISE)*

# INTRODUCTION (FRANÇAISE)

## *A. La sclérose systémique*

La sclérose systémique (sclérodermie) est une maladie chronique de possible origine auto-immune, multi-systémique, marquée par une activation immunitaire aberrante, une atteinte du tissu conjonctif et de la vascularisation, conduisant à terme à une dysfonction puis à une insuffisance des organes atteints. Le risque de survenue de la sclérodermie est 3 fois plus élevé chez la femme que chez l'homme et sa faible incidence et prévalence permet son classement dans les maladies orphelines. Par la diminution importante de l'espérance et de la qualité de vie des personnes atteintes, ainsi que par l'absence de traitement efficace non toxique à disposition, cette maladie pose un véritable problème de santé.

L'Association des Rhumatologues Américains (ACR) a proposé une série de critères pour la classification de la sclérodermie basée sur la distribution des lésions cutanées et le type d'atteinte des organes internes. Cette classification, combinée à la présence ou non d'auto-anticorps, en particulier la présence d'anticorps antinucléaires, est utile pour établir un diagnostic et pour prédire la sévérité, les risques de progression et l'atteinte d'organes à suspecter.

D'étiologie multifactorielle, la sclérodermie associe au terrain génétique de susceptibilité un désordre immunologique, une atteinte des cellules endothéliales et une activation des fibroblastes. Des éléments extérieurs comme des agents infectieux et environnementaux sont également suspectés d'intervenir dans le déclenchement de la maladie.

Les patients atteints de sclérodermie présentent classiquement une atteinte cutanée symétrique, progressant en sens centripète à partir des extrémités. Une phase oedémateuse précède l'installation graduelle de la rigidité et de l'épaississement de la peau. Un dépôt excessif de matrice extracellulaire peut être observé dans tous les organes et peut conduire à des dysfonctions majeures comme l'alvéolite fibrosante dans le poumon ou encore des troubles de motilité affectant le tractus gastro-intestinal. La dysfonction vasculaire se manifeste cliniquement

par le phénomène de Raynaud, souvent première manifestation de la maladie, des ulcérations digitales, une hypertension artérielle pulmonaire et la crise rénale sclérodermique.

## *B. La Fibrose*

Lorsque des biopsies de lésions cutanées précoces de patients atteints de sclérodermie sont examinées au microscope, un infiltrat inflammatoire péri-vasculaire est observé, consistant surtout en monocytes/macrophages, lymphocytes B et T et mastocytes. Autour de cet infiltrat, les fibroblastes démontrent une production accentuée de collagène, avec pour certains, une différentiation en myofibroblastes, cellules phénotypiquement distinctes des fibroblastes par leur propriété contractile et leur capacité accrue à produire des composants de la matrice extra-cellulaire. La protéine TGF-β (transforming growth factor-β) ainsi que son RNA messager sont également détectés. Une relation spatio-temporelle a été démontrée entre la déposition du collagène et la présence de cette cytokine, suggérant un rôle prépondérant du TGF-β dans la dérégulation de la matrice extra-cellulaire.

La pathogenèse de la fibrose est ainsi caractérisée par différents degrés d'inflammation, de prolifération cellulaire et par un déséquilibre entre déposition et dégradation de la matrice extracellulaire, conduisant à l'accumulation excessive de ses composants, y compris du collagène. Dans des circonstances physiologiques, la synthèse du collagène est en balance avec sa dégradation pour un maintien optimal de son niveau dans les tissus. Si cette balance est perturbée ou rompue, une accumulation excessive de collagène liée à l'activation des fibroblastes est observée et le tissu conjonctif, habituellement lâche, est remplacé par une matrice extracellulaire dense. Alors que la proportion relative et le phénotype du collagène dans le derme sont normaux, la quantité totale de collagène est augmentée, résultant en une augmentation de l'épaisseur du derme dans la peau et du tissu conjonctif dans les autres organes.

Le processus fibrotique peut ainsi être arbitrairement divisé en 4 phases :

1) phase de signalisation fibreuse, caractérisée par la libération de médiateurs comme le TGF-β, inducteur de fibrose
2) phase de migration cellulaire et d'activation des macrophages, lymphocytes T et fibroblastes dans l'interstice

3) phase fibreuse avec accumulation de protéines et enfin
4) phase de destruction tissulaire.

## *C. TGF-β*

Le TGF-β joue des rôles divers dans une multitude de processus biologiques, allant de la différentiation à la croissance cellulaire, en passant par la régulation de l'inflammation, la cicatrisation et la formation osseuse. Dans des circonstances idéales et physiologiques, le TGF-β contribue au maintien et à la restauration de l'intégrité des tissus en stimulant la synthèse, par les fibroblastes, du collagène et d'autres protéines de la matrice extra-cellulaire, comme la fibronectine et les protéoglycanes. De plus, il inhibe la dégradation de la matrice extra-cellulaire en diminuant la synthèse des métalloprotéinases de la matrice et en augmentant la synthèse des inhibiteurs de ces dernières.

D'un point de vue thérapeutique, la compréhension des mécanismes de régulation du TGF-β suscite un vif intérêt, car il pourrait constituer une cible thérapeutique anti-fibrotique potentielle. En effet, dans le cadre de la sclérodermie et d'autres conditions pathologiques caractérisées par une fibrose excessive, le TGF-β est considéré comme l'un des médiateurs principaux impliqués dans le processus inflammatoire et dans les dommages tissulaires.

Les travaux réalisés à ce jour sur des modèles animaux suggèrent que l'administration chronique et à haute dose de TGF-β recombinant par voie systémique induit des lésions fibrosantes dans de multiples organes (foie, rein, cœur,…). D'autre part, on a remarqué une amélioration ou la prévention de la fibrose tissulaire par l'injection d'anticorps neutralisants.

Par la production et la subséquente activation du TGF-β, les monocytes/macrophages peuvent délivrer un stimulus pro-fibrotique et jouer ainsi un rôle clé dans la pathogenèse des maladies fibrosantes. Par ailleurs, les expérimentations sur tissu humain ont confirmé l'infiltration précoce des lymphocytes T. Ces connaissances nous ont mené à l'hypothèse que la transcription du gène du TGF-β pourrait être induite par un contact de cellule à cellule entre monocytes/macrophages et lymphocytes T.

## *D. Conclusion*

L'objectif de ce travail de thèse a donc été double. D'une part, une revue systématique de la littérature concernant les aspects cliniques et les mécanismes physiopathologiques impliqués dans la sclérose systémique, modèle de pathologie fibro-prolifération. D'autre part un travail expérimental dont le but a été celui d'observer si les niveaux, à l'équilibre, de l'ARN messager du TGF-β sont modifiés dans les monocytes/macrophages activés par contact cellulaire avec les lymphocytes T. Une telle activation a bien été démontrée pour le TNF-α, pour lequel un contact cellulaire entre monocytes et lymphocytes T est reconnu comme mécanisme pro-inflammatoire, induisant une régulation massivement à la hausse de cette cytokine. Par analogie, nous avons voulu tester si un tel mécanisme pouvait se vérifier pour le TGF-β. Le but étant de mieux comprendre la régulation de cette cytokine, ayant un rôle primordial dans les processus fibrotiques.

Pour ce faire, trois techniques différentes ont été utilisées et comparées afin d'évaluer la l'expression à l'équilibre de l'ARN messager du TGF-β; 1) la « polymerase-chain reaction » (PCR) classique, 2) la « RNase protection assay » (RPA) et 3) la PCR quantitative. Bien qu'une franche augmentation par contact cellulaire ait pu être vérifiée dans nos expérimentations pour l'ARNm du TNF-α au niveau des monocytes/macrophages, nous n'avons pas pu mettre en évidence une modulation quelconque de l'expression de l'ARNm du TGF-β. Ces résultats vont contre l'hypothèse que nous voulions tester et donc contre la relevance du contact entre lymphocytes T et monocytes pour la production de TGF-β. Il est tout de même possible de postuler que le contact entre lymphocytes T et monocytes ne soit pas important pour la modulation de la transcription du gène du TGF-β mais, peut-être, pour favoriser la dissociation du TGF-β de sa protéine de latence (LAP), permettant ainsi au TGF-β de devenir biologiquement actif. L'hypothèse de la modulation au niveau de la protéine du TGF-β mérite évidemment une vérification expérimentale.

# *CHAPITRE 2 INTRODUCTION*

# INTRODUCTION

## *SSc – a disease model to enlighten the pathophysiology of tissue fibrosis*

### Introduction

Systemic sclerosis (SSc), also known as scleroderma, is a chronic multisystemic disorder of the interstitial connective tissue and vasculature. Scleroderma is derived from the Greek words **sklêros** (hard or indurate) and **derma** (skin). It is characterized by 1) an inflammatory and auto-immune disorder, 2) a fibroproliferative vasculopathy, especially of the microvasculature, and 3) an exuberant fibrosis throughout the body, leading to disruption of the normal architecture and ultimately, to dysfunction and failure of the affected organs[1]. SSc has a worldwide distribution, affecting all races, and is primarily a disease of women (female-to-male ratio, 3:1). Incidence increases with age, peaking in the 40-60 years age group. Disease onset in childhood and in young men is unusual. Degree and rate of skin and internal organs involvement vary among patients and symptoms result from progressive tissue fibrosis and occlusion of the microvasculature[2].

Immune activation, vascular damage and excessive synthesis of extra cellular matrix with deposit of increased amounts of structurally normal collagen and other extra cellular matrix proteins, including fibronectin, tenascin and glycosaminoglycans in skin and other organs are all known to be important in the development of SSc[3;4]. They result from abnormal cell-cell, cell-cytokine, and cell-matrix interactions. The abnormalities observed in SSc resemble those made in other pathological fibroproliferative disorders and SSc is considered to be a model to understand fibrosis development.

### SSc : a disorder of unknown etiology

The etiology of SSc is unknown, however, several pathogenic mechanisms are proposed : genetic predisposition, endothelial cell injury, fibroblast activation and immunologic derangement. Infectious and environmental agents have also been proposed as of etiologic significance in the

development of SSc including silica dust and solvent exposure as well as herpes and human cytomegalovirus[5;6].

Italian colleagues have found in the sera of SSc individuals antibodies recognizing simultaneously the CMV UL-94 and the NAG-2 proteins also expressed on the cell surface of human endothelial cells and fibroblasts, resulting in cross-reactive antibodies that have the potential to induce EC apoptosis and activating fibroblasts[7;8].

Of interest, graft-versus-host disease (GVHD) reproduces most important features of human scleroderma, including skin thickening, mononuclear cell infiltrates, lung fibrosis and up-regulation of cutaneous collagen and TGF-β1 mRNA. GVHD is a condition encountered after bone marrow transplantation, and rarely after kidney, heart, liver and skin graft, in which viable donor T lymphocytes react against the host tissuses[9]. Based on the observation that the incidence of SSc increases in women after the childbearing years[10-12], some investigators postulated that SSc could be a variant of GVHD. Indirect support for this hypothesis is provided by a frequent history of pregnancy compared to control groups and the finding of increased frequency and quantity of fetal cells among women with scleroderma. Fetal cells are found in the involved skin and blood for many years after childbirth, but also in other tissues like the spleen. All this suggests that a GVHD-like reaction, due to microchimerism, may be a predisposing factor in women with scleroderma after child bearing years, but may also be a predisposing factor in null gravid women and in men, since non-host cells may come from blood transfusion or maternal cells in utero[11;12].

This hypothesis has been tested in a mouse model in which vinyl chloride injections lead to activation of microchimeric cells, which causes the cells to divide and multiply. The correlation between the 48-fold increase in microchimeric cells and the appearance of dermal inflammation and fibrosis similar to that of GVHD, suggests that activated microchimeric cells, in this case by a chemical agent, may be a necessary event in the pathogenesis of autoimmune disease. The presence of fetal cells in the peripheral blood of normal individuals is in itself not enough and requires a second agent (pathogen or environmental) to initiate the chain of events that leads to the disease[13].

The genetics of SSc are complex, and although the disease is inherited, it is not inherited in a Mendelian fashion. SSc occurs substantially more frequently in families (1,6%) than in the general population (0,026%), and a positive family history represents the strongest risk factor for SSc yet identified[14]. Twins show a low disease concordance rate (<5%) that is similar between monozygotic and dizygotic twin pairs[15].

## The ACR classification

The distribution of skin lesions and the accompanying pattern of internal organ involvement form the basis of the most widely accepted classification system established by the American College of Rheumatology[16]. **Systemic sclerosis** comprise limited and diffuse cutaneous disease. A patient with slow progressive face involvement and symmetric skin thickening distal to elbow and knee is considered to have **limited cutaneous scleroderma** (lcSSc). In the past, this subset of patients was identified by the CREST acronym (Calcinosis, Raynaud's disease, Esophageal dysmotility, Sclerodactyly, and Telangiectasia). On the other hand, a patient with rapid development of symmetric skin thickening of proximal and distal extremities, face, thighs and/or trunk is considered to have **diffuse cutaneous scleroderma** (dcSSc) and increased risk of early development of kidney and other internal organ disease[17]. Patients who present with an incomplete expressed rheumatic disease, who do not fulfill ACR criteria, who have overlapping feature of two or more diseases or who have non specific auto antibodies associated with early Raynaud Phenomenon are designed as having **undifferentiated systemic rheumatic disease**. These pre-scleroderma affected patients have a high rate of spontaneous remission. Evolution to a discrete rheumatic disease is only slightly more common than resolution of the syndrome.

Beside de systemic sclerosis, the **localized sclerosis** forms, classically only skin diseases, comprise 1) *morphea* (single or multiple plaques of skin indurations), 2) *linear scleroderma* (involvement of extremity or face) and 3) *en coup de sabre* (linear scleroderma of one side of the forehead and scalp producing a disfiguration looking like a wound from a sword), while the **sine scleroderma** forms are referred to diseases with involvement of visceral organs, presence of characteristics auto-antibodies but absence of skin affection .

Beside the systemic and localized classification, **chemically induced scleroderma-like disorders** such as :

- Toxic-oil syndrome
- Vinyl chloride induced disease
- Epoxy-and aromatic hydrocarbons-induced fibrosis
- Eosinophilia-myalgia syndrome

**drug induced scleroderma-like disorders** such as :

- Chemotherapeutics such as bleomycin-induced fibrosis
- Analgesia such as pentazocine, ergot and methysergide induced fibrosis
- L-tryptophane
- Others like penicillin, cocaine and beta-blockers

and other **scleroderma-like disorders co-exist** :

- Digital sclerosis in diabetes
- Chronic GVHD
- Scleroderma adultorum of Buschke
- Scleromyx edema
- Eosinophilic fasciitis
- Nephrogenic fibrosing dermopathy
- Primary amyloidosis
- Dupuytren contracture
- Werner syndrome
- Porphiria cutanea tarda
- Pseudoscleroderma
- Phenylketonuria

These illnesses mimic SSc, but should be recognized as different as progression and treatment are not identic[18].

**Clinical manifestations (attachment 1)**

Raynaud's phenomenon and blood vessels abnormalities

The most obvious clinical manifestation of vascular dysfunction, and usually the first symptom among SSc patients is Raynaud's phenomenon (RP)[19]. Thus, about 95% of SSc patients will experience this phenomenon, which is defined by episodic and reversible vasospasm of small arteries and arterioles in the extremities. RP highlights the role of vascular dysfunction early in the disease process. Fingers, toes, and sometimes the tip of the nose and the earlobes are affected. Episodes are brought on by cold exposure, vibration or emotional stress. Patients experience color changes of pallor (white), acrocyanosis (blue) followed by reperfusion hyperemia (red) on rewarming. Cyanosis is usually associated with coldness and rubor with pain and tingling. Raynaud's phenomenon may precede skin changes by several months or even years in those patients who subsequently develop lcSSc. In dcSSc, skin changes are seen typically within a year of the onset of Raynaud's phenomenon. As an example, microcirculatory volume in the affected lcSSc finger pulp is more than 15-fold reduced compared with age- and gender–matched controls. Flow velocity is also 10-fold reduced and arteriovenous anastomoses open poorly if at all in SSc patients, suggesting both neuropathic (increased activity of the sympathetic nervous system) as well as structural micro vascular abnormalities in the digital artery vasospasm[20].

Loss of microvasculature is associated with tissue hypoxia, which normally induces strong expression of VEGF and its receptors. An apparent paradox is that, in the face of tissue hypoxia and ongoing angiogenic drive, SSc is associated with a lack of angiogenesis and progressive disappearance of blood vessels[21;22].

Debate continues as to whether endothelial injury is a primary or a secondary event. But in either event, RP leads to further injury by decreasing blood flow, leading to vascular insufficiency from both arterial intimal scarring and micro vessel obliteration in skin, but also in many other organs, compromising critical organ function. Intimal edema with deposition of collagen material and concentric proliferation of intimal cells is followed by thickening and fibrosis of the vessel. Intimal fibrosis is evident and capillary dilatation as well as destruction are also frequent. The

involved vessels are usually severely restricted in terms of blood flow and may be thrombosed[18;23].

The skin

In SSc, virtually all organs may be involved, but the skin is more often affected. Hands and face generally show the most marked changes, especially in the early stages of disease. In early disease, the skin is swollen. This edematous phase may last for a few weeks, months, or even longer, and precedes the fibrotic phase. Gradually, the skin becomes firm, thickened, discolored, tight and shiny, with a characteristic loss of hair, decreased sweating, and loss of ability to make skin fold. It may be tightly bound to underlying subcutaneous tissue, and is then referred to the sclerotic phase. In the extremities, the tighten skin over fingers gradually limits full extension, and flexion contractures develop. Beside face involvement, fibrosis of the skin usually progress from distal to proximal sites, i.e., fingertips first then progression to the fingers, hands, forearms and arms. By the time the skin of the arms is tight, there may be stiffness of the legs, thighs, and in some cases, chest and abdomen. The pattern of skin stiffness is usually bilateral and symmetrical.

Rapid progression of the skin affection (1 to 3 years period) is associated with a greater risk of visceral disease, particularly lung and kidney. In dcSSc, the skin changes usually peak in 3 to 5 years and then slowly improves. On the other hand, patients with lcSSc will usually have a more gradual progression of skin changes and may continue to worse. In both subsets, skin thickening is usually greater in the distal extremity, and ulcers may appear on the volar pads of the fingertips and over bony prominences. After many years of disease, the skin may spontaneously soften and return to normal thickness in the reverse order of original skin involvement or become thin and atrophic[18;23].

The gastrointestinal tract

Nearly 90% of patients with SSc have some degree of gastrointestinal involvement, with approximately 50% being symptomatic. Esophageal dysmotility and incompetence of the lower esophageal sphincter are the most common in SSc. Symptoms principally result in gastro-esophageal reflux, abnormal motility, and stricture formation. Ulceration of the mucosa is often

present and may be due to either SSc or superimposed peptic esophagitis. Chronic esophageal reflux can lead to metaplasia of the lower esophagus. The histological finding in the esophagus consists of a thin mucosa and increased collagen deposit in the lamina propria, submucosa, and serosa.

The degree of fibrosis is less than in the skin. Atrophy of the muscularis in the esophagus and throughout the involved portions of the gastrointestinal tract is more prominent than the amount of fibrotic replacement of muscle. Similar changes may be found throughout the gastrointestinal tract, especially in the second and third portion of the duodenum, in the jejunum, and in the large intestine. Atrophy of the muscularis of the large intestine may lead to the development of large-mouth diverticula. In the later stages of the disease, the involved portions of the gastrointestinal tract become dilated. Clinically, the patient may suffer from malabsorption, alternation of diarrhea/constipation, or develop diverticulities[18;23].

The lung

Pulmonary involvement occurs in at least two-thirds of SSc patients and is now the leading cause of death in SSc, replacing renal disease, that can usually be effectively treated. Pulmonary, as a visceral complication, is second in frequency, after esophageal involvement. The two main clinical manifestation are interstitial lung disease, also called “fibrosing alveolitis” (>75% of SSc) and pulmonary vascular disease (± 10% of SSc). Lung involvement is first characterized by interstitial inflammatory alterations, but as the disease progresses, fibrotic changes become evident. Diffuse interstitial fibrosis, thickening of the alveolar membrane, bronchiolar epithelial proliferation and peri-bronchial and pleural fibrosis are observed. The diffusion capacity for carbon monoxide is impaired, and the most common symptoms are breathlessness and dry cough. Pulmonary function tests are frequently abnormal and show a reduction in vital capacity, and decreased lung compliance resulting in a restrictive pattern. Intimal thickening of small pulmonary arteries and muscular hypertrophy may occur independently from interstitial pulmonary fibrosis and produce pulmonary hypertension, particularly in a subset of patients with lcSSc. Dyspnea is the most common symptom of pulmonary hypertension, but usually develops late in the course of disease[18;23].

The kidney

Autopsy studies suggest that 60 to 80% of patients with SSc have pathologic evidence of renal involvement. Clinically evident disease is less common, but a high risk of renal crisis is present in those patients who have rapidly progressive widespread skin thickening in the first 2 to 3 years of the disease. The vascular involvement of the kidneys in generalized SSc is responsible for the so-called "scleroderma renal crisis", characterized by the sudden onset of malignant hypertension and progressive renal insufficiency, with or without thrombotic microangiopathy. This affection can rapidly progress to renal failure and death. Renal involvement consists of intimal hyperplasia of interlobular arteries, fibrinoid necrosis of the afferent arterioles, including the glomerular tuft and thickening of the glomerular basement membrane. Small cortical infarctions and glomerulosclerosis may be present. The renal pathologic change is often indistinguishable from that observed in malignant hypertension. Renal vascular lesions, however, may be present in the absence of hypertension[18;23].

**Presence of characteristic autoimmune antibodies (attachment 2)**

The hypothesis of an auto-immune origin in SSc was postulated after the finding in approximately 75% of patients of circulating antibodies directed against a number of antigens Some of these antibodies induce endothelial cell apoptosis and fibroblast activation in cell culture assays, suggesting that they play a direct pathogenic role in tissue damage[8]. Two major serum antibodies have been recognized since 1980 : anti-topoisomerase I (anti-Scl-70) and anticentromere. Although not very sensitive, antitopoisomerse-1 antibodies are highly specific, present in 20-30% of patients with diffuse disease (dcSSc) and associated with an increased risk to develop pulmonary fibrosis[24]. Anticentromere antibodies, on the other hand, are present in about 60-90% of patients with limited disease (lcSSc), and associated with increased risk to develop peripheral vascular disease and digital necrosis[25].

**Prognosis**

The extent and severity of internal organ involvement are the more important factors influencing the disease outcome and prognosis in SSc. The course of disease is quite variable. Patients with lcSSc, especially those with anticentromere antibodies have a good prognosis, with the notable

exception of those who, late in disease, develop pulmonary arterial hypertension. On the other hand, the prognosis is generally worse for patients with dcSSc, particularly when the onset occurs at an older age. Visceral organ disease may develop early in the course of those patients with rapidly progressive generalized skin thickening. Survival is determined by the severity of visceral involvement, and death occurs most often from lung or kidney failure[18].

## Fibrosis

### Definition

Fibrosis is defined as a deposition of excessive extracellular matrix (ECM) components, most notably collagen, produced by cytokine stimulated fibroblasts. As the implicated cytokine are mostly produced during chronic inflammation phase, fibrosis is, in the first place, considered as an aspect of defense process. Thus, in chronic inflammation, fibrosis can be helpful by contributing to the walling off of infected areas as with building the outer wall of an abscess, or being the end-point of a wound healing process with a fibrous scar restoring the continuity of the tissues. However, fibrosis can also contribute to disease and impairment of an organ function when an excessive or inappropriate stimulus leads to interstitial fibrosis and tissue destruction.

The following discussion reviews the ECM and the main cells and pathway implicated in the inflammatory process leading to fibrosis and tissue destruction[26].

### Extracellular matrix

The ECM is a remarkably complex structure composed of a large number of distinct molecules, providing an exceptional extra cellular framework for all multicellular organisms. The ECM is secreted locally, assembled into a network in the spaces surrounding cells, and forms a significant proportion of the volume of any tissue. It provides a substratum for cells to adhere, migrate, and proliferate, and sequesters molecules such as water, which furnishes turgor to soft tissues, as well as minerals, which procures rigidity to skeletal tissues. But the tensile strength and structural support are not the only functions sub-served by the ECM. It also provides a reservoir for growth factors controlling cell proliferation, exchanges information with cells, thereby modulating a host of processes including cell migration, attachment, differentiation, organization and repair. ECM also plays a crucial role in wound healing through its chemotactic, opsonic, and attachment properties. Three groups of macromolecules are physically associated to form the ECM; (1) fibrous structural proteins such as collagen and elastin; (2) adhesive glycoproteins including fibronectin and laminin; and (3) a gel of proteoglycans and hyaluronans. These macromolecules assemble into two general organizations : basal membrane and interstitial matrix[27].

## Collagen : main ECM component over-expressed in fibrosis

Collagen is a major gene product of fibroblasts, and consists of a family of related proteins. Nineteen distinct human collagens, coded for by at least 25 distinct genes localized on different chromosomes have been reported to date. In human skin for example, 11 collagen types have been so far detected and the corresponding genes are known to be expressed in cultured human skin fibroblasts. Each distinct collagen type has an important functional role within its compartmental distribution and plays a major role in providing tensile strength to the skin. Types I, II, and III are the most abundant interstitial collagens, whereas types IV, V, and VI are nonfibrillar and present in interstitial tissue and basal membranes[28].

Under normal circumstances, the fibroblast repair program is self limited and the overall synthesis of collagen is in balance with the physiologic degradation to maintain optimal levels of collagen fibers in tissues. If this balance is perturbed, an excessive accumulation can occur do to sustained and amplified fibroblast activation, as seen in a variety of acquired and heritable fibrous disorders. Several different mechanisms can explain the excessive deposition of collagen in fibrous diseases. The accumulation can result from (a) an accelerated production of collagen by fibroblasts, (b) an enhanced collagen gene expression at the transcriptional level or, (c) a stabilization of the collagen mRNA[29;30]. Reduced degradation of collagen, on the other hand, can have similar consequences in terms of collagen deposition[31;32]. Histological examination of skin biopsy from patients affected by SSc demonstrates that the loose dermal and subdermal connective tissue is replaced by a dense extra cellular matrix[33-35]. While the relative proportion and phenotype of collagen in the dermis is normal, the total amount of collagen is increased, resulting in augmented thickness of the dermis.

## Fibroblast : a main effector cell in fibrogenesis

The fibroblast is normally a relatively quiescent cell that divides slowly and, in the absence of injury, elaborates little matrix. In normal wound healing, myofibroblasts can be detected transiently in the granulation tissue; their removal by apoptosis is a crucial step in wound resolution[36]. By contrast, fibroblasts taken from fibroproliferative disorders, such as keloids, hypertrophic scar, SSc and liver or kidney fibrosis induced upon injury (e.g. by toxins or chronic hepatitis)[37] are all characterized by excess accumulation of collagen within the wound or tissue[36;38]. In these conditions, abnormalities in cell migration and proliferation, inflammation,

synthesis and secretion of ECM proteins and cytokines, as well as abnormalities in matrix remodeling have all been described.

Fibroblasts migration to the perivascular spaces and proliferation is seen in the early settings of tissue fibrosis. The role of endothelial cells (EC) in this process is not precisely known, however, their contribution to fibrosis is likely to be multifactorial and may involve a spectrum of delicate mechanisms and array of chemical signals. The induction of fibroblasts chemotaxis and fibroblasts proliferation by chemotactic factors realized by the EC is one possible pathway of the fibroblasts participation to fibrous development. Another major mechanism involves EC mobilization, guidance and regulation of mononuclear cell infiltration in the perivascular spaces and the subsequent impact of that process on fibroblasts activation[30].

Increased collagen production has been shown by many groups not to be a feature of all SSc skin fibroblasts strains or of all fibroblasts within a culture population, but represents an expanded subpopulation that inherently expresses increased matrix genes and produces two to three times more collagen than other fibroblasts from the same tissue[39;40]. Thus fibroblasts represent an heterogeneous population of cells localized preferentially in perivascular areas, generally in the vicinity of activated inflammatory cells[41;42]. The differentiation from quiescent into typically activated, contractile and particular aggressive profibrotic phenotype with deleterious role in decreasing compliance of the affected organ results in a cell type called **myofibroblast**[43] and occurs through four distinct mechanisms[44]:

1) stimulation by direct cell-cell contacts with lymphocytes, macrophages, mast cells, or leukocytes, which may influence fibroblast metabolism in the profibrotic direction
2) stimulation by soluble mediators released from these cells such as growth factors and cytokines (TGF-β) able to regulate growth, proliferation and activation, as well as survival against apoptotic stimuli
3) stimulation by extra cellular matrix via integrins, and
4) stimulation by environmental conditions such as for example hypoxia. Once activated, the fibroblasts produce cytokines and growth factors, such as IL-1, prostaglandin E, TGF-β , CTGF and PDGF, which may in turn serve to activate other fibroblasts and express adhesion molecules ICAM-1 on their surface, which may be important in the adherence and activation of T cells and macrophages.

This activation includes functional implications as well as phenotypic changes such as the expression of alpha-smooth muscle actin, commonly used to identify this cell type. The main characteristics of myofibroblasts during the fibrotic response, from clinically involved SSc skin biopsies can be summarized as follows :

1) enhanced collagen type I expression, but also collagen type III, and other components of the ECM. Collagen mRNA was found increased, up to three-fold compared to control, indicative of increased collagen synthesis[42;45], persisting through many passages in tissue culture[46]

2) enhanced collagen mRNA stability, resulting in pro-collagen synthesis when compared to healthy control[47]

3) increased integrin expression, a cell-cell adhesion molecule, which play a major role in the homing of pathogenic lymphocytes to skin and their adhesion to tissue fibroblasts[48-51]

4) enhanced cytokine expression, such as PDGF, TGF-β, CTGF, IL-6 and MCP-1[32;43].

5) enhanced expression of tissue inhibitor of metalloproteinases (TIMP), leading to reduced collagen degradation and decreased ECM turn over[31]

Recent DNA micro array studies have revealed that fibroblasts from different anatomic locations differ markedly in their gene expression patterns and show site-specific variations in their transcriptional profiles that seem to be related to their location within the body [36].

**Monocyte/macrophage : a central role in tissue fibrosis**

When early skin lesions of SSc patients are microscopically examined, a perivascular inflammatory infiltrate is observed[52], consisting of monocytes, macrophages, T lymphocytes and mast cells, increased in numbers or activated state[53]. Around this infiltrate, TGF-β protein and messenger RNA have been localized by immunostaining and in situ hybridization studies and fibroblasts have been observed to display up-regulation of cutaneous collagen mRNA and enhanced collagen synthesis, suggesting that these MNC infiltrates may directly and/or

indirectly, through cytokine production or regulation, deregulate the ECM turnover in a profibrotic manner[54;55]

In lung fibrosis, the results obtained in animal models are consistent with results reported in human patients with idiopathic pulmonary fibrosis (IPF). Alveolar macrophages have been identified as the lung cells responsible for increased TGF-β mRNA level during the course of lung fibrosis development in bleomycin treated mice[56] and hamsters and in patients with advanced IPF[57]. The same result was obtained in an IL-13 transgenic mice model in which IL-13 over-expression causes pulmonary fibrosis through an increased TGF-β production by macrophages[58]. TGF-β mRNA and protein have been demonstrated in large quantities in macrophages and in lesser quantities in bronchiolar epithelial cells, alveolar type II cells, and eosinophils.

A temporal and spatial relationship between collagen and TGF-β production by macrophages has also been demonstrated by Khalil and co-worker[59], suggesting an important if not primary role for TGF-β in the pathogenesis of pulmonary fibrosis. The peak of lung TGF-β levels coincided with intense TGF-β staining of macrophages dispersed in the alveolar interstitium. Later in the course of the response, TGF-β was primarily associated with the maximum fibroblast collagen synthesis. Indeed macrophages infiltrating fibrous lesion are capable of inducing myofibroblasts development through TGF-β secretion. A direct relation between the presence of macrophages and myofibroblasts was demonstrated in hepatic and renal fibrosis by Ide et all.[38;38]. Of interest too, the murine Scl GVHD mode of SSc in which fibrosis can be prevented by anti-TGF-β antibodies, mainly produced by macrophages[54]. Taken together, these findings suggest that myofibroblasts and macrophages contribute to progressive fibrosis in the lung, skin, liver and kidney disease.

**T lymphocyte : a cell potentially initiating fibrosis**

Scleroderma and scleroderma-like lesions in patients with GVHD after bone marrow transplantation and mouse murine models of chronic GVHD are all conditions believed to be initiated by, and dependent upon T cells, as an inflammatory infiltrate rich in T lymphocytes expressing HLA class II molecules (activated T cells) precedes the development of fibrosis and dominate the inflammatory infiltrates in these tissues[60].

An emerging hypothesis for the pathogenesis of fibrotic disorders implicates an altered balance between Th1 and Th2 cytokines[61]. Recently, various soluble factors produced by activated, polarized T cells have been investigated for their capacity to regulate ECM production. In particular IFN-γ and IL-4 cytokines, produced in high or low level depending on Th1 or Th2 profile have been investigated with opposite effects on fibroblast growth and collagen production. These two immune mediators are mutually inhibitor and represent the prototypic cytokines which functionally define the CD4+ polarized T-cell in Th1 or Th2. Strong evidence suggests that Th1 and Th2 cells do not derive from distinct lineages, but rather develop from the same T helper cell precursor under the influence of both, environmental and genetic factors acting at the level of antigen presentation. Activated Th1 cells produce high IFN-γ, low IL-4 as well as IL-2 and TNF which activate macrophages, suppress fibroblast activation and inhibit collagen production. Th1 cytokines have been documented as useful antifibrous agents in human and renal fibrous disorders. On the other hand, activated Th2 cells produce high IL-4, IL-5, IL-13 and low IFN-γ, inhibit several macrophage functions and show enhanced rather than inhibited collagen production by skin fibroblasts[62]. Th2 responses are implicated in atopic disorders, idiopathic pulmonary fibrosis (IPF), radiation-induced pulmonary fibrosis, bleomycin lung, hepatic fibrosis and SSc. Thus activated Th1 and Th2 lymphocytes regulate collagen production in opposite direction.

The degree of inflammation and fibroblast activation/proliferation during the pathogenesis of chronic pulmonary inflammation may be depending on a Th1 and Th2-like cytokine balance which is expressed during the evolution of the disease[63]. The type 2 fibrosis cytokine hypothesis suggests that fibrosis occurs when cytokine balance shifts to a Th2 direction as in SSc patients, in which IL-4 protein and mRNA production by mononuclear cells from the blood and BAL are increased with little change in IFN-γ proteins and mRNAs[52;60;64]. Microchimeric male-offspring T cells present in the blood and skin of women with SSc react with maternal MHC antigens, have been demonstrated to exhibit a Th2-oriented profile[65].

Interestingly, in the skin of patients with SSc, both CD4+ and CD8+ T cells are found, but CD4+ T cell subpopulation appear to outnumber CD8+. In contrast, analyses of T cells within BAL (broncho-alveolar lavage) of SSc patients with alveolites show increased CD8+, and reduced CD4+. The dominance of distinct T cell subpopulations in the skin (CD4+) and lungs (CD8+) of

SSc patients suggests a complex regulation and different T cells contribution to the disease process in different organs[60].

## Inflammation

Inflammation precedes or at least accompanies most processes leading to fibrosis. This review has been recently challenged, particularly by authors interested in lung fibrosis. Available data points to different routes for developing diffuse pulmonary fibrosis[66] : **a)** the inflammatory pathway represented by almost all non-IPF interstitial lung diseases like SSc, where an early, clearly distinguishable phase of alveolitis precedes the subsequent fibrotic phase [67]and **b)** the epithelial pathway, represented by the IPF in which, according to some authors, an abnormal wound healing in response to multiple, microscopic injured sites are regions of fibroblastic proliferation, the "fibroblasts foci", associated with repair and evolving fibrosis[67;68].

### Inflammatory pathway

By analogy to wound healing, a form of controlled repair, an inflammatory phase appears to precede and provoke the fibrotic phase in SSc. Most current hypotheses of SSc pathogenesis focus on the interplay between early inflammatory events and microvascular changes, resulting in the generation of a population of activated fibroblasts[41;41;42;47]. The mononuclear inflammatory infiltrate precedes the development of increased ECM deposition and the characteristic vasculopathy changes[45;53] .

Recent studies on skin and lung suggest that in the early stage of SSc disease, aberrant expression of cell adhesion molecules on leukocytes and endothelial cells, in response to inflammatory cytokines (specifically TNF and INF-γ), result in the accumulation of specific, activated leukocytes, which may be involved in the fibrous process by the release of cytokines such as IL-4 or growth factors like TGF-β that stimulates the synthesis of ECM components. Indeed IL-4 has been reported to increase proliferation and chemotactic of fibroblasts as well as increase in type I collagen[69;70;70;71;71], whereas TGF-β is unambiguously a profibrotic cytokine. Thus the enhanced interaction between fibroblasts and the activated inflammatory cells such as monocytes/macrophages and lymphocytes in SSc may influence fibroblast metabolism in the profibrotic direction and thus contribute to the elevated synthesis of ECM[72;53].

Epithelial pathway

While research has largely focused on inflammatory mechanisms for initiating the fibroproliferative response and structural alterations of the lung, recent evidence strongly suggests that fibro-proliferation and fibrosis can occur independently of inflammation in idiopathic pulmonary fibrosis (IPF), an "epithelial-fibroblastic disease" caused by a mesenchymal, rather than an immune disorder. IPF is a chronic, progressive and usually fatal lung disease of unknown etiology characterized by epithelial injury and activation, disruption of the epithelial basement membrane, formation of distinctive sub-epithelial fibroblast/myofibroblast foci, profound defect in the ability to conduct appropriate re-epitheliazation and impaired ECM remodeling, which progresses to destruction of the lung parenchyma. Inflammation-independent fibrosis in IPF includes the following evidences[73] :

**1)** Intra-alveolar macrophage is a non-specific response to cigarette smoke and, accumulation is to be expected as most patients with IPF are current or former cigarette smokers[74;75]. In addition, interstitial lung disease such as hypersensitivity pneumonitis, in which inflammation is a prominent feature of early disease, often do not progress to end-stage fibrosis.

**2)** Epithelial injury in the absence of ongoing inflammation is sufficient to stimulate the development of fibrosis. Transgenic animals show that it is possible to dissociate the inflammatory response from the fibrous response. Sime and co-workers using two replication-deficient adenoviruses expressing active and latent TGF-β1 showed that over-expression of both trans-genes provokes transient inflammation. However, only the active form induced fibroblast proliferation and progressive ECM accumulation[76].

**3)** Inflammatory response to a lung fibrogenic insult is not necessarily related to the fibrous response, and clinical measurement of inflammation fail to correlate with stage or outcome[77;77].

**4)** Anti-inflammatory or immunosuppressive therapies fail to help patients with IPF[78;79]. Moreover, it also is consistent with the tendency of strong inducers of epithelial apoptosis (e.g. antineoplasic agents such as bleomycin, adryamycin and methotrexate) to induce lung fibrosis.

Of interest, epithelial apoptosis co localizes in regions of intense myofibroblast activity. These myofibroblats produce factors identified as angiotensin peptides capable of killing alveolar

epithelial cells[80] and leading to severe epithelial injury which is thought to drive a fibrous response through the elimination of the many "antifibrotic" functions of the epithelium[81]. These functions include among others constitutive synthesis of matrix metalloproteinases known to degrade interstitial collagens.

**SSc : a vascular disease and the role of ET-1**

It is proposed that repeated cycles of endothelial injury, followed by platelets aggregation, obstructive micro vascular lesions, and increased vascular wall thickness, lead to release of endothelium adhesion molecules, cytokines and growth factors as well as secondary products of platelets activation, all of which are capable of activating local inflammatory processes and inducing collagen synthesis present in all involved organs in scleroderma. The vascular pathology is associated with altered vascular function, with increased vasospasm, reduced vasodilator capacity and increased adhesiveness of the blood vessels to platelets and lymphocytes. The impact of the vascular pathology on the evolution of tissue fibrosis is not known. Still, cytokines (TGF-β, IL4), growth factors PDGF and vascular factors (endothelin) are possibly crucial signals that link the vascular disease to tissue fibrosis.

Endothelin-1 (ET-1), measured at high levels in BAL fluid from patients with fibrous lung diseases[82] and in the circulation of patients with SSc, has been implicated in the pathogenesis of systemic sclerosis as it is both, a potent vasoconstrictor and a fibroblast mitogen. ET-1 cytokine belongs to a family of 21-amino acid peptides, synthesized and secreted by endothelial cells, acting via 2 receptors : $ET_A$ on vascular smooth muscle cells and $ET_B$ on fibroblasts, vascular smooth muscle and endothelial cells. ET-1 exerts a wide range of biologic effects, including setting of inflammation, stimulation of extra cellular matrix accumulation and fibrosis. In addition to its chemo attractive activity, ET-1 induces a fibrogenic phenotype in normal dermal fibroblasts that resembles such seen in fibroblasts grown from lesional SSc skin[83]. In line with these findings, increased ET-1 levels were found in a rat model of bleomycin-induced pulmonary fibrosis and protective properties were obtained from an endothelin receptor antagonist.urrent research points to the imbalance between vasodilatation and vasoconstriction in the SSc disease process. Vasodilatation as well as vasoconstriction are dependent upon both, an intact endothelium and a neural control mechanisms. It is likely that both elements are dysfunctional and/or damaged in SSc, with a disturbed balance in favor of reduced vasodilatation or increased

vasoconstriction[84-86]. No unifying hypothesis has been accepted, anyway, as vascular damage progresses, the permeability and the thrombosis protection function of the endothelium are altered, exaggerated vasospasm appears, vascular bed in the skin and the other sites is diminished, resulting in a state of chronic ischemia. Initially, the vascular involvement of RP has been considered to be mainly microvascular[87], in recent years however, it becomes increasingly recognized that vascular dysfunction is widespread, affecting both, large as well as small vessels throughout the body[88].

**In conclusion**

Initially, fibrosis appears to follow a similar pathway to that of normal wound healing, consisting of a dynamic and interactive process involving soluble mediators, blood cells, extra cellular matrix, and parenchyma cells. Wound healing leads to restoration of integrity of an injured tissue and can be divided into three phases; **1)** an initial inflammation phase leads to the influx of inflammatory cells which attract phagocytes that remove dead cells and other debris that cumulate after injury and fibroblasts influx **2)** a granulation tissue phase with ECM deposition and 3) a tissue remodeling phase with scar constitution. Once an abundant collagen matrix has been deposited in the wound, the fibroblasts stop producing collagen, undergo apoptosis and the fibroblast-rich granulation tissue is replaced by a relatively acellular scar[89]. Apoptosis, in contrast to necrosis or "accidental" cell death, is an active form of cell death that requires the activation of specific enzymes and other components of signaling pathways, and represent the crucial step which distinguishes wound healing from a fibrotic disorder, resulting in an excessive deposition of ECM components without resolution.

Generally, fibrosis develops after an initial insult, which leads to cellular injury, as for example asbestos in the case of pulmonary fibrosis or viruses in the case of liver fibrosis. But fibrosis may also develop as a result of excessive matrix deposition surrounding normal parenchyma and compromising its function such as in SSc. Here, although opinions differ (see inflammatory and epithelial pathway), excessive matrix deposition seems not to be triggered by tissue injury, but rather by a direct stimulation from local factors.

Indeed, three factors appears to determine the fibrous response :

1) a continuous insult or stimulus, suggesting that the fibrotic process is an on-going event,
2) an excessive synthesis of collagen and other ECM components and
3) a decrease in resolution owing to down regulation of the degrading enzymes involved in removing the scared tissue.

And four arbitrary phases divide the fibrous process :

1) the fibrous signaling phase, characterized by the release of soluble factors, such as TGF-β that contribute to ongoing inflammation and have fibrosis-promoting effects
2) the cellular migration phase of macrophages, T lymphocytes and fibroblasts into the interstitium and its activation.
3) the fibrous phase when matrix proteins begin to accumulate. During this time, both increased matrix protein synthesis and impaired matrix turnover are evident and
4) the phase of tissue destruction, the ultimate sequel to excessive matrix accumulation.

Although the mediators involved in the healing and in the fibrous process appear to be the same, an over-production or activation of these factors or an excessive sensibility owing to a receptor up-regulation is postulated[26]. Pathologic fibrosis is thought not to be different from normal connective tissue formation, with identical molecular mediators implicated and can be therefore considered as a wound healing process that for a not yet defined reasons, escapes control.

## TGF-beta

### Introduction

Discovered as a growth factor[90], TGF-β, a multifunctional cytokine, has emerged as a pivotal immunoregulator and pro-fibrotic cytokine. Cytokines are proteins that regulate the immune system, participate in intercellular communication and cause a variety of responses in different target cells[91;92]. Additionally, they trigger the synthesis of other cytokines, which, in turn, may accentuate or abrogate their responses. As any cell can express TGF-β receptors and secrete TGF-β ligand, this cytokine has a wide range of action, affecting nearly every cell and tissue. It plays diverse roles in many biologic processes ranging from cellular differentiation and growth regulation to inflammation, wound healing and bone formation[93]. TGF-β contributes to the pathogenesis of disease as diverse as autoimmune diseases and carcinogenesis[90;94]. Its multiple, often contradictory facets in autoimmune and inflammatory processes, prompted Whal and co-workers in 1994 to entitle an editorial paper on TGF-β : "Transforming growth factor beta - The Good, the Bad and the Ugly"[95]. This chapter do not pretend to be complete, but is an attempt to highlight some of the aspects of TGF-β structure and function relevant to fibrotic processes.

Several cytokines including but not exclusively PDGF, TNF, CTGF, IL-4, IL-13 and IFN-γ have been examined as potential effectors of fibrosis, but TGF-β has received most attention and is considered to be the main fibrogenic growth factor implicated in fibrosis pathogenesis[96]. The marked TGF-β matrix stimulatory properties, in particular its role in initiating the typical SSc fibroblast phenotype, have implicated this protein as a potentially important mediator in scleroderma[96]. Skin biopsies, and broncho-alveolar lavage from patients with SSc have produced conflicting results on the levels of TGF-β mRNA and proteins, but TGF-β can be readily demonstrated in both, involved skin and fibrous lung[97-99].

### Three isoforms

Three TGF isoforms β1, β2, and β3 are present in mammals. Although, the three isoforms are biologically similar, there are differences in their potencies and some biological activities. The absence of TGF-β in knockout mice models, whether β1, 2 or 3, results in death within hours, days, or weeks. Interestingly, although all were ultimately lethal, the phenotypes of the 3

Isoforms-specific knockouts varied, revealing more isoform-specific functions than had been anticipated from in vitro studies. TGF-β1 null mice show no gross developmental abnormalities, but develop an excessive inflammatory response with massive infiltration of lymphocytes and macrophages in many organs, primarily in heart and lung leading to organ failure and death. Lesions resemble those found in autoimmune disorders, or GVHD. This phenotype suggests a prominent role for TGF-β1 in homeostatic regulation of immune cell proliferation and extravasations into tissues[98;100]. TGF-β2-null mice have multiple developing defects of the heart, lungs, skeleton, eyes, and ears that result in perinatal mortality[37]. This phenotype suggests a prominent role for TGF-β2 in embryogenesis, and morphogenesis[101]. Mice lacking TGF-β3 die from more limited and consistent features including delayed pulmonary development and defective palatogenesis[102].

The 3 isoforms share 64-82% amino acid sequence homology and in specific biological assays are largely interchangeable. However, each is encoded by a separate gene and unique promoter, and shows distinct spatial and temporal characteristics[103]. TGF-β1 is the most abundant isoform in tissues, particularly abundant in bone, lung, kidney and placental tissue. The β1 is produced in large quantities by platelets, released upon aggregation and during clot dissolution at sites of injury[104;105], by inflammatory infiltrating cells such as monocytes/macrophages and lymphocytes, by fibroblasts and epithelial cells[106-108]. TGF-β2 is present in body fluids such as saliva and breast, and TGF-β3 is the least abundant of the three.

**<u>Secretion of an inactive protein</u> (attachment 3)**

Most cells secrete TGF-β as a latent complex that is sequestered and modified intracellular prior to secretion[109]. One of the most relevant intracellular modifications is the proteolytic digestion of this precursor protein by the endopeptidase furin[110], resulting in the release of the biologically active C-terminal pro-region (mature TGF) and the subsequent non-covalent association of this fragment with the remainder of the precursor, referred to as the latency-associated peptide (LAP). The proteolysis yields two products that assemble into dimers. When mature TGF-β is associated with the LAP protein (L-TGF), secretion of either large or small latent complex from the cell is facilitated[111]. When L-TGF is associated with a latency TGF binding protein (LTBP) it is referred to as “large” latent TGF, while L-TGF alone is referred to as “small” latent TGF-β[112;113]. L-TGF-β cannot interact with its receptor as receptor binding site is hidden and is therefore

biologically inert. The unique ability of LAP to confer latency, prevents binding of secreted TGF to ubiquitously expressed receptors and assures an extra cellular reservoir of TGF that can be activated on demand. Activation results in converting LTGF-β to active TGF-β, a complex and multifactor regulation, regarding its ubiquitously distribution. LAP can be removed from mature TGF-β by plasmin, thrombospondin-1, β-6 integrin, extreme pH such as 2 or 8, heating to 100˚C, urea, etc…[93;114].

Although the SMAD pathway is considered to be the central conduit for signals from the TGF-β receptors, emerging evidence highlights the importance of non-SMAD pathways. Non-SMAD molecules activated by TGF-β include protein kinases (MAPKS p 38 and JNK and TGF-β activated kinase 1), lipid kinases and the calcium-dependent phosphatase calcineurin. These novel non-SMAD pathways interact with each other and with SMAD proteins in complex, cell lineage-specific signaling networks[115].

The regulation of TGF-β action is critical to maintain normal physiological functions, and deregulation implicating altered SMAD signalling[116] or altered TGF-β receptor signalling[117] can lead to pathogenesis of numerous diseases. Thus activation of L-TGF-β forms, is an important posttranscriptional check point in both physiological and pathological situations. The current literature supports two major mechanisms of L-TGF activation, and suggests that the mechanism of activation of L-TGF may be varied and context-dependent. For TGF-β to become biologically active, the LAP has to be either released from its association with L-TGF by proteolytic digestion of the precursor or to undergo conformational changes that allow TGF-β binding to its receptor without being released from the LAP complex[112;113;118;119].

**<u>Signaling pathway from cell surface to nucleus</u> (attachment 4)**

Mature TGF-β, once released into the local environment, induces its potent effect first by binding to its cognate receptor TGF-βRII. This binding activates the receptor which is then phosphorylated at specific serine and threonine residues, and, in turn, recruits TGF-βRI. The heterotetrameric receptor complex so formed activates the TGF-βRI kinase[120;121]. Upon activation, the cytoplasmic portion of the transmembrane receptor interacts with intracellular signal-transuding proteins, identified as Smads[122;123]. Certain Smad family members, can interact directly with the activated TGF-β RI, which signals from the cell membrane to the nucleus[122]. To

date, eight different mammalian Smad genes have been described, which fall into three distinct functional categories : receptor-activated Smads (R-Smad), which includes Smads 1,2,3,5 and 8 ; a single common mediator Smad 4 (C-Smad) and inhibitory Smads 6 and 7 (I-Smad)[124]. The current model for downstream signaling via the Smad pathway is as follow : 1) signal transuding Smad 2 and 3 bind to and are phosphorylated by TGF type I receptor kinase; 2) the phosphorylated pathway-specific Smads then hetero-oligomerize in the cytoplasm with the C-Smad 4; 3) the heteromeric Smad4-containing complex is then translocated into the nucleus, where it mediates transcriptional activation of target genes[125], as for example type I collagen gene promoters (COL1A1 and COL1A2). While Smad proteins can bind DNA directly, they likely also require the cooperation of multiple, diverse, sequence-specific-DNA binding proteins, as well as co-activator molecules to mediate their transcriptional effects[120]. Inhibitory Smads, function in a negative feedback loop to terminate or reduce the strength of the signal[126;127]. Smad 6 and 7 have the ability to interact both with type I receptors and with other Smads. The mRNAs for Smad6 and Smad7 are rapidly induced by treatment of cells with TGF-β suggesting that I-Smad are direct feedback effectors of a ligand-induced signal to suppress a response.

**TGF-β implicated in ECM homeostasis**

Biochemical active TGF-β stimulates the production of collagen adhesion receptors, and extracelullar matrix proteins (collagen type I, II,III, IV, V and VII, fibronectin, elastin, proteoglycan, thrombospondin, etc…), inhibits matrix degrading metalloproteinases (plasminogen activator, collagenase, elastase, etc…), up-regulates proteinase inhibitors, and in vivo induces fibrosis in mice[128-130]. TGF-β influences ECM deposition, but also inflammation, and angiogenesis, all critical components of the tissue repair process, which, under ideal circumstances, leads to the restoration of normal tissue architecture[129].

**TGF-β : a central role in wound healing and in tissue repair**

When tissue is injured, a repair process is initiated which almost invariably is associated with increased expression of TGF-β mRNA and protein by parenchymal cells and/or infiltrating lymphoid cells, suggesting a predominant role of this cytokine in tissue repair. Many groups have focused their attention on TGF-β implication in wound healing process. For instance application of collagen scaffolds impregnated with TGF-β to full thickness skin defects in rabbits results in

faster wound epithelisation and wound contraction than in control animals treated with plain collagen scaffolds[131]. TGF-β appears to markedly enhance the ability of fibroblasts to contract collagen gels and secure a provisional wound matrix[132;132]. Too much locally active TGF-β has also been demonstrated to result in fibrosis and overzealous tissue wound healing[133].

On the other hand, TGF-β null mice show markedly delayed healing of incisive wounds[134]. Inhibiting the activity of TGF-β using topical neutralizing AB applied to rodent cutaneous wounds has an anti-scarring effect, associated with reduced monocytic infiltrate[135;136]. These findings are similar to those observed during fetal wound healing where monocytes are largely absent, endogenous TGF-β levels is low, and healing occurs by scar-free regeneration[137;137-139]. Wound healing in fetal tissues is different from that seen in adult animals, in that fetal wounds heal without scar, thus inflammation, wound contraction, and fibrosis are absent[140;140;141]. Early murine fetal fibroblasts express less TGF-β than late fetal or adult fibroblasts, and it may be that this relative deficit promotes scar healing impairment. When cellulose discs impregnated with TGF-β were implanted subcutaneous into fetal rabbits, increased expression of collagen I mRNA and more fibrosis compared with control fetal rabbits was observed. Thus, the delivery of exogenous TGF-β caused fetal wound healing to resemble more closely that of adult rabbits[141]. TGF-β's ability to promote matrix deposition may also be beneficial in repairing destroyed matrix cartilage. Local administration of TGF-β stimulates proteoglycan synthesis and may restore prostaglandin content of depleted cartilage[142;143].

Physiologic wound healing is a self-limited process. Once a sufficient amount of matrix accumulates in the wound bed, fibroblasts either revert to their quiescent phenotype or undergo apoptosis, and matrix accumulation is terminated. Work done so far suggests that TGF-β contributes to maintain and restore tissue integrity, with fibrosis being the consequence of an overly exuberant repair process. In pathologic scarring, activated fibroblasts continue to made matrix components and profibrotic cytokines, even after complete healing has been achieved. Temporal and spatial restriction of repair is lost, and chronic progressive fibrosis ensues.

## TGF-β : a central role in tissue fibrosis

Overproduction of TGF-β, clearly underlies tissue fibrosis in numerous experimental and human diseases. The causal relationship between this cytokine, inflammation, and pathogenesis of

fibrosis has been demonstrated by antagonizing the activity of TGF-β with neutralizing antibodies. In vitro evidence comes from fibroblasts in which TGF-β added on culture plastic dishes represents one of the most potent pro-fibrotic stimuli to fibroblasts[144], and SSc fibroblast phenotype can be initiated or maintained by TGF-β in vitro. But the most persuasive evidence comes from human tissue biopsies and in vivo experiments. Elevated tissue expression of TGF-β mRNA correlates with measurements of procollagen mRNA in tissue and degree of histological injury. This has been demonstrated in human with idiopathic pulmonary fibrosis[145] and hepatic cirrhosis[146] as well as in animal models of pulmonary inflammation and fibrosis induced by the antineoplasic antibiotic, bleomycin[57] in liver[147], and in renal fibrosis[148]. Terrell and co-workers demonstrated that chronic systemic administration of high doses of human recombinant TGF-β1 in rabbit, rat and pig induces fibrotic lesions in multiple organs (liver, kidney, hart). Wound treated by doxorubicin, a potent antitumor drug, show impaired healing associated with decreased mRNA for TGF-β and collagen. Correction of the defect was observed by topical replacement of this growth factor[149].

TGF-β induced profibrotic cellular and transcriptional responses in vitro and in vivo can be effectively prevented by TGF-β inhibitors or antagonists. Injection of naturally occurring TGF-β antagonists such as decorin, which binds to TGF-β, and neutralizes its biological activites[150], neutralizing antibodies, or soluble TGF-β receptors have been shown to prevent or ameliorate tissue fibrosis in various animal models[151;152]. Mouse models such as murine sclerodermatous GVHD or bleomycin lung and skin induced fibrosis[151], considered models of SSc, show effective prevention of fibrosis by anti-TGF-β AB administration. Matrix accumulation is also suppressed in experimental glomerulonephritis by the injection of decorin, a proteoglycan which inhibit TGF-β action[153;154]. These results suggest that TGF-β :

1) plays a critical role in the initial physiopathologic process of fibrosis
2) stands in a dose relationship with fibrosis

Recent reports have demonstrated an increase in TGF-β receptors in cultured SSc fibroblasts compared to normal cells[155;155;156]. It has been proposed that in scleroderma, fibroblasts may over-express collagen due to extreme sensitivity to the effects of TGF-β[157]. This is supported by the ability of neutralizing anti-TGF-β AB to normalize elevated collagen production in sclerodermal fibroblasts[156].

**TGF-β1 isoform**

Each of the phenotypic attributes of scleroderma fibroblasts is reproduced by TGF-β1 treatment of normal fibroblasts. Fibroblasts have an exaggerated proliferation response to TGF-β1, and express high levels of collagen mRNA. TGF-β1 is the predominant isoform present at sites of inflammation/injury, and is the major isoform secreted by circulating monocytes and tissue macrophages[106;107;107]. Thus, in bleomycin-induced pulmonary fibrosis in mice, TGF-β1 mRNA expression predominates, while TGF-β2 and TGF-β3 RNA expression are unaffected[144]. In that model, monocyte-produced TGF-β1 is thought to drive the fibrosis. Compelling evidence implicated that only TGF-β1 is involved in the pathogenesis of the cutaneous and pulmonary fibrous process in SSc patients[97;99;158]. However other studies have reported a markedly elevated TGF-β1 and -β2 expression in tissue from patients with lcSSc and dcSSc [45;159].

Pro-inflammatory

TGF-β has pluripotential effects not only on extra cellular matrix homeostasis, but also on immune regulation[160;161]. At sites of tissue injury, initial platelets degranulation results in a concentrated source of local TGF-β[104]. This observation implicates TGF-β as an early mediator of the inflammatory response. In vitro, TGF-β induce a chemo attractant response on human peripheral blood monocytes[162]. Thus TGF-β has been shown in vivo to affect the recruitment of monocytes to the sites of inflammation/injury. For example intradermal or intraarticular injection of TGF-β stimulates monocytes infiltration and matrix deposition[163;164].

The influx of monocytes can be further modulated by a number of factors including state of cellular differentiation, receptor expression, cytokine and proteolytic milieu, and the expression of specific cell surface adhesion molecules[165;166]. In vivo evidence suggests that overproduction and/or activation of TGF-β contribute to profound pro-inflammatory effects. This emanates from studies where TGF-β activity has been blocked by specific antagonists and the local inflammatory response was markedly reduced. Antagonists of TGF-β delivered locally can break the cycle of leukocyte recruitment and influx of other inflammatory cells as well as fibrous sequel. Further evidence for the in vivo pro-inflammatory action of TGF-β stems from studies using transgenic mice over-expressing TGF gene. These animals exhibit elevated circulating levels of TGF-β and progressive hepatic fibrosis and renal disease, characterized by increased matrix deposition and inflammatory infiltrate[167].

Anti-inflammatory

During the early stages of inflammation, TGF-β acts locally within the site of injury as a pro-inflammatory agent by recruiting and activating neutrophils, monocytes, and lymphocytes. But as the recruited monocytes are activated and differentiated, they lose responsiveness to TGF-β, autocrine TGF-β release declines, and specific immunosuppressive actions of TGF-β predominate.

Current literature suggests that regulation of TGF-β activity lies in the balanced expression of the membrane TGF-β type I and II receptors, and that this balance is determined in part by cell differentiation. Resting blood monocytes constitutively express approximately 400 high-affinity type I/II TGF-β receptors which render these cells extremely sensitive to stimulation by TGF-β[162;168]. But down regulation of TGF-β receptor expression appears as monocytes are activated at sites of inflammation and differentiates into macrophages. Thus, one mechanism for down regulation of the inflammatory response involves cellular differentiation and marked reduction of the response to TGF-β, resulting in decreased cellular chemotaxis[94;169]. This may help to explain the paradox of TGF-β acting as both a pro-inflammatory agent and as a factor contributing to the resolution of inflammation.

The multiorgan infiltration by leukocytes in the TGF-β1 knockout mice illustrates the importance of TGF-β gradients at the sites of injury/inflammation[166;170;170;171]. After amplification of the early inflammatory response, the major role of TGF-β in vivo is the resolution and down regulation of such a response[172]. TGF-β mediates most of its anti-inflammatory action via modulation of cytokines and chemokines produced by macrophages. Recent studies utilizing TGF-β gene transfer to treat chronic inflammatory diseases revealed that increased TGF-β was associated with decreased MCP-1, decreased mononuclear cell infiltration, and improvement of tissue pathology. During this resolution phase, TGF-β production by macrophages is stimulated by the ingestion of apoptotic neutrophils, and TGF-β suppresses pro-inflammatory cytokine production by macrophages, including IL-8, GM-CSF and TNF-α[173].

# *RATIONALE AND HYPOTHESIS*

# RATIONALE & HYPOTHESIS

Fibrosis is characterized by increased ECM deposition and is preceded by fibroblasts proliferation and activation. The interplay between fibroblasts and adjacent recruited inflammatory cells may impact on ECM balance[174]. The mechanisms likely involved consist in the release of soluble mediators such as cytokines and growth factors and/or direct cell-to-cell contact via membrane associated molecules. Indeed, a complex relationship is postulated to take place between inflammatory cells and fibroblasts with a local network of cytokines strongly implicated in the ECM regulation.

Among inflammatory cells, T cells are found in increased number and activated state in early fibrotic processes. A complex facet of T cell-fibroblast interaction in human was demonstrated with differential sensitivity and opposite outcome in ECM deposition according to the implicated T-cell subpopulation. Indeed, T lymphocytes encomprise several subpopulations of which some can be identified based on the pattern of the cytokines they produce. Type 1 cells are defined as high IFN-gamma/low IL-4 producers and were shown to inhibit ECM deposition. Type 2 cells are defined as low IFN-gamma/high IL-4. Type 2 cells are thought to enhance ECM deposition mediated by an evident profibrotic activity of IL-4 and IL-13[175].

Through the production and/or regulation of TGF-β bioactivity, the macrophage is thought to play a pivotal role in tissue repair and fibrosis[176] and in the pathogenesis of idiopathic pulmonary fibrosis[145], hepatic cirrhosis[146], renal fibrosis[29] and SSc[54]. Indeed, an increased TGF-β transcription has been shown in comparison to controls and a temporal and spatial relationship been assessed between TGF-β synthesis and maximal fibroblast collagen production. Macrophages are targets of T cell cytokines as documented with a transgenic mouse model expressing high IL-13 level in bronchial epithelial cells[177;177]. In this experiment, the documented role of TGF-β up-regulation in macrophages was followed by enhanced collagen synthesis and lung fibrosis.

A recent study performed in our laboratory was undertaken to characterize T cells infiltrating lesional skin in early SSc and to investigate their capacity to affect type I collagen production by dermal fibroblasts through direct cell-to-cell interaction. The result indicate that skin infiltrating T cells appear to down-regulate rather than up regulate collagen production via a cell-to-cell interaction. In addition, this inhibitory function was dominant on the pro-fibrotic activity of exogenously added TGF-β.

These findings are difficult to reconcile with previously reported data[42;47], indicating that in early SSc skin lesions, collagen synthesis is higher in the proximity of the inflammatory infiltrate. Thus, I wanted to test whether a third party cell, namely the monocyte/macrophage also present in the inflammatory infiltrate in SSc early lesions can deliver profibrotic stimuli through the secretion of TGF-β, thus driving fibrosis when activated by T lymphocytes **(attachment 6)**. Indeed, several in vitro studies have investigated T cell modulation of cytokine synthesis by monocytes/macrophages upon cell-to-cell contact[178], but none, to our knowledge, has specifically investigated whether T cells may regulate the production of TGF-β1 by monocytes/macrophages[179].

# *EXPERIMENTAL APPROACH*

# EXPERIMENTAL APPROACH

1) To address the potential role of T cell contact in regulating TGF-β1 production by monocytes/macrophages, I specifically focused on the analysis of **TGF-β1 gene transcription in monocytes/macrophages**. To this aim I used and compared **three different techniques** (**attachment 5**):
    a) RT-PCR
    b) RNase protection assay
    c) Quantitative, real-time RT-PCR.

2) **T cells** used to activate monocytes/macrophages were from a human T cell line generated from a cutaneous T cell lymphoma (HUT-78). In order to minimize T cell mRNA contamination and to focus on cell surface mediated signals in the absence of soluble mediators, plasma T cell membrane preparations were used as effectors instead of living T cells. T cell membranes have been shown to maintain biological activities in similar experimental conditions[180] and were obtained by sonication[178;181] and separation from cytosol and nuclei.

3) **Monocytes** were purified from peripheral blood from healthy donors.

4) **Macrophages** were obtained by culturing peripheral blood monocytes for 7 days in the presence of GM-CSF[177].

5) **Culture conditions** : monocytes or macrophages were cultured alone or in the presence of T cell membranes from resting or activated T cells for 0, 0.5, 1, 2, 6 and 24 hours. Total RNA was purified thereafter[182;183].

6) As **internal positive control**, I studied **TNF-α gene transcription in monocytes/macrophages.** Indeed, TNF-α mRNA was shown to be induced in monocytes when activated by direct contact with stimulated T lymphocytes[184].

# *MATERIALS AND METHODS*

# MATERIALS AND METHODS

Reagents

-Phaseolus vulgaris leucophytohemagglutinin (PHA) (E-Y Laboratories, San Mateo, CA)
-Phorbol myristate acetate (PMA), polymyxin B sulfate, b-mercapto-ethanol and neuraminidase (Sigma Chemicals, St Louis, MO)
-RPMI-1640, phosphate-buffered saline (PBS) without Ca++ and Mg++, fetal calf serum (FCS), penicillin, streptomycin and L-glutamine (Gibco, Paisley, Scotland)
-Ficoll-Paque (Amersham-Pharmacia, Uppsala, Sweden)
-TRIzol reagent (Life Technologies, Basel, Switzerland)
Superscript II RNase H Reverse Transcriptase and recombinant Taq DNA Polymerase (Gibco BRL, USA)
-Oligo (dT)15 primer, recombinant Rnasin Ribonuclease inhibitor and 100pB ladder (Catalys Promega, Madison, USA)
- RPA kit (PharMingen, San Diego, CA)
-Rneasy Mini kit (50) (Qiagen Gmbh, Hilden, Germany)
-TaqMan Universal PCR Master Mix, specific real-time PCR primer (PDAR) human TGF-β1(20x), FAM - (PDAR) human TNF-α (20x), FAM and (PDAR) human 18S rRNA, VIC (AB Applied Biosystems, Warrington, UK)

Monocytes purification and differentiation into macrophages

Human peripheral blood monocytes were obtained from buffy coats of healthy donors (Blood Transfusion Centre, Geneva University Hospital) using density centrifugation on Ficoll-Paque followed by monocytes aggregation at 4°C for 40 minutes, and rosetting over-night[185]. Rosetting was performed with sheep red blood cells to deplete contaminating T cells. Purity of harvested mononuclear cells was assessed by flow cytometry (Beckton Dickinson Biosciences, FACS Calibur). Purity was > 85% with about 15% non-identified cells. Part of the freshly isolated monocytes (100-120 x $10^6$) were then plated on P10 plastic Petri dishes (10 x $10^6$/ dish) and

cultured in presence of GM-CSF (50 ng/ml) for 6 to 8 days at 37°C and 5% CO2-air for macrophages differentiation.

Preparation of T-cell plasma membranes

For the preparation of plasma cell membranes, a human cutaneous T lymphoma cell line (HUT-78) was obtained from the ATCC (American Type Culture Collection, Manaus, VA). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50µg/mL streptomycin, 50 IU/ml penicillin and 2 mM L-glutamine (complete RPMI medium) and 5% heat-inactivated pooled human AB serum in 5% CO2-air humidified atmosphere at 37°C. T cells were then activated (HUTs) for 6 hours by PHA (1µg/ml) and PMA (5ng/ml) or not (HUTu) before harvesting and membrane preparation. For membrane preparation, the cells were washed three times in PBS, then suspended in 1ml of PBS. Cell suspensions were kept on ice and sonicated with 5-s bursts of 40 W each. Nuclei were pelleted at low speed centrifugation, supernatants were collected and centrifuged at 100,000 X g for 45 min at 4°C. Membrane pellets were suspended in RPMI 1640 containing 10% FCS and 5mM iodoacetamide, and frozen at –70°C until use[179;181;186;187].

Monocytes/macrophages - T cell co-cultures

Freshly isolated monocytes or GM-CSF differentiated macrophages were plated in 24 well-plates at 1.5 - 3 x $10^6$ cells/well for various times (0.5, 1, 2, 6 and 24 hours) in **medium alone** in the **presence of rested (HUTu) or activated (HUTs) T cell membranes**. T cell membranes were used at 100 µl/well corresponding to 0.8 x $10^6$ cells/well. This amount of cells was shown in previous experiments to provide optimal monocytes/macrophages stimulation. RPMI was supplemented with 10% heat inactivated FCS, glutamine, penicillin, streptomycin, non essential amino-acids, sodium pyruvate and β-mercapto-ethanol. Polymyxin B 1mg/ml was added to avoid monocytes activation by contaminant LPS.

RNA extraction

Total RNA was obtained using two different methods. Total RNA was isolated from monocytes/macrophages using either TRIzol Reagent (Life Technologies) or Rneasy Mini Kits according to the manufacturer's instructions. With TRIzol reagent, total RNA was treated with an

RNas-free DNase for 30 minutes at 37°C before reverse transcription to avoid genomic DNA contamination. The absorbance was measured at 260 and 280 nm (PerkinElmer, spectrometer Lambda 25) in order to calculate RNA concentration and to assess total RNA quality.

Polymerase Chain Reaction (PCR)

Total RNA (1μg) was reverse-transcribed using 200U SuperScript II RNase H according to the manufacturer protocol. The resulting cDNA was diluted 10-fold in sterile water and aliquots were subjected to quantitative, real-time RT-PCR. The amplification parameters were 95°C for 50 seconds, 59°C for 60 seconds, 72°C for 60 seconds and 10 more min for the final extension in the presence of $MgCl_2$, dNTPs, buffer and 0.4 pmol commercially available specific TGF-β1 primer (5'-GCCCTGGACACCAACTATTGC-3', rev 5'-GCTGCACTTGCAGGAGCGCAC-3') and GAPDH (glyceraldehyde phosphate dehydrogenase) primer (5'-GGACCTGACCTGCCGTCTAG- 3', rev- 5'-CCACCACCCTGTTGCTGTAG-3') (Amplimmum AG, Madulain, CH) in a rapid thermocycler. Amplified PCR products were detected using electrophoresis on a 1.5 % agarose gel stained with ethidium bromide in TRIS-borate-EDTA buffer, running at 100 V for 30 min before being photographed. PCR sizes products were determined using DNA markers. Reactions performed in the absence of reverse transcriptase were negative. Preliminary experiments were performed to optimize TGF-β1 PCR conditions. 200 μL reaction mixture were divided into five tubes (20 μl each) in order to assure homogenous initial concentrations of enzymes and substrates. Single tubes were removed after completion of 20, 25, 30, 35 and 40 cycles to determine the range of cycles of amplification that yielded exponential increase in PCR products. Optimal primer concentration and $MgCl_2$ buffer concentration were also assessed.

Ribonuclease protection assay (RPA)

To detect and quantify the level of expression of TGF-β1 and TNF-α (mRNA) in monocytes, 3μg total RNA was submitted to "RNase protection assay system" using the RiboQuant RPA hCK-3 multiprobe template set kit from PharMingen BD, according to the supplier's instructions. The method is based on hybridisation of a target RNA to 32P-labeled anti-sense RNA probe in vitro. The probe and target RNA are resolved by denaturing polyacrylamide gel electrophoresis and

images are obtained by exposure to X-ray films. The results are then analyzed using β-imaging equipment. The PharMingen's hCK3 multiple probe template set is composed of a series of 10 templates including TNF-α, TGF-β1, β2, and β3 in addition to two housekeeping genes (L32 and GAPDH). The data were normalized by phospho-imaging quantification using the ratio of the amount of target gene (TGF-β1 or TNF- α) relative to endogenous control (GAPDH).

Quantitative, real-time RT-PCR

Gene expression quantification assays were performed in two-step reverse transcription-polymerase chain reactions (RT-PCR). In the reverse transcription step, cDNA is reverse transcribed from total RNA samples using random hexamers. In the polymerase chain reaction, PCR products are synthesized from cDNA samples. The TaqMan probe contains a reporter dye at the 5'-end of the probe and a quencher dye at the 3'-end of the probe. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye using ABI PRISM 7700 sequence Detection System (Perklin-Elmer; applied Biosystems, Foster City, CA). An automated photometric detector combined with a special software monitors the increasing reporter dye emission. Together with a melting curve analysis performed subsequently to the PCR, this technique provides a tool for specific product identification and quantification. Reverse transcription, performed with TaqMan Reverse Transcription Reagents, and real-time PCR, performed with SYBER Green double-stranded DNA binding dye, were carried out according to the manufacturer's protocol and were performed in separate tubes in triplicate. One μl of cDNA and oligonucleotides with a final concentration of 200nM of primers and 200 nM TaqMan hybridisation probe were added to 12.5 ul reaction mix. The thermocycler parameters were 50° for 2 min, 95°C for 10 min followed by 40 cycles of 95° for 15 sec and 55° for 1 min. Fold induction of mRNA was determined from the average threshold cycle values of each triplicate normalized for 18S expression. 18S mRNA was quantified in the same well (multiplex assay). NO-template controls and reverse transcription-negative samples were included as controls to ensure that amplification was not due to contaminating genomic DNA and were always negative.

# *RESULTS*

# RESULTS

## *RT-PCR detection of TGF-β1 mRNA*

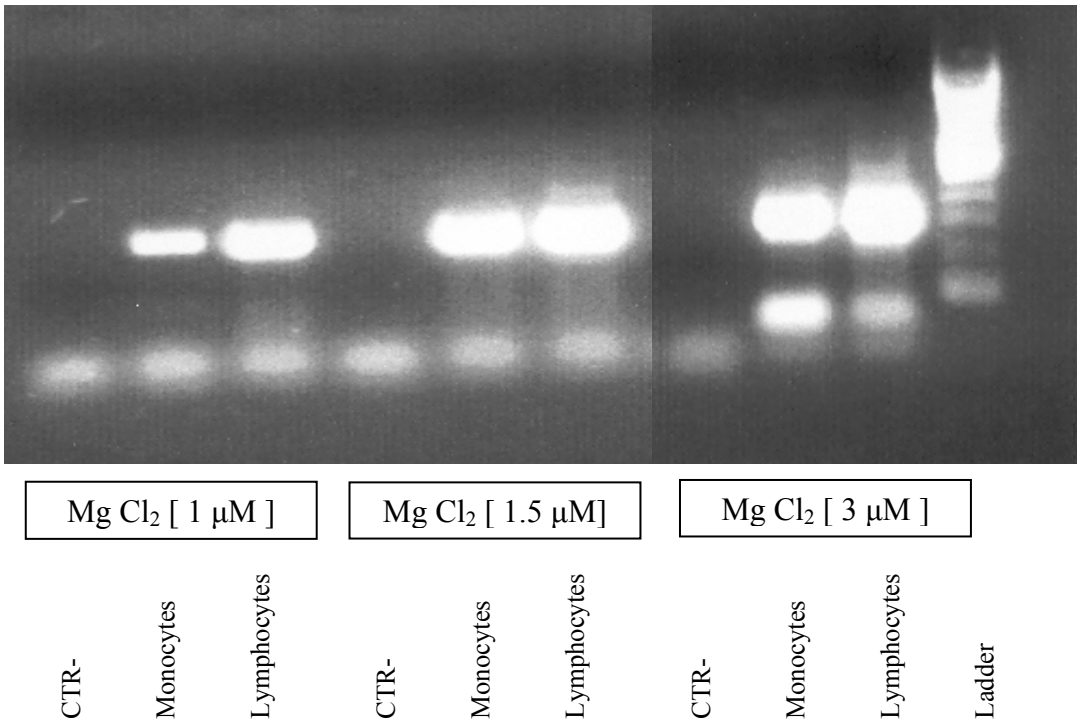
***RT-PCR set-up and optimization***

RT-PCR buffer concentration

The first effort was to set-up conventional RT-PCR in order to find optimal conditions for a high ratio of specific-to-non-specific annealing of primers. Several factors critically influence the efficiency, sensitivity and specificity of RT-PCR. Primer design and concentration, RT-PCR buffer composition, cycle number and starting cDNA quantity are important conditions to optimize. As commercial primers are of quality high enough to guarantee efficient template synthesis for the PCR phase, experimental parameters such as $MgCl_2$ and primer concentration were adjusted and repeated until satisfactory results were obtained. A satisfactory result was considered when a single band of the correct size was obtained, which intensity responds to the relative expression level of the corresponding gene, without saturating the signal. Non-optimal concentration of primer and buffer can cause a lack of amplification (no detectable DNA) or, to the opposite, non-specific amplification. Primer annealing is particularly influenced by cations contained in the RT-PCR buffer, since cations such as Mg++ neutralize the electro-repulsive forces between DNA strands and stabilize the primer-template complex.

To this aim, I tested different Mg++ concentrations during the PCR. TGF-β1 primers were tested under saturating cycles numbers using RNA from both monocytes and T lymphocytes. The width of the band constitutes the selection criteria. The presence of TGF-β1 mRNA was tested at Mg ++ buffer concentration ranging from 1 to 3 μM $MgCl_2$ and at primer concentration of 0.4 μM. As shown in ***figure 1.***, band width increased by increasing Mg ++ concentration from 1 to 3 μM. However, at 3 μM, the specificity of the reaction decreased as documented by the presence of additional bands of lower molecular weight. The best results were obtained at 1.5 μM since specific amplified reaction product was width with no unspecific amplification bands. Thus I selected 1.5 μM Mg++ for the following experiments.

***Fig. 1.*** *RT-PCR of the coding sequence of monocytes and lymphocytes TGF-β1 gene.*

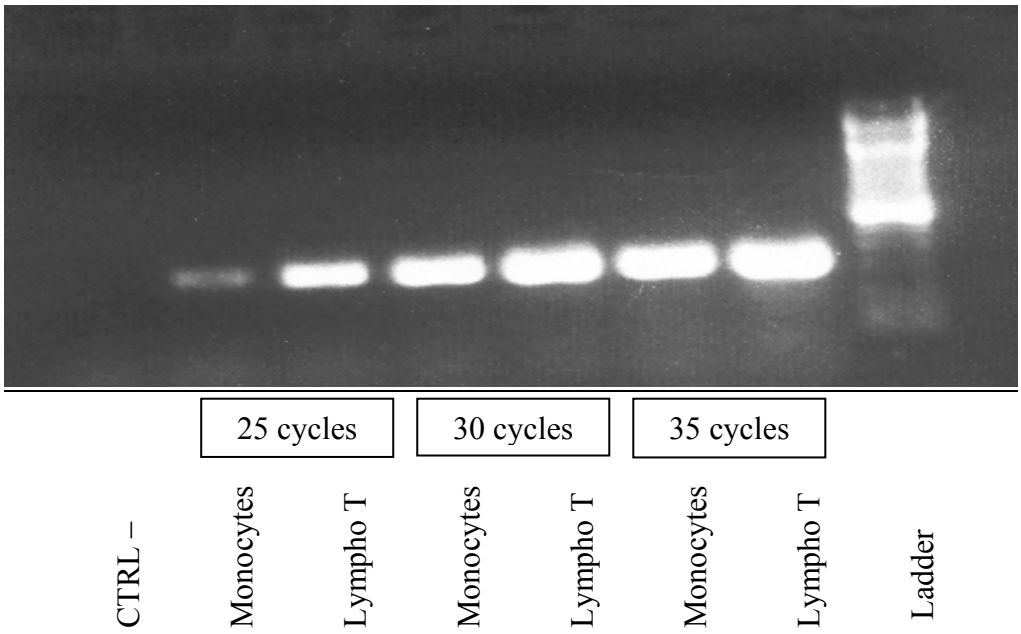



Reverse transcription was performed with total RNA isolated from monocytes or lymphocytes using TAQ Reverse Transcriptase and 1.0 μM of TGF-β1 primer. After reverse transcription, equal volumes of the reverse-transcription reaction were transferred into the PCR mix and amplified under $MgCl_2$ buffer concentration indicated. A band of 248 bp for TGF-β1 was observed in each sample in which RT-PCR was performed.
CTRL– denotes samples in which cDNA or RT was omitted.

cDNA concentration and cycle number optimization

Next, I optimized the amplification cycle numbers under identical primer and $MgCl_2$ conditions in order to detect limiting doses of starting cDNA. Generally, 25 to 35 cycles are sufficient to produce 1μg of RNA from 50 ng of genomic DNA. A ng of cDNA was submitted to different amplification cycles ranging from 25 to 35, thus allowing the determination of the amplification efficiency. Band intensity rose with cycle numbers and the width of the band reached a plateau after 30 cycles (***figure 2.***).

***Fig. 2.*** *RT-PCR of the coding sequence of monocytes and lymphocytes TGF-β1 gene*




Reverse transcription was carried out with total RNA isolated from monocytes or lymphocytes using TAQ Reverse Transcriptase and 1.0 µM of TGF-β1 primer. After reverse transcription, equal volumes of the reverse-transcription reaction were transferred into the PCR mix and amplified. A band of 248 bp was observed in each sample in which RT-PCR was performed.
CTRL – denotes samples in witch cDNA or RT was omitted.

**IN CONCLUSION**, these experiments allowed me to define the optimal RT-PCR conditions to detect TGF-β mRNA in monocytes and T lymphocytes.

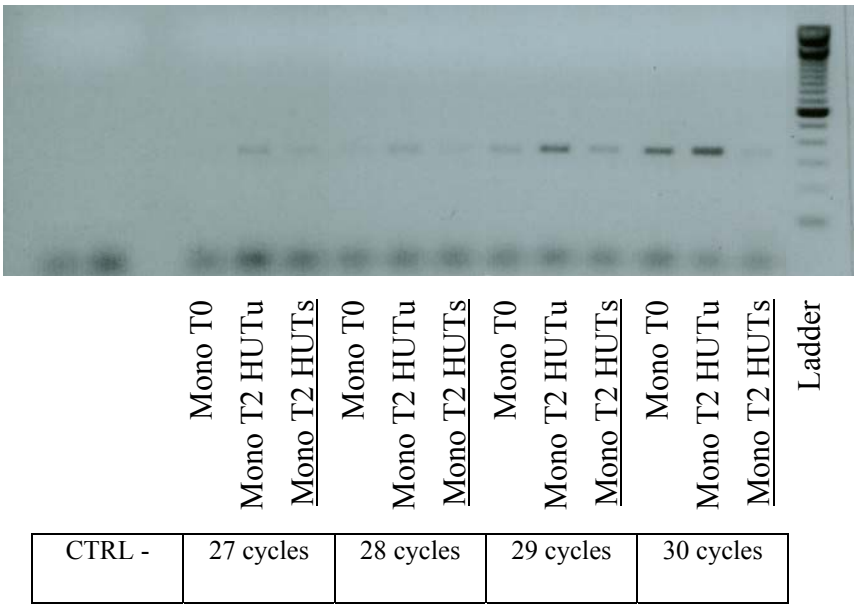
These conditions were :

- 0.4 µM TGF-β1 primer
- 1ng cDNA
- 1.5 µM $MgCl_2$ buffer
- and 30 cycles for amplification.

***TGF-β1 mRNA levels in resting and in monocytes cultured in presence of T cells***

The RT-PCR was used to determine constitutive TGF-β1 mRNA expression in resting monocytes and to determine TGF-β1 mRNA modulation in monocytes cultured in the presence of resting or activated T cells. To this aim, sub-saturating RT-PCR conditions (cycle number ranging from 27-30) were used since non saturating conditions should enhance the detection of changes among samples. As shown in ***figure 3***, TGF-β1 mRNA amplification products were observed when monocytes were cultured for 2 hours in the presence of resting T cells (HUTu) at 29 and 30 cycles of amplification as well as in resting monocytes (***fig. 3***). Similar results were obtained in four distinct experiments (***results not shown***).

***Fig. 3.*** *RT-PCR of the coding sequence of monocytes TGF-β1 gene*




**TGF-β1 mRNA detection by RT-PCR and modulation in monocytes cultured in the presence of resting or activated T cells.**
Monocytes purified from peripheral blood of a healthy individual were plated immediately after purification at $1x10^6$ cells/well before being exposed for 2 hours in the presence of activated (HUTs) or resting (HUTu) T cell membranes (equivalent to 0.8 x $10^6$ cells/well).
The upper band corresponds to the specific TGF-β1 mRNA amplification and the lower band to non specific amplification product.
**Mono T0** : freshly purified monocytes
**Mono T2 HUTu** : monocytes cultured for 2 hours with resting T cell membranes
**Mono T2 HUTs** : monocytes cultured for 2 hours with activated T cell membranes

The conclusions of this set of experiments are :

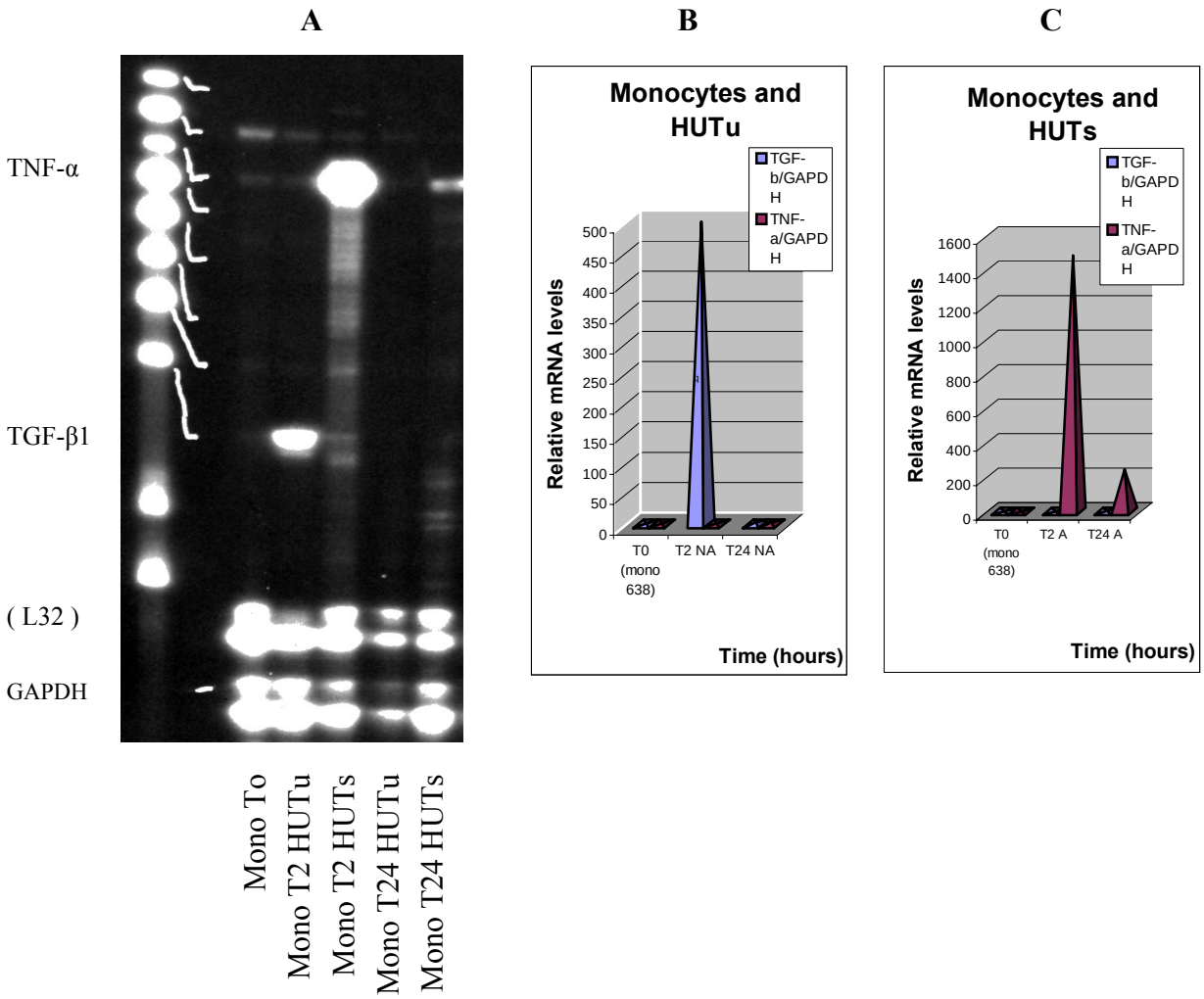
1. TGF-β1 mRNA is easily detected in resting monocytes by standard RT-PCR, thus indicating constitutive transcription of this cytokine.
2. The contact of monocytes with resting T cells for two hours results in TGF-β1 bands that appear to be slightly wider than those of resting monocytes. In addition, the band appears at lower cycle numbers, thus indicating that membranes from resting T cells may increase the steady state TGF-β1 mRNA levels.
3. On the opposite, TGF-β1 bands appeared to be slightly thinner compared to resting cells when monocytes were in contact with membranes from activated T cells, thus indicating that activated T cells decrease the steady state TGF-β1 mRNA levels.
4. The possibility to interpret these results are however strongly limited by the absence of internal controls such as GAPDH mRNA.

### *RNase protection assay detection of TGF-β1 mRNA*

In an attempt to confirm the RT-PCR results, I used the RNase protection assay on the same samples tested in *figure* 3.

The auto radiogram of the RNAse protection assay showed no band on resting monocytes for TGF-β1, but a wide TGF-β1 band in monocytes cultured for 2 hours with resting T cells, which returned to basal levels at 24 hours (***fig. 4.A***). On the other hand, TGF-β1 mRNA levels in monocytes were not affected when cultured in the presence of activated T cells. Of interest, and as expected, TNF-α mRNA levels increased sharply at two hours, declining thereafter when monocytes were cultured with activated but not resting T cells (***fig. 4.A and C***).

***Fig. 4***. *TGF-β1 mRNA detection in Monocytes*




**Auto radiogram and densitometry quantification of TGF-β1 mRNA detection by RPA in monocytes cultured in the presence of resting or activated T cells**

Monocytes purified from peripheral blood of a healthy individual were plated immediately after purification at $1x10^6$ cells/well before being exposed for 2 or 24 hours in the presence of activated (HUTs) or resting (HUTu) T cell membranes (equivalent to 0.8 X $10^6$ cells/well). Total RNA was extracted and probed as described in material and methods. The results were normalized to the GAPDH house-keeping gene. Basal level of cytokine production was given the value of 1, and the fold increase after T cell membrane exposure was calculated relative to the basal level. Time zero represent expression of cytokine at the beginning of the experiment. The upper light band corresponds to the specific GAPDH amplification and the lower band to non specific amplification product.

**(A)** Auto radiogram of a representative RNAse protection assay.

**(B, C)** Densitometry quantification of auto radiography RNase protection assay products. TGF-β1 (blue pyramids); TNF-α (brown pyramids).

**Mono T0** : freshly purified monocytes
**Mono T2 or T24 HUTu** : monocytes cultured for 2 or 24 hours with resting T cell membranes
**Mono T2 or T24 HUTs** : monocytes cultured for 2 or 24 hours with activated T cell membranes

Similar results were obtained in two additional experiments, except for the TGF-β1 mRNA basal level for which the auto radiogram showed a small band on resting monocytes (results not-shown). Interestingly, the extent of TGF-β1 modulation by resting T cells showed important individual variations in these different monocytes preparations.

The conclusions of this set of experiments are that :

1) TGF-β1 mRNA steady state levels increased transiently in monocytes cultured with resting T cells but not with activated T cells.
2) TNF-α mRNA steady state levels increased transiently and sharply when monocytes were cultured in the presence of activated T cells but not in the presence of resting T cells.

Taken together, these data substantiate the potential resting T cells to induce TGF-β1 mRNA transcription in monocytes and confirm the high potential of activated T cells in triggering TNF-α mRNA transcription by monocytes.

### *Quantitative, Real-time RT-PCR detection of TGF-β1 mRNA*

Quantitative, real-time RT-PCR was used as third techniques to further investigate the capacity of T cells to modulate TGF-β1 and TNF-α mRNA transcription in isolated monocytes and macrophages. It is indeed likely that, in vivo, T cells will interact with macrophages rather than monocytes at sites of inflammation. To quantify the relative level of mRNA encoding for TGF-β1 and to take into account differences in sample concentration and loading, cytokine mRNA levels were normalized using as internal standard, the 18S ribosomal RNA, considered to be constant in all culture conditions.

I first checked whether under my basal culture condition, the steady state levels of TGF-β1 mRNA changed over time in monocytes and macrophages. As shown in ***figure 5.A and 6.A***, this was not the case. Similar results were obtained when studying TNF-α mRNA levels (***fig. 7.A and 8.A***). I then assessed whether TGF-β1 mRNA levels were modulated when monocytes and macrophages were cultured in the presence of membranes from resting or activated T cells. To my surprise and in contrast with the observed up-regulation when standard RT-PCR and RNase protection assay were used to assess monocytes/macrophages responses, no substantial changes in TGF-β1 mRNA levels were observed in several distinct experiments (***fig. 5.B and 6.B***). However, in agreement with the results previously obtained with standard RT-PCR, activated T cells did not induced changes in TGF-β1 mRNA levels (***fig. 5.C and 6.C***).

In contrast, TNF-α mRNA steady state levels were strongly increased (up to 40 fold) in monocytes and macrophages exposed to activated T cells. This increase peaked at 2 hours (***fig. 8.B and 8.C***). Of interest, TNF-α mRNA levels increased, although at lower levels, also in monocytes and macrophages cultured with resting T cells (***fig. 7.B and 7.C***).

*Fig. 5.* ***TGF-β1 mRNA expression in Monocytes***

*FIGURE 5.A*

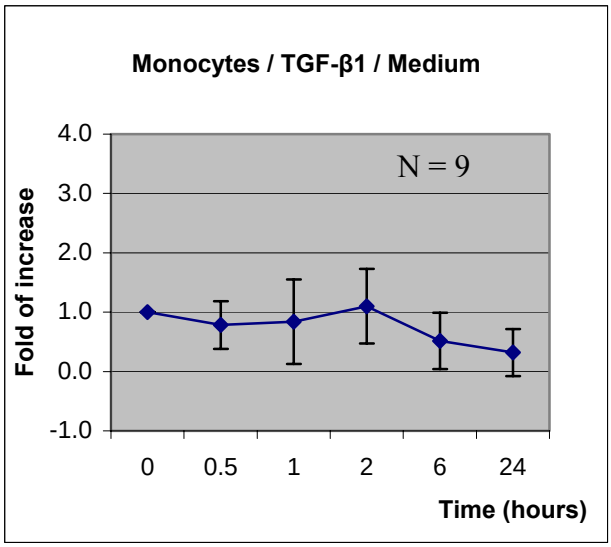



*FIGURE 5.B*

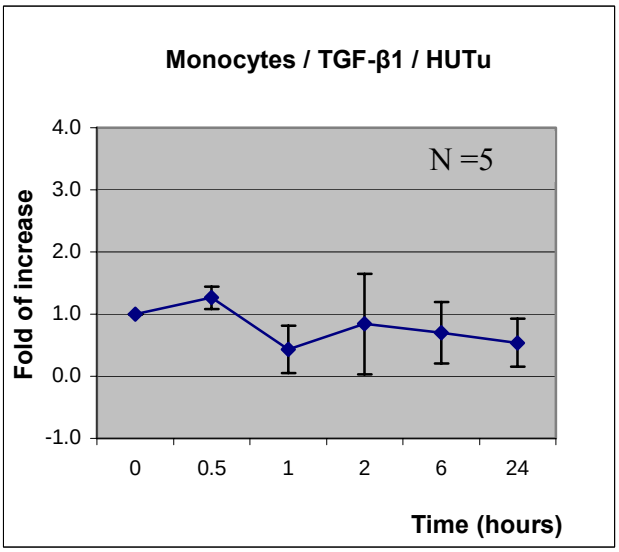
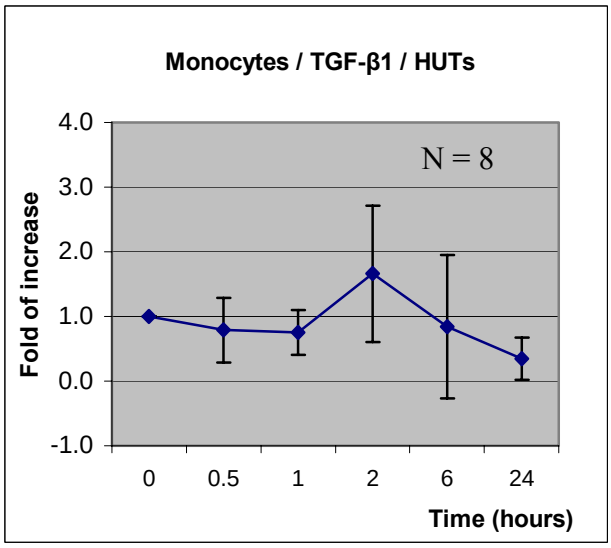



*FIGURE 5.C*




***A/B/C*. TGF-β1 mRNA detected by quantitative, real-time RT-PCR in monocytes cultured in the presence of resting or activated T cells.**
3 µg of total mRNA was extracted at base line and at 0.5, 1, 2 ,6 and 24 hours of culture. Culture conditions were as in *fig* 4. TGF-β1 mRNA and 18S ribosome RNA were measured simultaneously in each sample.
The results are expressed as relative mRNA levels (ratio of TGF-β1/18S). Fold induction was calculated relatively to the level at time 0 and the TGF-β1 mRNA level at time zero was given the value of 1.
The number of experiments (N) per condition is reported in each panel. Bars represent the standard error of the mean. Each point was assessed in triplicate.

*Fig. 6* ***TGF-β1 mRNA expression in Macrophages***

*FIGURE 6.A*

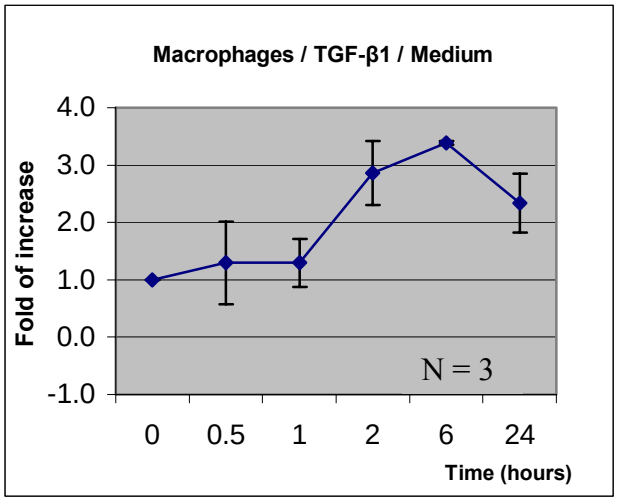



*FIGURE 6.B*

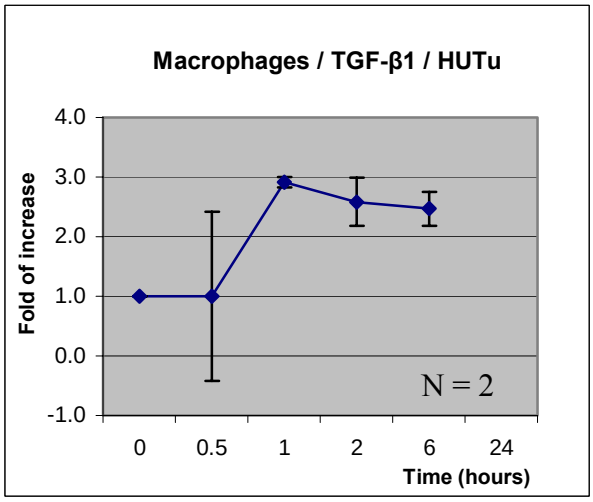
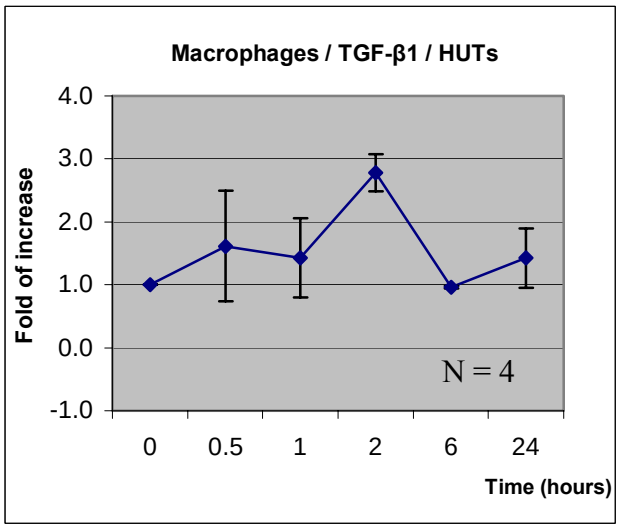



*FIGURE 6.C*




***A/B/C.*** **TGF-β1 mRNA detected by quantitative, real-time RT-PCR in macrophages cultured in the presence of resting or activated T cells.**
Macrophages were obtained after 7 days of culture in GM-CSF (50ng/ml). 3 µg of total mRNA was extracted at base line and at 0.5, 1, 2 ,6 and 24 hours of culture. Culture conditions were as in *fig* 4. TGF-β1 mRNA and 18S ribosome RNA were measured simultaneously in each sample.
The results are expressed as relatively mRNA levels (ratio of TGF-β1/18S). Fold induction was calculated relative to the level at time 0 and the TGF-β1 mRNA level at time zero was given the value of 1.
The number of experiments (N) per condition is reported in each panel. Bars represent the standard error of the mean. Each point was assessed in triplicate.

*Fig. 7.* ***TNF-α mRNA expression in Monocytes***

*FIGURE 7.A*

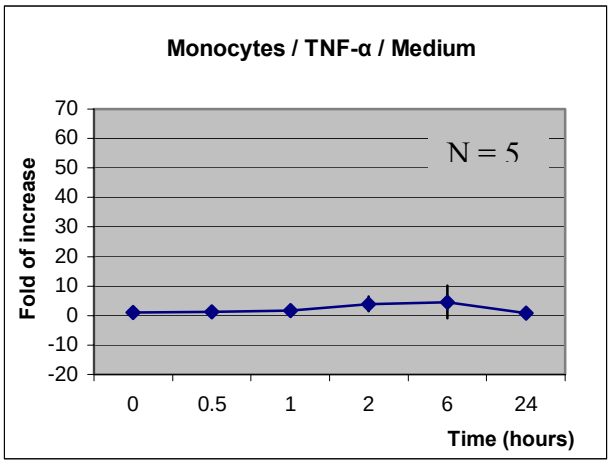



*FIGURE 7.B*

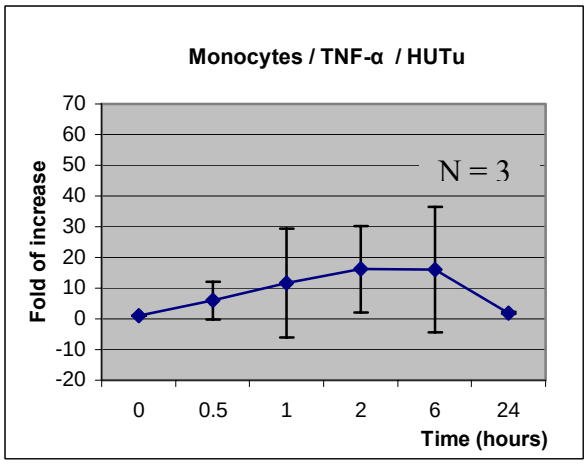
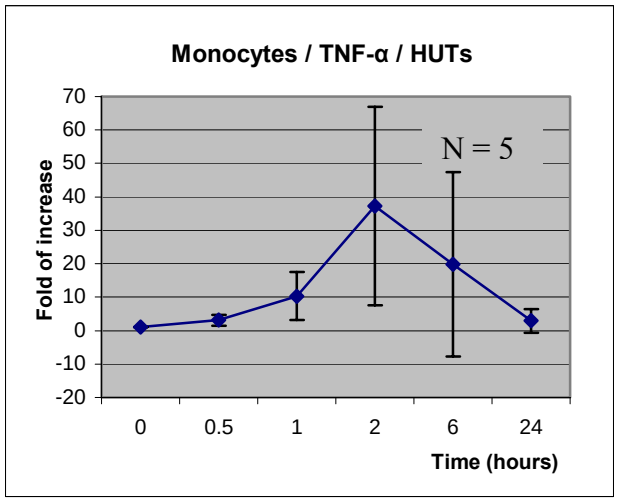



*FIGURE 7.C*




***A/B/C*. TNF-α mRNA and modulation detected by quantitative, real-time RT-PCR in monocytes cultured in the presence of resting or activated T cells.**
3 µg of total mRNA was extracted at base line and at 0.5, 1, 2 ,6 and 24 hours of culture. Culture conditions were as *figure* 4. TNF-α mRNA and 18S ribosome RNA were measured.
The results are expressed as relatively mRNA levels (ratio of TNF-α/18S). Fold induction was calculated relative to the level at time 0 and the TNF-α mRNA level at time zero was given the value of 1.
The number of experiments (N) per condition is reported in each panel. Bars represent the standard error of the mean. Each point was assessed in triplicate.

***Fig. 8. <u>TNF-α expression in Macrophages</u>***

***FIGURE 8.A***

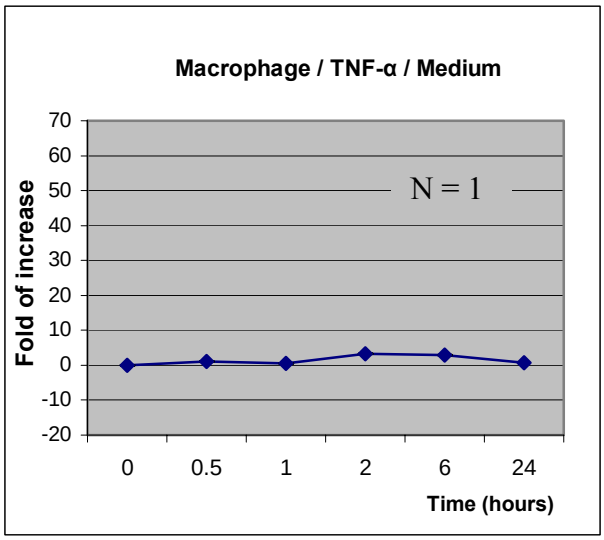



***FIGURE 8.B***

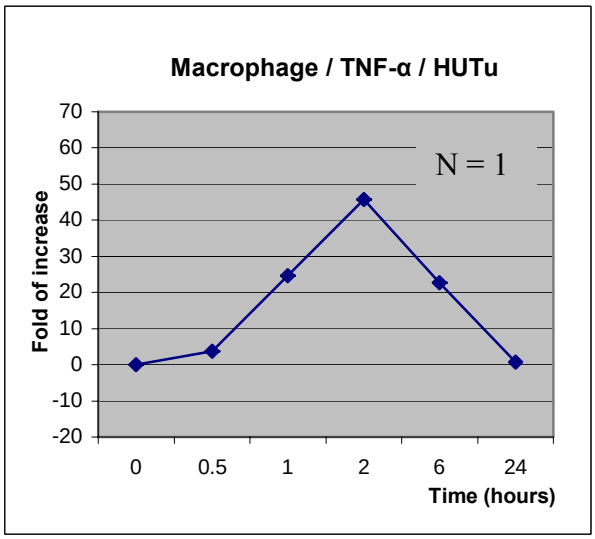
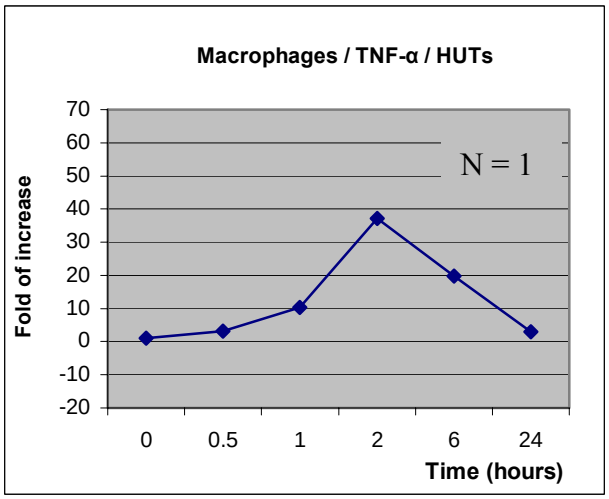



***FIGURE 8.C***




***A/B/C*. TNF-α mRNA and modulation detected by quantitative, real-time RT-PCR in macrophages cultured in the presence of resting or activated T cells.**
Macrophages were obtained after 7 days of culture in GM-CSF (50ng/ml). 3 µg of total mRNA was extracted at base line and at 0.5, 1, 2 ,6 and 24 hours of culture. Culture conditions were as in *fig* 4. TNF-α mRNA and 18S ribosome RNA were measured simultaneously in each sample.
The results are expressed as relatively mRNA levels (ratio of TNF-α/18S). Fold induction was calculated relative to the level at time 0 and the TNF-α mRNA level at time zero was given the value of 1.
The number of experiments (N) per condition is reported in each panel. Bars represent the standard error of the mean. Each point was assessed in triplicate.

The conclusions of this set of experiments are that :

1) No significant modulation of TGF-β1 mRNA was observed in monocytes and macrophages when cultured with resting or activated T cell.
2) Significant modulation of TNF-α mRNA was observed in monocytes and macrophages, whether cultured with resting or activated T cells.

# *DISCUSSION*

# DISCUSSION

The aim of this study was to evaluate whether the production of TGF-β1 by monocytes/macrophages, a key cytokine mediator in fibroproliferative disorders, could be affected by T cell contact. The main characteristics of this study were the following :

- First, to minimize the effect of soluble mediators and to asses the biological effect of T cell contact, T cell membranes preparations were used as effectors rather than intact T cells.
- Second, TGF-β biological activity depends mainly on the cleavage of the active polypeptide from its latent precursor. In the set of experiments here described, it was preferred to assess whether monocytes/macrophages could modulate the TGF-β1 transcription upon contact by T cells rather than assessing the TGF-β biological activity .
- Third, to determine messenger modulation, three distinct methods were used for assessing TGF-β mRNA levels : standard RT-PCR, RNase protection assay and quantitative, real-time RT-PCR.
- Fourth, since according to existing literature, contact mediated signaling of monocytes/macrophages by human stimulated T lymphocytes trigger massive up regulation of TNF-α, it was of interest to use this cytokine as an internal control[188;189].

In most of the experiments, TGF-β1 mRNA was detected in resting monocytes/macrophages indicating constitutive expression. But whereas standard RT-PCR assay and RNase protection assay experiments were indicating that TGF-β1 mRNA steady state levels were up regulated at 2 hours, when monocytes were exposed to membranes of resting T cells, quantitative, real-time RT-PCR experiments did not confirm such results. In the same experimental conditions, a sharp increase in TNF-α mRNA levels was consistently observed in monocytes/macrophages exposed to T cells. These latter results were in accordance with previous publications[184;190;191].

The data obtained with standard RT-PCR and RNase protection assay on one hand and quantitative, real-time RT-PCR on the other hand, are obviously difficult to reconcile, but the failure of quantitative, real-time RT-PCR experiment to confirm observed results in RT-PCR and RNase protection assay can be explained by several hypothesis taking into account experimental design and application techniques used for mRNA quantitation as well as cytokine modulation itself.

Thus, under optimal conditions, standard RT-PCR analysis is a good method for determining the presence of specific RNA sequences. The first effort was therefore set to increase sensitivity of this method to obtain a high ratio of specific-to-non-specific annealing primers by testing the best Mg++ concentrations, affecting enzyme activity, and by defining amplification efficiency through cycle number variation. Standard RT-PCR results are usually expressed in term of positive/negative and semi-quantitative evaluation is possible on the basis of intensities aspect of the target amplicons (weak/strong positive). ***In figure 3*** for instance, the band size and intensity of TGF-β1 at 2 hours with resting T cells seems slightly less intense at 29 than 30 cycles, suggesting sub-saturating condition at 29 cycles. On the other hand, the absence of a TGF-β1 band in monocytes stimulated by activated T lymphocytes at 30 cycles could reflect the absence of activating signal under these culture conditions.

However, in the first set of assay, experiments at sub-saturating conditions were poorly reproducible and failed to detect small differences in the amount of specific mRNA transcript. Keilholz and all. demonstrate that the standard RT-PCR reaction is fraught with hazards[192] and is subjected to significant variation and non-reproducibility, even with identical samples between different laboratories[193]. Its reproducibility is inevitably compromised by the variable efficiency of the RT itself and the need for two sequential enzymatic steps[194]. The exponential nature of PCR amplification, together with the small quantities of target molecules means that trivial variations in reaction components and thermal cycling conditions and mispriming events during the early stages of the PCR can greatly influence the final yield of the amplified products[195].

Although there is evidence that standard RT-PCR can be performed in a semi-quantitative manner[196], quantitative evaluation of standard RT-PCR results approach is, as suggested above, strongly subjective, depending on examinator's reading. Sensibility is therefore very

low. In my experiments, the standard RT-PCR quantification is also limited by the lack of a standard internal control. The use of an internal control helps to monitor RT and PCR efficiencies and solves problems of variation in the starting amounts of templates and loading errors of the operator. The accepted method for minimizing these errors and correcting for sample to sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalized. Anyway, the minimum error in competitive standard RT-PCR reactions is approximately 10%, even when competitor and target are present in equal amounts and is usually significantly greater as demonstrated by Souaze and all[197].

The main advantage of RNase protection assay compared to the standard RT-PCR was the incorporation of an internal control, the GAPDH, which minimizes dyshomogeneity among the samples tested. When corrected for the intensity of the internal standard (GAPDH), the results of the RNase protection assay experiments were in agreement with those of the standard RT-PCR, indicating that TGF-β mRNA levels were higher in monocytes stimulated by resting than by activated T cells.

Standard RT-PCR and RNase protection assay are accurate for determining the presence of specific RNA sequences but lack the ability to detect small differences in the amount of a specific RNA transcript. These limitations can be overcome with the application of quantitative, real-time RT-PCR, nowadays shown as the most sensitive, specific and reproducible quantification method of mRNA detection. In my experience using Taqman, the variability between replicates within the same run was usually between 1 and 3%. These results are consistent with those reported in the literature indicating a coefficient of variation lower than 2% for the Taqman[198], which is significantly better than the 14% for standard RT-PCR[199]. In this technique, target gene mRNA levels are measured relative to their respective competitor templates and are normalized to reference genes to control for cDNA loaded into the reaction. The result of each gene expression measurement is reported as a numerical value that allows direct and precise comparison between experiments[200]. An other advantage of real-time PCR is the run as closed-tube systems in which quantification requires no post amplification manipulation. This avoids problems of contamination, results in short turn-around times for data acquisition and analysis and minimizes hands-on time. The entire process, starting at the RT and ending with full quantification is automated.

The almost unaffected TGF-β1 mRNA expression by the contact of T cell in the monocytes/macrophages tested by quantitative, real-time RT-PCR method was however on contradiction with the standard RT-PCR and RNase protection assay results. This discrepancy may be related to the fact that for quantitative, real-time RT-PCR, results are expressed as a rate of product formation instead of a total amount product generated by the end of the reaction.

Among quantitative, real-time RT-PCR, all experiments, up to 8 per condition, were concordant. Results are also concordant between standard RT-PCR and RNase protection assay experiments, although less experiments were performed per condition with these techniques. Results are obviously difficult to reconcile, but because variability among quantitative, real-time RT-PCR is significantly less than for any standard RT-PCR procedure and because results are concordant within 8 quantitative, real-time RT-PCR experiments, absence of TGF-β1 cytokine modulation by T cell contact seems more credible.

A weakness in the experimental conditions adopted to perform quantitative, real-time RT-PCR was that the fluorescent signal first recorded as statistically significant above back-ground was measured about 6 cycles earlier for the internal control 18S mRNA than for TGF-β1 mRNA. An internal control should be amplified with the same kinetics as the target gene under investigation to limit quantitative dys-balance between internal control and target gene[201;202]. An alternative explanation of the discrepancies between standard RT-PCR, RNase protection assay and quantitative, real-time RT-PCR could be related to differences in the cells that were from different donors. Indeed, subtle differences in monocytes purified from different donors may be responsible for the differences observed.

TNF-α results in RNase protection assay and quantitative, real-time RT-PCR were consistent with those reported in the literature and demonstrated that T cell membranes were biologically active although the use of a membrane model instead of living T cells has some limitation. Thus, cell membrane preparation may not entirely respect the structural polarity of intact cell surfaces, since the normal three-dimensional conformation of the membrane is lost, and membranes can reversibly bind to the substrate on either side. Interaction domains may be hidden due to new cell surface arrangements and membrane associated molecules usually directed inward may be exposed and become effectors for contact. Activation (or

deactivation) of membrane proteins can also be influenced by orientation or conformation changes[203].

In conclusion, contact-mediated activation of monocytes by T lymphocytes is known to be a potent pro-inflammatory mechanism that triggers massive up regulation of the pro-inflammatory cytokine TNF-α. A similar cell-cell contact-mediated activation does not appear to be relevant for TGF-β1. The almost unaffected TGF-β1 mRNA expression in monocytes/macrophages when T cell were used as effectors is consistent with differential regulation of TGF-β1 and TNF-α gene transcription.

As already mentioned, TGF-β is generated as a propeptide, processed intracellular and secreted as biologically inactive precursors with latency-associated peptide (LAP). The complex LAP-TGF-β can bind to latency TGF-β binding protein (LTBP), which is referred to as « large » latent TGF-β1. The unique ability of LAP to confer latency prevents binding of secreted TGF-β to ubiquitously expressed receptors and assures an extra cellular reservoir of TGF-β that can be activated on demand. TGF-β expression in the monocytes/macrophages may therefore be regulated either by changes in the secretion of latent TGF-β, as well as changes in the synthesis and/or stability of the TGF-β protein[204]. One of the hypothesis is that contact with T cells can lead to a detachment of LAP, rendering TGF-β available. Therefore, further work on TGF-β needs to be done to verify whether TGF-β biological activity can be modulated upon T cell contact. Advancement in this area of investigation may provide better understanding of mechanisms, that play a role in the pathogenesis of SSc.

# *ATTACHMENTS*

# ATTACHMENTS

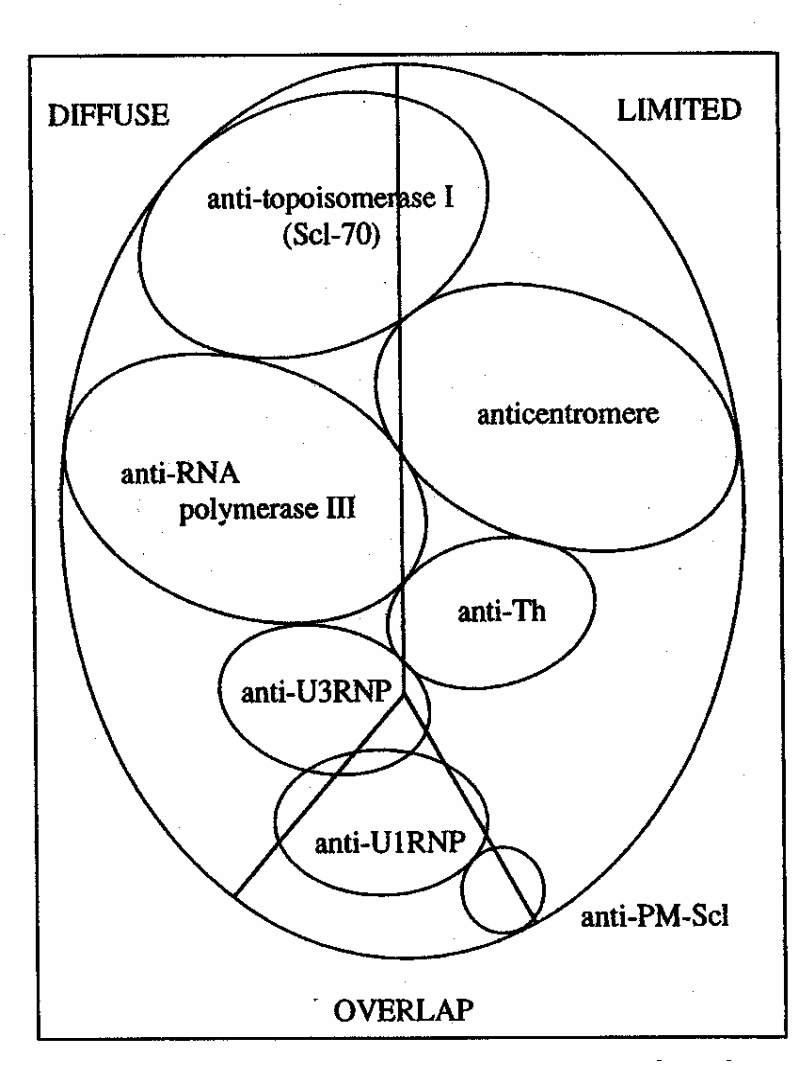
## Attachment 1

**Clinical Features of the Major Systemic Sclerosis Subsets**

| Diffuse cutaneous | Early (< 3 years after onset) | Late (> 3 years after onset) |
|---|---|---|
| Constitutional | Fatigue and weight loss | Minimal, weight gain typical |
| Vascular | Raynaud's often relatively mild | Raynaud's more severe, more telangiectasia |
| Cutaneous | Rapid progression involving arms, trunk, face | Stable or regression |
| Musculoskeletal | Prominent arthralgia, stiffness, myalgia, muscle weakness, tendon friction rubs | Flexion contractures and deformities, joint/muscle symptoms less prominent |
| Gastrointestinal | Dysphagia, heartburn | More pronounced symptoms, midgut and anorectal complications more common |
| Cardiopulmonary | Maximum risk for myocarditis, pericardial effusion, intersitital pulmonary fibrosis | Reduced risk of new involvement but progression of existing esta-blished visceral fibrosis |
| Renal | Maximum risk period for scleroderma after 5 years | Renal crisis less frequent, uncommon after 5 years |
| **Limited cutaneous** | **Early (< 10 years after onset)** | **Late (> 10 years after onset)** |
| Constitutional | None | Only secondary to visceral complications |
| Vascular | Raynaud's typically severe and longstanding telangiectasia | Raynaud's persists, often causing digital ulceration or gangrene |
| Cutaneous | Mild sclerosis with little progression on trunk, face | Stable, calcinosis more prominent |
| Musculoskeletal | Occasional joint stiffness | Mild flexion contractures |
| Gastrointestinal | Dysphagia, heartburn | More pronounced symptoms, midgut and anorectal complications more common |
| Cardiopulmonary | Usually no involvement | Lung fibrosis may develop, but often progresses slowly, Anti-scl-70 predicts increased risk of severe fibrosis. Maximum risk for developing isolated pulmonary hypertension and secondary right ventricular failure |
| Renal | No involvement | Rarely involved, anti-RNA polymerase predicts increased risk of renal involvement |

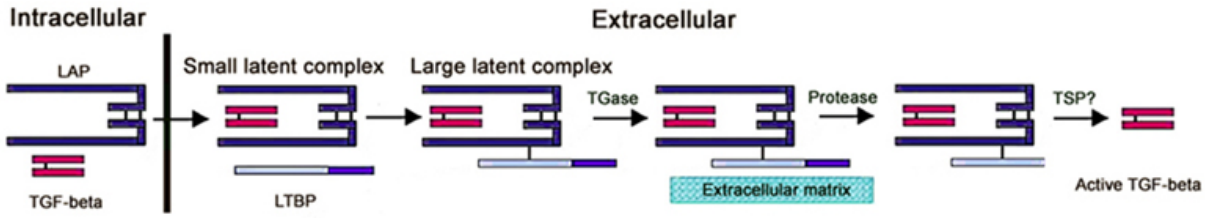
*UpToDate « classification of systemic sclerosis (scleroderma) », 01/2006*

## Attachment 2



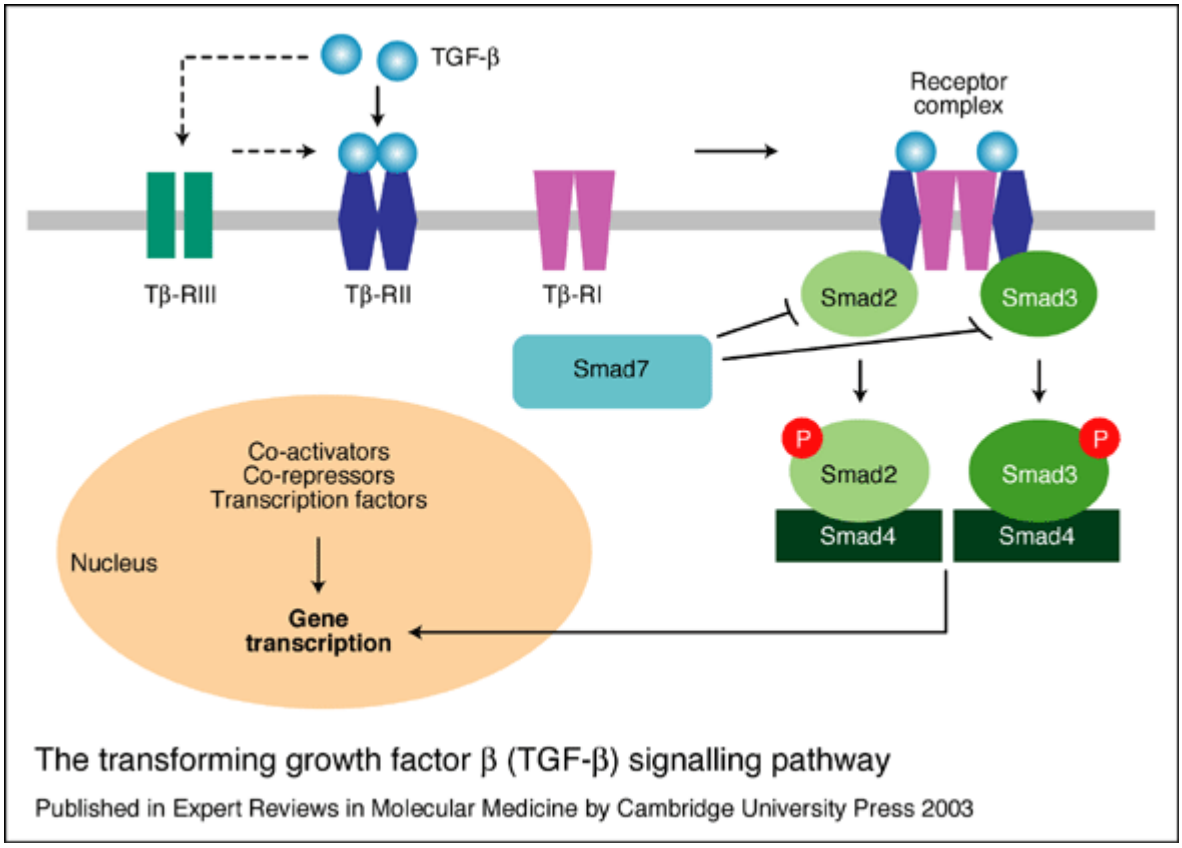

*Thomas A., Medsger JR, Virginia D. «classification, Prognosis »*

## Attachment 3




*Burger, D Eur Cytokines Netw 2000*

## Attachment 4




The transforming growth factor β (TGF-β) signalling pathway

Published in Expert Reviews in Molecular Medicine by Cambridge University Press 2003

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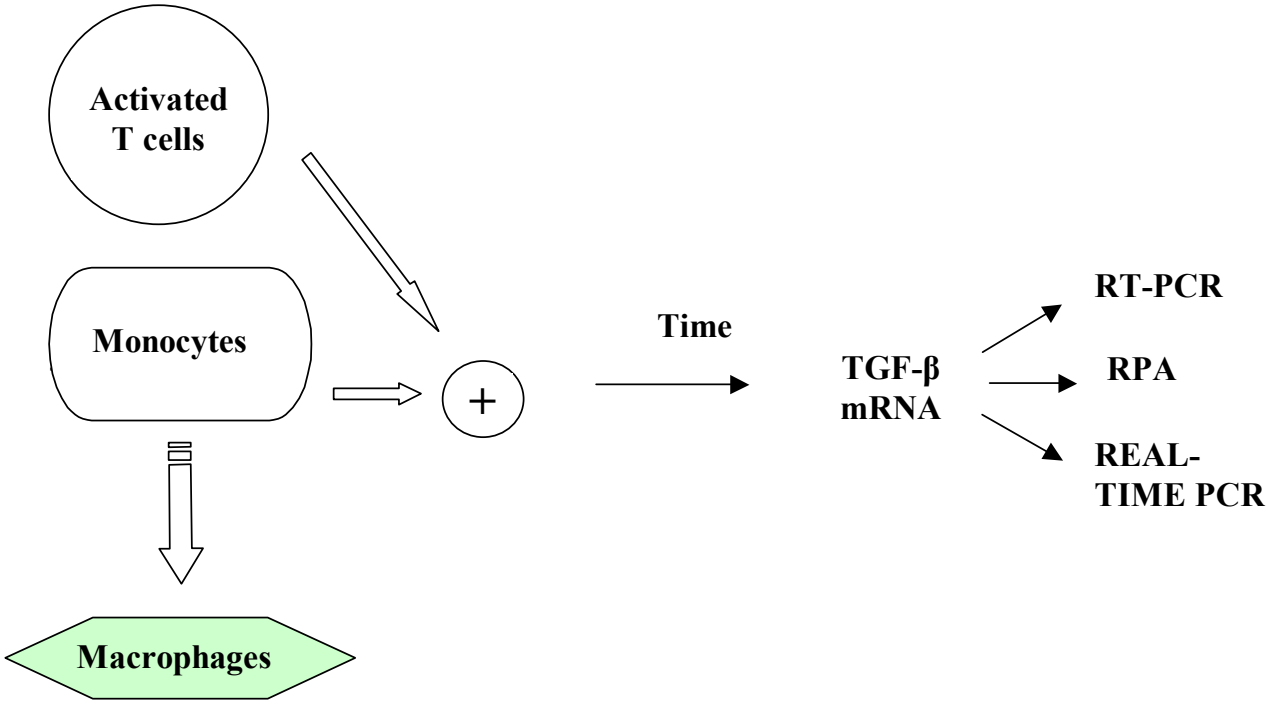


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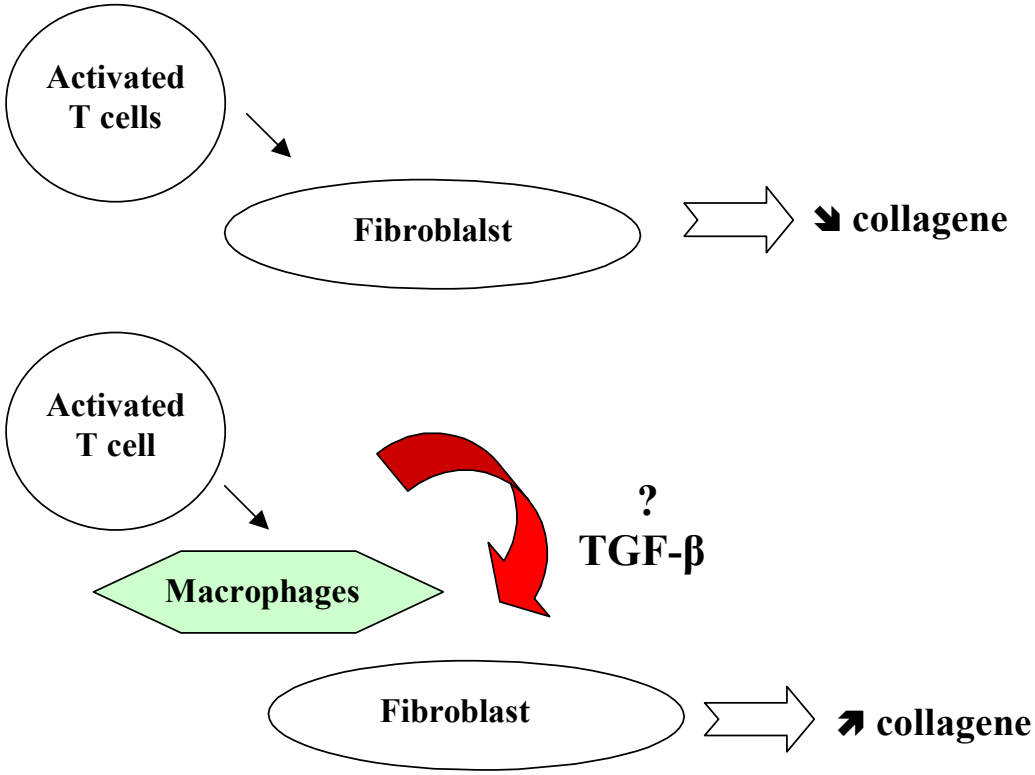

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# REFERENCES

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