Chemokines in Wegener's granulomatosis

Clinical and experimental studies

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Eirik A. Torheim

Abstract

BACKGROUND: Wegener's granulomatosis (WG) is a severe and potentially life-threatening disease characterized by necrotizing granulomatous vasculitis of small- to medium-sized blood vessels. Chemokines are powerful inflammatory mediators, contributing to inflammation through attraction of immune cells, and are, consequently, involved in several inflammatory and autoimmune disorders, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). It has never been established, however, whether chemokines are also involved in the pathophysiology of WG.

METHODS: 1) Serum from 14 WG patients and 9 healthy controls was analyzed with respect to circulating levels of the CC chemokines monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and regulated on activation normal T-cell expressed and secreted (RANTES), and the CXC chemokines epithelial cell-derived neutrophil-activating factor (ENA)-78 and interleukin (IL)-8. 2) Peripheral blood mononuclear cells (PBMC) from 9 WG patients and 9 healthy controls were stimulated *in vitro* with staphylococcal enterotoxin B (SEB) and methylprednisolone (MP), and the resultant levels of chemokine gene expression and secretion were measured by real-time reverse transcriptase – polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assays (ELISA), respectively.

RESULTS: 1) The serum levels of MCP-1 and RANTES were significantly elevated in WG patients, compared to healthy controls. Moreover, patients with active disease displayed a trend for lower RANTES levels and higher ENA-78 levels in the systemic circulation than patients in remission. 2) In PBMC from healthy controls all chemokines investigated were down-regulated following treatment *in vitro* with MP, considering both protein and mRNA levels. On the other hand, MP-mediated suppression of chemokine secretion from PBMC isolated from WG patients was only evident for IL-8, whereas the gene expression of IL-8, ENA-78, and RANTES was also down-regulated. While effective in inhibiting elevated levels of chemokine secretion following stimulation with SEB, MP failed to counteract the SEB-induced up-regulation of gene expression, in both WG patients and healthy controls.

CONCLUSION: The elevated circulatory chemokine levels observed in WG patients and the *in vitro* down-regulation of chemokine levels following treatment with MP *in vitro* suggest that chemokines may indeed be involved in the pathophysiology of Wegener's granulomatosis.

List of abbreviations

6Ckine	6 cysteine chemokine
AC	adenylyl cyclase
Ag	antigen
AIDS	acquired immune deficiency syndrome
AKT	protein kinase B
ANCA	anti-neutrophil cytoplasmic antibodies
AP	activating protein
APC	antigen-presenting cell
AZA	azathioprine
BCA	B-cell-activating chemokine
BSA	bovine serum albumin
BVAS	Birmingham vasculitis activity score
C (as in CC and CXC)	cysteine
cAb	capture antibody
cDNA	complementary deoxyribonucleic acid
Chemokine	chemoattractant cytokine
CNS	central nervous system
CR	chemokine receptor
CRP	C-reactive protein
CYC	cyclophosphamide
dAb	detection antibody
DARC	Duffy antigen receptor for chemokines
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELR	glutamic acid – leucine – arginine
ENA	endothelial cell-derived neutrophil-activating factor
ERK	extracellular signal-regulated kinase
ESR	erythrocyte sedimentation rate
FAK	focal adhesion kinase
FCS	foetal calf serum
GAPDH	glyceraldehyde phosphate dehydrogenase
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
H_2SO_4	sulphuric acid
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
hGR	human glucocorticoid receptor
HIV	human immunodeficiency virus
HSP	heat shock protein
IL	interleukin
IP	immunophilin
IP-10	γ-interferon-inducible protein
IvIg	intravenous immunoglobulin
JAK	janus activated kinase

LAL	Lumulus amoebocyte lysate
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MOPS	3-morpholinopropanesulfonic acid
MP	methylprednisolone
MPO	myeolperoxidase
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
mRNase	messenger ribonuclease
MS	multiple sclerosis
MTX	methotrexate
NaCl	sodium chloride
NaN_3	sodium azide
NF	nuclear factor
NSAID	non-steroid anti-inflammatory drug
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIK	phosphoinositide kinase
PLC	phospholipase C
PR3	proteinase-3
PRE	prednisolone
P value	the probability that the observed results are simply due to chance
PYK	protein tyrosine kinase
qPCR	quantitative polymerase chain reaction
RA	rheumatoid arthritis
RANTES	regulated on activation normal T-cell expressed and secreted
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institue
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
SAg	superantigen
S.aureus	*Staphylococcus aureus*
SDF	stromal cell-derived factor
SEB	staphylococcal enterotoxin B
S.E.M.	standard error of the mean
S-HRP	streptavidin horse-radish peroxidase
SLE	systemic lupus erythematosus
Spl	sample
STAT	signal transducer and activator of transcription
Std	standard
SYBR	Synergy Brands Inc.
Taq	*Thermus aquaticus*
TARC	thymus- and activation-related chemokine
TBS	Tris-buffered saline
TCR	T-cell receptor

T_m	melting point
Trim-Sulpha	trimethoprim-sulphametoxazole
Tris	tromethamine; 2-amino-2-hydroxymethyl-1,3-propanediol
UV	ultraviolet light
WG	Wegener's granulomatosis
X (as in CXC)	any amino acid besides cysteine

1. INTRODUCTION

1.1 WEGENER'S GRANULOMATOSIS

Wegener's granulomatosis (WG) is a severe disease, probably with autoimmune aetiology, that may affect as many as 1 in 20,000 people in Norway (1, 2), and at least 1 in 30,000 in the United States (3). Although it has a clinical predilection of affecting the upper airways, lungs and kidneys, WG is a multi-system disease, and mortality is high without specific therapy.

1.1.1 Clinical features

Patients with WG present with a wide variety of symptoms, commonly mimicking other disorders. The upper respiratory tract is usually the first site of clinical disease in most patients, the typical lung findings being necrotizing vasculitis and focal granulomatous inflammation affecting capillaries or medium-sized vessels, or both. Another characteristic of WG is the nasal deformity ("saddle nose") caused by sinusitis and nasal chondritis, which may appear early or late in the course of the illness (Fig. 1.1). (4)

The kidneys are also commonly affected in generalized disease, resulting in a focal, segmental, thrombotic, and necrotizing glomerulonephritis which progresses to a crescentic glomerulonephritis with periglomerular and focal interstitial inflammatory infiltrates. WG may also involve joints, skin, peripheral nerves, skeletal muscle, heart, brain and eyes (Fig. 1.2). (5)

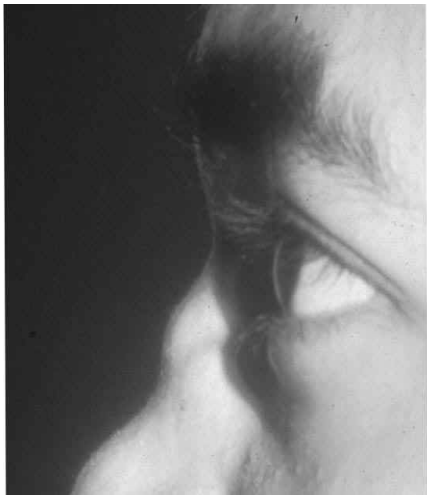


Figure 1.1: Nasal deformity ("saddle nose") commonly seen in Wegener's granulomatosis patients. Adapted from Waheed, 1996. (6)

A strict diagnosis of WG depends on 1) characteristic clinical signs and symptoms, 2) histological demonstration of characteristic features with necrotizing granulomatous vasculitis that cannot be attributed to infection, and/or 3) compatible clinical features and the detection in serum of anti-neutrophil cytoplasmic antibodies (ANCAs) that are usually specific for the neutrophil-derived cytoplasmic granule enzyme proteinase-3 (PR3). The diagnosis should not rely on any one of these variables alone. However, many patients do not have classical features at disease onset, and early manifestations may be diagnosed as

sinusitis due to allergy or infection, pneumonia, lung cancer or other malignancies, rheumatoid and other forms of arthritis, or idiopathic nephritis. In such cases, the diagnostic challenge may be considerable. (5)

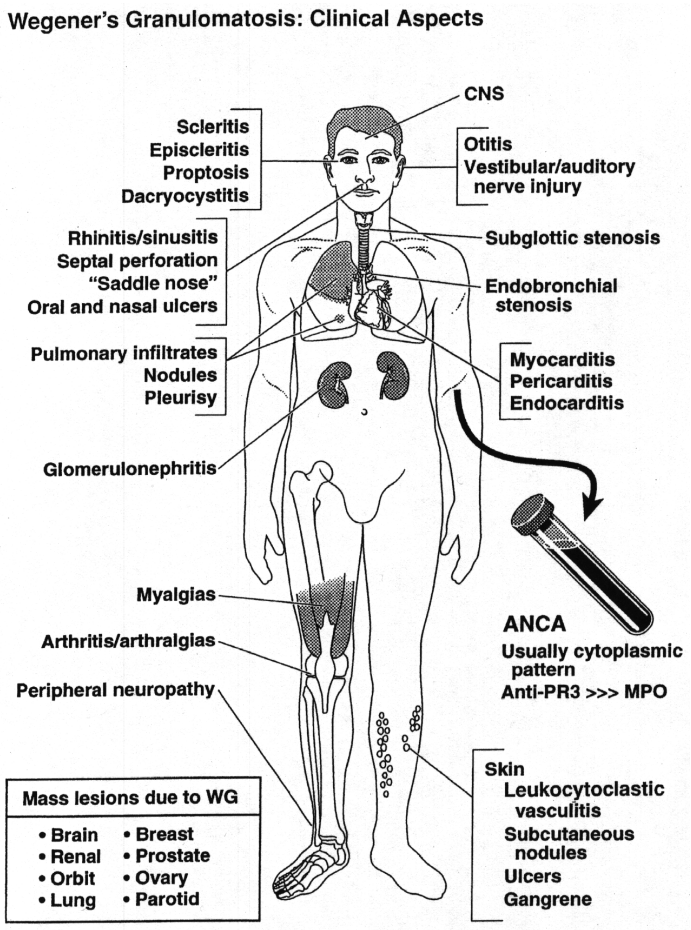


Figure 1.2: Clinical features of Wegener's granulomatosis (WG). CNS, central nervous system; ANCA, anti-neutrophil cytoplasmic antibodies; PR3, proteinase-3; MPO, myeloperoxidase. Adapted from Hoffmann and Gross, 2002. (4)

1.1.2 Aetiology and pathogenesis

WG is a necrotizing, granulomatous vasculitis belonging pathologically to the small- to medium-sized vessel systemic vasculitides (7). Like most of the other primary vasculitides, its aetiology remains unknown, and the theories dealing with its pathogenesis are currently based on circumstantial evidence. For instance, presence of ANCAs directed against neutrophilic proteinases correlates with disease activity in the small-vessel vasculitides, and is utilized in the diagnosis of WG (8-10). Although their role in the small-vessel vasculitides is supported by substantial evidence, the particular role of the ANCAs, that is, whether they are causing the disease or resulting from it, and how this is done, is presently a matter of speculation (11).

Furthermore, a number of genetic predispositions have been identified as possible determinants of disease occurrence and clinical course. For example, heterozygosity in the PiZ allele of the (α_1-PI) gene, producing subnormal levels of α_1-PI, a protein that is protective against the effects of proteolytic enzymes released from leukocytes undergoing degranulation, has been shown to correlate with PR3-ANCA-positive vasculitis and may increase the risk of

fatal outcome (5). Predominant involvement of the airways and the presence of neutrophilic alveolitis at disease onset have led to speculation that an inhaled agent may trigger the disease (4). Moreover, relapses of disease often follow systemic infections, suggesting a role for infection in priming neutrophils and activating endothelial cells. Finally, nasal carriage of *Staphylococcus aureus* has been proposed as a risk factor for development of relapse, possibly as a result of the actions of staphylococcal superantigens (5). Further elucidation of the possible mechanisms underlying WG is required.

1.1.3 Treatment and prognosis

As in most of the other systemic vasculitides, aggressive immunosuppressive therapy forms the foundation of WG treatment. There are marked variations in presentation, course of illness, and tendency for relapse of disease, and treatment is therefore tailored to each patient. Daily oral cyclophosphamide (2 mg/kg/day) or high-dose monthly intravenous cyclophosphamide (500 mg/m^2) in combination with glucocorticoids (1 mg/kg/day) has proven the most effective treatment for patients with active WG, and is the treatment of choice for inducing remission in critically ill patients (12). This regimen is, however, commonly associated with severe side-effects, and is substituted with less toxic maintenance therapy regimens as soon as possible. Such regimens include the combination of methotrexate and glucocorticoids, which have also proven effective in inducing remission in WG-patients (13, 14), and the utilization of other immunosuppressive agents, such as azathioprine or cyclosporine, in combination with glucocorticoids, although these regimens have not been found to induce remission successfully (12). Regardless of the type of medication employed in the alleviation of active WG, tapering of dosage in accordance to clinical improvement is recommended because of the inherent toxicity of immunosuppressive drugs.

Although once considered a disease with very high short-term mortality, the introduction of effective treatment options, and in particular the introduction of cyclophosphamide, has converted WG into a chronic disorder, the most common cause of death now being infections secondary to the drug-induced suppression of host defences (15). In chronic illness, as compared to life-threatening disease, the acceptable levels of treatment-associated morbidity are markedly lowered. There is, therefore, a strong need for new treatment modalities in the handling of chronic WG.

1.2 CHEMOKINES

Cytokines are peptides which mediate cell-to-cell interactions via specific cell surface receptors and regulate activation, differentiation, growth, death, and acquisition of effector functions of immune cells (16). The chemoattractant cytokines, or chemokines for short, constitute a class of cytokines with chemoattractant properties, inducing cells that carry the appropriate receptors to migrate toward the chemokine source. Chemokines are released by many different types of cells, serving to guide cells involved in innate, as well as adaptive, immunity (17).

1.2.1 Classification

The chemokines are highly basic proteins of 70-125 amino acids with molecular masses ranging from 6 to 14 kDa. All chemokines are related in amino acid sequence and display similar protein structures. Based on the configuration of a pair of intramolecular disulfide bridges, they are assigned to one of two major groups: CC (α-) chemokines, in which the cysteines engaged in bridge formation are adjacent, and CXC (β-) chemokines, in which the equivalent two cysteine residues are separated by another amino acid. In addition, there has been found one C (γ-) chemokine – lymphotactin – which has only one disulfide bridge, and one CX_3C (δ-) chemokine – fractalkine – where the bridge-forming cysteines enclose three amino acids (Fig. 1.3). The CXC chemokines can be divided further by the presence or absence of an amino acid triplet (ELR; glutamic acid − leucine − arginine) preceding the first of the invariant cysteines. All the CXC chemokines that attract neutrophils have this motif, while the other chemokines lack it. The ELR-motif is also distinctive for the chemokines entailing angiogenic properties. (18, 19)

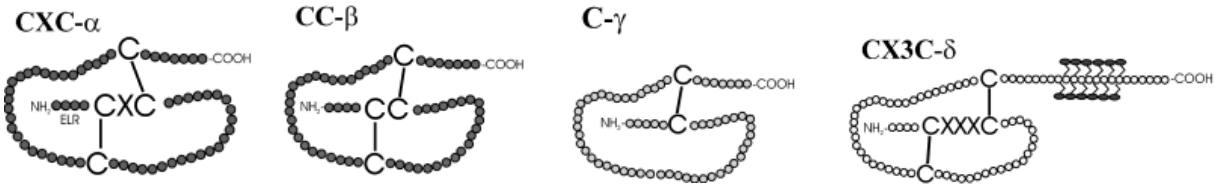


Figure 1.3: Structural characteristics of the chemokine subclasses. Fractalkine (CX_3CL) is currently the only chemokine known to be membrane-bound. Adapted from Bajetto *et al.*, 2002. (20)

A new chemokine nomenclature is presently being implemented world-wide, providing each of the 50 chemokines detected so far with systematic names based on its structural characteristics (21). However, in this thesis all chemokines are referred to primarily by their

original names. A complete list of chemokines (anno 2000) with both new and old names can be found in the Appendix, Table 1.

1.2.2 Chemokine receptors

The chemokine receptors are G-protein-linked integral membrane proteins containing seven membrane-spanning helices. CC and CXC chemokines act on different groups of receptors designated CC and CXC receptors, respectively. Each chemokine may bind and interact with one or more receptors within the matching receptor subgroup, and vice versa. The distribution of receptors thus determines the subset of cells on which each chemokine may exert its effects. (18) For a complete outline on the redundancy of chemokine – chemokine-receptor interactions the reader is referred to the Appendix, Table 1.

The effects of chemokine ligation are mediated through several intracellular signalling pathways, mainly originating from the activation of G-protein. The main signal transduction pathways are depicted in Fig. 1.4.

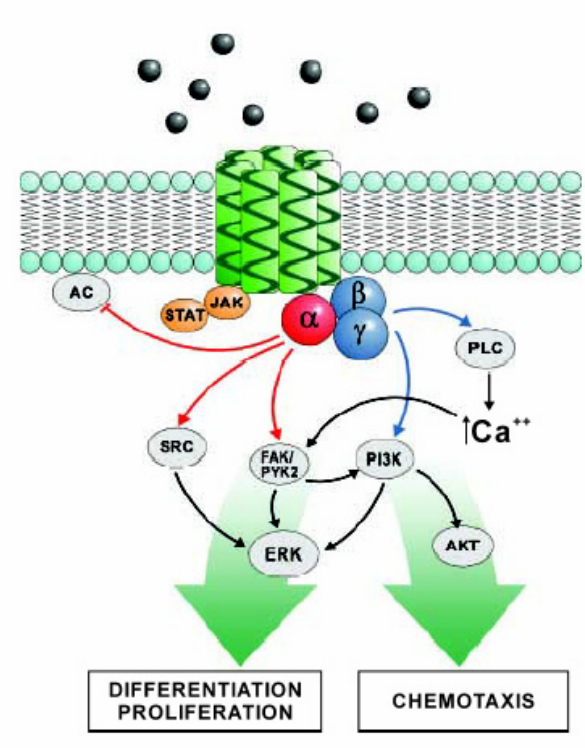


Figure 1.4: Schematic presentation of the main signal transduction pathways activated by chemokines. AC, adenylyl cyclase; JAK, janus activated kinase; STAT, signal transducer and activator of transcription; α, β, γ, subunits of G-protein; PLC, phospholipase C; FAK, focal adhesion kinase; PYK2, protein tyrosine kinase 2; PI3K, phosphoinositide 3-kinase; ERK extracellular signal-regulated kinase; AKT, protein kinase B. Red arrows indicate pathways mediated by α subunits of G-proteins, blue arrows by the βγ-complex. Adapted from Bajetto *et al.*, 2002. (20)

1.2.3 Biological functions of chemokines

The primary chemokine functions are chemoattraction of leukocytes to sites of inflammation and tissue injury, and activation of leukocyte effector functions at arrival to these sites (Fig. 1.5). Chemoattraction of immune cells is a property shared by the entire chemokine family, resulting from the receptor-mediated modulation of actin-dependent cell propagation and up-regulation of adhesion proteins. Several chemokines, such as interleukin (IL)-8 and monocyte

chemoattractant protein (MCP)-1, trigger integrin-dependent firm adhesion of rolling cells, an important step in the trafficking of leukocytes to sites of inflammation (22). Some chemokines also serve to activate effector functions of both leukocytes and resident tissue cells. For instance, certain CC chemokines, including MCP-1, macrophage inflammatory protein (MIP)-1α, regulated on activation normal T-cell expressed and secreted (RANTES) and eotaxin, can mediate allergic reactions and increase oxidative stress by inducing the release of histamine and reactive oxygen species from eosinophils, mast cells, and basophils (19, 23). Furthermore, some chemokines, such as MIP-3β, 6 cysteine chemokine (6Ckine), and B-cell-activating chemokine (BCA)-1 are critical for the development of secondary lymphoid tissues in mice (17), whereas others, the CXC-chemokines in particular (e.g., stromal cell-derived factor (SDF)-1), have been found to have important functions in angiogenesis and organ development (24). Thus, many pivotal roles of chemokines, not only confined to orchestration of immune responses, have been characterized. However, the chemokine network and its extensive applications are far from fully elucidated.

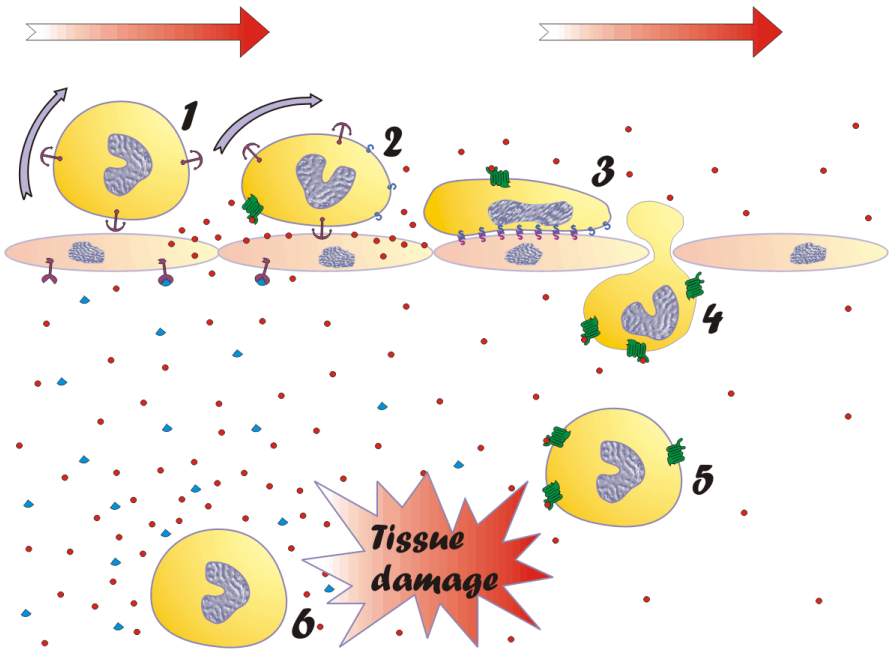


Figure 1.5: Chemokine regulation of leukocyte movement. Chemokines (red dots) are secreted at sites of inflammation and infection by resident tissue cells, resident and recruited leukocytes, and cytokine-activated endothelial cells. 1) Leukocytes rolling on the endothelium in a selectin-mediated process are brought into contact with chemokines retained on cell surface heparin sulphate proteoglycans. 2) Chemokine signalling activates leukocyte integrins, leading to 3) firm adhesion and 4) extravasation. 5) The leukocytes follow the chemokine gradient to the source of chemokine secretion, and 6) initiate effector functions.

1.2.4 Chemokines in disease

Chemokines are associated with a number of diseases, ranging from atherosclerosis to acquired immune deficiency syndrome (AIDS) (25). The malarial parasite *Plasmodium vivax* is known to utilize the Duffy antigen receptor for chemokines (DARC), which is a chemokine binding protein that binds both CC and CXC chemokines with equal affinity, as a portal of entry into human erythrocytes. Furthermore, the chemokine receptors CCR5 and CXCR4 are involved in the pathogenesis of AIDS, as they are employed by the human immunodeficiency virus (HIV)-1 to promote cellular fusion and infection. Other viruses, including members of the herpes and poxvirus classes, have pirated chemokine receptors from their hosts, presumably to help them overcome the immune response. (17)

Chemokines have also been linked to several autoimmune disorders, such as multiple sclerosis (MS) and rheumatoid arthritis (RA). Moreover, a role for chemokines has been suggested in coronary heart disease, asthma, and organ transplant rejection (25-27). Given their important role in orchestrating responses of the immune system, it is not surprising that dysregulation of the chemokine network may lead to dysfunction of the immune apparatus and, ultimately, disease. Based on the potential role of chemokines in the pathophysiology of a number of diseases, several chemokine receptor antagonists are presently being investigated for therapeutic properties. (28)

1.2.5 Chemokines in Wegener's granulomatosis

The role of chemokines in inflammation suggests that they may also be involved in WG, which is, in fact, characterized by inflammation and necrosis of blood-vessel walls. This assumption is sustained by several experimental findings, including the observations that production of RANTES in pulmonary WG lesions is elevated (29) and that the expression of IL-8 in monocytes is induced by ANCAs, which are believed to be influential in the development and exacerbation of this disease (30). PR3, the major antigen of WG-associated ANCAs, has been shown to enhance endothelial-cell production of IL-8 and MCP-1 (31). Furthermore, a number of studies have shown the presence of chemokines specific for neutrophils (IL-8) (32) and mononuclear cell subsets (MCP-1, RANTES, MIP-1α, MIP-1β) (33, 34) in renal biopsies from patients with WG. Although these findings are indicative of a role for chemokines in WG, additional investigations are required to further elucidate their implication. In particular, few studies have examined the effects of immunosuppressive medication on the chemokine network.

1.3 GLUCOCORTICOIDS

Glucocorticoids (GCs) are employed in the therapy of a wide spectre of disorders, mostly characterized by inappropriate immune responses or autoimmunity. These include, amongst others, allergic disorders, asthma, autoimmune and inflammatory disorders, cancer, and organ transplantation (35). In most cases, systemic GC therapy is highly effective in ameliorating disease symptoms and activity. However, the actions of GCs are mediated through receptors found in almost every cell of the body, resulting in powerful and widespread effects, and commencing not only therapeutic, but also toxic responses.

1.3.1 Clinical aspects

The endogenous glucocorticoids belong to a group of hormones collectively known as the corticosteroids. The corticosteroids are categorized as glucocorticoids, affecting intermediary metabolism, inflammation, immunity, wound healing, myocardial and muscle integrity, or mineralocorticoids, regulating salt, water, and mineral metabolisms. However, most corticosteroids, both endogenous and synthetic, produce a combination of both glucocorticoid and mineralocorticoid effects. (36)

The adverse effects commonly accompanying systemic GC therapy (Table 1.1) may sometimes represent a limiting factor to the treatment, especially when treatment is not life-saving. When the aims of therapy are confined to relieving symptoms, severe treatment-associated morbidity is unacceptable, calling for cessation of treatment or implementation of less toxic regimens, such as alternate day dosage (preferably in the morning, to comply with the endogenous diurnal rhythm of hormone secretion) or local/compartmental administration (e.g., topical or intra-articular). The concurrent administration of other immunosuppressants (so-called "steroid-sparing" agents), such as azathioprine, methotrexate, and cyclosporine, may allow for a reduction in GC-dose, thus reducing overall toxicity. (38)

Very common side effects:
- Weight gain
- Mood swings

Common side effects:
- Mild weakness in limbs
- Easy bruising of skin
- Impaired wound healing
- Acne
- 'Moon face'
- Slowed growth in children and adolescents
- Osteoporosis
- Cataracts
- Immunosuppression
- Hypothalamic-pituitary-adrenal axis suppression

Occasional side effects:
- High blood pressure
- Elevated blood sugar, worsening of diabetes mellitus
- Red/purple stretch marks
- Stomach irritation, ulcers

Table 1.1: Side effects of systemic glucocorticoid (GC) therapy. Adapted from Matsen (2002). (37)

Due to the negative feedback imposed by high levels of circulatory GCs on the hypothalamic-pituitary-adrenal axis, the endogenous production of corticosteroids is commonly down-regulated during systemic GC treatment. Furthermore, long-term treatment with GCs is associated with down-regulation of glucocorticoid receptor (GR), and, moreover, decreased function of other genes that are GC-sensitive (39). Owing to this suppression of the normal regulation of GC activity, abrupt termination of GC treatment must be avoided. Instead, a gradual tapering of dosage, including implementation of alternate-day dosing regimens, is recommended (35).

1.3.2 Cellular effects

The powerful immunosuppressive actions of GCs are the result of a variety of cellular effects. These include inhibition of chemotaxis and bactericidal activity in neutrophils and monocytes, lymphopenia, decreased macrophage function, and disturbed complement activation. The GCs reduce the number of circulating immune cells through apoptosis and redistribution, and restrain several of their functions, such as cytokine-release. The sum of these effects is impaired inflammatory function of immune cells, resulting in reduced immune activity and alleviation of inflammation, thus producing the principal effects of GC treatment. (38, 40)

1.3.3 Mechanisms of action

Most of the effects of GCs are thought to be mediated via the cytoplasmic GR (Fig. 1.6). The non-activated GR resides in the cytosol in the form of a hetero-oligomer with other highly conserved proteins, including heat-shock proteins (HSPs) and immunophilin. Upon binding of GC, which is facilitated through interaction with HSP 90, the GR dissociates from the rest of the hetero-oligomer and translocates into the nucleus. Before or after the translocation, the receptor forms homodimers. Inside the nucleus, the hormone-receptor complexes bind to specific DNA sequences called “GC responsive elements” (GREs), with ensuing inhibition or enhanced transcription of the associated genes. (38, 41)

One major way of GC-mediated gene suppression is through interaction of the hormone-receptor (GC-GR) complex with the c-Jun/c-Fos heterodimer, which binds to the activating protein (AP)-1 site of genes of several growth factors and cytokines. The GC-GR complex prevents the c-Jun/c-Fos heterodimer from stimulating the transcription of these genes. Cross-talk between the GC-GR complex and other transcription factors is further exemplified by its interaction with nuclear factor (NF)-κB, which is known to regulate several important

inflammatory mediators, including the chemokines IL-8, MCP-1, and RANTES, and which may contribute significantly to the GC resistance discussed later. Furthermore, the GC-GR complexes may also act by inducing transcription of certain mRNases, thereby indirectly reducing the stability of their respective substrate mRNA. All these mechanisms are collectively termed "genomic" mechanisms, as they directly or indirectly affect gene transcription. (38, 41)

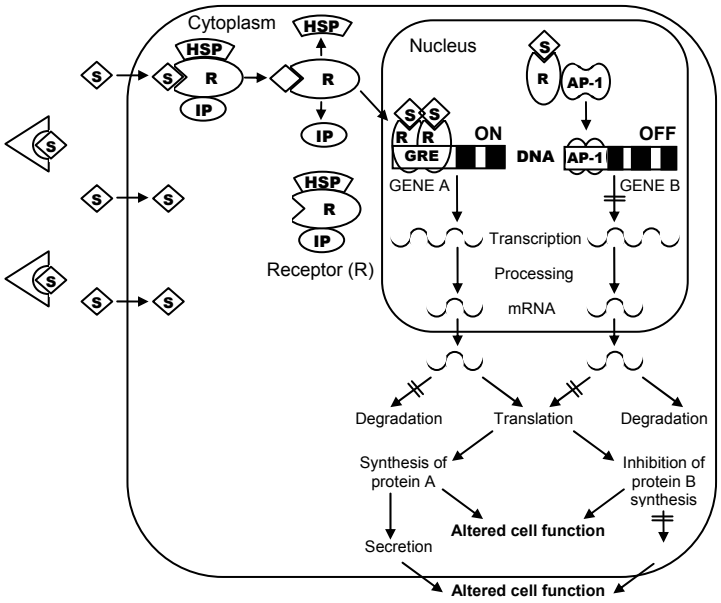


Figure 1.6: Mechanisms of action for the genomic glucocorticoid signalling pathway. Steroid hormone (S) circulates as a free molecule or as a complex with plasma-binding protein. After the steroid enters the cell, it binds to receptors (R) that reside in the cytosol complexed to heat-shock protein (HSP) and immunophilin (IP). Binding of the ligand to the complex causes dissociation of HSP and IP. The receptor-ligand translocates into the nucleus where it binds at or near the 5'-flanking DNA sequences of certain genes (glucocorticoid responsive elements (GRE)). Receptor binding to the regulatory sequences of the responsive genes increases or decreases their expression – illustrated by ON and OFF, respectively. Glucocorticoids may also exert their effects post-transcriptionally by either increasing the degradation of messenger RNA (mRNA) or by inhibiting the synthesis or secretion of the protein. AP, activating protein; DNA, deoxyribonucleic acid; RNA, ribonucleic acid. Adapted from Boumpas *et al.*, 1993 (38).

In addition to the genomic mechanisms, some of the effects of GCs may also be mediated through so-called "non-genomic" mechanisms, which operate independently of gene transcription (Fig. 1.7).

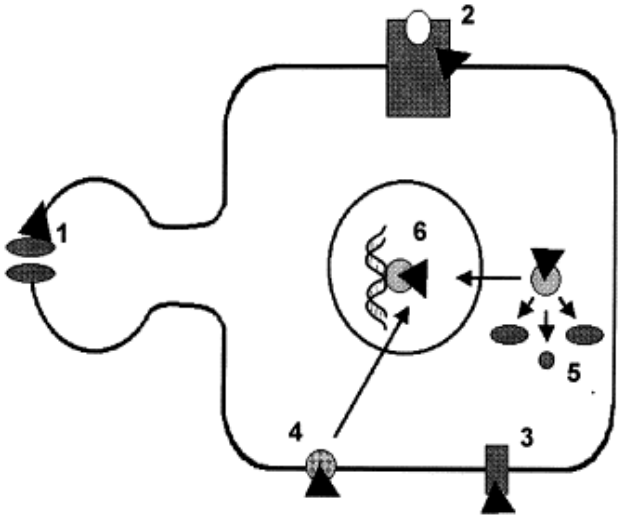


Figure 1.7: Schematic presentation of non-genomic binding sites of glucocorticoids (GCs). The closed triangle represents the GC molecule. The following mediators of non-genomic GC effects have been identified so far: 1) ion channels (e.g. the synaptic voltage-dependent calcium channel); 2) neurotransmitter receptors (e.g. the acetylcholine and the kappa opioid receptors); 3) specific, non-genomic membrane receptors (that may still represent a protein with other functions); 4) modified membrane GC receptors (GRs) (although genomically active, data suggest that these modified GRs may mediate non-genomic effects as well); 5) active proteins released from the GR complex upon binding the ligand. 6) Represents the classical, genomic way of action. Adapted from Makara and Haller, 2001. (42)

The non-genomic mechanisms are characterized by a rapid response (seconds/minutes), and are otherwise distinguished from the genomic mechanisms by 1) independency of corticosteroid receptors (including both the mineralocorticoid (MR) and glucocorticoid (GR) receptors), and 2) ability to execute their actions in the absence of a genomic apparatus (42).

1.3.4 Glucocorticoid effects on chemokines

The expression of many pro-inflammatory chemokines, including IL-8, MIP-1β, MIP-3β, MCP-1, MCP-2, MCP-3, thymus- and activation-related chemokine (TARC), and eotaxin, is down-regulated in response to GC therapy (43). Other GC-regulated chemokines are RANTES, MIP-1α, and MCP-1 (44). Although GCs also affect other pro- and anti-inflammatory mediators in a similar fashion, the modulation of chemokines is believed to contribute markedly to the effects of this class of drugs (31, 43).

1.3.5 Glucocorticoid resistance

Although GCs support and regulate several physiological functions that are essential to life, there are considerable inter-individual variations in GC responsiveness (45), and up to 30 % of the normal population may be unresponsive to anti-inflammatory GC treatment, as suggested in a study of lymphocyte steroid sensitivity in healthy volunteers (46). GC treatment is one of the factors that may contribute to GC resistance, but the underlying mechanisms for this phenomenon are largely unknown. However, some proposed mechanisms include (Fig. 1.8): 1) reduction in total GC binding sites through homologous down-regulation (39); 2) Up-regulation of the levels of β-type GR (GRβ), which is formed by an alternative splicing of the GR pre-mRNA and functions as an endogenous inhibitor of the genomically active α-type GR (47); 3) NF-κB-mediated trans-repression of the transcriptional activities of GR (45). GC resistance may interfere severely with the effects of GC therapy, necessitating the introduction of more powerful, but also more toxic, agents in the management of inflammatory diseases.

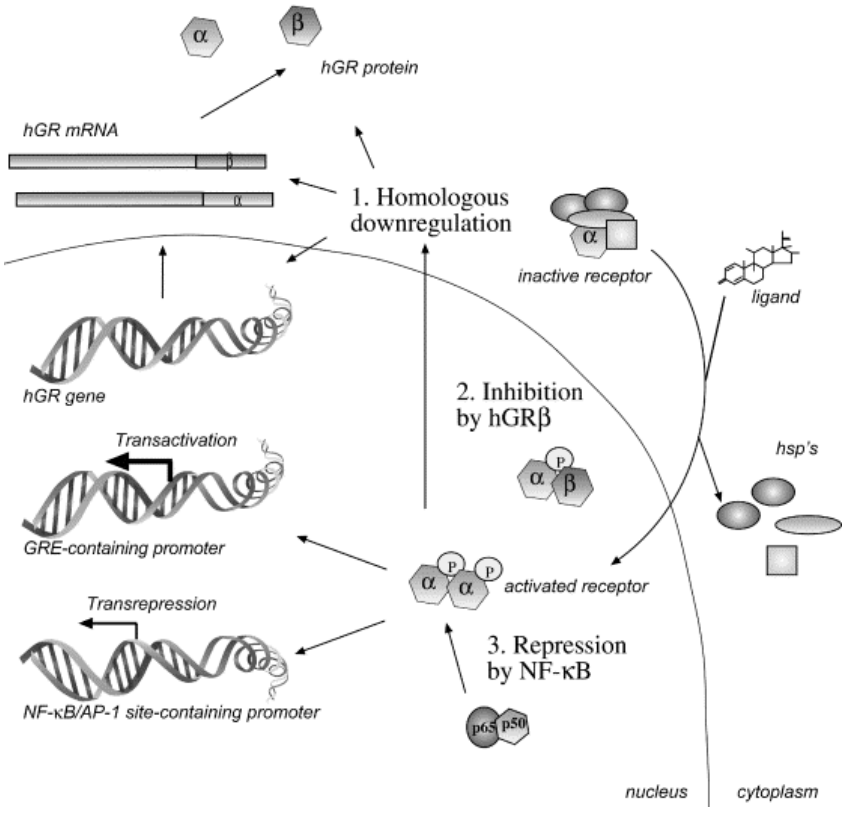


Figure 1.8: Mechanisms of glucocorticoid resistance. NF, nuclear factor; AP, activating protein; GRE, glucocorticoid responsive element; hGR, human glucocorticoid receptor; HSP, heat-shock protein Adapted from Schaaf and Cidlowski, 2002 (45).

1.3.6 Glucocorticoids in the management of Wegener's granulomatosis

The GCs have played a central role in the treatment of WG since their introduction in this condition some 40 years ago. They alleviate effectively the detrimental effects of the vascular inflammation associated with WG, and have a central position in inducing disease remission. However, their effects are widespread and pleomorphic, and the exact mechanisms underlying their therapeutic properties in WG remain uncertain, although the down-regulation of pro-inflammatory mediators most likely plays a role. (43)

As mentioned above, GCs are commonly co-administered with other immunosuppressive agents, such as cyclophosphamide, azathioprine or methotrexate. Although the current regimens relatively effectively induce and sustain disease remission, a majority of the patients experience relapses. In particular, several investigations have demonstrated an unacceptably high relapse rate in the course of tapering corticosteroids and/or decreasing the frequency of cyclophosphamide administration to less than once monthly. Another shortcoming of the current therapy is the presence of a considerable level of treatment-related morbidity. Considerable effort is therefore made to develop improved immunosuppressive drugs, and there are several studies in progress exploring the utility of biological agents that may have both precise immunoregulatory effects and diminished toxic properties. (4)

1.4 AIMS OF THE STUDY

The aims of the present study are to:

1. Investigate the role of chemokines in the pathogenesis of Wegener's granulomatosis

2. Identify effects of glucocorticoid therapy on the chemokine network that may possibly be of importance to its therapeutic efficacy

2. MATERIALS AND METHODS

2.1 PATIENTS AND CONTROLS

All patients included in our studies fulfilled the American College of Rheumatology 1990 classification criteria and the Chapel Hill Consensus Conference on the Nomenclature of Systemic Vasculitis 1992 definition of WG (48, 49). WG patients were classified as having active disease or being in remission based on clinical judgement, the Birmingham Vasculitis Activity Score (BVAS) (48), and levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). Signed informed consent was obtained from each individual. Our investigations conform to the principles outlined in the Declaration of Helsinki (50).

Patients and controls were included for two separate part studies. The first study population, which consisted of 14 patients with WG and 9 healthy controls, were included for measurement of serum chemokine levels. Demographic and clinical data are given in table 2.1 and 2.3, respectively.

Table 2.1: Fourteen patients with Wegener's granulomatosis were included in the study of serum chemokine levels. Seven were suffering active disease at the time of blood-sampling, whereas the rest were in remission.

No.	Sex	Age (years)	Disease activity	ESR (mm/h)	CRP (mg/l)	Medication
#1	Female	21	Remission	n.d.	<5	PRE (10 mg)
#2	Male	33	Remission	18	5	PRE (20 mg)
#3	Female	29	Remission	10	<5	Trim-Sulpha (160+800 mg)
#4	Male	54	Active	47	16	CYC (110 mg) PRE (25 mg)
#5	Male	65	Remission	11	<5	PRE (20 mg)
#6	Male	59	Remission	17	<5	Trim-Sulpha (160+800 mg)
#7	Male	60	Active	34	11	PRE (20 mg)
#8	Male	23	Active	61	29	CYC (150 mg) PRE (15 mg)
#9	Male	36	Active	>100	161	PRE (7.5 mg) Trim-Sulpha (160+800 mg)
#10	Female	78	Active	21	13	PRE (10 mg)
#11	Female	35	Remission	110	14	None
#12	Female	54	Active	96	62	CYC (250 mg) PRE (20 mg)
#13	Female	52	Remission	8	57	None
#14	Female	25	Active	28	62	CYC (250 mg) PRE (20 mg)

Medication is expressed in terms of daily dosage. AZA, azathioprine; CYC, cyclophosphamide; PRE, prednisolone; Trim-Sulpha, trimethoprim-sulphametoxazol; n.d., not determined; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

From the second study population, consisting of 9 WG patients and 9 age- and sex-matched healthy controls, peripheral blood mononuclear cells (PBMC) were isolated (see chapter 2.2) for analysis *in vitro*. Demographic and clinical data on this population are given in tables 2.2 and 2.3.

Table 2.2: Patients with Wegener's granulomatosis that were included in the second study, conserning chemokine levels *in vitro*. Four of the patients were suffering active disease at the time of blood-sampling.

No.	Sex	Age (years)	Disease activity	ESR (mm/h)	CRP (mg/l)	Medication
#1	Male	20	Remission	7	<5	AZA (150 mg) PRE (5 mg) Trim-Sulpha (320+1600 mg)
#2	Female	33	Remission	7	<5	MTX (15 mg weekly) PRE (10 mg) Trim-Sulpha (160+800 mg)
#3	Male	62	Remission	13	<5	IvIg (40 g monthly)
#4	Female	31	Remission	26	8	IvIg (30 g monthly) PRE (5 mg)
#5	Male	38	Active	49	13	Trim-Sulpha (320+1600 mg)
#6	Female	72	Active	64	97	Trim-Sulpha (160+800 mg) PRE (30 mg)
#7	Male	38	Active	18	26	IvIg (25 g monthly) PRE (25 mg)
#8	Female	60	Active	28	16	IvIg (40 g monthly) PRE (5 mg) Trim-Sulpha (160+800 mg)
#9	Male	26	Remission	5	<5	PRE (20 mg) Trim-Sulpha (160+800 mg)

Medication is expressed in terms of daily dosage, unless stated differently. AZA, azathioprine; PRE, prednisolone; Trim-Sulpha, trimethoprim-sulphametoxazol; IvIg, intravenous immunoglobulin infusion; MTX, methotrexate.

Table 2.3: Characteristics of the healthy controls included in the present studies. In the *in vitro* study, healthy controls were matched to WG patients with respect to age and sex in order to eliminate sex- and age-related bias.

No.	**Serum study**		***In vitro* study**	
	Age (yrs)	*Sex*	*Age (yrs)*	*Sex*
#1	33	Male	23	Male
#2	42	Male	34	Female
#3	24	Female	59	Male
#4	64	Female	31	Female
#5	54	Male	37	Male
#6	47	Female	64	Female
#7	27	Female	34	Male
#8	50	Male	55	Female
#9	47	Male	25	Male

2.2 ISOLATION OF PBMC

Materials

- Heparinized whole blood (Rikshospitalet University Hospital, Norway)
- Lymphoprep™ (Axis-Shield PoC AS, Norway; prod.no: 1053980)
- Sodium chloride solution, 9 mg/ml (B. Braun Melsungen AG, Germany)
- RPMI 1640 with HEPES (PAA Laboratories GmbH, Austria; cat.no: E15-842)
- Staining solution for the counting of leukocytes (NMD AS, Norway; cat.no: 327395)
- Labolux 11 100x microscope (Leitz Wetzlar AG, Germany)
- Assistent™ Bürker counting chamber (Karl Hecht KG, Germany)
- Centrifuge with cooler (Megafuge™ 1,0R; Heraeus Sepatech GmbH, Germany)
- LS 4800 nitrogen tank (Taylor-Wharton-Cryogenics Inc., Alabama, USA)

Method

Peripheral venous blood was drawn into pyrogen-free tubes with heparin as anticoagulant. PBMC were isolated within 45 minutes of respite in room temperature.

PBMC were isolated using Isopaque-Ficoll (Lymphoprep™) density gradient centrifugation. The density of Isopaque-Ficoll is lower than that of erythrocytes and granulocytes, and greater than that of lymphocytes and monocytes, collectively designated PBMC. During centrifugation, therefore, the erythrocytes and granulocytes will form a pellet in the bottom of the centrifugation tube, whereas the PBMC will form a layer on the interface between the Isopaque-Ficoll and medium layers.

Whole blood was diluted 1:1 with 9 mg/ml NaCl aquatic solution in a 50 ml centrifugation tube. Isopaque-Ficoll was carefully layered at the bottom of the tube, and the tube was centrifuged at 500 × *g* for 25 minutes at 20 °C with minimum brake. The PBMC layer was collected and centrifuged at 650 × *g* for 15 minutes at 4 °C. The pellet was resuspended and washed twice in RPMI 1640 with HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), followed by centrifugation at 650 × *g* for 10 minutes at 4 °C. Finally, the resulting pellet of isolated PBMC was resuspended in RPMI, and the concentration and total amount of cells were assessed using a Bürker counting chamber.

PBMC isolated from whole blood were resuspended in RPMI 1640 with HEPES and distributed into Nunc-tubes in appropriate volumes (equivalent to approximately 12×10^6 cells). The tubes were centrifuged at 650 × *g* for 10 minutes at 4 °C and, after discarding of

the supernatant, the pellets were stored in liquid nitrogen for subsequent RNA isolation and gene expression analysis (by real-time RT-PCR, see chapter 2.8).

2.3 PREPARATION OF PLASMA AND SERUM

2.3.1 Preparation of serum

Materials

- Whole blood (Rikshospitalet University Hospital, Norway)
- Centrifuge with cooler (Megafuge™ 1.0R; Heraeus Sepatech GmbH, Germany)
- Ultra-freezer, -80 °C (Forma Scientific Inc., Ohio, USA)

Method

Whole blood drawn in collecting-tubes with no additive was put on ice for 1.5 hours to coagulate. The tube was then centrifuged at 1300 × *g* for 10 minutes at 4 °C. Serum was partitioned into several Nunc-tubes and stored in a -80 °C freezer.

2.3.2 Preparation of plasma

Materials

- Whole blood (Rikshospitalet University Hospital, Norway)
- Centrifuge with cooler (Megafuge™ 1.0R; Heraeus Sepatech GmbH, Germany)
- Ultra-freezer, -80 °C (Forma Scientific Inc., Ohio, USA)

Methods

Whole blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) in order to prevent coagulation. The blood samples were subjected to centrifugation (1300 × g for 10 minutes at 4 °C) within 30 minutes. Plasma was partitioned into Nunc-tubes and stored in a -80 °C freezer.

2.4 IN VITRO EXPERIMENTS

PBMC were cultured and activated *in vitro* in order to investigate the cellular effects of an experimentally induced immune activation and its alleviation with MP. Culturing of PBMC was performed through re-suspension of the cells in culture medium, and required no pre-treatment other than the separation from whole blood depicted above (chapter 2.2). A mixture of 5 % foetal calf serum (FCS) in RPMI with HEPES is suitable as culture medium for sustaining viable cultures of PBMC. Experimental immune activation was induced by addition of staphylococcal enterotoxin B (SEB).

2.4.1 Staphylococcal enterotoxin B

SEB belongs to a class of pathogens known as superantigens (SAgs). SAgs are a class of bacterial and viral proteins exhibiting highly potent lymphocyte-transforming (mitogenic) activity towards human and/or other mammalian T lymphocytes. Unlike conventional antigens, SAgs bind to certain regions of major histocompatibility complex (MHC) class II molecules of antigen-presenting cells (APCs) outside the classical antigen-binding groove and concomitantly bind in their native form to T cells at specific motifs of the variable region of the β chain (Vβ) of the T-cell receptor (TCR) (Fig. 2.1). This interaction triggers the activation (proliferation) of the targeted T lymphocytes and leads to the *in vivo* or *in vitro* release of large amounts of various cytokines and other effector substances by immune cells. The human T-cell repertoire comprises about 24 major types of Vβ elements. Any SAg binds specifically to a characteristic set of receptor sequences, and may bind to several distinct Vβ elements. As a consequence, SAgs stimulate at nano- to picogram concentrations up to 30 % of T cells while only 1 in 10^5-10^6 T cells are activated upon conventional antigenic peptide presentation to the TCR in the immune response. (51)

As mentioned previously, chronic nasal carriage of *Staphylococcus aureus* constitutes a risk factor for the development of exacerbations in WG. Circulating T cells of WG patients are persistently activated, suggesting the presence of chronic stimulus. A causal link between chronic carriage of *S. aureus* and chronic T-cell activation in WG is conceivable, because *S. aureus* produces SAgs, which are potent T-cell stimulators. (52)

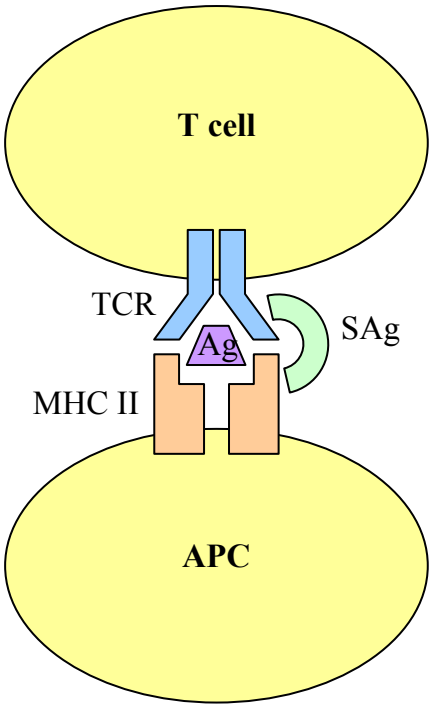


Figure 2.1: Model of T-cell activation by a conventional peptide antigen (Ag) or by a superantigen toxin (SAg). TCR, T-cell receptor; APC, antigen-presenting cell; MHC II, major histocompatibility class II complex molecule. Adapted from Müller-Alouf *et al*., 2001. (51)

2.4.2 Harvesting of cells and supernatant

Materials

- Centrifuge with cooler (Megafuge™ 1,0R; Heraeus Sepatech GmbH, Germany)
- Cell scrapers (Costar®, New York, USA; cat. no: 3010)
- Dulbecco's™ phosphate buffered saline (PAA Labs GmbH, Austria; cat.no: H15-001)
- LS 4800 nitrogen tank (Taylor-Wharton-Cryogenics Inc., Alabama, USA)

Method

Following centrifugation at 450 × *g* and 4 °C for 5 minutes, the supernatant was collected by carefully pipetting from the top of the wells, thereby avoiding cell contamination, and stored in Nunc-tubes in a -80 °C freezer. The cells were subsequently detached from the surface with a cell scrape, washed twice with ice-cold phosphate-buffered saline (PBS), and transferred to Nunc-tubes which were immediately put on ice in order to halt cell metabolism. The Nunc-tubes were centrifuged at 1800 × *g* for 10 minutes at 4 °C, and the supernatant was discarded. Then the pellets were snap frozen in liquid nitrogen and stored at -80 °C while awaiting further preparation and analysis.

2.4.3 Pilot study I

Materials

- Whole blood (Rikshospitalet University Hospital, Norway)
- Culture medium:
  - 5 % FCS (TCS BioSciences Ltd., UK; cat.no: CF018)
  - RPMI with HEPES (PAA Lab. GmbH, Austria; cat.no: E15-842)
- 6α-Methylprednisolone 21-hemisuccinate sodium (Sigma-Aldrich; cat.no: M 3781)
- Staphylococcal enterotoxin B (Sigma-Aldrich Co., St. Louis, USA; cat.no: S 4881)
- Incubator (Forma Scientific Inc., Ohio, USA)
- 96 well cell culture plates (Costar®, New York, USA; cat.no: 3596)
- Centrifuge with cooler (Megafuge™ 1.0R; Heraeus Sepatech GmbH, Germany)

Method

PBMC were isolated from whole blood by Isopaque-Ficoll density gradient centrifugation (described in chapter 2.2) of single blood samples from 2 patients with Wegener's granulomatosis and 1 healthy control and resuspended in culture medium to a cell concentration of 2×10^6 cells/ml. The resulting cell culture was partitioned into a culture plate and stimulated or not with staphylococcal enterotoxin B (SEB) or methylprednisolone (MP) or both. Three different concentrations of SEB (0.01 ng/ml, 1 ng/ml, and 100 ng/ml) and MP (10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M*) were used in order to assess a possible dose-response

relationship. In addition, the stimulants were added either before (-1 hour), concurrent, or after (+1 hour) in order to assess possible effects of contiguous administration. The plate was incubated for 24 hours at 37 °C and 5 % CO_2. After incubation, the plate was centrifuged at 450 × *g* and 4 °C for 5 minutes. The supernatants were harvested and stored in Nunc-tubes at -80 °C.

2.4.4 Pilot study II

Materials

- Whole blood (Rikshospitalet University Hospital, Norway)
- Culture medium:
  - 5 % FCS (TCS BioSciences Ltd., UK; cat.no: CF018)
  - RPMI with HEPES (PAA Lab. GmbH, Austria; cat.no: E15-842)
- 6α-Methylprednisolone 21-hemisuccinate sodium (Sigma-Aldrich; cat.no: M 3781)
- Staphylococcal enterotoxin B (Sigma-Aldrich Co., St. Louis, USA; cat.no: S 4881)
- Incubator (Forma Scientific Inc., Ohio, USA)
- 24 well cell culture plates (Costar®, New York, USA; cat.no: 3596)
- Cell scrapers (Costar®, New York, USA; cat.no: 3010)
- Centrifuge with cooler (Megafuge™ 1.0R; Heraeus Sepatech GmbH, Germany)

Method

This study was conducted in order to assess temporal variations in the expression of inflammatory chemokines following stimulation with SEB and MP. PBMC were isolated from whole blood from one healthy control and resuspended in culture medium to a cell concentration of 2×10^6 cells/ml. The resulting cell culture was partitioned into four culture plates and stimulated or not with 1 ng/ml SEB or 10^{-6} *M* MP or both (added contiguously), as determined in the first pilot study (see Results, chapter 3.2.1). The plates were subsequently incubated for 6, 8, 12, and 24 hours, respectively, at 37 °C and 5 % CO_2. After incubation, cells and supernatants were collected as described in chapter 2.4.2.

2.4.5 Experimental modulation of the production and secretion of chemokines in PBMC

Materials

Equal to pilot study II.

Method

PBMC were isolated from whole blood by Isopaque-Ficoll density gradient centrifugation (described in chapter 2.2) of single blood samples from 9 patients with WG (Table 2.2) and 9 healthy controls (Table 2.3). The cells were subsequently resuspended in culture medium to a

cell concentration of 2×10^6 cells/ml. The resulting cell culture was partitioned into a culture plate and stimulated or not with 1 ng/ml SEB or 10^{-6} *M* MP or both, as determined in the first pilot study. The plates were incubated for 6 or 24 hours at 37 °C and 5 % CO_2, based on the findings of the second pilot study (see Results, chapter 3.2.2). After incubation, cells and supernatant was collected as described in chapter 2.5.2.

2.5 ISOLATION OF RNA

RNA was isolated from PBMC pellets using the RNeasy® mini kit according to the manufacturer's instructions. In general, frozen samples are disrupted in buffer containing guanidine isothiocyanate and homogenized. Ethanol is subsequently added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. The sample is then applied to the RNeasy mini column. Total RNA binds to the membrane, while contaminants are washed away in the sequential washing steps, and total RNA is eluted by addition of water.

Materials

- RNeasy® Mini Kit (Qiagen™, Maryland, USA; cat.no: 74104)
- RNA Storage Solution (Ambion® Inc., Texas, USA; cat.no: 7000)
- Diax 100™ rotor-stator homogenizer (Heidolph Instruments AG, Germany)
- β-mercaptoethanol (Sigma-Aldrich Co., St. Louis, USA; cat.no: M 6250)
- 100 % ethanol (Arcus AS, Norway)
- RNase-free water (Ambion® Inc., Texas, USA; cat.no: 9937)
- Sodium acetate anhydrous (Sigma-Aldrich Co., St. Louis, USA; cat.no: S 2889)
- Linear acrylamide (Ambion® Inc., Texas, USA; cat.no: 9520)

Method

The following protocol was employed for pellets containing less than 5×10^6 cells. All binding, washing, and elution steps were performed by centrifugation in a microcentrifuge. Frozen pellets were dissolved, and the cells disrupted, in 350 µl Buffer RLT (lysis buffer containing β-mercaptoethanol and guanidine), followed by homogenisation with a rotor-stator homogenizer. One volume (350 µl) of 70 % ethanol (100 % ethanol diluted in distilled water) was added to the homogenized lysate, which was subsequently filtered on an RNeasy mini column placed in a 2 ml collection tube. For pellets containing more than 5×10^6 cells, the required volumes of Buffer RLT and 70 % ethanol were 600 µl. Filtration was forced by high-speed centrifugation (15 *s* at ≥8000 × *g*). The column was washed by the sequential addition of 700 µl Buffer RW1 (washing buffer containing guanidine) and two additions of 500 µl

Buffer RPE (washing buffer). Each addition was followed by a high-speed centrifugation, the last centrifugation (following the second addition of Buffer RPE) lasting for 2 minutes to make sure that the column's silica-gel membrane was completely dried. Subsequently, 50 µl of RNase-free water was added to the column, and the RNA was eluted by high-speed centrifugation. This step was repeated once, the last centrifugation lasting for 1 minute to ensure complete elution.

RNA was precipitated with the addition of 2.5 volumes (250 µl) ice-cold 100 % ethanol, 1/10 volume (10 µl) of a 3 *M* aqueous solution of sodium acetate and 4 µl linear acrylamide, followed by incubation in a -80 °C freezer for at least 1 hour. Afterwards, the RNA suspension was centrifuged at maximum speed (≥8.000 × *g*) and 4 °C for 30-60 minutes. The supernatant was discarded, and the pellet was washed with 300 µl 70 % ethanol. After another 15-30 minutes of high-speed centrifugation in 4 °C, the supernatant was thoroughly removed, and the pellet was dissolved in 10 µl RNA Storage Solution. RNA was stored at -80 °C while awaiting further analysis.

2.6 CONCENTRATION AND INTEGRITY OF RNA SAMPLES

RNA samples are susceptible to degradation by RNases, a family of enzymes present in virtually all living cells. These enzymes can degrade RNA molecules through both endonucleolytic and exonucleolytic activity, and are fairly resistant to most denaturants. Therefore, although the development of better RNA-isolation procedures has reduced the risk of RNase contamination, cautious handling of RNA samples is important. Besides RNase-mediated degradation, DNA and protein contamination resulting from incomplete RNA purification are the most prominent reasons for impairment of RNA analysis. The laborious and expensive nature of RNA analyses underlines the necessity of preliminary quality assessment. Gel electrophoresis and optical densitometry are convenient and common methods used in this respect. Positive results in both of these tests indicate good sample quality.

2.6.1 Gel electrophoresis

Electrophoresis on an agarose gel is the single best diagnostic available for validating the quality of purified RNA. Denatured RNA produces a characteristic banding profile upon gel electrophoresis, good quality samples presenting with a minimum of smear above, between, and below the bands of the abundant 28S and 18S ribosomal (r) RNAs. Lack of definition of

the 28S and 18S rRNA bands usually implies that the sample has suffered nuclease attack, especially if the smearing is confined to the lower portion (i.e., the leading edge) of the gel. However, smearing may also result from incomplete denaturation of secondary structures prior to electrophoresis or presence of detergents or excess salt in the sample. Further investigative steps should be commenced in order to determine the cause of a poor outcome of this test, and, if proved necessary, additional measures should be undertaken in the future to avoid DNA or protein contamination, or RNase activity.

Materials

- Casting tray, 10 x 15 cm (Bio-Rad™, Richmond, USA)
- Wide Mini-SUB® gel box (Bio-Rad™, Richmond, USA)
- Model 200/2.0 Power supply (Bio-Rad™, Richmond, USA)
- Seakem® LE Agarose (FMC® BioProducts, Maine, USA; cat.no: 50004)
- 37 % (12.3 *M*) Formaldehyde (Sigma-Aldrich Co., St. Louis, USA; cat.no: F 8775)
- 10x MOPS (3-morpholinopropanesulfonic acid)-buffer:
  - 0.2 *M* MOPS, pH 7.0 (MOPS hemisodium salt: Sigma-A Co., St. Louis, USA; cat.no: M 1254)
  - 50 m*M* Sodium acetate (0.3 *M* solution: Sigma-Aldrich Co., St. Louis, USA; cat.no: S 8388)
  - 10 m*M* EDTA, pH 8.0 (powder: Sigma-Aldrich Co., St. Louis, USA; cat.no: E 9884)
- 10x loading buffer:
  - 50 % glycerol (Sigma-Aldrich Co., St. Louis, USA; cat.no: G 5516)
  - 1 m*M* EDTA, pH 8.0 (powder: Sigma-Aldrich Co., St. Louis, USA; cat.no: E 9884)
  - 0.25 % bromophenol blue (powder: Sigma-Aldrich Co., St. Louis, USA; cat.no: B 0126)
- NorthernMax® Formaldehyde Load Dye (Ambion® Inc., Texas, USA; cat.no: 8552)
- Diethyl pyrocarbonate (Sigma-Aldrich Co., St. Louis, USA; cat.no: D 5758)
- Ethidium bromide (Sigma-Aldrich Co., St. Louis, USA; cat.no: E 8751)
- 100 % ethanol (Arcus AS, Norway)
- Block heater (Stuart Scientific Co., United Kingdom; cat.no: SH1200D)
- Gibco BRL UV Transilluminator TFX-35M (Life Technologies™, Rockville, USA)
- Kodak® Image Station 440 CF (Kodak Digital Science™, New York, USA)

Method

The casting tray was mounted and levelled. A comb-shaped device designed to form 15 wells of 5.5 x 1.5 mm was placed in the tray, traversing it a few centimetres from the edge. A denaturing agarose gel was prepared: 1 g Seakem® LE Agarose was added to 95 ml 1 % MOPS-buffer in an erlenmeyer (lidded), and allowed to swell at room-temperature for one minute. The agarose was then dissolved completely during microwave heating for 3-4 minutes in the lidded container, which was subsequently placed in a 55-60 °C water bath.

Water-loss was compensated with sterile DEPC-treated water (1 µl diethyl pyrocarbonate (DEPC) per ml distilled water, stored overnight and autoclaved), followed by re-tempering to 55 °C. Five ml of formaldehyde, also tempered to 55 °C, was added to the solution, yielding a final concentration of approximately 0.6 *M*. Immediately thereafter, and in a fume-hood to prevent circulation of toxic fumes, the solution was poured onto the center of the casting tray until completely covering its bottom. Any bubbles were removed with a pipette, and the gel was allowed to set. Once solidified, the gel was transferred to the gel box and soaked in MOPS-buffer.

Approximately 1 µg of sample RNA was added to a mixture of 2 µl 0.5 µg/ml ethidium bromide and 25 µl formaldehyde load dye, and heated for 2 minutes at 95 °C on a heating block. This treatment disrupts intra- and inter-molecular RNA structures, thereby preventing these from interfering with the electrophoretic migration. After heating, the samples were put directly on ice for 2 minutes, before being inserted into the wells of the agarose gel with a micro-pipette. The samples were electrophoresed at 95 V for 30 minutes or as long as necessary for complete separation of the bands. Due to the negatively charged backbone of nucleic acid polymers, the RNA (and any DNA contaminants) migrates in direction of the cathode (the positive electrode). After electrophoresis, the gel was inspected visually on a UV transilluminator. The result was also recorded with a digital camera (Fig. 2.2).

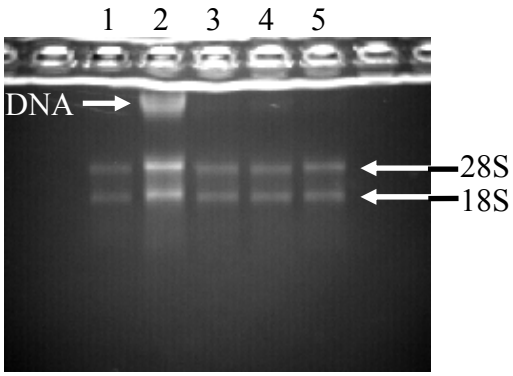


Figure 2.2: Picture showing the 18S and 28S ribosomal RNA (rRNA) bands of 5 RNA samples that have been separated on an agarose gel to assess the integrity of the sample. Note the absence of smear around the rRNA bands, which indicates that these samples are of good quality. Sample number 2 displays a broad band of residual DNA close to the starting well.

2.6.2 Spectrophotometry

This procedure is performed in order to assess the sample RNA concentration, permitting the normalization of samples in relation to RNA content. In addition, the results of this test may provide some information on sample purity. The concentration and purity of RNA samples are determined readily by taking advantage of the ability of nucleic acids to absorb ultraviolet light, maximally at 260 nm. Absorbance measurements at this wavelength permit the direct calculation of nucleic acid concentration in a sample, according to Beer's law (53):

[RNA]µg/ml = A_{260} **× dilution × 40.0**, where A_{260} = absorbance at 260 nm
Dilution = dilution factor
40.0 = average extinction coefficient of RNA

Calculations based solely on A_{260} afford little information on sample quality and purity, however. For this reason, absorbance is also measured at 280 nm, and the A_{260}/A_{280} ratio is calculated. A pure sample of RNA has an A_{260}/A_{280} ratio of 2.0 ± 0.15. Repeated steps of purification may be required if the samples are found to contain impurities such as proteins or DNA, which can both affect optical density (OD) readings.

Materials

- GeneQuant® densitometer (LKB Biochrom, England)
- 1 *M* Tris (Trizma®)-HCl (Sigma-Aldrich Co., St. Louis, USA; cat.no: T1819)

Method

Prior to sample analysis, the densitometer was zeroed to pure Tris-HCl. The RNA samples, diluted 1:40 in 10 m*M* Tris-buffered HCl to provide a stable pH of approximately 7.5, were analyzed on the densitometer according to the manufacturer's instructions. The machine automatically estimated the RNA content and the A_{260}/A_{280} ratio based on readings at 260 and 280 nm.

2.7 ANALYSIS OF GENE EXPRESSION

Analysis of gene expression was performed using real-time reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR is a two-step procedure designed for the quantification of minute amounts of messenger RNA (mRNA). In the first step, the mRNA is converted to complementary DNA (cDNA) by the use of an enzyme called reverse transcriptase. The second step of RT-PCR entails amplification of the previously formed cDNA by DNA polymerases. In real-time RT-PCR, the amount of PCR-product produced during the amplification-step is measured continuously, providing more accurate data. This information can subsequently be used to calculate the original levels of mRNA in the sample.

2.7.1 Methodological principles

Isolated RNA is converted to cDNA through the actions of reverse transcriptases, acting upon an array of random primers that anneal to various sequences throughout the RNA molecule. The resulting cDNA must be greatly amplified in order to reach detectable amounts. This is accomplished with the polymerase chain reaction (PCR) technique. The PCR is typically divided into cycles, each cycle consisting of three components; sample denaturation, primer annealing, and primer extension. The successive application of these functionally distinct components, typically in the form of 30-40 cycles, results in exponential amplification of the template molecules.

Sample denaturation, i.e. strand separation, facilitates hybridization of primers to the template in the following step of primer annealing. The denaturation is achieved by heating the reaction tube to 95 °C for 1-10 minutes (depending on the complexity and nature of the target), and includes denaturation of double-stranded template material as well as any secondary structures that may have formed within/between primers. Complete denaturation is an absolute requirement if any PCR product is to be observed.

Annealing, or hybridization, of the primers to its specific targets is accomplished by lowering the temperature of the reaction tube. Depending on the size and composition of the primer, its melting point (T_m) typically lies within 42 – 65 °C, above which annealing will not commence. Annealing is usually attempted at least 1-2 °C below the lowest calculated T_m of the primers involved, for 0.5-2 minutes time. Thermostable DNA polymerases, which are able to withstand the temperatures of up to 95 °C that must be applied in order to achieve complete

sample denaturation, elongates the annealed primer by sequentially adding nucleotides to its 3'-end.

When performing quantitative PCR, an internal control, such as glyceraldehyde phosphate dehydrogenase (GAPDH) or β-actin, must be established in order to normalize the results to variations in template abundance. The transcripts used as internal controls should show minimal modulation during the course of the experimental manipulations, and are often referred to as housekeeping genes. The internal control may be assessed in the same or separate reactions, and utilize the same or different primers as the target. (53)

2.7.2 Reverse transcription

Materials

- Mastercycler gradient thermal cycler (Eppendorf® GmbH, Germany)
- Optical 96-well reaction plates (AB Inc., Foster city, USA; part no: N801-0560)
- High Capacity cDNA Archive Kit (AB Inc., Foster city, USA; part no: 4322171)
  - · 10x RT Buffer
  - · 10x RT Random Primers
  - · 25x dNTP Mix, 100 m*M*
  - · MultiScribe™ reverse transcriptase, 50 U/µl
- Nuclease-free water (Ambion® Inc., Texas, USA; cat.no: 9937)

Method

A mastermix containing random primers, deoxynucleotides (dNTPs), reverse transcriptase enzyme, and buffer was prepared according to Table 2.5. The enzyme (Multiscribe™) was added lastly due to its vulnerability to shear stress. Approximately 1000 ng RNA, isolated from PBMC and dissolved in RNA Storage Solution, was transferred to 0.5 ml eppendorf tubes and diluted with nuclease-free water to a total volume of 50 µl. Fifty µl of mastermix was added to each sample tube, followed by vortex and pulse centrifugation. The tubes were subsequently inserted into the PCR-machine, which was programmed to run for 10 minutes at 25 °C and 120 minutes at 37 °C, before cooling to 4 °C. cDNA was stored at -20 °C while awaiting PCR.

Ingredients	Final concentration
10x RT-buffer	1x
25x dNTP	4 m*M*
10x Random Primers	1x
Multiscribe™	2.5 U/µl

Table 2.5: Mastermix for reverse transcription of RNA. RT, reverse transcriptase; dNTP, deoxynucleotides.

2.7.3 Primer design

Designing primers is perhaps the most critical parameter for a successful PCR. The primer sequence determines the length of the product, its melting temperature, the yield, and, most importantly, the efficiency of the PCR reaction. A poorly designed primer can, for example, result in little or no product due to non-specific amplification and/or primer-dimer formation. Hence, care should be taken when designing primer and probes for real-time RT-PCR and it is strongly recommended to use software specially developed and validated for these purposes.

Primers and probes for real-time RT-PCR were designed using the Primer Expression version 2.0 software package (Applied Biosystems). This software is designed to identify primers and probes that require a minimum of optimization when performing real-time RT-PCR on a sequence detection system from Applied Biosystems with default cycling parameters.

First, nucleotide sequences were collected from GenBank via LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/). Exon-intron boundaries were identified for each gene using information available via the same website. The nucleotide sequence was subsequently imported into Primer Express. Primers and probes were designed according to the User's manual and with the default parameters for ideal primer and amplicon melting point (T_m), length and GC content. Briefly, all primers had T_m between 58 and 60 ºC, and the probes were designed to have a T_m 8-10 ºC higher than the primers, i.e., 68-70 ºC. One of the primers was designed to span an exon-intron boundary to avoid amplification of genomic DNA. Moreover, primers were selected to give an amplified fragment (amplicon) between 70 and 120 base pairs. All primers and probes were further evaluated to avoid oligonucleotides with advanced secondary structures and high potential for primer-dimer formation. Finally, a BLAST search against GenBank was performed to assure the specificity of the designed real-time PCR assay, i.e., avoid primers or probes with strong homologies to genes other than the intended target.

TaqMan probes were 5'-labeled with the fluorophore FAM (reporter) and 3'-labeled with TAMRA for quenching. High-purity oligonucleotides were ordered from Eurogentec. Details of the real-time RT-PCR assays used in the study are given in Table 2.6.

Table 2.6: Characteristics of the real-time RT-PCR assays used in the study.

Target	Sequence (5'→3')	Acc. nr.
β-actin*	(+)-AGGCACCAGGGCGTGAT	NM_001101
	(–)-TCGTCCCAGTTGGTGACGAT	
ENA-78	(+)-AAGTGGTAGCCTCCCTGAAGAAC	X78686
	(–)-CCTTGTTTCCACCGTCCAA	
	FAM-AGGAAATTTGTCTTGATCCAGAAGCCCCT-TAMRA	
GR-α*	(+)-TTGGATTCTATGCATGAAGTGGTT	X03225
	(–)-TATCTGATTGGTGATGATTTCAGCTAA	
IL-8	(+)-GCCAACACAGAAATTATTGTAAAGCTT	Y00787
	(–)-CCTCTGCACCCAGTTTTCCTT	
	FAM-CTGATGGAAGAGAGCTCTGTCTGGACCC-TAMRA	
MCP-1	(+)-AAGCTGTGATCTTCAAGACCATTGT	S69738
	(–)-TGGAATCCTGAACCCACTTCTG	
	FAM-CCAAGGAGATCTGTGCTGACCCCAA-TAMRA	
MIP-1α	(+)-CTGCATCACTTGCTGCTGACA	M23452
	(–)-CACTGGCTGCTCGTCTCAAAG	
	FAM-TTCAGCTACACCTCCCGGCAGATTCC -TAMRA	
RANTES	(+)-CCCAGCAGTCGTCTTTGTCA	M21121
	(–)-TCCCGAACCCATTTCTTCTCT	

* SYBR Green assays; (+), forward primers; (–), reverse primers; Acc. nr., GenBank accession number. ENA, epithelial cell-derived neutrophil-activating factor; GR, glucocorticoid receptor; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T-cell expressed and secreted.

2.7.4 Real-time RT-PCR

In real-time PCR, the PCR products are assayed as they accumulate, as opposed to performing a fixed number of cycles and assessing the amount and variety of products at the end of the reaction. The instantaneous quantification of product requires techniques involving fluorescent labelling reagents, of which there are currently four competing techniques available that detect amplified product with about the same sensitivity: 1) The molecular beacons; 2) DNA-binding dyes (SYBR Green); 3) hybridization probes; 4) hydrolysis probes (Taqman). We have used both the SYBR Green and the Taqman chemistries in our analyses.

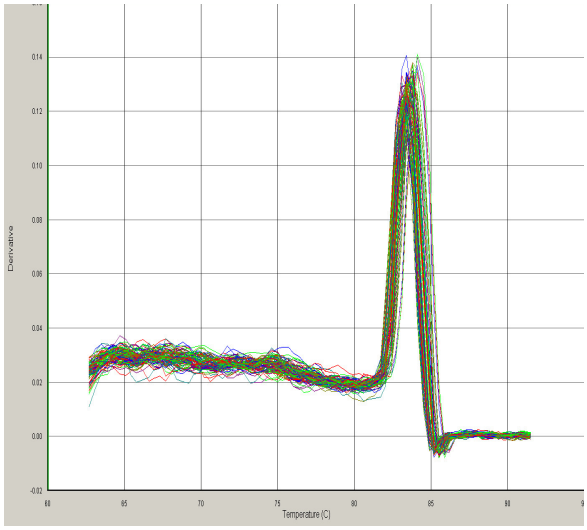


Fig. 2.3: Melting curve obtained for a SYBR Green assay. The one clear-cut top indicates that the amplification has been performed with high target specificity.

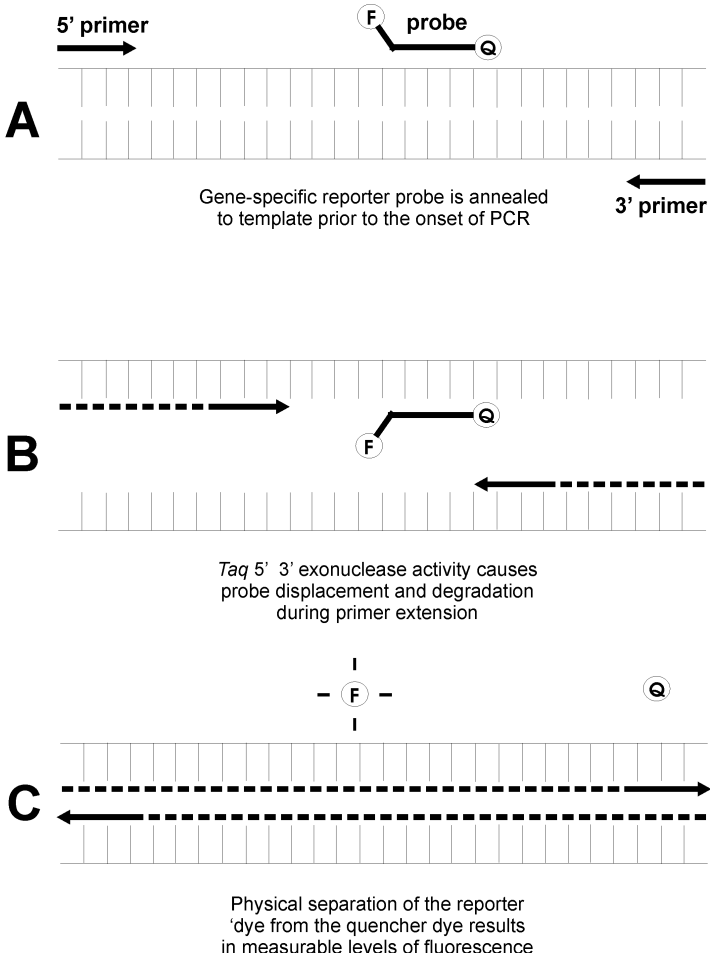
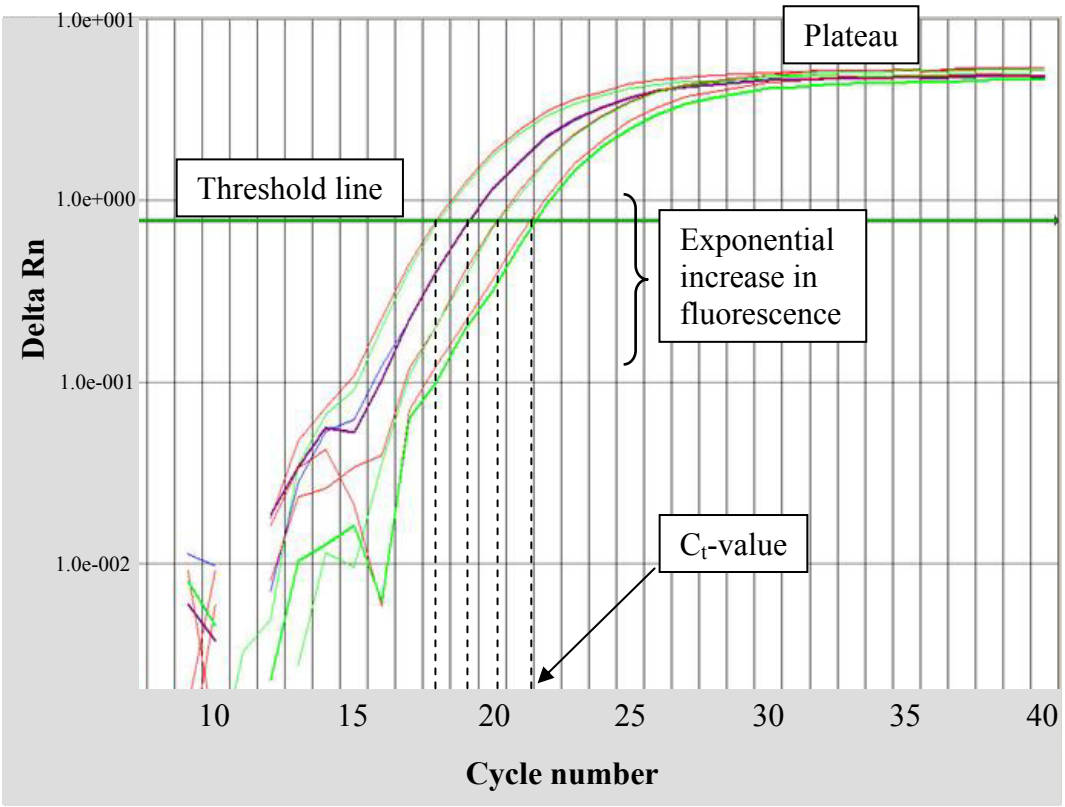


Figure 2.4: Presentation of the mechanisms involved in Taqman real-time PCR. Adapted from Farrell, 1998 (53).

The SYBR Green method involves detection of the binding of a fluorescent dye (SYBR Green) to DNA. The unbound dye exhibits little fluorescence, but during elongation increasing amounts of dye binds to the new-formed double-stranded DNA. When monitored in real-time, this results in an increase in the fluorescence signal that can be observed during the polymerisation step and that falls off when the DNA is denatured. Consequently, fluorescence measurements at the end of the elongation step of every PCR cycle are performed to monitor the increasing amount of amplified DNA. This method obviates the need for target-specific fluorescent probes, but its specificity is determined entirely by its primers (in contrast to the Taqman assay, where specificity is increased by the use of sequence-specific probes). Hence, the accumulation of unspecific double-stranded DNA, such as primer-dimers, is also measured. In order to assure the specificity when performing SYBR Green-assays, fluorescence can be plotted as a function of temperature, thereby generating a melting curve of the amplicon. As the T_m of the amplicon depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product (Fig. 2.3).

While SYBR Green is a fluorescent intercalator dye, the Taqman assay utilises the 5'-nuclease activity of the *Taq* DNA polymerase to hydrolyse a hybridisation probe bound to its target amplicon (Fig. 2.4). In the Taqman assay, an oligonucleotide probe hybridizes specifically to the target amplicon during the annealing/extension phase of the PCR. The probe contains a fluorescent reporter dye at its 5' end, the emission spectrum of which is

quenched by a second fluorescent dye at its 3' end. As the 5'-exonuclease activity of *Taq* polymerase is double-strand-specific, unbound probe remains intact and no reporter fluorescence is detected. Conversely, if the correct target DNA is present in the reaction, the probe hybridizes to it after the denaturation step, and remains hybridised until cleaved by the DNA polymerase during primer elongation. This separates the reporter and quencher dyes and releases quenching of reporter fluorescence emission. As the polymerase will cleave the probe only when it is hybridized to its complementary strand, the temperature conditions of the polymerisation phase of the PCR must be adjusted to ensure probe binding. Most probes have a T_m of around 70 °C; therefore, the Taqman system uses a combined annealing and polymerisation step at 60-62 °C.



Figur 2.5: Fluorescence detection during real-time PCR. The curves illustrate amplification of four 1:1 dilutions of pooled material, constituting a standard curve. For each dilution, the threshold cycle (C_t) increases with one unit.

The more templates present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background. This point is defined as the C_t (threshold cycle). The reporter signal is normalized to the fluorescence of an internal dye (i.e., ROX) to adjust for changes in

concentration or volume, and a C_t value is reported for each sample. This value can be translated into a quantitative result by constructing a standard curve (Fig. 2.5). (53, 54)

In the present study, sample mRNA levels were quantified in reference to a standard curve constructed from pooled material containing equal amounts of each of the samples included in the analysis. A series of 1:1 dilutions of the pooled material is analyzed along with the samples, producing a standard curve to which the other samples are compared by means of their respective C_t-value. Hence, the results reflect the relative abundance of target mRNA in the study population.

In addition to normalizing for the amount of total RNA, sample-to-sample variation was normalized by comparison to an internal standard mRNA. The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by the experimental treatment. Moreover, the endogenous control should also be expressed at roughly the same level as the RNA under study. The mRNAs commonly used as so-called housekeeping genes entail glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin and ribosomal RNAs (rRNA).

Materials

- 2x qPCR™ SYBR® Green m.mix (Eurogentec Ltd., Belgium; cat.no: RT-QP2X-03)
  - Includes SYBR® Green I dye, 1/2000
- 2x qPCR™ TaqMan® m.mix (Eurogentec Ltd., Belgium; cat.no: RT-SN2X-03)
- TaqMan® probes, 5 μ*M* (see Table 2.6; Eurogentec Ltd., Belgium)
- Primers, 30 μ*M* (see Table 2.6; Eurogentec Ltd., Belgium)
- Nuclease-free water (Ambion® Inc., Texas, USA; cat.no: 9937)
- Optical 96-well reaction plates (AB Inc., Foster city, USA; part no: N801-0560)
- ABI PRISM 7000 Sequence Detection System (AB Inc., Foster city, USA)

Table 2.7: Mastermix for real-time RT-PCR.

Ingredients	Final concentration
Mastermix	1x
Forward primer	300 n*M*
Reverse primer	300 n*M*
Taqman probe (when used)	200 n*M*
SYBR Green dye (when used)	1/67,000

Method

A mastermix containing forward and reverse primers, a fluorescent labelling reagent (either SYBR Green dye or TaqMan probe), assay-specific mastermix (depending on the labelling reagent), and nuclease-free water was prepared (see Table 2.6). Ten µl cDNA of each sample was diluted to 100 µl with nuclease-free water, mixed on vortex, and spun down (quick-spin). Standards were prepared from a pool consisting of 10 µl cDNA from all samples. Two parallels of each sample were transferred to an optical 96-well reaction plate and mixed with mastermix; 3.8 µl diluted cDNA and 21.2 µl mastermix, respectively. The plate was covered with an adhesive strip of optical quality and the contents spun down (quick-spin). The ABI PRISM 7000 thermal cycler was programmed to run 2 minutes at 50 °C and 10 minutes at 95 °C (in order to activate the polymerase), followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. A melting curve was obtained in all SYBR Green runs. After the run was completed, the threshold line was adjusted to an optimal position in the area of exponential amplification (see Fig. 2.5), whereupon the samples were assigned a value by comparison with the standard curve. This value was normalized to the corresponding value for β-actin, and used to assess the relative chemokine expression in the present sample, compared to the other samples.

2.8 ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-linked immunosorbent assays (ELISAs) are frequently utilized in the quantification of proteins in biological samples. This method provides semi-quantitative data on sample protein content, and, depending on the sensitivity and specificity of the antibodies employed, affords a lower detection limit in the order of pg/ml.

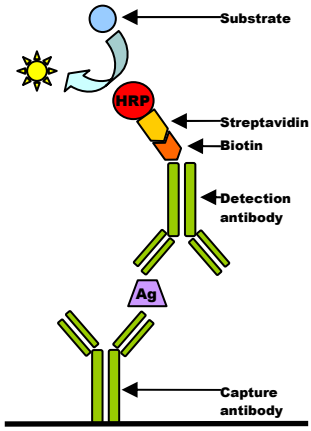


Figure 2.6: Model of sandwich ELISA. HRP, horse-radish peroxidase; Ag, antigen.

Monoclonal capture antibodies (cAb) fixed to the bottom of the wells of a microtiter plate captures specifically their cognate antigen (Ag) when incubated with sample (Fig. 2.6). A secondary antibody, the detection antibody, binds the resulting protein-cAb complex. In order to enhance the assay sensitivity, the detection antibody is linked covalently to a biotin residue, which binds streptavidin with high affinity. A peroxidase enzyme (horse-radish peroxidase; HRP) covalently bound to streptavidin consequently associates 1:1 with antibody-captured antigens and will, upon incubation with substrate solution,

produce quantities of coloured product that are directly proportional to the amount of complexed protein available. Optical densitometry is employed in the quantification of product, and semi-quantitative results are obtained by comparison with a standard curve generated with known amounts of antigen.

Materials

- 96-well microtiter plates (Costar®, New York, USA; cat.no: 9018)
- ELISA kits and reagents from R&D Systems Inc., Minneapolis, USA as depicted in Table 2.7 (catalogue numbers in parentheses)
- Tecan® Washer 430 (Organon Teknika Inc., Austria)
- Multiscan RL spectrophotometer (Labsystems Inc., Finland)

Table 2.7: Enzyme-linked immunosorbent assays used in the present study. RANTES, regulated on activation normal T-cell expressed and secreted; ENA, epithelial cell-derived neutrophil-activating factor; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; IL, interleukin; PBS, phosphate-buffered saline; BSA, bovine serum albumine; TBS, Tris-buffered saline.

Chemokine	Reagents	Diluent	Working concentration	Chemokine	Reagents	Diluent	Working concentration
RANTES - CCL5 (DY278)	cAb	PBS	1 µg/ml	**MCP-1** - CCL2 (DY279)	cAb	PBS	1 µg/ml
	dAb	1 % BSA in PBS	10 ng/ml		dAb	1 % BSA in PBS	100 ng/ml
	Std		1000 pg/ml		Std		1000 pg/ml
	Spl		1/50[a], 1/20[b], 1/50-1/100[c]		Spl		1/2[a], 1/10[b], 1/100[c]
	S-HRP		1/200		S-HRP		1/200
ENA-78 - CXCL5 (DY254)	cAb	PBS	2 µg/ml	**IL-8** - CXCL8 (DY208)	cAb	PBS	4 µg/ml
	dAb	1 % BSA in PBS	100 ng/ml		dAb	0.1 % BSA, 0.05 % Tween 20 in TBS	20 ng/ml
	Std		1000 pg/ml		Std		2000 pg/ml
	Spl		1/2[a], 1/5-1/50[b], 1/20-1/100[c]		Spl		1/2[a], 1/100[b], 1/100[c]
	S-HRP		1/200		S-HRP		1/200
MIP-1α - CCL3 (DY270)	cAb	PBS	0,4 µg/ml	cAb = Capture antibody dAb = Detection antibody Std = Standard (high) Spl = Sample S-HRP = Streptavidin horse-radish peroxidase			
	dAb	1 % BSA in PBS	200 ng/ml				
	Std		500 pg/ml				
	Spl		1/2[a], 1/25[b], 1/300[c]				
	S-HRP		1/200				

[a] Sample dilutions utilized when assessing serum samples

[b] Sample dilutions utilized when assessing cell supernatant samples stimulated or not with MP

[c] Sample dilutions utilized when assessing cell supernatant samples stimulated with SEB alone or SEB and MP together

Method

The levels of the CC-chemokines MCP-1, MIP-1α, and RANTES and the CXC-chemokines IL-8 and ENA-78 in cell-culture supernatants and serum were analyzed by ELISA according to the manufacturer's instructions. Reagents were diluted as depicted in Table 2.7. All incubations were made at room temperature, during which the plates were covered with an adhesive strip.

A 96-well microtiter plate was coated by incubation overnight with capture antibody (cAb). The wells were subsequently washed three times with 400 µl 0.05 % Tween 20 in phosphate buffered saline (PBS), using an automated washer. Following washing, the plate was blocked (to prevent unspecific binding of dAb) by adding 300 µl of 1 % BSA, 5 % sucrose and 0.05 % NaN_3 in PBS to each well, whereupon the plate was incubated for a minimum of 1 hour. After a second washing, 100 µl of sample or standard was added per well, and the plate was incubated for 2 hours. The washing-procedure was repeated, and 100 µl of detection antibody (dAb) was added to each well, followed by 2 more hours of incubation. Subsequent to another round of washing, 100 µl of a 1:1 mixture of hydrogen peroxide and tetramethylbenzidine was added to each well. The plate was incubated for 20 minutes, while protecting the photosensitive reactions from direct light exposure. Finally, 50 µl of 2 *M* H_2SO_4 was added to each well to stop the reaction. The optical density of each well was determined immediately thereafter, using a microplate reader set to 450 nm, with wavelength correction at 540 nm.

2.9 STATISTICAL ANALYSIS

As our data could not be expected to be normally distributed, all analyses were performed with nonparametric tests. Three different statistical methods were employed in evaluating the results: 1) Mann-Whitney test; 2) Wilcoxon signed rank test; 3) Spearman correlation. The Mann-Whitney test, also called the rank sum test, is a nonparametric t-test that compares two unpaired groups. The Wilcoxon matched pairs test is a test to compare two paired groups. It is also called the Wilcoxon matched pairs signed ranks test. Spearman correlation is a method for correlating nonparametric data.

In assessing differences between patient and control groups the Mann-Whitney *U*-test was used. Data are presented as scatter-plots, with the median indicated by a horizontal line. Probability values are two-sided and taken as statistically significant at $P < 0.05$. The correlation studies were performed using the nonparametric Spearman correlation method. When comparing differences between groups of differently treated PBMC within the same individual, the Wilcoxon signed rank test was used. Comparisons between the patient and control subpopulations were performed using the Mann-Whitney *U*-test. Data is given as mean ± S.E.M. if not otherwise stated. Probability values are two-sided and taken as statistically significant at $P < 0.05$.

3. RESULTS

3.1 SERUM LEVELS OF CHEMOKINES IN WG PATIENTS

3.1.1 Chemokine levels differ in WG patients and healthy individuals

The serum levels of IL-8, MIP-1α, MCP-1, RANTES, and ENA-78 in 14 WG patients and 9 healthy controls were assessed by ELISA (Fig. 3.1). RANTES and MCP-1 were found to be significantly up-regulated in WG-patients, compared to healthy controls. In addition, there was a strong tendency towards increased levels of IL-8 in the patient population ($P = 0.057$). There were no significant differences in the serum levels of MIP-1α and ENA-78.

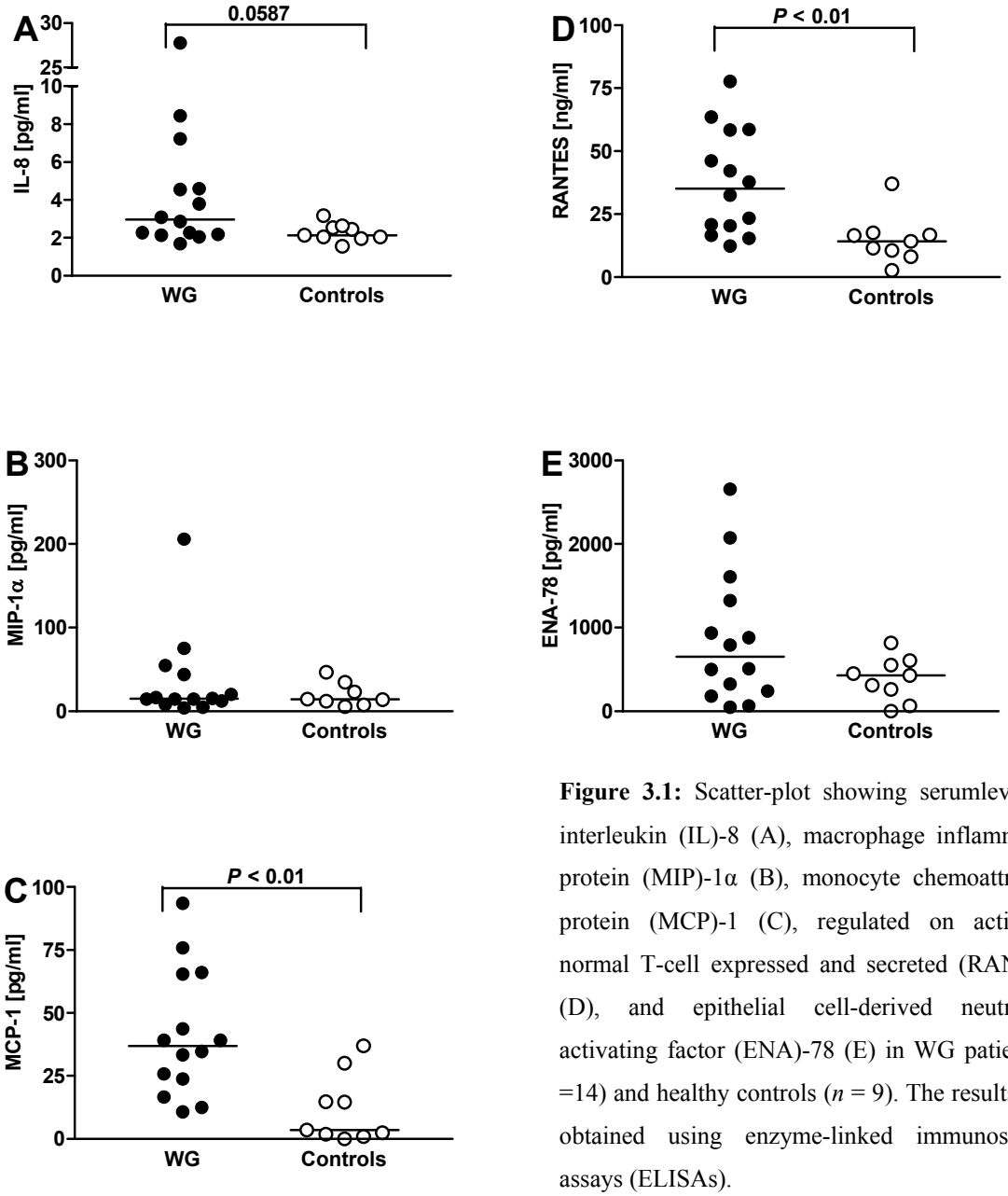


Figure 3.1: Scatter-plot showing serumlevels of interleukin (IL)-8 (A), macrophage inflammatory protein (MIP)-1α (B), monocyte chemoattractant protein (MCP)-1 (C), regulated on activation normal T-cell expressed and secreted (RANTES) (D), and epithelial cell-derived neutrophil-activating factor (ENA)-78 (E) in WG patients (n =14) and healthy controls (n = 9). The results were obtained using enzyme-linked immunosorbent assays (ELISAs).

3.1.2 Chemokines in WG – Relation to clinical markers of inflammation

We wanted to investigate whether the enhanced serum chemokine levels could reflect systemic inflammation. Hence, we performed correlation analyses to determine the relationship between serum chemokine levels and clinical inflammation-markers, i.e., CRP and ESR. Both ESR and the levels of CRP are widely used clinically in assessing the patient's immune status, and are also employed in the evaluation of disease activity in WG. Correlation factors and *P*-values were calculated using GraphPad Prism® software, and the results are listed in Table 3.1.

Chemokine	**ESR**	
	Spearman *r*	***P*-value**
RANTES	-0.29	0.3855
MIP-1α	-0.33	0.3176
IL-8	0.49	0.1252
MCP-1	-0.17	0.6115
ENA-78	0.24	0.4669

Chemokine	**CRP**	
	Spearman *r*	***P*-value**
RANTES	-0.59	0.0411
MIP-1α	-0.44	0.1456
IL-8	0.12	0.6992
MCP-1	-0.48	0.1131
ENA-78	0.017	0.9561

Table 3.1: Data from correlation-studies conducted on the findings in serum of 14 WG patients. Serum chemokine levels are compared to erythrocyte sedimentation rate (ESR) and the levels of C-reactive protein (CRP). Correlation factors and *P*-values were calculated using GraphPad Prism® software. RANTES, regulated on activation normal T-cell expressed and secreted; MIP, macrophage inflammatory protein; IL, interleukin; MCP, monocyte chemoattractant protein; ENA, epithelial cell-derived neutrophil-activating factor.

Only RANTES is displaying significant correlations with any of the two inflammation-markers investigated, being inversely correlated with serum CRP levels ($P = 0.0411$). Considering the small number of subjects investigated, however, some of the other results may also prove to be of interest: Both MIP-1α and MCP-1 tend towards being related to the levels of CRP in an inverse manner ($P = 0.1456$ and $P = 0.1131$), which might have proven significant in a larger population. Furthermore, IL-8 tends, although weakly, to be positively correlated with ESR ($P = 0.1252$).

3.1.3 Serum chemokine levels in active and remissive disease

As mentioned above, the disease activities of the WG patients included in the present study were classified as either "active" or "in remission". To further explore the role of chemokines in WG, we went on to investigate whether chemokine levels differed between patients classified as "active" or "in remission (Fig. 3.2). No statistically significant differences were revealed, although tendencies were evident for RANTES and ENA-78, with up-regulated levels of ENA-78 in active patients (P = 0.1419) and up-regulation of RANTES in the WG patients that were in remission (P = 0.1812).

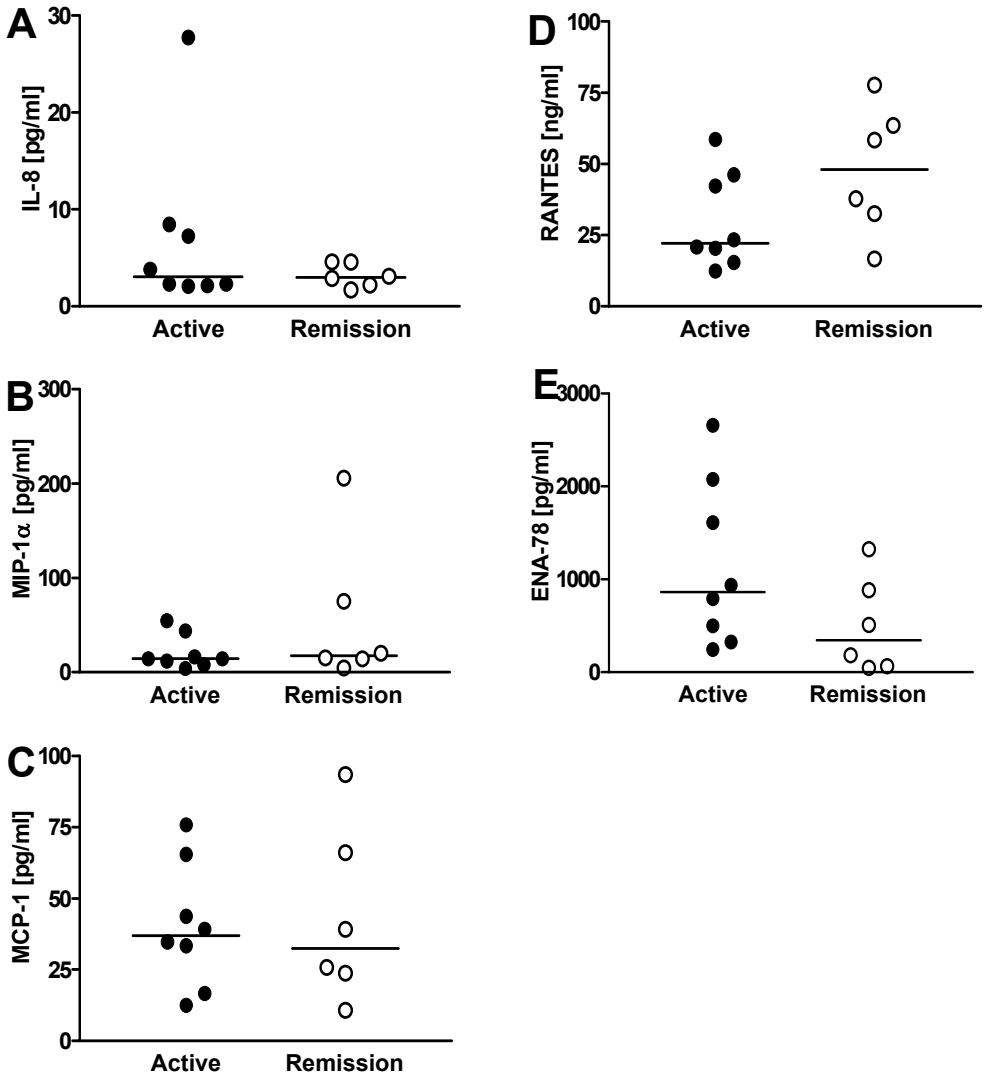


Figure 3.2: Scatter-plot showing serum levels of interleukin (IL)-8 (A), macrophage inflammatory protein (MIP)-1α (B), monocyte chemoattractant protein (MCP)-1 (C), regulated on activation normal T-cell expressed and secreted (RANTES) (D), and epithelial cell-derived neutrophil-activating factor (ENA)-78 (E) in WG patients with active (n = 8) or remissive (n = 6) disease. The results were obtained using enzyme-linked immunosorbent assays (ELISAs).

3.2 IN VITRO MANIPULATION OF PBMC

Having demonstrated significantly elevated serum levels of several chemokines in patients with WG, we wanted to investigate the effects of MP on basal and SEB-stimulated chemokine expression in PBMC from patients with WG and healthy controls. In order to establish the appropriate conditions to be used in the main study, preliminary experiments were conducted.

3.2.1 Pilot study I

The first pilot study was performed to examine the effects of stimulation of PBMC with different doses of SEB and MP and the outcome of adding the substances at different time-points (chapter 2.4.3). This experiment revealed that SEB markedly up-regulated the release of MCP-1, whereas MP significantly reduced it (Fig. 3.3). Furthermore, the effects of SEB were seemingly independent of the concentrations utilized, indicating that 0.01ng/ml SEB is sufficient to reach maximum stimulation of PBMC. On the other hand, MP counteracted SEB in a dose-dependent manner, 10^{-6} *M* producing the greatest effect. In light of the results from pilot study I, 1 ng/ml SEB and 10^{-6} *M* MP were used in the following experiments.

3.2.2 Pilot study II

With this second pilot study, we were aiming to find the most appropriate timing of cell harvesting (chapter 2.4.4). In order to assess the temporal variations in mRNA levels following stimulation, PBMC that had been cultured with medium containing SEB and/or MP were harvested at four intervals (6, 8, 12, and 24 hours, respectively). When looking into the gene expression of biologic mediators, one has to realize that the levels of mRNA only represent an instantaneous image of cellular conditions. Therefore, it is expected that the levels of mRNA will vary considerably during a period of 24 hours stimulation. The kinetics of different chemokines expectedly varies a lot, and, consequently, this second preliminary study focused on the effects of SEB and/or MP on two different chemokines, MCP-1 and MIP-1α, and also a chemokine receptor, CCR5, one of the receptors for MIP-1α. Messenger RNA levels were analyzed using real-time RT-PCR and normalized to the concurrent levels of β-actin mRNA.

Stimulation with SEB greatly up-regulated the levels of MIP-1α mRNA at all the selected intervals, but the induction, and also, apparently, the MP counteraction, were greatest at 12 hours (Fig. 3.4A). The levels in PBMC treated with MP alone, and in non-stimulated cells, were markedly lower than for the SEB-treated cells. The effects on MCP-1 were equally

profound as those on MIP-1α (Fig. 3.4B). However, the response maximum occurred much earlier than for the previous. Already at 6 hours, the effects of both SEB and MP were maximal. The levels of expression in non-induced cells were low, but the levels in MP-treated cells were even lower, although seeming to approach the levels of the non-stimulated cells during prolonged incubation. The time-dependent effects on the chemokine receptor, CCR5, were less clear-cut than for its ligands (Fig. 3.4C).

In conclusion, it is difficult to predict at which time the levels of all chemokines are maximal, and, consequently, we decided to stimulate the PBMC for both 6 and 24 hours in the subsequent studies. Hopefully, this would provide more precise results in our evaluation of effects of MP and SEB on the production of chemokines in PBMC.

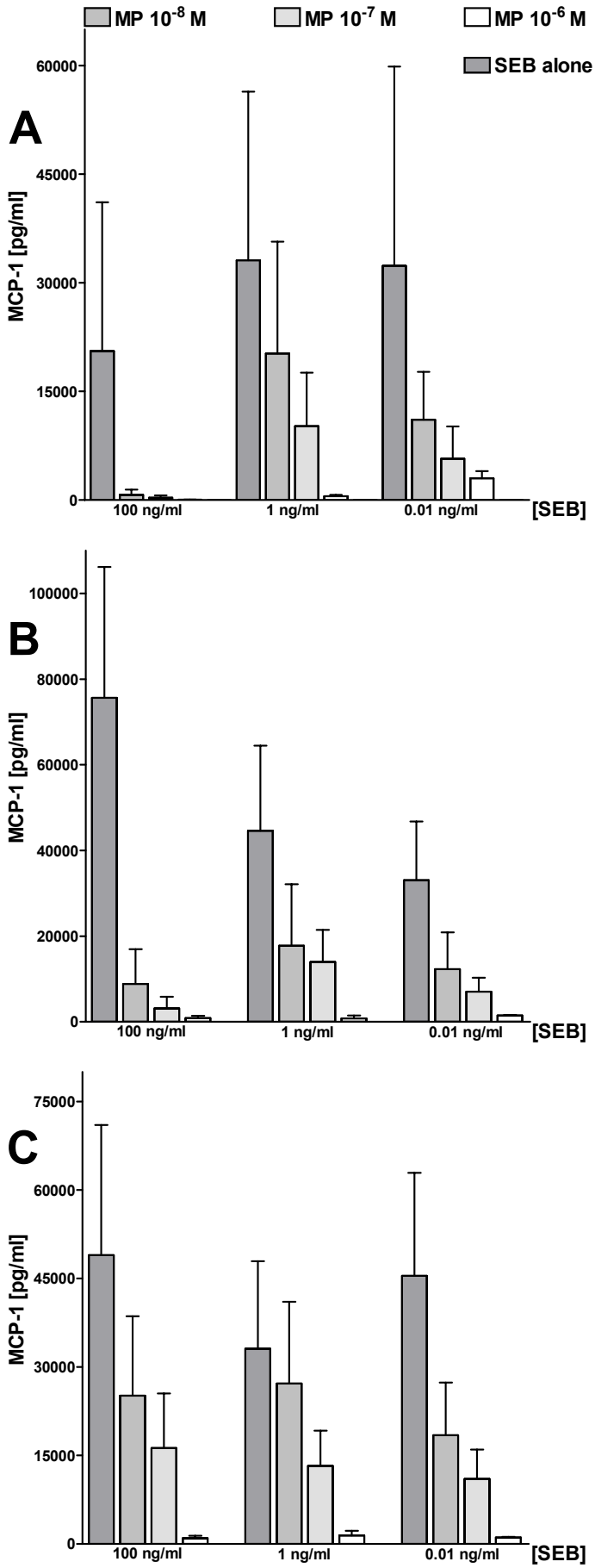


Figure 3.3: Bar graphs showing the secretion of monocyte chemoattractant protein (MCP)-1 by peripheral blood mononuclear cells (PBMC) from 2 patients with Wegener's granulomatosis (WG) and one healthy control in response to treatment with staphylococcal enterotoxin B (SEB) or methylprednisolone (MP) or both at three different concentrations (SEB: 0.01 ng/ml, 1 ng/ml, and 100 ng/ml; MP: 10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M*) for 24 hours, adding SEB either before (A), concurrently (B) or after (C) addition of MP. MCP-1 secretion was assessed using an enzyme-linked immunosorbant assay (ELISA).

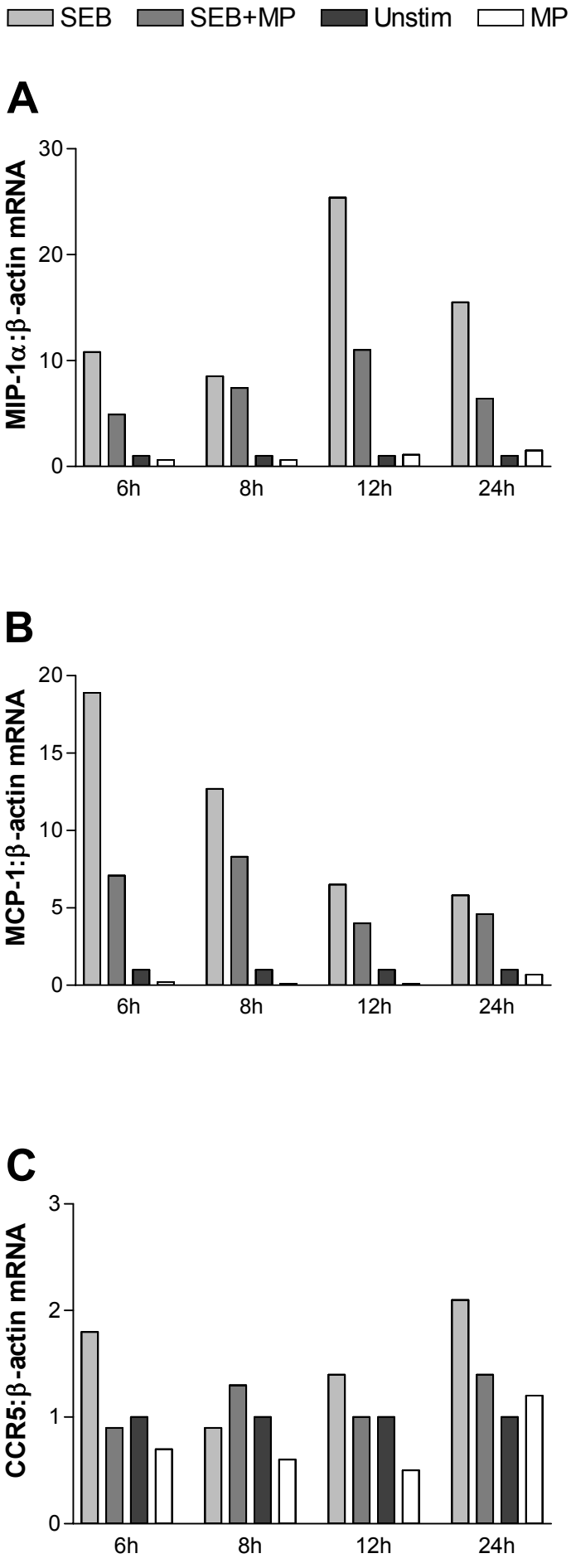


Figure 3.4: Bar graphs showing the effects of stimulation of peripheral blood mononuclear cells (PBMC) from a healthy subject with 1 ng/ml staphylococcal enterotoxin B (SEB) and/or 10^{-6} *M* methylprednisolone (MP) on the gene expression of macrophage inflammatory protein (MIP)-1α (A), monocyte chemoattractant protein (MCP)-1 (B), and CC-chemokine receptor 5 (CCR5) (C). PBMC were stimulated for 6, 8, 12, or 24 hours, respectively. Gene expression was quantified using real-time reverse transcriptase polymerase chain reaction (RT-PCR) and related to the gene expression of β-actin.

3.3 MODULATION OF THE PRODUCTION AND SECRETION OF CHEMOKINES IN PBMC

Following the preliminary studies referred above, we concluded that the most favourable conditions for stimulation of PBMC in the subsequent analysis of chemokine production and secretion would be 1 ng/ml SEB, 10^{-6} *M* MP, and incubation for both 6 and 24 hours.

3.3.1 Effects on protein secretion from PBMC

Subsequent to incubation for 24 hours with or without 1 ng/ml SEB and/or 10^{-6} *M* MP, the secretion of IL-8, MIP-1α, MCP-1, RANTES, and ENA-78 was analyzed with ELISA. The secretion of IL-8 was reduced to a third following treatment with MP ($P < 0.005$), whereas the SEB-induced up-regulation of chemokine secretion was nearly halved in response to MP in both WG patients and healthy controls ($P < 0.005$; Fig. 3.5). MIP-1α displayed a similar pattern (Fig. 3.6), with highly significant modulation of chemokine levels in response to both MP and SEB, although not displaying down-regulation in response to MP treatment in the patient population.

While the effect of MP on release of MCP-1 paralleled that observed for IL-8 and MIP-1α in healthy controls (Fig. 3.7), it did not apply for WG patients, as it failed to produce significant down-regulation of both unstimulated and SEB-conditioned PBMC. There was, however, a tendency towards a reduction in secreted levels following treatment of MP alone ($P = 0.0742$). On the other hand, MP had profound effects on the secretion of RANTES (Fig. 3.8) from PBMC from both WG patients and healthy controls, although the effects on the spontaneous release of RANTES in PBMC from WG patients were not significant. Interestingly, PBMC from healthy controls produced significantly lower levels of RANTES than PBMC from WG patients when treated with MP alone ($P < 0.05$), although the spontaneous release of RANTES was the same.

The effects of MP/SEB on secretion of the CXC-chemokine ENA-78 (Fig. 3.9) markedly differed from the other chemokines, including the CXC-chemokine IL-8. No significant effects were evident in the patient population, neither upon MP- nor SEB-stimulation, although there was a tendency towards down-regulation of chemokine secretion following MP-treatment of unstimulated PBMC ($P = 0.0977$). In the control population treatment with MP alone produced a highly significant down-regulation ($P < 0.005$), but no effect was seen on SEB-conditioned PBMC.

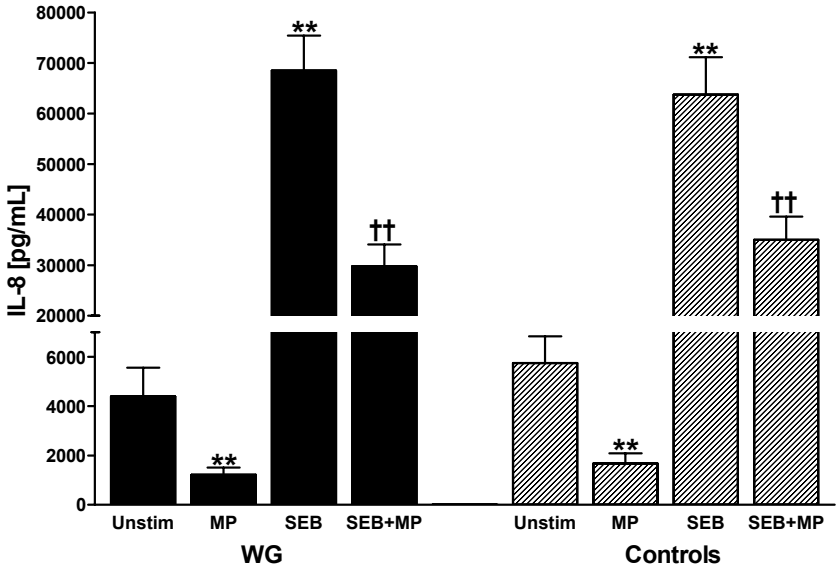


Figure 3.5: Bar graph showing the secretion of interleukin (IL)-8 from cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} *M* methylprednisolone (MP) or both for 24 hours. The results were obtained using enzyme-linked immunosorbent assay (ELISA). Values are presented as mean ± S.E.M. ** P < 0.005 when compared to unstimulated levels; †† P < 0.005 when compared to the SEB-induced levels.

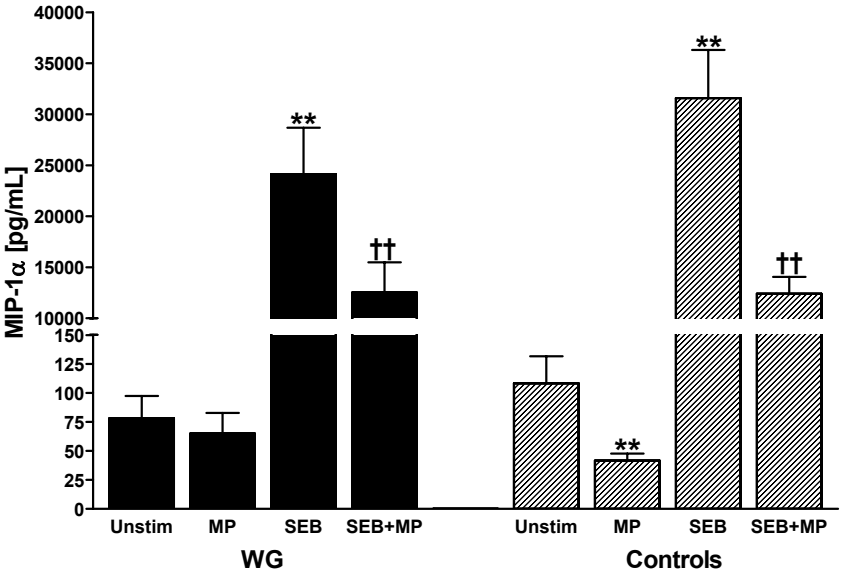


Figure 3.6: Bar graph showing the secretion of macrophage inflammatory protein (MIP)-1α from cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} *M* methylprednisolone (MP) or both for 24 hours. The results were obtained using enzyme-linked immunosorbent assay (ELISA). Values are presented as mean ± S.E.M. ** P < 0.005 when compared to unstimulated levels; †† P < 0.005 when compared to the SEB-induced levels.

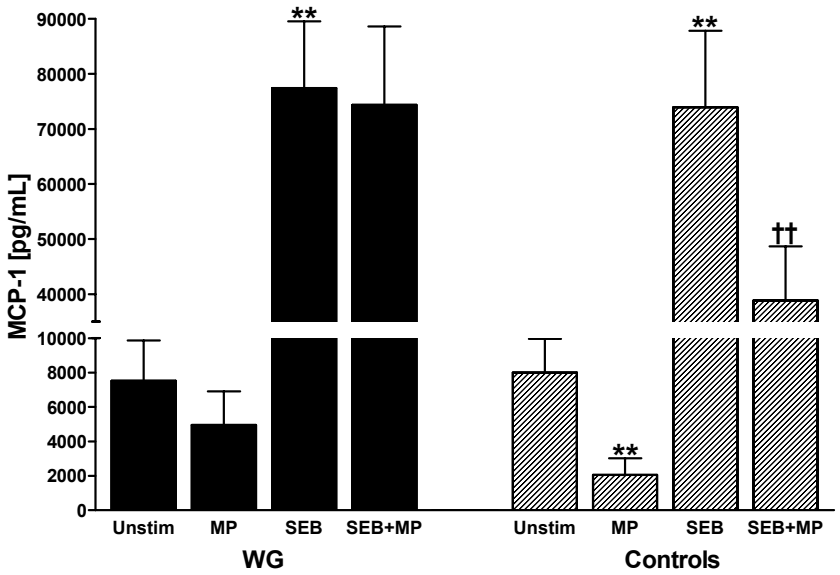


Figure 3.7: Bar graph showing the secretion of monocyte chemoattractant protein (MCP)-1 from cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} *M* methylprednisolone (MP) or both for 24 hours. The results were obtained using enzyme-linked immunosorbent assay (ELISA). Values are presented as mean ± S.E.M. ** $P < 0.005$ when compared to unstimulated levels; †† $P < 0.005$ when compared to the SEB-induced levels.

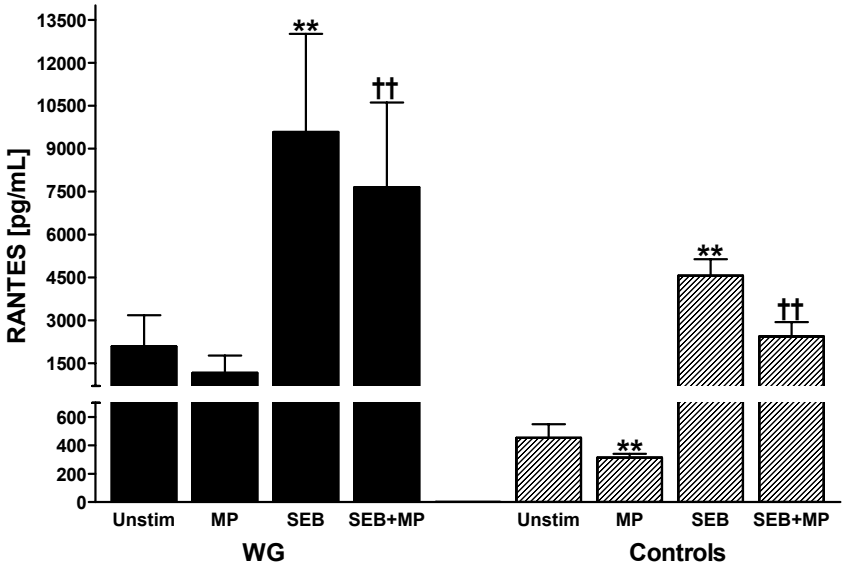


Figure 3.8: Bar graph showing the secretion of regulated on activation normal T-cell expressed and secreted (RANTES) from cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} *M* methylprednisolone (MP) or both for 24 hours. The results were obtained using enzyme-linked immunosorbent assay (ELISA). Values are presented as mean ± S.E.M. ** $P < 0.005$ when compared to unstimulated levels; †† $P < 0.005$ when compared to the SEB-induced levels.

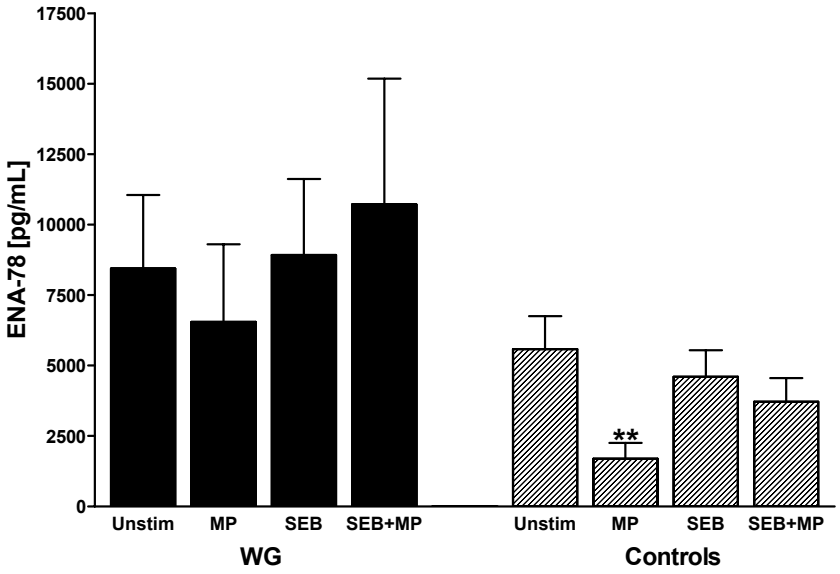


Figure 3.9: Bar graph showing the secretion of epithelial cell-derived neutrophil-activating factor (ENA)-78 from cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} M methylprednisolone (MP) or both for 24 hours. The results were obtained using enzyme-linked immunosorbent assay (ELISA). Values are presented as mean ± S.E.M. ** $P < 0.005$ when compared to unstimulated levels.

3.3.2 Effects on gene transcription of PBMC

Following 6 hours of stimulation with MP in the presence or absence of SEB, the effects on gene transcription in PBMC from both patients and healthy controls were assessed by real-time RT-PCR. It turned out that the expression of several of the chemokines studied was profoundly modulated by the treatment. The expression of IL-8, for instance, was significantly reduced upon MP-treatment in both patients and controls ($P < 0.05$ and 0.005, respectively) (Fig. 3.10). In spite of the weighty effect of MP on secreted levels of the chemokine (Fig. 3.5), however, MP was unable to neutralize the potent up-regulation of IL-8 gene expression following stimulation with SEB. A similar pattern was observed for the expression of MIP-1α and MCP-1, differing only in respect to the effects of MP alone, which failed to produce significant down-regulation of gene expression in the patient population.

Interestingly, the levels of RANTES mRNA were only modestly influenced by stimulation with SEB or MP, contrasting sharply with our findings on the protein level. Whereas the secretion of RANTES was vigorously up-regulated following SEB-treatment, this treatment failed to induce a response on gene transcription in the patient population, and, furthermore, resulted in down-regulation in the control population ($P < 0.05$). The same perplexing picture was evident for ENA-78; no discernible effect of SEB was monitored. However, MP showed

significant effect on the expression of ENA-78 in both WG patients and healthy controls. Thus, for several of the chemokines (i.e., IL-8, MIP-1α, and MCP-1), the down-regulatory effect of MP on the SEB-stimulated chemokine response was not accompanied by a corresponding decrease in mRNA levels, suggesting post-transcriptional modulation of MP.

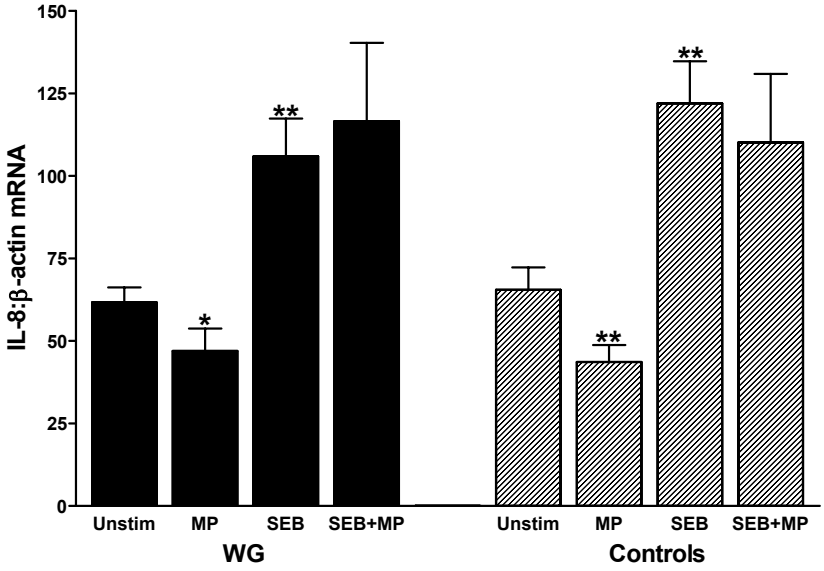


Figure 3.10: Bar graph showing the gene expression of interleukin (IL)-8 in cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} *M* methylprednisolone (MP) or both for 6 hours. The results were obtained using real-time reverse transcriptase – polymerase chain reaction (RT-PCR). Values are presented as mean ± S.E.M. * $P < 0.05$; ** $P < 0.005$ when compared to unstimulated levels.

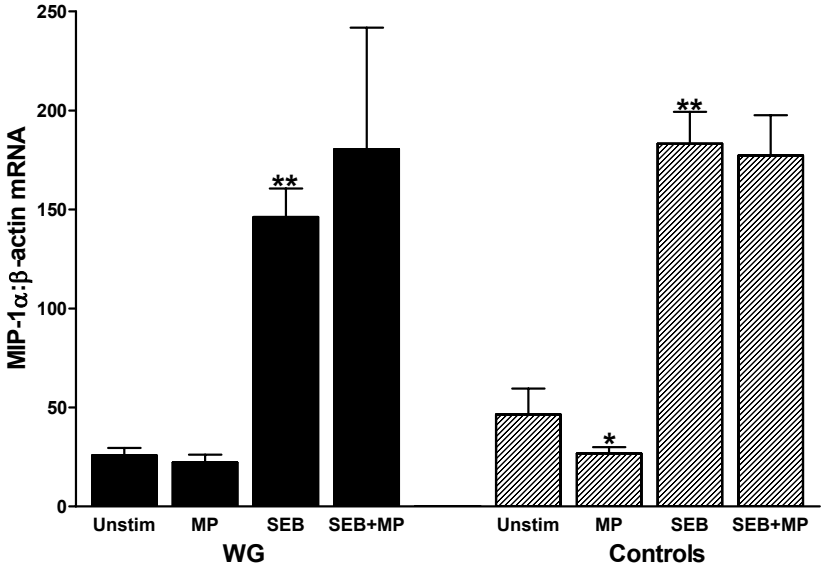


Figure 3.11: Bar graph showing the gene expression of macrophage inflammatory protein (MIP)-1α in cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} M methylprednisolone (MP) or both for 6 hours. The results were obtained using real-time reverse transcriptase – polymerase chain reaction (RT-PCR). Values are presented as mean ± S.E.M. * $P < 0.05$; ** $P < 0.005$ when compared to unstimulated levels.

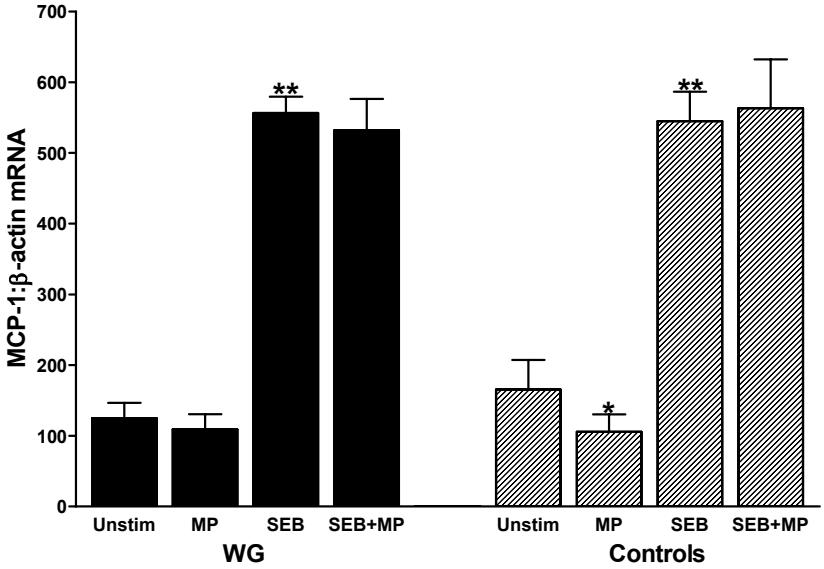


Figure 3.12: Bar graph showing the gene expression of monocyte chemoattractant protein (MCP)-1 in cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} M methylprednisolone (MP) or both for 6 hours. The results were obtained using real-time reverse transcriptase – polymerase chain reaction (RT-PCR). Values are presented as mean ± S.E.M. * $P < 0.05$; ** $P < 0.005$ when compared to unstimulated levels.

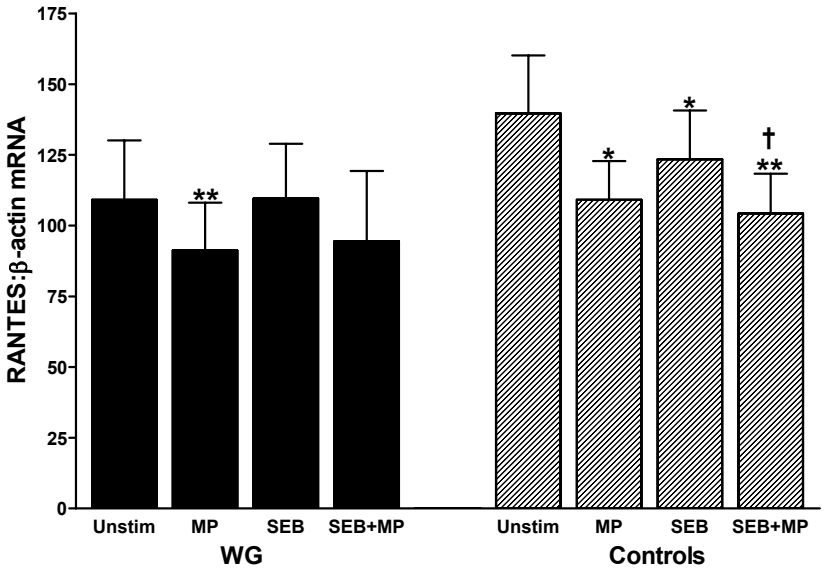


Figure 3.13: Bar graph showing the gene expression of regulated on activation normal T-cell expressed and secreted (RANTES) in cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} M methylprednisolone (MP) or both for 6 hours. The results were obtained using real-time reverse transcriptase – polymerase chain reaction (RT-PCR). Values are presented as mean ± S.E.M. * $P < 0.05$; ** $P < 0.005$ when compared to unstimulated levels; † $P < 0.05$ when compared to the SEB-induced levels.

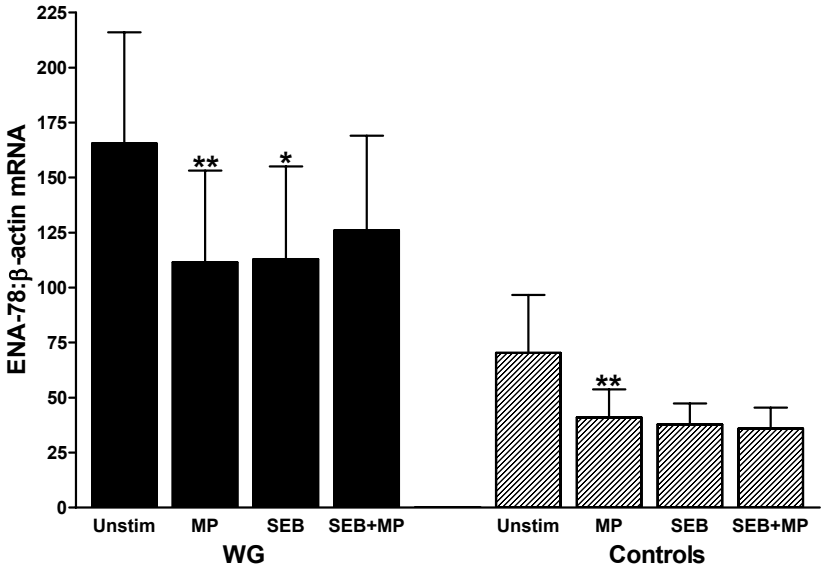


Figure 3.14: Bar graph showing the gene expression of epithelial cell-derived neutrophil-activating factor (ENA)-78 in cultured peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} M methylprednisolone (MP) or both for 6 hours. The results were obtained using real-time reverse transcriptase – polymerase chain reaction (RT-PCR). Values are presented as mean ± S.E.M. * $P < 0.05$; ** $P < 0.005$ when compared to unstimulated levels.

3.4 INFLUENCE OF EXPERIMENTAL TREATMENT ON THE EXPRESSION OF GLUCOCORTICOID RECEPTOR MRNA

The expression of glucocorticoid receptor (GR)-α in PBMC was not significantly affected by stimulation with MP. However, SEB produced significant up-regulation of the GR-α mRNA levels. There were no significant differences between the patient and control populations regarding the level of gene expression of GR-α, although a strong tendency towards elevated levels in controls following treatment with SEB+MP was evident ($P = 0.0625$).

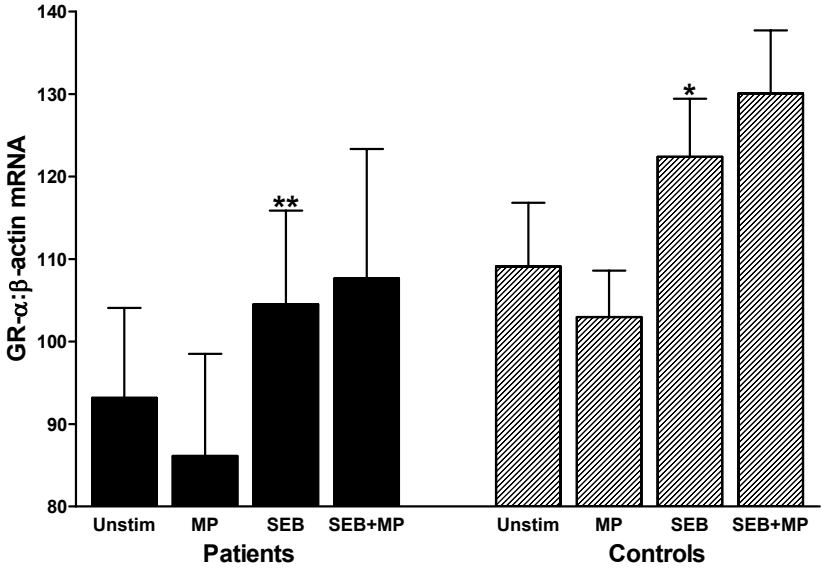


Figure 3.15: Bar graph showing the gene expression of glucocorticoid receptor (GR)-α in cultured peripheral blood mononuclear cells (PBMC) from WG patients ($n = 9$) and healthy controls ($n = 9$) in response to stimulation with 1 ng/ml staphylococcal enterotoxin B (SEB) or 10^{-6} *M* methylprednisolone (MP) or both for 6 hours. The results were obtained using real-time reverse transcriptase – polymerase chain reaction (RT-PCR). Values are presented as mean ± S.E.M. * $P < 0.05$; ** $P < 0.005$ when compared to unstimulated levels.

4. DISCUSSION

4.1 SUMMARY OF RESULTS

In the present study we demonstrate that patients with WG have elevated circulating levels of the chemokines RANTES and MCP-1. When classifying the patients according to disease activity, i.e., as active or in remission, we found a trend for lower RANTES levels and higher ENA-78 levels in patients with active disease compared to patients in remission.

We also examined the effects of MP on the *in vitro* gene expression and release of chemokines in PBMC isolated from WG patients and healthy controls. Notably, MP reduced both basal and SEB-stimulated chemokine release in PBMC from healthy controls. However, the effects of MP on chemokine secretion from PBMC isolated from WG patients were variable, suggesting that these patients may have altered glucocorticoid sensitivity. Furthermore, the chemokine mRNA levels in PBMC stimulated with SEB were unaffected of MP treatment.

Table 4.1: Summary of the effects of methylprednisolone on the release of chemokines from peripheral blood mononuclear cells (PBMC) from WG patients (n = 9) and healthy controls (n = 9) that were concomitantly stimulated with or without staphylococcal enterotoxin B (SEB).

Chemokine	WG Patients		Controls	
	Unstim	*SEB*	*Unstim*	*SEB*
IL-8	↓	↓	↓	↓
MIP-1α	—	↓	↓	↓
MCP-1	—	—	↓	↓
RANTES	—	↓	↓	↓
ENA-78	—	—	↓	—

IL, interleukin; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; RANTES, regulated on activation normal T-cell expressed and secreted; ENA, epithelial cell-derived neutrophil-activating factor. Data were obtained using enzyme-linked immunosorbent assays (ELISA).

4.2 INTERPRETATION OF RESULTS

4.2.1 Potential pathogenic role for chemokines in WG

Very few studies have addressed the involvement of chemokines in WG. However, a pathogenic role has been attributed to IL-8 in ANCA-associated glomerulonephritis (32). Correspondingly, Mayet *et al.* (1999) showed that endothelial IL-8 synthesis was up-regulated following stimulation *in vitro* with anti-PR3-antibodies (55), whereas Ralston *et al.* (1997) found that ANCAs induced monocytic IL-8 release (30). Monocytes are an integral part of granulomas and glomerular crescents in active WG (8), while neutrophils, being one of the

main targets of IL-8, are suggested to be major effector cells in the development of inflammatory lesions in active WG. PR3-ANCAs have been established as an important pathogenic factor in WG, and the levels of perinuclear ANCAs (ANCAs showing a perinuclear distribution pattern in indirect immunofluorescence, as opposed to cytoplasmic ANCAs, which are widely distributed throughout the cytoplasm) are used as markers of disease activity (8). In the present study, IL-8 shows a strong tendency for being up-regulated in WG patients, compared to healthy controls, further supporting a pathogenic role for this chemokine in WG.

Although not concretely associated with WG, the serum levels of RANTES and MCP-1 have been shown to be elevated in relation to other autoimmune disorders, including multiple sclerosis (MS), rheumatoid arthritis (RA) (27), and systemic lupus erythematosus (SLE) (56). Interestingly, Kaneko *et al.* (1999) observed a decrease in RANTES with the progression of disease activity in patients with SLE (56). In the present study, RANTES was negatively correlated with CRP, indicating a similar regulation in WG. Furthermore, there was a tendency towards down-regulation of the serum levels of RANTES in active WG patients compared to patients in remission, substantiating the assumption that RANTES may indeed be involved in WG, although in a little surprising manner, which cannot presently be explained. However, caution is needed when interpreting data from plasma/serum analyses as they may not necessarily reflect the activity in pathologically involved tissues. The levels of RANTES in lung tissues have indeed been found to be elevated in WG patients (57).

It has previously been shown that both cytoplasmic and perinuclear ANCAs induce MCP-1 release from PBMC *in vitro*, and that MCP-1 is largely responsible for the ANCA-induced chemoattraction of mononuclear leukocytes (58). Our demonstration of elevated serum MCP-1 levels in WG patients is consistent with these findings, and a possible role in determining disease activity is substantiated by the inverse correlation of MCP-1 to CRP. Collectively, these data suggest that MCP-1 may be involved in the pathophysiology of WG.

The effects of immunosuppressive therapy may partly result from modulation of cytokine and chemokine levels. The present study reveals modulation of chemokine levels following *in vitro* stimulation with MP, a widely used immunosuppressant. Moreover, Lamprecht *et al.* (2002) demonstrated that elevated monocytic IL-12 and TNF-α, but not IL-8, in WG was normalized by cyclophosphamide and corticosteroid therapy (59). In addition, intravenous

pulse methylprednisolone (PMP) treatment of patients with rheumatoid arthritis was shown by Wong *et al.* (2001) to reduce MCP-1 and MIP-1α expression in the synovial lining layer within 24 hours of administration (60). This may be due to either a direct effect on chemokine production or an indirect effect on other proinflammatory mediators that regulate chemokine expression. TNF-α may be important in this context, influencing the production of several chemokines, including IL-8 and MCP-1 (61, 62), and playing an important role in granuloma formation and the induction of vasculitis in WG, as demonstrated by several clinical and *in vitro* studies (59, 63).

Chemokine up-regulation may also be localized to certain tissues, therefore not necessarily producing elevated levels in systemic circulation. Zhou *et al.* (2003) demonstrated that RANTES, MIP-1α, and MIP-1β, all of which are ligands of the chemokine receptor CCR5, were enriched in lung lesions from patients with WG, producing localized accumulation of CCR5-positive cells (57). Moreover, Lamprecht *et al.* (2003) recently found that a larger fraction of T lymphocytes in localized WG than in generalized WG displayed CCR5 production, suggesting that these chemokines are primarily involved in the early phases of disease (64).

Immunosuppressive therapy of the WG patients may have influenced both serum and PBMC levels of chemokines found in the present study. Moreover, the diminished responses experienced in PBMC from WG patients to stimulation with MP may result from down-regulation of GRs as a consequence of long-term GC treatment. Additional mechanisms, including up-regulation of GR-β, may also contribute to this unresponsiveness. Furthermore, the diverging responsiveness observed may also result from GR-independent post-transcriptional and post-translational mechanisms. The individualized therapy of WG patients may complicate the comparison of active and remissive patients, and inherent differences in chemokine levels between WG patients and healthy individuals may be obscured by drug-induced effects.

4.2.2 MP modulates chemokines in vitro

MP had substantial effect on the secretion of chemokines from PBMC isolated from healthy controls. In the patient population, however, a significant effect of MP alone was only seen for IL-8, although similar tendencies were evident for both MCP-1 and ENA-78. Interestingly, while PBMC from the same pair of patients are consistently opposing the down-

regulatory effects of MP in MIP-1α, RANTES, and MCP-1 (the CC chemokines), every patient, except for one, are resistant to modulation by MP in respect of at least one chemokine. This suggests a complexity within the regulation of chemokine secretion that we are, presently, unable to grasp. Neither medication nor disease activity could explain these manifestations in the present study. Correlation studies were indeed performed to reveal possible connections between medication and disease activity and PBMC chemokine production. However, relating serum levels of chemokines to oral drug dosage must be considered a highly inaccurate method, and was only attempted because these were the only data available regarding the patients' medication. A much more precise approach would be to compare serum chemokine levels with levels of MP in serum, a feature that should be procured in future studies.

The effects of MP were also manifest on the level of gene expression, although not always in consistence with our findings on the protein level. The most remarkable difference between the two is the lacking effect of MP on mRNA-levels in SEB-conditioned PBMC, an outcome that is apparent in both patients and healthy controls and, moreover, for all chemokines assessed in the present study, except RANTES. MP produces significant effects when used alone, however, ruling out the possibility that the effects seen on chemokine secretion are exlusively produced by non-genomic mechanisms. There are no significant differences between the effects seen in the two study populations, indicating that the possible existence of treatment-induced GC resistance in WG patients submitted to long-term GC therapy is without importance in this setting. On the other hand, the GC unresponsiveness seen in SEB-conditioned PBMC may result from SEB-induced GC resistance, although further investigations are required to establish such a connection.

The gene expression of RANTES does not conform to the pattern seen with the other chemokines. While down-regulated by MP, the gene expression of RANTES is also down-regulated with SEB, compared to unstimulated PBMC. Furthermore, the concomitant stimulation with SEB+MP results in a RANTES mRNA expression that is significantly lower than the levels experienced in unstimulated cells. A down-regulation of mRNA levels following SEB-stimulation is also seen with ENA-78, suggesting that different regulatory mechanisms may be involved in the production of RANTES and ENA-78. There may be several mechanisms in play, influencing differently the production of divergent chemokines.

The effects of superantigens on PBMC are non-controversial, a fact that was confirmed further in the present study, as chemokine production was generally up-regulated in response to stimulation with SEB. SEB-stimulation also resulted in up-regulation of GR-α. This is consistent with the findings in a study of Hauk *et al.* (2000), which concluded that both GR-α and GR-β were significantly up-regulated in response to SEB (100 ng/ml for 48 hours), although GR-β somewhat more than GR-α (65). Moreover, they found that microbial superantigens (SEB and PHA) induced GC insensitivity in PBMC from healthy volunteers, probably resulting from the elevated levels of GR-β, which are believed to inhibit the actions of GR-α (47). This does not explain, however, the diminished effect of MP on PBMC from WG patients. On the other hand, unresponsiveness to GC treatment may also be induced by long-term systemic GC therapy, which is the mainstay of maintenance therapy in WG. There are also other mechanisms than up-regulation of GR-β that may contribute to this effect, and it is presently unclear to what extent up-regulation of GR-β is involved.

4.3 METHODOLOGICAL CONSIDERATIONS

4.3.1 In vitro activation of viable cells

In vitro studies on viable cells may be confounded by preparative stimulation, either by contamination of activating agents, or by inappropriate sample handling. Common contaminants include bacteria or bacterial surface products (e.g., endotoxins), which provoke a state of alertness in leukocytes, reflected by secretion of inflammatory products and up-regulation of adhesion molecules. These activities may have profound influence on the parameters under investigation, and are therefore sought eliminated. In order to minimize the risk of contamination, and thereby the hazard of *in vitro* activation, all handling of viable cell samples was performed aseptically in sterile laminar air flow (LAF) cabinets. Furthermore, all reagents were routinely checked for endotoxin content by the Lumulus amoebocyte lysate (LAL) test. Moreover, our *in vitro* experiments were designed in such a way that potential *in vitro* activation would not influence the results. For instance, samples that were to be compared in respect of cellular function (i.e., chemokine gene expression or secretion) were treated identically up to the point of addition of stimulant.

4.3.2 Experimental stimulation

Stegeman *et al.* demonstrated in 1994 that chronic nasal carriage of *Staphylococcus aureus* could be a risk factor for WG relapse, and concluded that this could be due to activation of neutrophils, followed by surface-expression of ANCA-antigens, or that staphylococcal

superantigens or other immunogenic surface structures (such as peptidoglycan) could stimulate lymphocytes independently of T-cell help (10). Therefore, SEB, possibly contributing to the pathophysiology of WG, was chosen above phytohemagglutinin (PHA), LPS, and CD3, which could otherwise have been equally competent stimulating agents. Recently, however, the same group has concluded that the effects of *S. aureus* are probably mediated by a mechanism other than superantigenic T-cell activation because the T cell expansion experienced in WG patients was not associated with the presence of either *S. aureus* or its SAgs (52). However, more plausible mechanisms have not yet emerged.

MP was a natural agent of choice when seeking to elucidate the effects of immunosuppressive therapy, as this agent is used for induction of remission in WG patients with active disease.

4.3.3 Assay evaluation

The assays employed in this study, including both ELISA and real-time RT-PCR applications, are previously well-tested for assessing chemokine levels in the medias presently encountered (i.e. serum and cell supernatants, and purified RNA, respectively). The analyses in some applications were performed repeatedly, but the reproducibility and accuracy of the methods employed were not systematically subdued to further inquiry.

The relatively small size of the study populations included in the present thesis limits the possibility of finding statistically significant relationships. On the other hand, variations that are found to be significant in smaller populations generally are substantial. Furthermore, observations that are not statistically significant in smaller populations may as well be so when assessed in a larger material, and are said to be tendencies, representing interesting areas for future investigation.

High doses of SEB have been reported to induce apoptosis in T lymphocytes (66). The topical report utilized SEB in concentrations of 5 µg/ml, which is much higher than the concentrations we have used in our study. However, we have reason to believe that apoptosis may be seen already at 100 ng/ml, which is partly the reason why a concentration of only 1 ng/ml was employed in our study. Although we feel confident that apoptosis has not influenced the results of our study, this has not been confirmed experimentally.

4.3.4 Longitudinal variations in chemokine levels

The data obtained in this study are based on measurements taken at a single time point only. Therefore, longitudinal (over time) variation in chemokine levels can affect the data obtained, possibly also influencing the conclusions drawn on basis of the data. Longitudinal changes in the circulating levels of some chemokines (IP-10, Mig, eotaxin, and TARC) and chemokine receptors (CXCR3, CCR5, and CCR3) have previously been investigated in healthy individuals (67). These results indicated a reliable baseline of *in vivo* expression of chemokines and chemokine receptors among healthy subjects. Furthermore, Kivisakk *et al.* (2003) have resently completed a two-year longitudinal study of the constitutive levels of the chemokine receptors CCR2, CCR5, and CXCR3 on CD4-positive T cells in healthy volunteers, concluding that major fluctuations in chemokine-receptor levels within healthy individuals are probably uncommon (68). In WG patients, on the other hand, the chemokine levels may fluctuate considerably, most likely in accordance with disease activity. If the chemokines we have investigated are involved in the pathophysiology of WG, the condition of the patient at sample collection may affect our results considerably. We have, therefore, tried to assess this possibility by comparing serum chemokine levels with CRP and ESR.

4.3.5 Inter-subject variations in chemokine levels

The study of Campbell *et al.* (2001) (67) also assessed inter-individual variations in chemokine expression. In our study, this is not of importance except for the assessment of serum chemokine levels in WG patients versus healthy controls, as our conclusions for the most part are based on differences in chemokine expression in aliquots of the same cell population, handled identically up to the point of stimulation with inducing agents. Nonetheless, Campbell *et al.* found a high inter-subject variability in the chemokine levels in plasma, indicating that large sample populations would be required to identify significant differences between disease and control groups. In contrast, we, in the present study, have revealed differences between WG patients and healthy controls in the serum levels of RANTES and MCP-1 that are sufficiently large to be assessed even in the relatively small population included.

4.3.6 Presentation of data

Some ways of presenting data may preclude important information. Although perspicuous, bar charts may mislead the interpretation of the results by omitting individual values. The figures presented below (Fig. 4.1) will aid in the interpretation of some of the results from this study.

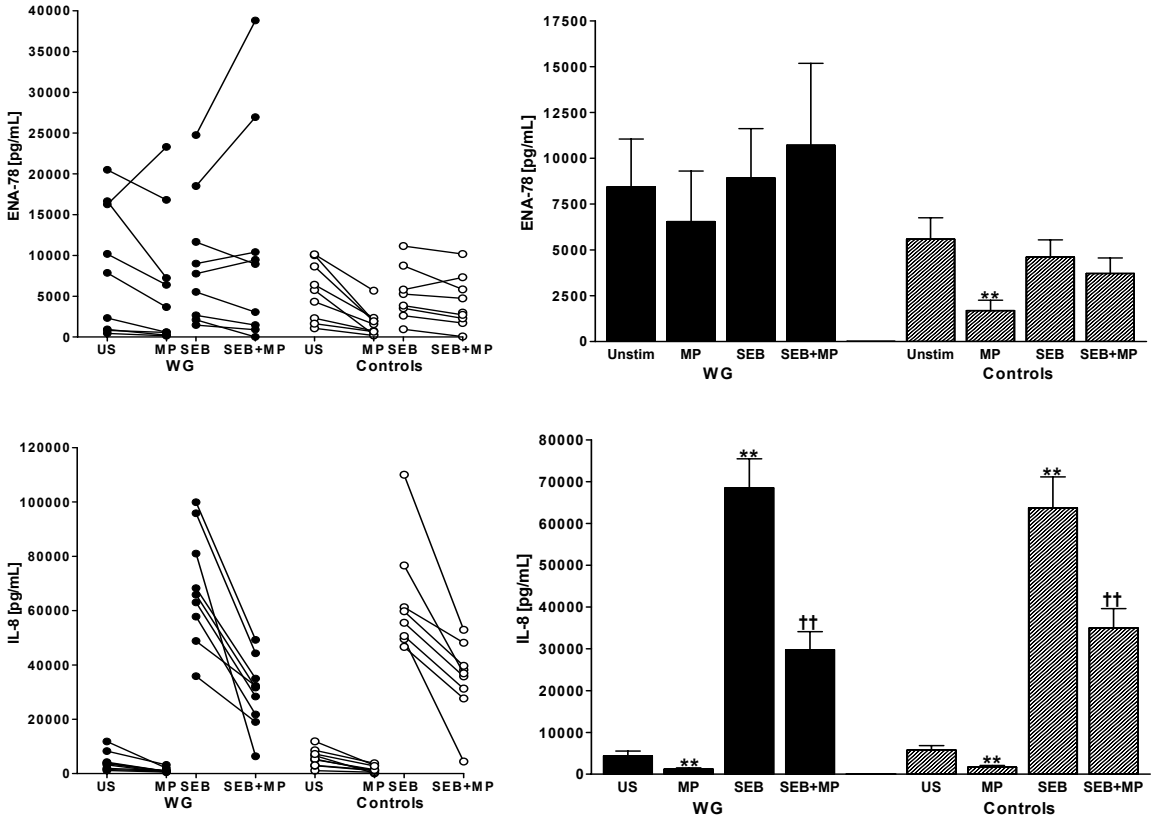


Figure 4.1: Two ways of presenting the secretion of epithelial cell-derived neutrophil-activating factor (ENA)-78 (upper panel) and interleukin (IL)-8 (lower panel) from peripheral blood mononuclear cells from 9 patients with Wegener's granulomatosis and 9 healthy controls in response to 24 hours stimulation with staphylococcal enterotoxin B (SEB) or methylprednisolone (MP) or both. The results are obtained using enzyme-linked immunosorbent assays (ELISA). Values are presented as connected scatter-plots (left) and bar graphs (right).

The figures above may provide a more nuanced picture of the modulation of IL-8 and ENA-78 experienced in this study. Whereas IL-8 is profoundly and unidirectionally modulated by the prevailing conditions, the levels of ENA-78 seemingly remains stable upon stimulation, particularly in the patient population. However, this is hardly the case; by closer investigation of the data, it is revealed that the reason why MP seemingly has no effect on unstimulated PBMC from WG patients is that one single sample deviates from the general trend. Due to the small study population, no statistical significance is achieved. This does not mean, however, that MP has no effect on the secretion of ENA-78.

4.3.7 Drawing conclusions

The results experienced in this study, obtained by analysis on protein as well as mRNA levels, depend on a variety of cellular regulatory mechanisms that will not be completely elucidated unless further investigations are performed. Figure 4.2 illustrates the many points at which chemokine expression may be modulated within the cells analyzed.

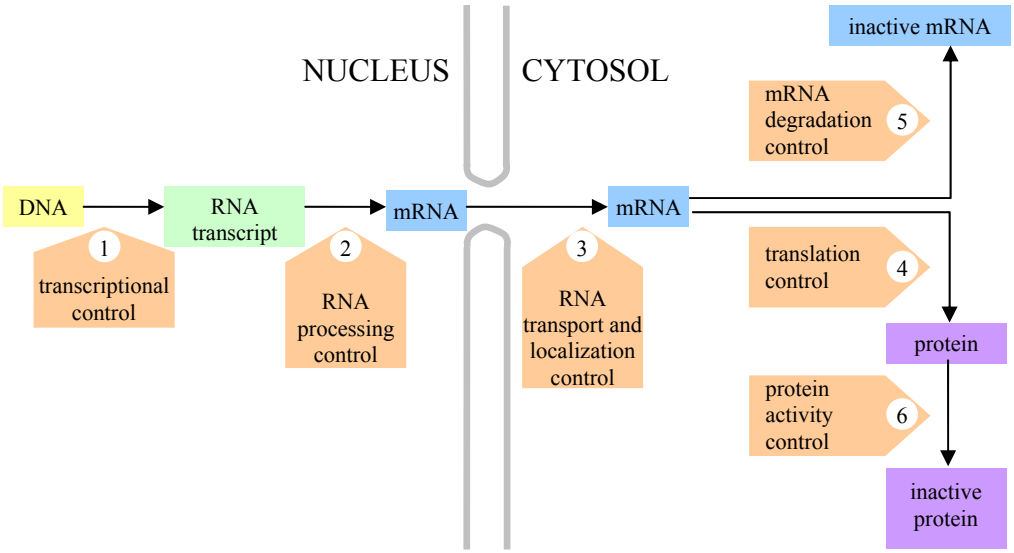


Figure 4.2: Six steps at which gene expression can be controlled. Step 6, the regulation of protein activity, includes reversible activation or inactivation by protein phosphorylation as well as irreversible inactivation by proteolytic degradation. The figure is modified after Alberts *et al.*, 2002. (69)

Thus, there are a variety of steps on which MP and SEB can act to produce their effects. The observation that the secretion of a certain chemokine is modulated in response to one of these agents may consequently result from increased gene expression or translation, including post-transcriptional and post-translational modulation, as well as up-regulated secretion of the chemokine. Furthermore, effects observed on the gene expression of a specific chemokine do not always imply increased secretion of the chemokine, due to several instances of post-transcriptional control.

4.4 FUTURE CONCERNS

The pathophysiology of WG is far from elucidated and should therefore, because of the possibly fatal nature of this disease, be intensively investigated in the future. We and others have suggested a role of chemokines in WG, reflecting either causal or resultant involvement in the pathology of this disease. The next step will be to further elucidate the functions of the involved chemokines *in situ*, as well as investigating other factors that may contribute to both chemokine levels and disease activity. Both *in vitro* and *in vivo* actions of the GCs must be further illuminated. Moreover, if indeed the effects on chemokine production are linked to the therapeutic effects of immunosuppression, the development of drugs that more selectively interact with chemokines or chemokine receptors should be a goal for the distant future. Such drugs are, in fact, in the pipeline for the treatment of HIV-infection (70).

For the near future, on the other hand, further studies of PBMC are warranted, including investigation of the effects at other stimulation intervals in order to assess temporal variations in the gene expression and secretion of chemokines following treatment with MP. Samples are indeed in store that have been stimulated for 24 hours, and will be investigated presently. Furthermore, the levels of GR-α and -β will be more closely explored, hopefully leading to a better understanding of the mechanisms underlying GC resistance. It proved too laborious to include these investigations in the present thesis.

5. CONCLUSIONS

The results of the present study suggest a role for chemokines in WG; RANTES, IL-8, and MCP-1 in particular. MIP-1α and ENA-78 may be involved as well, although these were less convincingly regulated in the present study. We found elevated levels of RANTES and MCP-1, and also a strong tendency for up-regulation of IL-8, in serum of WG patients. In addition, there were tendencies for differential levels of RANTES and ENA-78 in patients with active and remissive disease.

The chemokines may also constitute important targets for therapy – clearly, the current immunosuppressive therapy, in our study represented by the glucocorticoid MP, influences the production of chemokines in PBMC isolated from healthy individuals *in vitro*. However, there is a diminished response in patients, suggesting that potentially important pathogenic mechanisms remain unmodulated by the current treatment strategies.

Presently, the treatment of WG is based on drugs with wide-spread and pleomorphic effects, resulting in substantial treatment-associated morbidity. Moreover, the development of therapy resistance severely impairs treatment efficacy in a significant proportion of the patients. Specific modulation of chemokine levels therefore constitutes a promising foundation for future treatment strategies in WG.

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7. APPENDIX

Table 1: Characteristics of the chemokines. Adapted from Olson and Ley, 2002 (19).

Systematic Name*	Human Common Names	Mouse Common Names	Receptors Bound	Chemokine Type	Expression	Proposed Functional Expression Sites
CXCL1	GROα, MGSA	MIP-2, KC	CXCR2	ELR+	Inducible	Neutrophilic inflammatory sites; atherosclerotic lesions
CXCL2	GROβ, MIP-2$^{\alpha}$	KC	CXCR2	ELR+	Inducible	
CXCL3	GROγ, MIP-2β	KC	CXCR2	ELR+	Inducible	
CXCL4	PF4	PF4		ELR–		
CXCL5	ENA-78	LIX	CXCR2	ELR+	Inducible	Neutrophilic inflammatory sites Neutrophilic inflammatory sites Neutrophilic inflammatory sites
CXCL6	GCP-2	CKα-3	CXCR1,2	ELR+	Inducible	
CXCL7	NAP-2		CXCR2	ELR+	Inducible	
CXCL8	IL-8		CXCR1,2	ELR+	Inducible	Neutrophilic inflammation; liver, acute lung injury; atherosclerotic lesions
CXCL9	Mig	Mig	CXCR3	ELR–	Inducible	Th1 inflammation; CNS, intestinal lesions
CXCL10	IP-10	IP-10, CRG-2	CXCR3	ELR–	Inducible	
CXCL11	I-TAC		CXCR3	ELR–	Inducible	Th1 inflammation
CXCL12	SDF-1	SDF-1	CXCR4	ELR–	Constitutive	Bone marrow; thymus; lung; lymphoid organs
CXCL13	BLC, BCA-1	BLC, BCA-1	CXCR5	ELR–	Constitutive	Lymphoid follicles
CXCL14	BRAK, bolekine			ELR–		
CXCL15		lungkine		ELR–		
CXCL16	CXCL16	CXCL16	CXCR6	ELR–, TMD+		Th1 inflammation
CCL1	I-309	TCA-3	CCR8	4 cysteines	Inducible	Th2 inflammation
CCL2	MCP-1, MCAF	JE	CCR2	4 cysteines	Inducible	Th1 inflammation; liver, CNS, allergic lung injury; atherosclerotic lesions
CCL3	MIP-1α	MIP-1α	CCR1,5	4 cysteines	Inducible	Th1 inflammation; lung, CNS, atherosclerotic injury
CCL4	MIP-1β	MIP-1β	CCR5,8	4 cysteines	Inducible	
CCL5	RANTES	RANTES	CCR1,3,5	4 cysteines	Inducible	Th1, Th2 inflammation; Lung, CNS, skin injury; atherosclerotic lesions
CCL6		MRP-1		4 cysteines		
CCL7	MCP-3	MARC	CCR1,2,3	6 cysteines	Inducible	Th1, Th2 inflammation; CNS, lung injury
CCL8	MCP-2	MCP-2	CCR3	4 cysteines	Inducible	
CCL9		MRP-2, MIP-1γ		6 cysteines		
CCL10		CCF-18		4 cysteines		
CCL11	eotaxin	eotaxin	CCR3	4 cysteines	Inducible	Th2 inflammation; allergic lung, skin disease
CCL12		MCP-5	CCR2	4 cysteines	Inducible	Th1, Th2 inflammation; allergic lung disease
CCL13	MCP-4		CCR2,3	4 cysteines	Inducible	

CCL14	HCC-1, CKβ-1		CCR1	4 cysteines		
CCL15	HCC-2, Lkn-1, MIP-5		CCR1,3	6 cysteines		
CCL16	HCC-4, LEC, Mtn-1	LCC-1	CCR1	4 cysteines		
CCL17	TARC	TARC	CCR4	4 cysteines	Inducible	Th2 inflammation in skin
CCL18	DC-CK1, PARC			4 cysteines	Constitutive	Lymphoid T cell zones
CCL19	MIP-3β, ELC, CKβ-11	MIP-3β, ELC	CCR7	4 cysteines	Constitutive	Lymphoid T cell zones
CCL20	MIP-3α, LARC	MIP-3α, LARC	CCR6	4 cysteines	Constitutive	Intestinal villi; skin
CCL21	6Ckine, SLC, CKβ-9	SLC, TCA-4	CCR7	6 cysteines	Constitutive	Lymphoid organs, HEV
CCL22	MDC, STCP1	abcd-1	CCR4	4 cysteines	Both	Thymus; allergic lung disease; Th2 inflammation
CCL23	MPIF-1, CKβ-8		CCR1	6 cysteines		
CCL24	MPIF-2, eotaxin-2		CCR3	4 cysteines	Inducible	Th2 inflammation
CCL25	TECK, CKβ-15	TECK, CKβ-15	CCR9	4 cysteines	Constitutive	Small intestine; thymus
CCL26	eotaxin-3, MIP-4α		CCR3	4 cysteines	Inducible	Th2 inflammation
CCL27	CTACK, ILC, ESkine	ALP, skinkine	CCR10	4 cysteines	Constitutive	Skin
CX3CL1	fractalkine	neurotactin	CX3CR1	TMD+	both	Ubiquitous
XCL1	lymphotactin, ATAC	lymphotactin	XCR1			

* Systematic nomenclature and mouse/human correlation defined by Zlotnik and Yoshie (71). GRO, growth-related oncogene; MGSA, melanoma growth stimulatory activity; MIP, macrophage inflammatory protein; PF, platelet factor; ENA, epithelial cell-derived neutrophil-activating factor; LIX, lipopolysaccharide-induced CXC human chemokine; GCP, granulocyte chemotactic protein; CK, chemokine; NAP, neutrophil-activating protein; IL, interleukin; Mig, monokine induced by γ-interferon; IP-10, γ-interferon-inducible protein; CRG, chemokine responsive to gamma; I-TAC, interferon-inducible T-cell chemoattractant; SDF, stromal cell-derived factor; BCA-1, B-cell-activating chemokine; BLC, B-lymphocyte chemoattractant; BRAK, breast and kidney expressed chemokine; TCA, T-cell-activation protein; MCP, monocyte chemoattractant protein; MCAF, monocyte chemotactic and activating factor; RANTES, regulated on activation normal T-cell expressed and secreted; MRP, MIP-related protein; CCF, CC chemokine factor; HCC, hemofiltrate CC chemokine; Lkn, leukotactin; LEC, liver expressed chemokine; Mtn, monotactin; LCC, liver-specific CC chemokine; TARC, thymus- and activation-related chemokine; DC-CK, dendritic cell chemokine; PARC, pulmonary- and activation-regulated chemokine; ELC, Epstein-Barr virus-induced receptor ligand chemokine; LARC, liver- and activation-induced chemokine; 6Ckine, 6 cysteine chemokine; SLC, secondary lymphoid tissue chemokine; MDC, macrophage-derived chemokine; STCP, stimulated T-cell chemotactic protein; MPIF, myeloid progenitor inhibitory factor; TECK, thymus-expressed chemokine; CTACK, cutaneous T cell-attracting chemokine; ILC, interleukin 11 receptor alpha-locus chemokine; ESkine, embryonic stem cell chemokine; ALP, amino-terminal alanine-leucine-proline chemokine; KC, JE, I-309, MARC, abcd-1, ATAC, derived from gene names; TMD, transmembrane domain.