VARIABILITY OF TNF-a SECRETION BY PERIPHERAL BLOOD MONONUCLEAR CELLS FROM HEALTHY MALES

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Variability of TNF-α Secretion by Peripheral Blood Mononuclear Cells from Healthy Males

by

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ABSTRACT

Tumor necrosis factor-α (TNF-α) is a cytokine with inflammatory and regulatory properties produced by monocytes and activated T-cells. Levels of TNF-α secretion in vitro by peripheral blood mononuclear cells (PBMC) stimulated with bacterial lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA) plus concanavalin A (ConA) have been reported to vary among individuals and to be associated with HLA-DR alleles, namely HLA-DR2 with low levels of TNF-α secretion and HLA-DR3 and DR4 with high levels.

In this study we have measured levels of TNF-α secreted by LPS-stimulated PBMC from 72 healthy young males. There was a large degree of variation in the level of secreted TNF-α and this interindividual variation was relatively stable in four individuals tested 2 to 4 times. The study panel was also typed for HLA-DR, -A, -B and -C antigens. A weak association was shown between HLA-DR3 and low levels of TNF-α secretion ($p = 0.014$, uncorrected). No association was shown between levels of TNF-α secretion and HLA-DR2, -DR4 or HLA-B antigens.

We further explored the possibility that differences in levels of TNF-α secretion might be due to (1) individual differences in the kinetics of TNF-α secretion and/or (2) differences in the relative number of TNF-α secreting cells when different stimulants were used. The kinetics of 20μg/ml LPS-induced and 1ng/ml LPS-induced TNF-α secretion by PBMC were very similar but the total amounts secreted were higher with 20 μg/ml LPS. The kinetics of TNF-α secretion

were quite different when PBMC were stimulated by LPS or ConA plus PMA. LPS-induced TNF-α peaked at 3 to 6 hours while ConA/PMA-induced TNF-α secretion did not reach an obvious peak in our experiments but was low at 3 hours and continued to rise at 53 hours. Five individuals were classified as high to low secretors using the 3-hour, 20 μg/ml LPS, stimulation protocol. This classification changed dramatically when the ConA/PMA, 53-hour protocol was used and three individuals classified high became low or vice versa. This is an important observation and must be considered when investigating associations between levels of TNF-α secretion and HLA antigens.

To investigate if differences in secretion might be due to individual differences in the number of TNF-α secreting cells, a two-color immuno-fluorescence assay was developed to monitor cell phenotype and cytoplasmic TNF-α by flow cytometry. There was no consistent relationship between the proportion of CD14 monocytes in the samples staining positive for cytoplasmic TNF-α and the levels of secreted TNF-α by LPS-stimulated PBMC from two individuals tested, but this may have been due to technical difficulties. However, differential counts of the male study panel PBMC samples by flow cytometry for the subsets CD14 monocytes, CD4 T-cells, CD8 T-cells and CD45RO T-cells showed no correlation between the relative numbers of cells in each subset and secreted TNF-α levels.

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACD	Acid-Citrate-Dextrose
BSA	Bovine Serum Albumin
Con A	Concanavalin A
ELISA	Enzyme Linked Immunosorbent Assay
FcR	Fc Receptor
FITC	Fluorescein Isothiocyanate
GAM-HRPO	Goat anti-mouse-Horse Radish Peroxidase
GAR-HRPO	Goat anti-Rabbit-Horse Radish Peroxidase
HLA	Human Leukocyte Antigen
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-4	Interleukin 4
IL-6	Interleukin 6
kb	kilobases
kDa	kilo Daltons
KLH	Keyhole Limpet Hemocyanin
LBP	LPS Binding Protein
LPS	Lipopolysaccharide
M	molar
MHC	Major Histocompatibility Complex
ml	millilitres
mM	millimolar
mRNA	Messenger Ribonucleic Acid
ng	nanograms
NHS	Normal Human Serum
NK	Natural Killer Cells
NZB	New Zealand Black
NZW	New Zealand White
OD	Optical Density
OPD	o-Phenylenediamine
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
pg	picograms
PHA	Phytohaemagglutinin
PI	Propidium Iodide
PMA	Phorbol 12-Myristate 13-Acetate
RA	Rheumatoid Arthritis

RFLP	Restriction Fragment Length Polymorphism
rTNF-α	Recombinant TNF-α
SAC	Staphyloccus aureus Cowan Strain 1
SD	Standard Deviation
SEA	Staphlococcal Enterotoxin A
SLE	Systemic Lupus Erythematosis
TcR	T Cell Receptor
TNF-α	Tumor Necrosis Factor-alpha
TNF-β	Tumor Necrosis Factor-beta
TNF-BP	TNF Binding Protein
TNF-R	Tumor Necrosis Factor Receptors
TNF-Rβ	TNF Receptor Type 1
TNF-Rα	TNF Receptor Type 2
TNF-R1	TNF Receptor Type 1
TNF-R2	TNF Receptor Type 2
Tris	Tris(Hydroxymethyl) aminomethane
U	Units
μg	micrograms
μl	microlitres

Chapter 1

Introduction

1.0 Historical Background

During the latter part of the last century physicians noticed that if patients with tumors contracted erysipelas, a Streptococcus pyrogenes infection of the skin, their tumours shrank during the course of the infection. Dr. Wm. B. Coley (Coley, 1893) discovered that he could prepare crude bacterial extracts and use them as a treatment for some types of tumors. This preparation became known as Coley's Toxins and was the only approved systemic treatment for cancer until the advent of chemotherapy and radiation in 1934. The treatment was eventually abandoned because of its toxicity to the patients.

Interest in Coley's Toxins was revived during the 1930's when researchers were interested in isolating and purifying the bacterial factor that induced tumor necrosis and separating it from the toxic material in the crude bacterial extracts. Shears and colleagues (Shears, et al. 1936; Hartwell, et al. 1943) purified a "bacterial polysaccharide" which was very effective in inducing tumor necrosis. This material however, was very toxic and its use as an anti-tumor agent was not carried any further.

By the 1960's work on the necrosis caused by "bacterial polysaccharides", now called lipopolysaccharide (LPS), started again because it was now hypothesized that the mediator of LPS-induced necrosis was not LPS itself but

some endogenous material produced in response to LPS (O'Malley et al. 1962). O'Malley demonstrated that serum derived from endotoxin-treated mice could induce haemorrhagic necrosis of tumors of untreated mice. A similar observation was made by Carswell et al. (1975) with serum from mice treated with Bacillus Calmette-Guerin (BCG). The factor responsible was heat labile and was termed tumor necrosis factor (TNF). Meanwhile, in the 1970's, Rouzer and Cerami (1980), working with rabbits, demonstrated that animals with Trypanosoma brucei infections developed hypertriglyceridemia and "wasting" or cachexia. The elevated triglycerides were shown to result from a deficiency in lipoprotein lipase. In a series of experiments with endotoxin-resistent (C3H/HeJ) and endotoxin-sensitive mice (C3H/HeN), Kawakami and Cerami (1981) demonstrated that a serum factor, which they called cachectin, was responsible for cachexia.

By the mid-1980's both tumor necrosis factor-α and cachectin had been sequenced and the chromosomal location found (Beutler et al. 1985, Caput et al. 1986, Nedwin et al. 1985). These studies showed that TNF-α and cachectin were in fact the same molecule.

1.1 TNF-α and TNF-β

Two biological molecules, TNF-α and TNF-β, now use the name tumor necrosis factor. TNF-β, originally called lymphotoxin, was thought to be of different cellular origin than TNF-α. TNF-β was considered to be mainly a lymphocyte

product and TNF-α a monocyte/macrophage product (see section 1.5 for review). It is now clear that TNF-α is produced by a wider variety of cell types than is TNF-β. Some cells can make both, which one depending upon the signal the cell receives.

TNF-α also has a much broader spectrum of activity than TNF-β. For example, vascular smooth muscle cell expression of HLA-DR (Stemme et al. 1990) and macrophage production of macrophage colony-stimulating factor (Oster et al. 1987) are influenced by TNF-α but not TNF-β. In some cases where there are shared effects, one may have a greater effect than the other.

More information on profound differences between the two molecules is starting to emerge. Most of these differences are at the production level, such as mode of production, transcription rates and different regulatory sequences (Ruddle 1992). TNF-α and TNF-β show a limited amino acid sequence homology of about 30% (Pennica et al. 1984), but have an almost identical structure (see section 1.2). Both genes are single copy genes of roughly the same size closely linked on chromosome 6.

1.2 TNF-α Structure

TNF-α is secreted as an unglycosylated, 157-amino acid polypeptide with a molecular weight of 17 kDa (Jones et al. 1989). The biologically active form of the secreted molecule is a 52-kDa trimer in which monomers are held together by

single, interchain, disulfide bonds (Aggarwal et al. 1985). The tertiary structure of each subunit is unusual because it has a typical jelly roll-β structure, a structure common to viral capsid proteins. The most striking structural similarities are with the capsid proteins of eicosahedral plant viruses, particularly the Satellite Tobacco Necrosis Virus (STNV), with 70% structural homology (Fiers 1991). TNF-α is the first non-viral protein found to have this structure.

The complete carboxyl terminal end of the molecule is essential for bioactivity (Beutler and Cerami, 1988). Genetic manipulation of the primary structure has shown that the amino terminal can lose up to eight residues without loss of bioactivity but the carboxyl terminal must remain intact.

TNF-α also exists in a 26-kDa integral transmembrane form (Krigler et al. 1988). In addition to the 157 residues of the monomers of the secreted form, the membrane-bound form has an additional 76 residues which traverse the membrane. The 17-kDa secreted protein is derived from the 26-kDa form by proteolytic cleavage from the 76 residue intramembrane portion.

The 26-kDa membrane bound form has been shown to be biologicaliy active (Perez et al. 1990). Mutant cells that are unable to secrete TNF-α but express the membrane-bound form, have been shown to kill tumor cells and virus-infected cells directly by cell to cell contact. The same mutant cell type not expressing TNF-α on the membrane did not have this cytotoxic activity.

1.3 Actions on Cells

TNF-α is a multifunctional cytokine affecting many different cell types and eliciting a plethora of different activities in different cells. This diversity of activity can be ascribed to multiple signal transduction pathways and activation of a large number of genes. The following sections will describe some of these activities and the role of TNF-α in the immune response.

1.3.1 TNF Receptors

TNF exerts its activity upon a cell by first binding to specific TNF receptors (TNF-R). Two distinct receptors have been identified, TNF-R1 (TNF-Rβ) and TNF-R2 (TNF-Rα) (Brackhaus et al.,1990), with different molecular weights, immunoreactivities and glycosylation patterns. There is only 28% homology between the extracellular domains of these molecules and no homology between intracellular domains, suggesting that the receptors have different signalling pathways (Lewis et al. 1991). Hohmann et al. (1990) however, presented evidence that both receptors activate a common transcription factor, NF-KB. Both TNF-α and TNF-β can bind to either receptor.

Both receptors are found on most cell lines (Kull et al. 1985;Baglioni et al. 1985; Aggarwal et al. 1985) and on nearly all cells of somatic tissues, except erythrocytes (Beutler & Cerami, 1988). Using flow cytometry to quantitate the number of receptors per cell, Gehr et al. (1992) have shown that there are no

significant differences among peripheral blood mononuclear subsets in numbers of either receptor. This observation also holds true after phytohaemagglutinin (PHA) stimulation, which activates CD3-positive lymphocytes.

The signals from each receptor may not be redundant; each may be responsible for different activites. Using receptor-specific antibodies, it was demonstrated that TNF-R1 signals cytotoxicity and induces several genes while TNF-R2 signals proliferation in primary thymocytes and cytotoxic T-cells (Tartaglia et al. 1991). Gehr et al. (1992) however, somewhat contrary to these findings, showed that both receptors can elicit a proliferative cell response and that the response is greatest if both receptors are activated. In this study the proliferative response was diminished by blocking either of the receptors with antibodies.

One very important feature of TNF receptors is their ability to be secreted. Secreted forms have a lower molecular weight but retain the ability to bind TNF. The secreted forms were first identified as TNF-binding protein (TNF-BP) in urine (Engelmann et al. 1990; Seckinger et al. 1988) and serum of cancer patients (Gatanega et al. 1990). Aggarwal et al. (1988) has shown that, in vitro, membrane TNF-R's on monocytes and macrophages are rapidly downregulated by phorbol esters (phorbol 12-myristate 13-acetate (PMA)), IL-1 and TNF-α . Gatangea et al. (1991) went on to show that part of the downregulation that occurred when cells were stimulated with PMA and LPS was due to secretion or shedding of the receptor into the supernatant.

1.3.2 Tumor Cells

Historically, TNF-α was first identified and named for its anti-tumor activity. This activity was observed in vitro with the fibroblast-like L-929 cell line and in vivo in the necrosis of Meth A sarcomas implanted in mice (O'Malley et al. 1962). Because of these initial findings there was great enthusiasm for the potential use of TNF-α as an anti-tumor therapy.

Further research in the therapeutic uses of TNF-α as an anti-tumor treatment has shown that the early enthusiasm may have been premature. Not all tumor cells respond to TNF-α, and those that do, show a wide variety of responses (Ortaldo et al. 1986; Tsujimoto et al. 1985). Resistance to TNF-α appears not to be due to a lack of receptors on the cell but to the absence of a biochemical signal after TNF-α binds to the receptor (Sugarman et al. 1985). Tumors that are resistant to TNF-α in vitro can sometimes be susceptible to necrosis in vivo if the tumor is vascularized. In this case it appears that TNF-α acts indirectly by destroying the vascular endothelium in the tumor, thus cutting off blood flow to the tumor (Palladina et al. 1987).

TNF-α can also act as a growth factor for some malignancies. Chronic B-cell malignancies, such as hairy cell leukaemia, have been shown to proliferate and to produce TNF-α when treated with TNF-α (Cordingley et al. 1988a).

1.3.3 Septic Shock

It has long been observed that patients suffering from acute bacterial infections can very quickly go into septic shock. Symptoms include fever, chills, nausea and in severe cases, may rapidly result in death. Patients suffering from chronic disease, such as cancer or tuberculosis, suffer cachexia or wasting, characterized by a loss of muscle and adipose tissue. In the last century when Coley was treating patients with Coley's toxins, it was noted that many of the patients suffered symptoms similar to those of bacterial infections, and in some patients, severe septic shock (Spooner et al. 1992).

Research into the role of TNF-α in septic shock has taken several approaches. One was to measure plasma TNF-α levels in critically ill septic patients, such as those with meningococcal (Waage and Espevik, 1987) and other diseases (Debets et al. 1989). Not all the septic patients had detectable levels of TNF-α, but those that did had a much higher mortality rate than TNF-α negative patients. The lack of detectable circulating TNF-α does not preclude its effects. The detection limit of the assays used in these studies may not have been low enough and TNF-α has a short circulating half-life. Also, cell-associated forms of TNF-α may act via paracrine mechanisms (Perez et al. 1990). Another approach was to infuse animals with rTNF-α. Both rats (Tracey and Beutler 1986) and dogs (Tracey et al. 1987) given quantities similar to those produced endogenously in response to LPS died within minutes to hours.

Cancer patients with advanced tumors treated with TNF-α suffer

hypotension, leukopenia, and renal impairment, symptoms associated with septic shock (Selby et al. 1987). In fact, the maximum tolerable doses are much lower than those required for maximum cytoxicity to the tumor cells.

1.4 Effects on the Immune System

It is now generally accepted that TNF-α secreted at low levels in localized infections acts as an immune modulator. In this context it probably has a protective role in helping to eliminate the infectious agent.

TNF-α is primarily an inflammatory cytokine, acting as an early response cytokine in cellular activation and propagation of the inflammatory response. Its major role is to initiate and resolve a local inflammatory response. One of the first events in a local inflammatory response is the trapping of neutrophils in the area. TNF-α stimulates the vascular endothelial cells to release a neutrophil chemotactic factor (Moser et al. 1988) and may be chemotactic itself in attracting leukocytes (Ming et al. 1987). Neutrophils also have increased phagocytic properties and increased superoxide production in the presence of TNF-α (Klebanoff et al. 1986). At the same time TNF-α increases the adhesiveness of neutrophils to endothelial cells by upregulating the expression of adhesion molecules such as intercellular adhesion molecule (ICAM) and endothelial-leukocyte adhesion molecule (ELAM) (Griffiths et al. 1989). TNF-α also upregulates Class I MHC molecules (Pohlman et al. 1986) and attracts macrophages to the site of inflammation. TNF-α can then

stimulate these macrophages to produce more TNF-α and stimulate endothelial cells and macrophages to produce IL-1. Both TNF-α and IL-1 can trigger fever. TNF-α may also function as a co-stimulator for T-lymphocytes (Zucali et al. 1987), may modulate B-lymphocyte differentiation (Kashiwa et al. 1987) and may exert anti-viral activity similar to that of IFN-γ (Ohase et al. 1986; Wong and Goeddel 1986).

The observed effects of TNF-α on the immune response are not necessarily a direct result of TNF-α activity, but may be the result of a second or third mediator produced in response to TNF-α activity. It is now commonplace to discuss these phenomena as the "cytokine network," where one cytokine stimulates the production of a second and so on. TNF-α, for example, can stimulate the production of interleukin-1 (IL-1) by macrophages. Both of these cytokines can stimulate the production of interleukin-6 (IL-6). IL-6 can then stimulate lymphocytes, activate haematopoietic cells, and induce acute phase proteins (reviewed in Van Snick 1990).

1.5 TNF-α Secretion

When TNF-α and TNF-β were first identified, it was thought that TNF-α was a monocyte/macrophage product (Carswell et al. 1975) and TNF-β was a lymphocyte, particularly T-lymphocyte, product. It is now known that TNF-α can be produced by many different cell types of immune origin, including lymphocytes, NK

cells, and Kupffer cells, as well as cells of nonimmune origin, such as endothelial cells (Aggarwal & Vilek 1992) and smooth muscle (Warner & Libby 1989).

1.5.1 Monocytes/Macrophages

Macrophages are the primary source of TNF-α (Beutler & Cerami 1988). This is not surprising considering the role TNF-α plays in inflammation and the fact that macrophages are among the first cells to arrive at a localized site of infection. All macrophages that have been tested thus far, including macrophages derived from peripheral blood monocytes, Kupffer cells and peritoneal macrophages, produce TNF-α.

Both the secreted soluble 17 kDa form and the cell-surface bound 26-kDa transmembrane form are produced by monocytes (Kriegler et al. 1988). It is suggested that surface-bound TNF-α may play a role, such as killing of target cells, by cell-to-cell contact. Most TNF-α research however, has been on the secreted form.

In vitro experiments have shown that a variety of agents can induce macrophages to produce TNF-α, but lipopolysaccharide (LPS) from gram negative bacteria is the most potent stimulus (Beutler & Cerami 1987). Not all types of LPS are equally effective. Bacteria expressing the rough LPS phenotype are better inducers of TNF-α than bacteria with smooth LPS, possible because rough LPS is shed more readily (Kelly et al. 1991). Also, long chain polysaccharides attached

to the Lipid A moiety reduce the ability to induce TNF-α. Very small amounts of LPS, as low as 12 pg/ml, can stimulate monocytes to secrete TNF-α and the amount of TNF-α secreted is dependent on the LPS concentration (Molvig et al. 1988). Concentrations of a few picograms up to micrograms per millilitre are routinely used (Cuturi et al. 1987; Aderka et al. 1989; Jacob et al. 1990). The use of microgram quantities of LPS to induce TNF-α secretion by cells in culture has been criticised because such large quantities may be greater than levels achieved in vivo. Lynn et al. (1993) point out however, that LPS concentrations up 10 μg/ml can occur with fecal soiling of the peritoneum and in abscess cavities.

For monocyte activation, exposure to LPS need only be a few minutes. LPS binds to monocytes very quickly (Gallay et al. 1993). Within 5 minutes near maximum binding to the cell has occurred, and the cells continue to secrete TNF-α after extensive washing to remove the LPS. A rapid burst of secreted TNF-α is seen within 20 minutes of LPS exposure (Hofsli et al. 1988).

TNF-α is not secreted constitutively (Hofsli et al. 1988; Beutler et al. 1986; Lonnemann et al. 1989), but there are conflicting reports as to whether non-stimulated, circulating monocytes contain non-translated TNF-α mRNA. Some researchers have shown that resting monocytes contain a small pool of non-translated TNF-α mRNA and that this mRNA may be translated in the first few minutes of cellular stimulation to account for the initial burst of secreted protein (Hofsli et al. 1988). This initial burst can be inhibited by cycloheximide, a protein

synthesis inhibitor, but not by actinomycin, an RNA synthesis inhibitor. Beutler et al. (1986) also report finding low TNF-α mRNA levels in fresh monocytes but question whether it was induced by the isclation procedures of the experiment. Both groups report not finding any TNF-α from lysates of resting cells, suggesting that TNF-α mRNA in resting cells is not translated. After LPS stimulation, there is a rapid increase in TNF-α mRNA demonstrated by nuclear run-on transcription assays. In contrast to these studies, Sariban et al. (1988) reported that TNF-α mRNA was absent in resting monocytes but was quickly induced after stimulation.

LPS stimulates monocytes to produce TNF-α by first binding to receptors on the cell surface. There are a number of reports of the different mechanisms by which LPS can bind to monocytes, and more recently the finding of more than one type of receptor (reviewed by Wright, 1991) (See table 1 for a list of receptors).

CD14, a surface protein found on monocytes, macrophages and polymorphonuclear leukocytes, is the classical receptor for LPS. The existence of CD14 as a monocyte antigen has been known since the mid-1980's (Goyert et al. 1986) but was only recently shown to be a receptor for LPS (Wright et al. 1990). LPS does not bind CD14 directly, but first complexes with a serum protein called LPS-binding protein (LBP). LBP was first discovered in the mid-1980's and shown to have a role in LPS-induced sepsis and acute phase reaction (Tobias et al. 1986; Tobias et al. 1988) but its mechanism of action was unknown. It is now known that monocytes are activated when CD14 binds LPS-LBP complexes.

LBP appears not to be the only serum protein that can complex with LPS to bind CD14. A second serum factor, called septin, has been shown to opsonize LPS-bearing particles and then bind to CD14 (Wright et al. 1992). It is distinct from LBP because its activity is not blocked by anti-LBP antibodies. The molecule (or molecules) also appears to have proteolytic activity because its activity is blocked by protease inhibitors.

New evidence indicates that neither CD14 nor serum factors are essential for monocyte activation by LPS to produce TNF-α (Lynn et al. 1993). THP-1 cells, a human monocytic cell line, can still produce TNF-α in response to LPS stimulation when grown in serum-free media. At low LPS concentrations in serum free conditions, the amount of TNF-α secreted is much lower than when serum is present at the same LPS concentrations. In this situation, the increase in TNF-α by adding serum to the culture can be blocked by anti-CD14 antibodies. At high LPS concentrations in serum-free media however, there is increased TNF-α secretion and the levels of TNF-α secreted is affected very little by the addition of serum. Secretion cannot be blocked by anti-CD14 antibodies at high LPS. Thus, anti-CD14 affects TNF-α secretion only if serum or LBP is present. These results clearly point to the existence of other LPS receptors that are not LBP-dependent.

There is a growing pool of evidence that other LPS receptors do exist. The CD11/CD18 family of adhesion molecules on leukocytes have been shown to bind LPS (Wright & Jong 1986) There is some doubt, however as to whether these

receptors are involved in LPS-mediated signal transduction because CD18-deficient cells still respond to LPS (Wright et al. 1990b). The most promising candidate as a second LPS receptor is a 70-80 kDa protein found to bind LPS to mouse splenocytes (Lei et al.1988) and more recently to human monocytes, lymphocytes and polymorphonuclear leukocytes (Halling et al. 1992). Another membrane associated protein, MW 30-40 kDa, also binds LPS and is a possible receptor candidate. A 95 kDa "scavenger" receptor may also play a role in LPS activation of monocytes (Morrison et al. 1993). (Table 2)

These non-CD14 LPS pathways may be important at sites of infection where LPS concentrations are very high (Lynn et al. 1993). CD14-LBP dependent pathways may be more important for circulating monocytes where the LPS concentration would be very low.

Monocytes express receptors (FcR) for the Fc portion of immunoglobulin molecules. To date there are three FcR's known to exist on monocytes. FcRI (CD64), a high affinity receptor, and FcRII (CD32), a low affinity receptor, are found on all monocytes (Debets et al. 1988). More recently, FcRIII (CD16) has been reported on a subset of monocytes (Passlick et al. 1989).

Crosslinking FcRI with immune complexes or IgG bound to plastic wells can stimulate monocytes to secrete TNF-α (Debets et al. 1988; Szabo et al. 1990). FcRII appear to bind the immune complexes or immobilized IgG only after treatment with proteases (Debets et al. 1990). After treatment of these receptors

they can be crosslinked and induce TNF-α secretion. TNF-α secretion stimulated by FcR crosslinking may be important when monocytes are presented with antibody-coated antigens, such as viruses and parasites, and in antibody-mediated cellular cytotoxicity.

Table 1: List of Reported Possible LPS Receptors

Receptor	Cell Type	LPS-Serum Factors Complex*	References
CD14	Monocyte	Yes	Wright *et al.* (1990a) Goyert *et al.* (1986)
CD11/CD18	Leukocytes	No	Wright *et al.* (1990b) Wright & Jong (1986)
70 to 80-kDa protein	Murine splenocytes Human leukocytes	? No	Lei *et al.* (1988) Halling *et al.* (1992)
30 to 40-kDa protein	Murine lymphocytes	?	Reviewed in Morrison *et al.* (1993)
95-kDA scavenger receptor	Macrophages	?	Reviewed in Wright *et al.* (1991)

* Indicates whether LPS first binds to a serum factor to form a complex and the complex then binds to the receptor

A variety of other agents have been used experimentally to induce the secretion of TNF-α. The most commonly used is a phorbol ester, 4β-phorbol 12-myristate 13-acetate (PMA) or O-tetradecanoylphorbol 13-acetate (TPA). PMA is a nonspecific cell activator of a wide variety of genes in a wide variety of cell types (Castagna et al. 1982). Cells of the monoblastoid line, U937, are not responsive to LPS but will produce TNF-α within 2-4 hours after exposure to PMA (Hass et al. 1991). Freshly isolated human monocytes will also secrete TNF-α after PMA exposure (Nedwin et al. 1985;Saribon et al. 1988). Using nuclear run-on assays, TNF-α mRNA has been detected within 20 minutes of PMA exposure. Secreted protein is detectable after 3 hours and increases progressively.

1.5.1A Monocyte Subsets

Subsets of monocytes may differ in TNF-α production. In 1989 there was a report of the finding of a new subset of monocytes (Passlick et al. 1989). These cells had lower levels of CD14 expressed on the surface, higher MHC Class II molecule expression and, unlike "regular" monocytes, expressed CD16 (FcRIII) on their surface. These cells were also not adherent to plastic and were not phagocytic when tested with antibody-coated erythrocytes. They were smaller and less granular than other monocytes.

Wang et al. (1992) also reported the existence of two sets of monocytes, based on size alone. One group was roughly twice the size of the other but still

displayed monocyte characteristics, such as non-specific esterase staining. When stimulated with LPS, the smaller cells secreted lower levels of TNF-α than the larger cells. Ziegler-Heitbrock et al. (1992) reconfirmed the existence of smaller, CD14/CD16-positive, monocytes and showed that, when stimulated with LPS, they produced 10-fold lower levels of TNF-α than regular CD14-positive monocytes.

This new CD14/CD16-positive subset, representing about 9 % of peripheral blood monocytes, has been further characterized by immunofluorescent assays for surface antigens and slot blot RNA hybridizations for transcript levels (Ziegler-Heitbrock et al. 1993). Because of their lower CD14 expression they are generally denoted as $CD14^{+}$/CD16 monocytes while regular monocytes are denoted $CD14^{+++}$ monocytes. $CD14^{+}$/CD16 monocytes have four-fold lower levels of CD14 expression but approximately eight fold higher levels of MHC Class II expression. There is an increase in VLA-4 expression, a surface molecule involved in leukocyte-endothelial cell interactions and in expression of ICAM-1 on some $CD14^{+}$/CD16 cells but not on $CD14^{+++}$ cells.

$CD14^{+}$/CD16 monocytes show many of the same characteristics as alveolar macrophages. In fact, Passlick et al. (1989) reported that these cells could be found in large numbers in alveolar space. The authors suggested that they may represent $CD14^{+++}$ monocytes at a further differentiation stage because when $CD14^{+++}$ monocytes are left in culture for 5 days they show many of the characteristics of $CD14^{+}$/CD16 monocytes. Scheibenbogen & Andreesen (1991)

reported that up to 70 % of fresh monocytes that had been left in culture for 14 days were expressing CD16. It had been reported several years prior to the discovery of this novel monocyte subset that the ability of monocytes to produce TNF-α decreases when the cells are left to differentiate into macrophages in vitro (Burchett et al. 1988).

1.5.2 Lymphocytes

While monocytes/macrophages appear to be the primary source of TNF-α, both T- and B-lymphocytes can produce TNF-α, at least in vitro, in varying amounts depending on the stimulant used. Lymphocytes, in culture, are unresponsive to LPS. They are responsive to phorbol esters and cross-linking of various surface molecules with immobilized immunoglobulin.

1.5.2A T Cells

Both human T-cell lines and fresh peripheral blood T-lymphocytes produce TNF-α when stimulated with phorbol esters and calcium ionophores (Kinkhabwata et al. 1990; Cuturi et al. 1987). These studies have shown that the effects of phorbol esters and calcium ionophores are additive. Both TNF-α mRNA and secreted protein can be detected earlier than when cells are stimulated with either phorbol ester or calcium ionophore alone. When cells are stimulated with PMA alone TNF-α mRNA can be detected within one hour after stimulation (Sung et al.

1988).

There are a number of molecules on the surface of T-cells that are important in cell-to-cell interactions and antigen presentation. CD3 for example, is associated with the T-cell receptor and is involved in T-cell activation during antigen presentation by antigen-presenting cells. Because these surface molecules are involved in cell activation, and cell activation often involves one or several cytokines, there has been a flurry of research in TNF-α production by cell activation through these molecules. There have been a number of studies demonstrating that cross-linking CD3 on T-cells with immobilized anti-CD3 antibodies can stimulate TNF-α production (von Fliedner et al. 1992; Chong et al. 1992; Sung et al. 1988; Turner et al. 1987). Soluble anti-CD3 antibody has no affect on TNF-α production. Again, co-stimulation with PMA can increase TNF-α to higher levels than stimulating with PMA or anti-CD3 alone. Co-stimulating with anti-CD28 antibodies also augments TNF-α production over CD3 stimulation alone.

CD2 is a molecule found on most thymocytes and mature peripheral blood T-cells. It is an adhesion molecule involved in interactions with endothelial cells, connective tissue and most blood cells. Santis et al. (1992) demonstrated that T-cells could be stimulated through CD2 to produce TNF-α. Two different monoclonal anti-CD2 antibodies, each recognizing different epitopes, were required for sustained TNF-α production. Co-stimulation with PMA or calcium ionophore greatly augmented the amount of TNF-α produced.

CD69 is a surface molecule not found on resting lymphocytes. It is the earliest inducible surface antigen during lymphocyte activation. Fresh peripheral blood T-cells can be induced to express CD69 with PMA. Stimulating the cells with immobilized anti-CD69 can induce the cells to produce small amounts of TNF-α within 24 hours (Santis et al. 1992b). Co-stimulating with PMA stimulates the cells to produce more TNF-α than stimulation with anti-CD69 or PMA alone.

Lymphokine-activated killer T-cells (LAK-T), T-cells that can kill target cells in a non-MHC restricted manner, can also produce TNF-α (Chong et al. 1992). LAK-T cells were stimulated by co-culture with anti-CD3 and K562, a FcR expressing tumor cell line. This tumor cell line would immobilize the anti-CD3 through Fc-FcR interactions. Immobilizing anti-CD3 by absorbing onto plastic plates gave the same results. However, co-culture with non-FcR expressing tumor cells could also augment TNF-α production. Antibodies to adhesion molecules, such as LFA-1, LFA-2, CD44 and CD45 could also augment TNF-α production when the cells were cultured with anti-CD3. The interactions with these adhesion molecules on the non-FcR expressing tumor cells could account for their enhancement of TNF-α production.

Staphylococcal enterotoxin A (SEA) can induce both monocytes and T-cells to produce TNF-α if the cells are cultured together (Fisher et al. 1990). SEA alone does not stimulate either T-cells or monocytes to produce TNF-α. Addition of other cytokines, such as IL-2 or IL-4 to monocyte/SEA cultures does not induce

TNF-α, therefore an unknown mediator or direct cell-to-cell interaction is required in this situation. Only $CD4^+$ T-cells can induce monocytes to produce TNF-α in the SEA stimulation system (Fisher et al. 1990).

Not all T-cell subsets produce TNF-α to the same extent. Both CD4 and CD8 T-cells can produce TNF-α but CD4 T-cells produce levels 3-fold higher than CD8 cells (Cuturi et al. 1987). $CD45RO^+$ T-cells (memory cells) when stimulated with anti-CD3 and PMA produce 10-fold higher levels than $CD45RO^-$ T-cells (von Fliedner et al. 1992).

Physiologically, TNF-α from T-cells may act more in a paracrine manner at localized sites of infection and/or tissue injury. Adhesion molecules for example, such as LFA's, help hold cells together for TcR-CD3 engagement during antigen presentation. Engagement of the adhesion molecules may enhance TNF-α secretion. $CD69^+$ T-cells are localized at sites of tissue injury. TNF-α secreted through the CD69 activation pathway may contribute to tissue injury, such as in the synovial membranes of rheumatoid arthritis.

1.5.2B B Cells

B-cells are now recognized as a source of TNF-α depending on the stimulation pathway. PMA stimulates tonsillar B-cells to secrete very low levels of TNF-α, in comparison to T-cells and monocytes, but if co-stimulated with Staphylococcus aureus Cowan Strain 1 (SAC) there is an increase in both secreted

TNF-α and mRNA (Sung et al. 1988). Tonsillar B-cells also secrete TNF-α when co-stimulated with SAC and IL-2 (Rieckmann et al. 1991). Pre-B cells lines, such as Josh-7 and Nalm-6, do not produce TNF-α when stimulated with SAC and PMA, suggesting a developmental regulatory mechanism.

From a physiological perspective, TNF-α secretion from B-cells activated through surface immunoglobulin binding to antigen may be more significant. Goldfield et al. (1992) has shown that cross-linking surface immunoglobulin molecules on B-cells with anti-human Ig (IgG, IgM, IgA) can stimulate TNF-α secretion. This TNF-α could be an important mediator in Ig secretion because TNF-α can enhance Ig secretion.

1.5.3 Peripheral Blood Mononuclear Cells

Some researchers have used PBMC stimulated in vitro to study TNF-α secretion. This system avoids some unnecessary manipulation of cells because they are subjected to only one separation process and mimics physiological conditions as closely as possible. In mixed cell populations however, only total secreted TNF-α can be measured. It is difficult to determine the relative contributions of each cell type to the total TNF-α secreted. Because we know that different cell types are activated by different agents, the choice of stimulating agent becomes important in interpreting the data.

For example, Bendtzen et al (1988) stimulated their cultures of PBMC

with LPS, therefore the TNF-α secreted was most likely by monocytes. Malave et al (1989) and Jacob et al (1990) stimulated cells with a combination of PMA and Concanavalin A (Con A) for up to 3 days. Con A is a plant derived lectin that is known to stimulate T-cells to proliferate. The secreted TNF-α in this situation is probably derived from several sources, monocytes and T-cells. The relative contributions of each may change over time as the T-cell numbers increase.

1.5.4 Methods for Measuring TNF-α

In the study of TNF-α production, a number of different experimental systems have been used. In addition to using PBMC, or subsets of PBMC, different cell lines have been used. The two most commonly used are THP-1, a monocyte line and U937, a pre-monocyte line. THP-1 cells can be stimulated by LPS to produce TNF-α but U937 cells must be pretreated with PMA to induce further differentiation.

Both TNF-α mRNA and secreted protein have been measured in different systems to monitor TNF-α production. Secreted protein has been measured by two main methods, with a bioassay and with an Elisa. The bioassay is a cellular cytotoxicity based assay that uses cell lines sensitive to the cytotoxic activity of TNF-α, such as the mouse fibroblast cell line L-929. The bioassay measures only biologically active TNF-α while the ELISA can measure total protein. The main disadvantage of the bioassay is its lack of specificity. It will measure other cytotoxic

substances that may be present with TNF-α in the sample, such as TNF-β.

1.6 Variation in Secreted TNF-α Levels

There are a number of reports demonstrating that there are stable interindividual differences in secreted TNF-α from fresh peripheral blood mononuclear cells. Molvig et al. (1988) first reported that, when fresh monocytes from healthy males were stimulated in vitro with LPS, there were differences among individuals in the amount of TNF-α secreted and these differences were stable over time. Jacob et al. (1990) also made a similar observation for males using a combination of Con A and PMA as stimulant. Secreted TNF-α from males and post-menopausal females when tested several times was highly reproducible. Pre-menopausal females however, showed large interindividual differences but the differences were not stable over time.

Most reports describing TNF-α secretion by freshly purified peripheral blood monocytes stimulated with LPS report peak secretion levels between 3 and 6 hours (Lonnemann et al. 1989; Beutler & Cerami, 1988). The amount of TNF-α in the culture supernatant slowly diminishes after about 6 hours. There are no clear kinetic studies of TNF-α secretion when peripheral blood monocytes are stimulated with PMA. Sariban et al. (1988) reported a peak secretion level at 15 hours and Nedwin et al. (1985) reported a peak secretion level at 24 hours. However, these experiments were not carried beyond 15 and 24 hours. With a monocyte-like cell

line, U937, a plateau of secreted TNF-α secretion was reached at 48 - 72 hours when stimulated with PMA (Hass et al. 1991).

The kinetics of TNF-α secretion by lymphocytes is less clearly understood than secretion by monocytes. In most experiments secreted TNF-α was measured at only one or two time periods (Goldfield et al. 1992; Santis et al. 1992). In experiments where cells were stimulated for 2 - 3 days secreted TNF-α was measured only once or twice. TNF-α mRNA however, was measured more frequently in these experiments so there are better kinetic studies of mRNA synthesis than secreted protein. B-cells, when stimulated with SAC for example have peak TNF-α mRNA levels at 32 - 48 hours (Sung et al. 1988). Even though there are no true kinetic studies of secreted TNF-α by lymphocytes, we do know that both T- and B-lymphocytes when stimulated can sustain TNF-α secretion for at least up to 48 hours after stimulation (von Fliedner et al. 1992; Sung et al. 1988).

Some researchers have chosen to use whole blood or mixed peripheral blood mononuclear cell culture because the results may be more relevant to physiological conditions. Whole blood stimulations with LPS had peak TNF-α secretion at 4 - 8 hours (Allan et al. 1992; Strieter et al. 1990). Purified peripheral blood mononuclear cells stimulated with LPS in the same experiments had peak secreted TNF-α bioactivity at 24 hours.

1.7 TNF-α and HLA

In humans, the TNF-α gene maps to the short arm of chromosome 6 (Nedwin et al. 1985). It is found within the MHC cluster of genes, about 230 kb centromeric to HLA-B and approximately 870 kb telomeric to HLA-DR genes (Carroll et al. 1987) (Figure 1).

The mapping of the TNF-α gene within the MHC Class III region prompted speculation about the role of TNF-α in the etiology of MHC-linked diseases. Molvig et al. (1988) first suggested a possible association between both low TNF-α and low IL-1 responses and HLA-DR2 when monocytes from healthy males were stimulated with low LPS for 20 hours. It is not stated whether the subjects were typed for HLA-B. Bendtzen et al. (1988), using a panel of 39 subjects of both sexes ranging in age from 25 to 52, demonstrated that PBMC from HLA-DR2 individuals secreted less TNF-α in vitro when stimulated with 1 ng/ml and 100 ng/ml LPS for 20 hours than non-HLA-DR2 individuals. Subjects were also typed for HLA-A, -B, -C, and -DP but no association was found. In contrast to the Molvig et al (1988) data, there were no observed correlations with HLA-DR and the

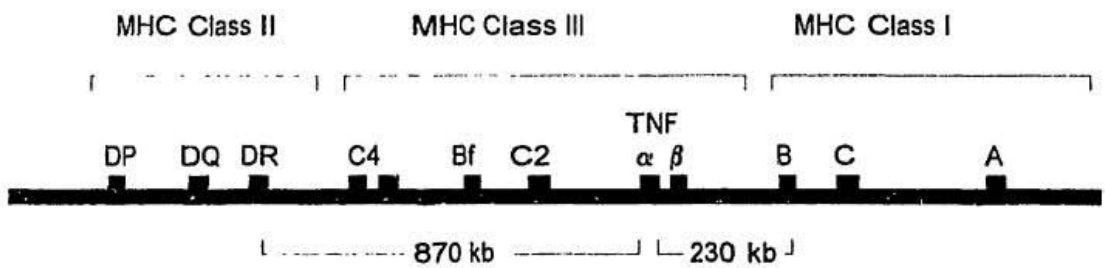


Figure 1: Molecular map of the MHC region showing the distances between TNF-α and HLA-DR and HLA-B. (Adapted from Trowsdale *et al.* 1991)

IL-1 response. Preincubation of the cells with IFN-γ for 16 hours augmented the LPS-induced TNF-α secretion in both HLA-DR2$^+$ and HLA-DR2$^-$ individuals and caused the difference in secreted TNF-α levels between the two groups to disappear.

Jacob et al. (1990) reported a similar observation for a panel of healthy males whose mononuclear cells were stimulated in vitro with a combination of Con A (10 μg/ml) and PMA (2 ng/ml) for approximately 60 hours. In addition to the low TNF-α secretion and HLA-DR2 association, they reported that HLA-DR3 and -DR4 individuals were high TNF-α secretors. DR2/3 and DR2/4 heterozygotes were also high secretors. There were no HLA-A, -B, or -C associations observed. Although the data reported were for ConA/PMA stimulations because it gave the highest levels of secreted TNF-α, the authors report that other reagents were used to induce TNF-α, such as 10 μg/ml LPS for ~60 hours, and the interindividual differences between high and low secretors were the same for all methods. (Table 2).

Table 2 : Reported HLA-DR Associations with TNF-α Secretion Levels

Author	Cell Type	Stimulant	Time	Associations		
				HLA-DR2	HLA-DR3	HLA-DR4
Molvig *et al.* (1988)	Monocyte	LPS 12.5 pg/ml to 250 ng/ml	20 hrs	low	NT	NT
Bentzen *et al.* (1988)	PBMC	LPS 1 ng/ml 100 ng/ml	20 hrs	low	no	NT
Jacob *et al.* (1990)	PBMC	ConA/PMA	~60 hrs	low	high	high
Molvig *et al.* (1990)	Monocyte	LPS 13 pg/ml to 250 ng/ml	6 hrs & 20 hrs	no	no	NT
Sachs *et al.* (1990)	PBMC	PHA	72 hrs	no	no	no
Picot *et al.* (1993)	Monocyte	LPS 250 pg/ml	20 hrs	low	high	high

low = low levels of secreted TNF-α
high = high levels of secreted TNF-α
NT = not tested or not reported inpaper
no = no association found

1.8 TNF-α and Disease

There are a number of reasons why there is interest in the role TNF-α may play in disease. The initial area of research was in the role TNF-α may play in infectious diseases, such as in inflammation, and in the elimination of infectious agents. A second area of research was prompted after the mapping of the TNF-α gene to the human and murine MHC regions. This initiated speculation that TNF-α may play a role in HLA-linked autoimmune diseases, or serve as a better marker for autoimmune disease. The following two sections will address the role of TNF-α in infectious diseases and its possible role in autoimmune disease.

1.8.1 TNF-α and Infectious Diseases

There are many infectious disease causing agents that stimulate the production of TNF-α, including both Gram negative and positive bacteria, fungi, parasites and viruses. The result may be beneficial with the elimination of the agent, or deleterious, depending on the quantity produced and time period over which production is sustained. Septic shock is the most common disease state from the overproduction of TNF-α in response to an infectious agent, usually Gram negative bacteria. Malaria is also a potent inducer of TNF-α and infected individuals with high serum TNF-α levels are at risk of cerebral malaria (Kwiatowski et al. 1990).

TNF-α plays an important role in the protection from a number of

infectious agents. Mice for example, when passively immunized against TNF-α with anti-TNF-α antibodies, readily succumb to Mycobacterium bovis. (Beutler & Grau, 1993). A similar protective effect of TNF-α is seen during infections with Listeria monocytogenes, Legionella pneumophila, and Plasmodium berghei.

1.8.2 TNF-α and Autoimmune Disease

There are a number of autoimmune diseases that are associated with different MHC alleles. Ankylosing spondylitis, for example, has a very strong association with HLA-B27 and SLE has an association with HLA-B8 and DR3 (Tiwari & Terasaki 1985). The role of the MHC Class I and II genes in the disease state is still not completely understood. They may be the predisposing genes but equally may be markers for other closely linked genes. TNF-α is a candidate for one of those marker or predisposing genes because it is found within the MHC cluster and is a potent modulator of the immune system. Muller et al. (1987) in fact, hypothesised that a dysregulated TNF-α gene may be involved in autoimmunity.

Jacob & McDevitt (1988) reported a possible link between TNF-α and disease in the (NZB x NZW)F_1 mouse, a model for SLE. The NZW mouse is phenotypically normal but the NZB has mild glomerulonephritis. The F1 hybrids, however, suffer severe glomerulonephritis and 95% die within the first year. Peritoneal exudate cells from the NZW animals secrete lower levels of TNF-α than

cells from NZB mice, and the F1 hybrids have an intermediate level which is closer to levels secreted by NZW. Treating the (NZB x NZW)F_1 with TNF-α early resulted in prolonged life. At 11 months 90% were still alive while 80% of the non-treated animals were dead. The authors suggested that the NZW parent contributed a gene that produced low levels of TNF-α.

Malave et al. (1989) stimulated PBMC's from healthy individuals as well as SLE patients with active and inactive disease. When stimulated with Con A plus PMA there was maximum TNF-α secretion between 48 and 72 hours for all groups. Con A alone caused very little TNF-α secretion. With Con A/PMA stimulations, patients with active SLE secreted less TNF-α than controls. There was no significant difference between patients with inactive SLE and controls. In addition, spontaneous TNF-α secretion in unstimulated cultures was higher in the SLE group.

SLE nephritis has a strong association with HLA-DR2 (Tiwari & Terasaki, 1985). The combined data of the (NZB xNZW)F1 studies and the work of Jacob et al. (1990) showing low TNF-α production associated with HLA-DR2 suggests TNF-α may play a role in SLE nephritis. In fact, the Jacob et al. (1990) report also pointed out that HLA-DR3 and -DR4 individuals have high TNF-α production and SLE patients with these haplotypes are not predisposed to nephritis. Jacob (1992) has suggested that, with SLE, TNF-α may have a primary involvement in the autoimmune process rather than acting mainly as the effector arm of the

inflammatory process in organ destruction.

Rheumatoid arthritis (RA) has a strong association with HLA-DR4 but no association with TNF-α production levels has been demonstrated (Maury & Teppo 1989). TNF-α probably plays an important role in tissue destruction in RA because TNF-α has the ability to stimulate collagenase and cause bone and cartilage resorption (Beutler & Cerami, 1987). Maury & Tempo (1989) did show that RA patients have elevated serum TNF-α levels and Buchan et al. (1988) reported that TNF-α could be detected in the synovial fluid of RA patients. TNF-α does not appear to play a role in the predisposition of a person to develop RA. In this case it appears to be more of the effector arm of the inflammatory process that leads to bone and cartilage destruction.

1.9 Aims and Objectives

This project began in the context of (i) inconclusive data regarding HLA associations with level of TNF-α production and (ii) a system in place in this lab for studying TNF-α induction and quantitation which had been optimized for another study. Individuals in this laboratory had been using 20 μg/ml of LPS to stimulate THP-1 cells and a cytotoxicity assay to measure secreted TNF. In this system TNF production peaked between 3 and 6 hours after stimulation. The initial objectives of this project were :

(a) to verify, using 20 μg/ml LPS for 3 hours as inducer and human PBMC as the source of TNF-α, that the level of TNF-α production by PBMC of different individuals varied and that the levels were stable over time,

(b) to determine whether the reported associations between HLA and TNF-α levels could be verified using the experimental conditions described in (a),

and because the cytotoxicity assay was very sensitive to, but not specific for TNF-α,

(c) to develop an ELISA to quantify TNF-α in (a) and (b).

Because we were unable to verify the report that HLA-DR2 was associated with low TNF-α expression and, in our experimental conditions, found that DR3 was associated with low expression, we hypothesized that "high

expression" and "low expression" might be affected by the conditions under which TNF-α was induced. Thus, one objective was then to :

(d) to carry out kinetic studies to investigate

(i) the pattern of TNF-α secretion over time in "high" and "low" individuals

(ii) the differences in TNF-α secretion induced by different inducers and different concentrations of the same agents. For example, high LPS, low LPS and Con A/PMA.

Peripheral blood mononuclear cells are a mixed population of cells in which the sizes of subsets vary from individual to individual and possibly also in the same individual over time. An ideal experiment might compare, in a group of individuals, the TNF-α producing capacity of a known number of cells of each subset. Such an experiment would be arduous and also subject to technical difficulties, such as the possible TNF-α induction in some cells by the reagents, such as antibodies, used to purify them. Thus, the final objectives were:

(e) to develop a two-color flow cytometry assay to monitor the cell types and the number of each cell type in a sample of PBMC that are producing TNF-α at a given time when different inducing agents are used to induce TNF-α production

(f) to determine the relative number of cells in each PBMC subset of different individuals to determine if the number of cells of a particular

subset correlated with levels of TNF-α secretion.

Chapter 2

TNF-α Secretion and HLA Associations in Healthy Males

This chapter describes the development of an ELISA for TNF-α, experiments to determine interindividual variation of TNF-α secretion by a group of healthy males and experiments to investigate associations between levels of TNF-α secretion and HLA.

2.1 Methods and Materials

2.1.1 Reagents

2.1.1A Cell Culture Materials

Cell culture medium was RPMI 1640 (Gibco/BRL, Burlington) with the following additives:

10 % fetal calf serum (FCS) (Gibco/BRL)
2 mM L-glutamine (Gibco/BRL)
50 U Penicillin G (Gibco/BRL)
50 µg/ml Streptomycin sulfate (Gibco/BRL)
5 µM 2-mercaptoethanol (2-ME) (Sigma, St. Louis)

2.1.1B Buffers

(1) Phosphate Buffered Saline (PBS) pH 7.2, 10X

80 g sodium chloride (Fisher Scientific)
2 g potassium chloride
14.4 g sodium phosphate, dibasic
2.4 g potassium phosphate, monobasic
Add distilled water up to 1000 ml

To make 1x PBS, 10X stock was diluted by adding 1ml to 9 ml distilled water

(2) Coating Buffer for ELISA, pH 9.2

0.159 g sodium carbonate (Fisher Scientific, Montreal)
0.293 g sodium bicarbonate (Fisher Scientific, Montreal
100 ml distilled water

(3) Blocking Buffer (1 % BSA-PBS)

1 g Bovine Serum Albumin (BSA) (Sigma, St. Louis)
100 ml PBS

(4) Citrate Buffer for ELISA Substrate, pH ~5.1

a. 0.1 M citric acid : 0.96 g citric acid (Fisher Scientific) + 100 ml distilled water

b. 0.2 M sodium phospahte dibasic : 1.41 g sodium phosphate dibasic (Fisher Scientific) + 100 ml distilled water

The buffer was then prepared by mixing 4.9 ml of (a), 5.1 ml of (b) and 10 ml distilled water.

(5) Substrate for ELISA

8 mg o-phenylenediamine (OPD) (ICN Biomedicals, Mississauga)
20 ml citrate buffer
8 µl 30 %-hydrogen peroxide (Fisher Scientific, Montreal) - added immediately before use

(6) Washing Buffer (PBS-0.5 % Tween)

500 µl Tween-20 (Fisher Scientific, Montreal)
1000 ml PBS

2.1.1C Antibodies

(1) Purified Mouse Monoclonal anti-TNF-α (IgG_1) (Endogen, Boston)

(2) Purified Rabbit Polyclonal anti-TNF-α (IgG) (Endogen, Boston)

(3) Purified Goat anti-Rabbit - Horseradish Peroxidase Conjugate (GAR-HRPO) (Southern Biotechnology, Alabama)

(4) Purified Goat anti-Mouse - Horseradish Peroxidase Conjugate (GAM-HRPO) (Southern Biotechnology, Alabama)

(5) Purified Normal Rabbit IgG (Southern Biotechnology, Alabama)

(6) Purified Normal Mouse IgG (Southern Biotechnology, Alabama)

2.1.1D Other Materials

Flat bottomed 96-well polystyrene plates, o-phenylenediamine (OPD) and normal goat serum were purchased from ICN Biomedicals, Mississauga. Bovine serum albumin (BSA), lipopolysaccharide (LPS) (E. coli Serotype O26-B6), Tween-20 and 2-mercaptoethanol were purchased from Sigma, St. Louis. Lymphoprep™, a sterile solution of 9.6 % sodium Metrizoate and 5.6 % polysaccharide, was purchased from Gibco/BRL, Burlington. Recombinant TNF-α (rTNF-α) was purchased from Boehringer Mannheim, Montreal.

2.1.2 TNF-α ELISA

The ELISA used to measure secreted TNF-α was a modification of the indirect sandwich ELISA by McLaughlin et al. (1990). All volumes added to the wells were 50 µl and all washes were with PBS-Tween unless otherwise noted.

The inner 60 wells of flat bottomed 96-well polystyrene plates were coated with mouse monoclonal anti-TNF-α at 2 μg/ml by adding 50 μl of antibody diluted in coating buffer at pH 9.2. Plates were covered and incubated overnight at 4°C.

After coating, coating buffer was removed by flicking the plate and blotting on paper towel. Any remaining protein-binding sites were blocked with 200 μl/well of blocking buffer for 1 hour at room temperature.

A rTNF-α standard curve was generated by preparing serial dilutions from 7 ng/ml to 0.054 ng/ml rTNF-α in culture medium. The first dilution was prepared by transferring 1.4 μl of a 10 μg/ml stock rTNF-α solution into 2 ml of diluent to give a concentration of 7 ng/ml. Doubling dilutions were prepared in 500 μl of diluent.

After blocking, blocking buffer was removed and the plates were washed 3 times. Each standard dilution, sample, and negative control, consisting of only culture medium, were added in duplicate wells. The plates were covered and incubated overnight at 4°C

After washing the plate 3 times, the secondary antibody was added to each well and incubated for 2.5 hours at room temperature. The secondary antibody was a polyclonal rabbit anti-TNF-α diluted 1:300 in 1% goat serum in PBS.

The conjugate was a horseradish peroxidase conjugated goat anti-rabbit

(GAR-HRPO) diluted in 1% goat serum-PBS. The dilution factor had been previously determined and had to be determined for each new lot. Generally the dilution factor was from 1:3000 to 1:4000. After washing the plate 3 times, GAR-HRPO was added to each well and incubated for 90 minutes at room temperature.

The color was developed with o-phenylenediamine (OPD) and hydrogen peroxide in citrate buffer as substrate. 100 µl of the substrate was added to each well including the first column of wells to act as a blank. The plate was incubated at room temperature in the dark for 30 minutes. The reaction was stopped by adding 50 µl of 2.5 M H_2SO_4 to each well. The optical densities (OD) were read at 490 nm on a Bio-Rad Microplate Reader (Model 3550) interfaced with a computer with Microplate Manager software (Bio-Rad, Mississauga, ON) for data analysis.

Standard curves were generated with OD's plotted against log standard rTNF-α concentrations. TNF-α concentrations in the samples were determined by reading directly from the standard curve. The limits of detection of the ELISA were 54 pg/ml to 6 000 pg/ml TNF-α but the useful range for reliable measurements were 54 pg/ml to 3500 pg/ml.

2.1.3 Development of TNF-α ELISA

The following sections describe the steps that were taken to optimize sensitivity of the ELISA for the particular antibodies and different lot numbers of

antibodies that were used.

2.1.3A Determination of Conjugate Dilution - Goat anti-rabbit-HRPO

Thirty of the inner 60 wells of a flat bottom 96-well polystyrene plate were coated with 50 µl/well of normal rabbit IgG and the remaining 30 wells were coated with normal mouse IgG at 5 µg/ml in coating buffer (pH 9.2) overnight at 4°C.

The following dilutions of GAR-HRPO were prepared in 1% goat serum-PBS: 1:500, 1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:3500, 1:4000, and 1:4500. Each dilution as well as a negative control, which was diluent alone, were tested in triplicate against both the rabbit IgG and mouse IgG.

After coating, the plate was washed twice with PBS, blocked for 1 hour with 200 µl/well of blocking buffer, followed by washing 3-times with PBS-Tween. Aliquots of the GAR-HRPO dilutions were added to the plate at 50 µl/well. The plate was incubated at room temperature for 90 minutes.

Colour was developed with 100 µl/well of substrate buffer for 30 minutes in the dark. The reaction was stopped with the addition of 50 µl 2.5 M H_2SO_4 and OD's read at 490 nm.

The conjugate was routinely tested in this manner and the working dilution adjusted accordingly (see Results). In addition, each new lot was also tested before use.

2.1.3B Determination of Concentration of Coating Antibody

Of the inner 60 wells of a 96-well polystyrene plate, 36 were coated with monoclonal mouse anti-TNF-α at 5 concentrations from 1 μg/ml to 5 μg/ml in coating buffer. Six wells were coated with each of the different concentrations and six wells were not coated but received only buffer. The plate was incubated over night at 4°C.

The following day the plate was washed with PBS, blocked for 1 hour with 200 μl/well of blocking buffer followed by washing 3 times with PBS-Tween. For each of the five sets of six wells coated with the different antibody concentrations, three received 50 μl/well of GAM-HRPO diluted accordingly and the other three wells received GAR-HRPO for 90 minutes at room temperature. Three of the uncoated wells received GAM-HRPO and three received GAR-HRPO. GAR-HRPO was included to determine if it bound non-specifically to the coating antibody because in the TNF-α ELISA, GAR-HRPO would be the conjugate used. The plate was then washed 5 times with PBS-Tween and colour developed with OPD as described previously.

2.1.3C Determination of Secondary Antibody Concentrations

McLaughlin et al. (1990) reported using polyclonal rabbit anti-TNF-α at 1:300 dilution, therefore several dilutions in this range was chosen to test. The dilutions were 1:100, 1:200, 1:300 and 1:400 in 1% goat serum-PBS. In addition,

because the sensitivity of the ELISA was not known at this point several rTNF-α concentrations were used in testing the secondary antibody. Three concentrations, 10 ng/ml, 5 ng/ml, 1 ng/ml were arbitrarily chosen and 700 µl of each were prepared in culture media. The range of Mclaughlin's ELISA was 0.08 ng/ml to 6 ng/ml, but the useful range was from 0.08 ng/ml to about 2-3 ng/ml.

The inner 60 wells of a flat bottomed plate were coated with monoclonal anti-TNF-α as previously described. After blocking, 50 µl aliquots of the three rTNF-α concentrations were each put into 15 wells and the remaining 15 of the 60 wells received 50 µl of culture medium. The plate was covered and incubated overnight at 4°C.

Each of the polyclonal rabbit anti-TNF-α dilutions were tested in duplicate against each of the rTNF-α concentrations and against the wells coated with culture medium. In addition, normal rabbit IgG diluted 1:100 and 1:400 and dilution buffer alone were tested against each of the rTNF-α concentrations. After adding 50 µl/well of these materials the plate was incubated at room temperature for 2.5 hours.

The plate was washed three times and GAR-HRPO, diluted as in section 2.1.3A, was added to each well for 90 minutes at room temperature. After washing an additional five times color was developed with OPD substrate.

2.1.3D Determination of Range of Sensitivity

To determine the useful range of the ELISA, rTNF-α concentrations ranging from 10 ng/ml to 0.013 ng/ml were tested in triplicate using the antibody concentrations previously determined. When the upper and lower sensitivity limits were determined, the standard curve was tested 3 times to determine if it was reproducible before assaying samples.

2.1.3E Coefficient of Variation (CV)

The CV was determined by testing a sample with a known concentration of TNF-α each time the ELISA was run. This sample had been prepared by diluting rTNF-α to a concentration of 2500 pg/ml in culture medium and storing at -70°C in 100 µl aliquots. The concentration of the sample read from the standard curve was used to calculate the CV. As a control, this sample could test both reliability of the standard curve and the variability in the ELISA from day to day.

2.1.4 Collection of Samples

Healthy male volunteers between the ages of 20 and 30 years were chosen. Most were medical students that were also part of another study. Care was taken to exclude individuals with know current infections and taking medication. Blood samples were collected by venipuncture between 8:30 am and 12:00 noon. A maximum of 5 donors were bled on any given day. A total of 6 x 10-ml Vacutainer (Becton-Dickinson, Rutherford, NJ) tubes with ACD (acid-citrate-

dextrose) as an anticoagulant were collected from each subject. One tube was used for TNF-α experiments and the remaining blood was used for HLA-typing, differential blood cell counts and other cytokine studies.

As part of a similar study, blood was collected from a panel of pre-menopausal females, 20 - 30 years of age, once a week for a 4 to 8 week period. At each sampling time TNF-α production was induced using the same protocol as used for the males. This latter work was done by two summer students, Paula Rowe and Nancy Sampson.

2.1.5 Preparation of Cells

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood using Lymphoprep™. 10 ml of blood was diluted with 10 ml of sterile PBS then layered onto 4 ml of Lymphoprep™ in each of two 15 ml centrifuge tubes at room temperature. The tubes were centrifuged at 800 x g for 30 minutes. The PBMC form a distinctive layer which was removed with a Pasteur pipet. The cells were washed twice with culture medium, counted and resuspended at 1 x 10^6/ml in culture media.

2.1.6 Induction of TNF-α Secretion

TNF-α secretion was induced with 20 μg/ml LPS for three hours. Previous work in the lab by Claire Simpson had shown that the monocytic cell line,

THP-1, secreted the highest amounts of TNF-α when stimulated with 20 μg/ml LPS for 3 and 5 hours. LPS concentrations from 1μg/ml to 20 μg/ml had been tested in that study and secreted TNF-α had been measured by a bioassay which measured biologically active TNF-α.

Freshly isolated PBMC were plated out at 200 μl/well (200 000 cells/well) in 3 wells for each subject in a sterile 96-well round bottomed plate. The cells were incubated at 37°C in a humidified CO_2-incubator overnight.

The following day the plates were centrifuged at 800 x g for 10 minutes. Supernatants were removed with a sterile pipet and discarded. Fresh media with 20 μg/ml LPS was added to two of the wells and fresh medium without LPS was added to the third well to serve as a negative control and to determine if the cells were secreting TNF-α spontaneously. The cells were then incubated at 37°C for three hours. After incubation the plates were centrifuged at 800 x g for 10 minutes, the supernatants were removed and TNF-α was measured by ELISA.

The cells were stimulated in 200 μl of media so there would be enough supernatant to test each in duplicate both undiluted and diluted 1:2. It was important to test samples diluted because the upper reliable limit of the ELISA was 3.5 ng/ml TNF-α and if a sample contained more TNF-α than 3.5 ng/ml it would not be measured accurately in an undiluted sample. Because the ELISA was designed to test 50 μl/well, 100 μl of undiluted supernatant was required to test the sample in duplicate. The dilute sample was prepared by diluting 50 μl of

undiluted supernatant with 50 µl of culture medium. Thus, a total of 150 µl of undiluted supernatant was required. The supernatants from the non-stimulated cells were only tested undiluted.

2.1.7 HLA-Typing

HLA-A, -B, -C, -DR and -DQ typing was done using standard complement mediated cytotoxicity assays by two medical students, Christian Pugh and Ken Power, as part of a another project.

2.1.8 Statistical Methods

The coefficient of variation for the ELISA was determined by the formula:

$$\frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

The standard deviation and mean were calculated using the statistical software InStat (GraphPad, San Diego).

For HLA associations, secreted TNF-α levels were compared by the Mann-Whitney test using the statistical software InStat.

2.2 RESULTS

2.2.1 Elisa Development

This section describes the results for the development of the ELISA.

2.2.1A GAR-HRPO Dilution

OD's were plotted against the dilution factor. The titration plots were generally very steep with a shallow portion somewhere in the middle (Figure 2.1). The working dilution chosen was the one which was in the shallow portion of the curve. The working dilution was usually from 1:2500 - 1:4000. In the figure shown it was 1:3000.

2.2.1B Coating Antibody Dilution

The concentration of coating antibody chosen was the one above which higher concentrations did not give a significant increase in OD and concentrations below gave a significant OD decrease (Figure 2.2). Based on these results 2 μg/ml of coating antibody was chosen.

2.2.1C Secondary Antibody Concentration

OD's were plotted against rabbit anti-TNF-α dilution factor. A dilution of 1:300 was chosen because it was not in the steep section of the curve and the background was almost zero. However, at 1:300 dilution there was little difference in OD's between 10 ng/ml and 5 ng/ml (Figure 2.3). The upper limit of the ELISA at this dilution would therefore be between 5 ng/ml and 10 ng/ml.

2.2.1D Range of Sensitivity

The sensitivity range of the ELISA is 54 pg/ml to 7000 pg/ml TNF-α. At 54 pg/ml the OD's are 2 to 3 times above background but below 54 pg/ml TNF-α the OD's are at background levels. Above 7000 pg/ml the curve reaches a plateau. However, the useful range of the ELISA for measuring TNF-α is 3500 pg/ml to 100 pg/ml because outside of this range the curve begins to plateau (Figure 2.4).

2.2.1E Coefficient of Variation

The coefficient of variation for the ELISA was less the 15 %. Figure 2.5 shows the variation in concentration for the control sample that was calculated from the standard curves for 15 different experiments.

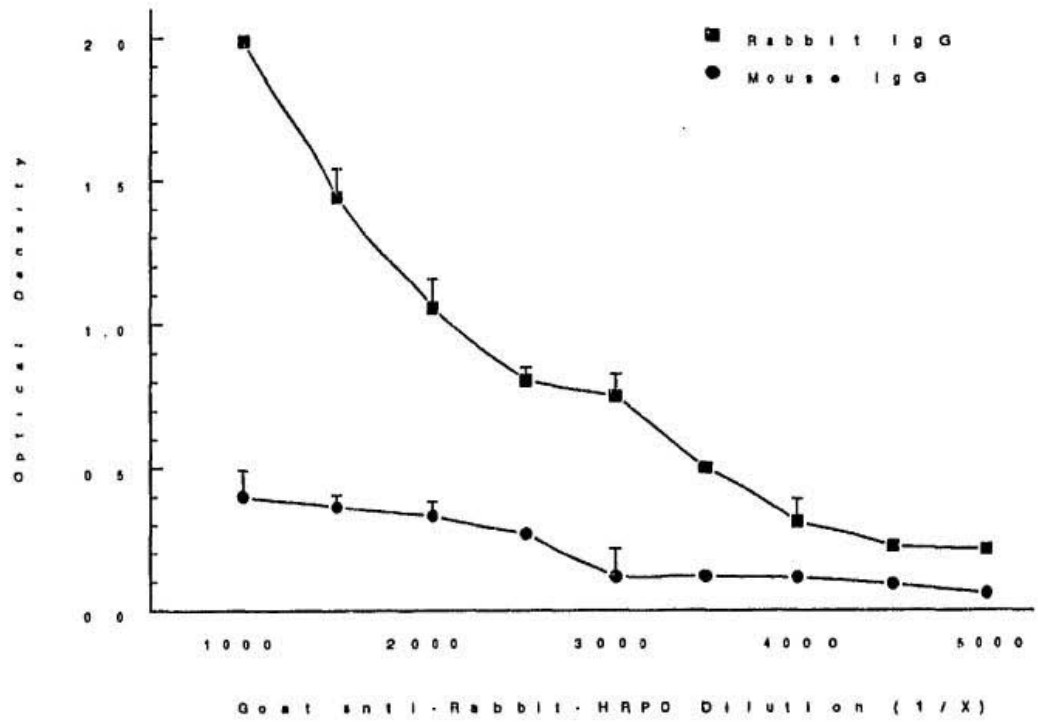


Figure 2.1 : Sample titration curve for goat anti-rabbit-HRPO. The plate was coated with both rabbit IgG (■) and mouse IgG (●).

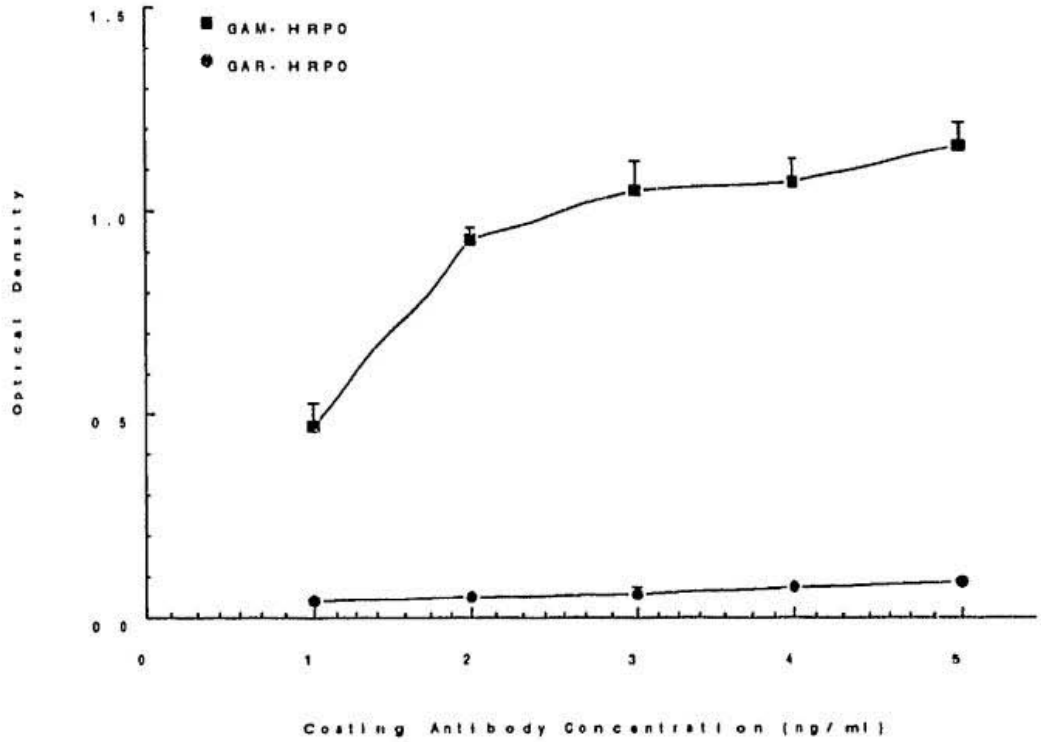


Figure 2.2 : Binding of mouse monoclonal anti-TNF-α to polystyrene plate under different concentrations. The (■) shows the binding of the antibody to the plate by using a goat anti-mouse-HRPO as labelling antibody. The (●) shows the binding of goat anti-rabbit-HRPO to the mouse monoclonal anti-TNF-α to test for nonspecific binding.

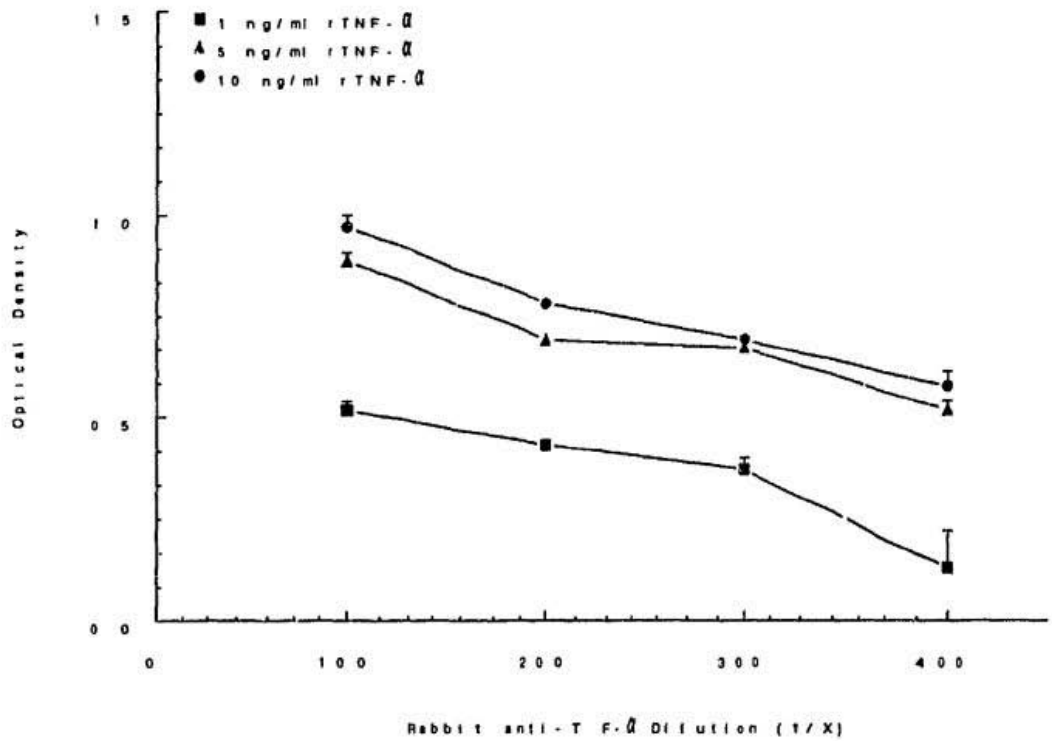


Figure 2.3 : Titration of the secondary antibody, rabbit anti-TNF-α, against 3 concentrations of *r*TNF-α, 10 ng/ml (●), 5 ng/ml (▲), and 1 ng/ml (■).

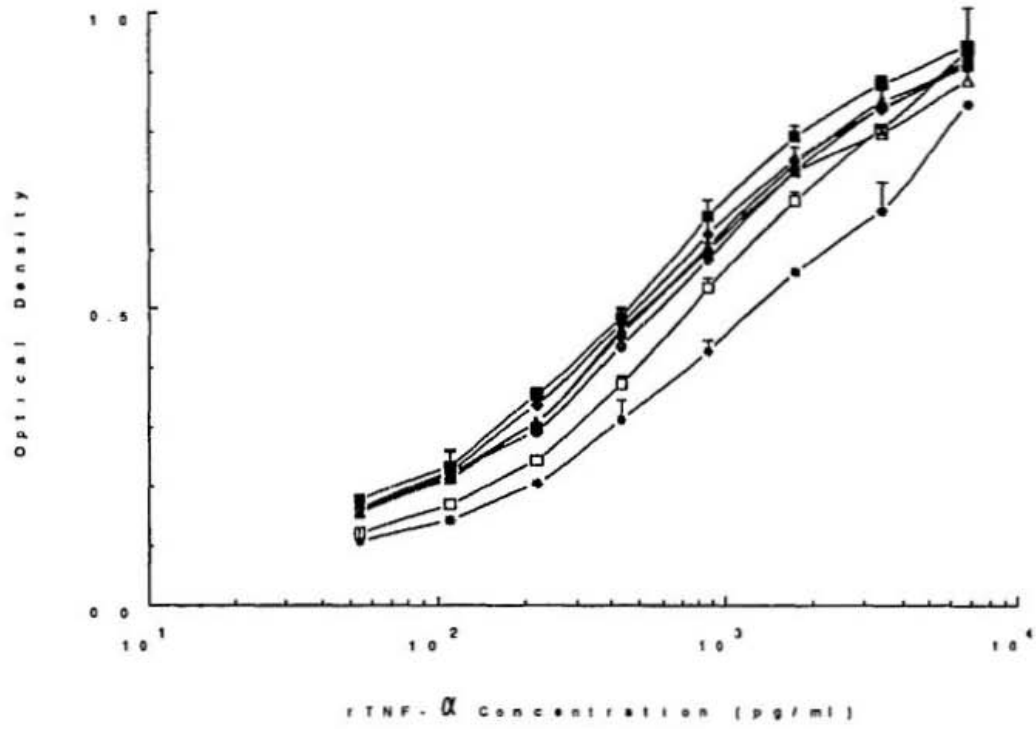


Figure 2.4 : Standard curves from the TNF-α ELISA run at six different times. Standard deviation bars show the standard deviation for duplicate wells.

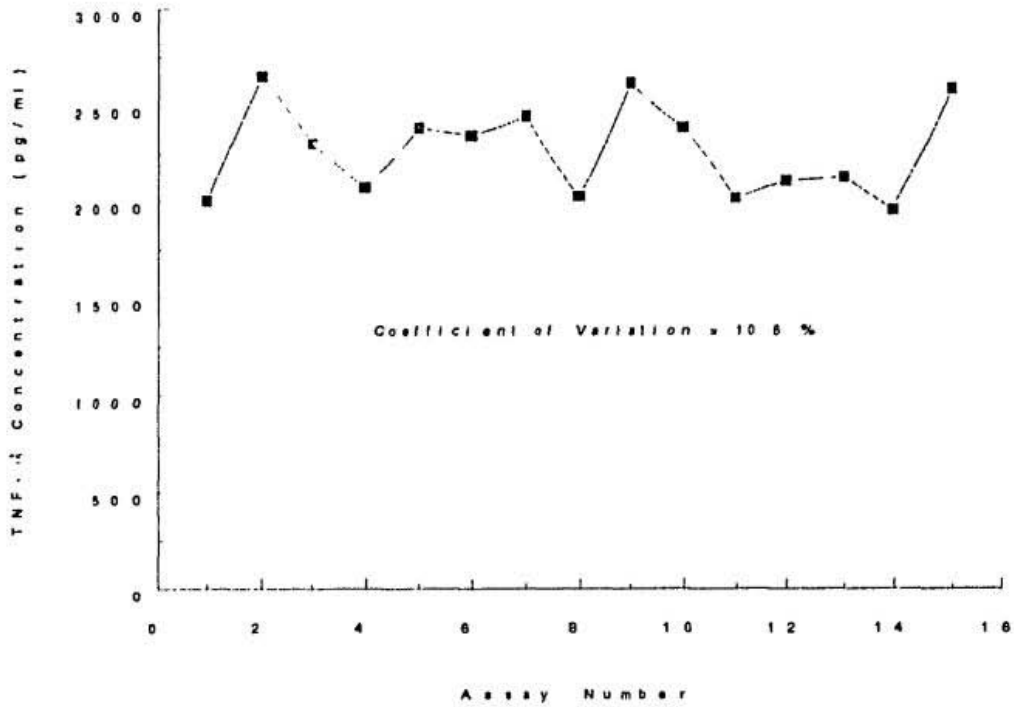


Figure 2.5 : Variation between runs of the TNF-α ELISA. The data are derived from running a sample of 2500 pg/ml rTNF-α each time and then calculating the concentration from the standard curve. These values were then used to calculate the coefficient of variation.

2.2.2 Variation in Secreted TNF-α Levels in vitro

Peripheral blood mononuclear cells from young males show large interindividual differences in the levels of TNF-α secreted when stimulated with 20 μg/ml LPS for 3 hours. The range for the group was 157.7 pg/ml to 3900 pg/ml in a frequency distribution that was skewed to the left (Figure 2.6). The mean for the group is 1213.9 pg/ml with a standard deviation of 673.6.

Because the distribution is skewed to the left, the high secretors stand out. This group is also outside of a population defined by the mean ± 2 x standard deviation (2SD = 1347.2). The low secretors however, are within the limits set by the mean ± 2SD.

2.2.3 Stable Interindividual Differences

The levels of secreted TNF-α in four male subjects that were tested from 2 to 4 times are stable (Table 3). The standard errors were low for SP, LS and DB. DB and VJ were repeated only once. Although the second reading for VJ was almost double the first, the amount of TNF-α secreted is still at the lower end of the range for the panel of males.

Secreted TNF-α levels were not as stable for the females. The standard errors were high for all 8 females tested (Table 4) with a variation range from 28.5% to 100.5%.

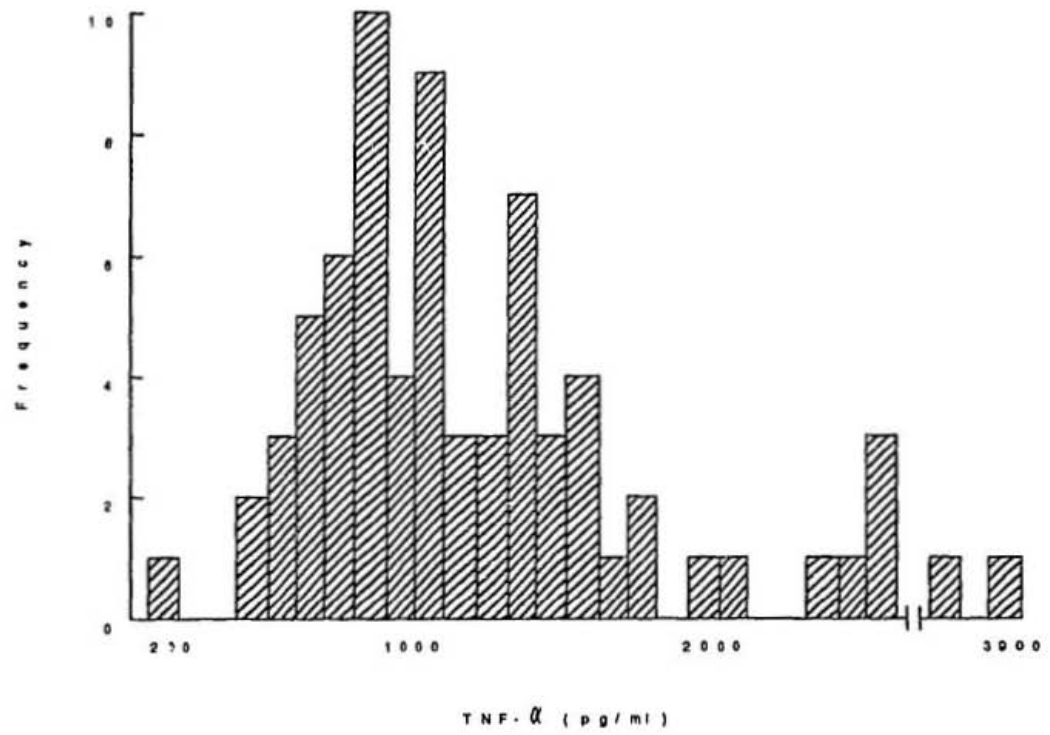


Figure 2.6 : Frequency Distribution of TNF-α Secretion in 72 Healthy Males 20 - 30 Years Old.

Table 3: Stable Intra-individual Levels of TNF-α Secretion by 4 Individuals Tested from 2 to 4 Times.

Subject	TNF-α (pg/ml)				Mean	SD	
	1	2	3	4			
SP	1173	1400	1191	1477	1310.3	151.6	(11.6%)
LS	3358	3567	3000	--	3308.5	286	(8.6%)
DB	1083	1032	--	--	1057	36	(3.4%)
VJ	432	848	--	--	640	293	(44.8%)

Table 4 : Variation in Secreted TNF-α Levels by PBMC from 8 Pre-menopausal Females Tested 3 to 4 times.

Subject	TNF-α (pg/ml)				Mean	SD
	1	2	3	4		
1	1485.5	706.5	711.3	--	967.7	448.4 (46.3%)
2	319.5	677.5	1075.6	--	690.9	378.2 (54.7%)
3	716.8	1507	1788	699	1177.7	554.5 (47.1%)
4	576.2	1627	413.2	62.3	669.7	673.3 (100.5%)
5	1119.5	380.5	648.9	--	716.3	374.1 (52.2%)
6	1187	2563	1644	--	1798	700.8 (39%)
7	424	614.5	669.2	1573	820.2	512.8 (62.5%)
8	870.1	1369	920	1550.5	1177.4	335 (28.5%)

2.2.4 HLA Associations with Secreted TNF-α

HLA-DR data are available for only 49 of the 75 males in the study group. The HLA-B data are nearly complete, with 62 individuals typed. The complete results are shown in Appendix A. The data were analyzed for HLA-DR2, -DR3, and -DR4 individuals (Figure 2.7) and only for those HLA-B groups where $n \geq 5$, or, in other words, only for those HLA-antigens which were present in 5 or more individuals in the sample tested. Eight groups were tested for HLA-B antigens (Figure 2.8). In each case pairwise comparisons were made between levels of TNF-α in, for example, DR2-positive and DR2-negative individuals, HLA-B8 positive and HLA-B8 negative individuals, etc. The Mann-Whitney Test was used for these analyses.

HLA-DR2 was not found to be associated with low TNF-α secretion. The mean TNF-α secreted by HLA-DR2 individuals was higher than that of non-HLA-DR2 individuals (Figure 2.7) but not significantly ($p=0.16$) (Table 5). The data were also analyzed after removing from the analysis the DR2 individuals who were also DR3 or DR4. Again there was no statistical difference between those DR2 individuals and non-DR2 individuals ($p=0.12$).

The HLA-DR3 subjects had lower mean TNF-α secretion than non-DR3 subjects as well as lower than the HLA-DR2 subjects (Figure 2.7). When the DR3-positive and DR3-negative subjects are compared by the Mann-Whitney test, the difference between the two groups is significant ($p=0.014$) (Table 5

). The probability remained barely significant after correction for the number of comparisons made in the HLA-DR analysis (0.014 x 3 comparisons = 0.042). The results suggest that individuals with the HLA-DR3 antigen show less variability and lower levels of TNF-α secretion than other individuals in the sample. However, the number of HLA-DR3$^+$ individuals is relatively small (n=10) and there was only one HLA-DR3 homozygous individual.

There was no association found between HLA-B antigens and levels of TNF-α secretion. HLA-B5 individuals had the lowest mean secreted TNF-α levels but the difference is not significant when compared to the non-B5 individuals (p=0.4) and only 6 of 62 were HLA-B5 positive. HLA-B17 individuals had the highest mean secreted TNF-α levels but again the difference is not significant when compared to the non-B17 individuals (p=0.44).

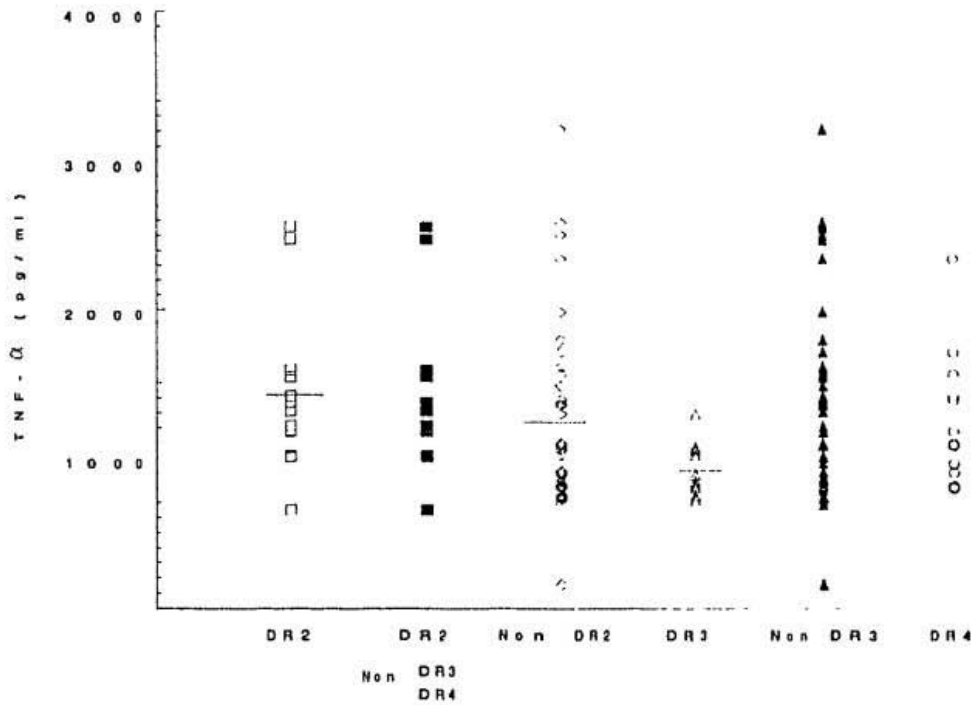


Figure 2.7 : HLA-DR Distribution and TNF-α Secretion in Healthy Males(n=49). Each point represents a single individual and the horizontal bars mark the average TNF-α secretion for the group.

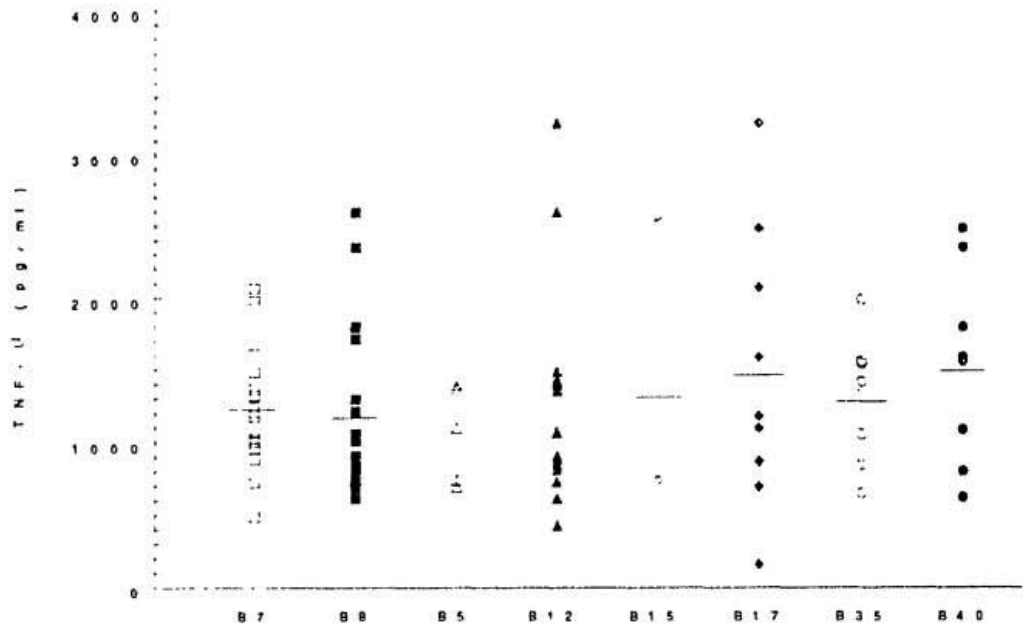


Figure 2.8 : HLA-B Distribution and TNF-α Secretion in Normal Males (n=62). Each point represents a single individual and the horizontal bars mark the mean TNF-α secretion for the group.

Table 5: Analysis of HLA-DR associations with secreted TNF-α from healthy males

HLA Class II	Number of Subjects	Mean TNF-α (pg/ml)	Std.Dev.	Mann-Whitney p value
DR2 (non 3 or 4)	11	1446	590	p=0.16
Non DR2	36	1244	637	
DR2	13	1423.5	543.8	p=0.12
DR3 (non DR2)	10	927	179.5	p=0.014
Non DR3	39	1389.7	647.3	

2.3 Discussion

This chapter has shown that there is variability in levels of TNF-α secreted by PBMC from young males under the experimental conditions used and that there is a possible association of low TNF-α secretion and HLA-DR3. The levels of TNF-α secreted are relatively stable in five of the males that were tested more than once.

2.3.1 Inter-individual Variability in Secreted TNF-α Levels

The results of this analysis has confirmed those of Jacob et al. (1990) and Molvig et al. (1988) that males have stable interindividual differences in TNF-α secretion, although experimental conditions were different in the three sets of experiments.

Jacob et al. (1990) arbitrarily divided their panel of subjects into three groups, high, medium and low TNF-α secretors. Sachs et al. (1990), however, in PHA stimulated PBMC experiments said that for TNF-α levels, subjects could not be statistically divided into high and low secretor groups. They had used the standard skewness test for normality and then the likelihood ratio test to see if two populations, high and low secretors, existed.

This would imply that there are not two groups, high and low secretors, but simply a wide variation about the mean. An individual, therefore, on the high or low end of the distribution would stay that way when sampled again. This variability in

the distribution could be due to (i) differences, or polymorphisms in the TNF-α gene which affect levels of expression, (ii) differences in secretion, (iii) differences in kinetics which a single time sample would not detect, (iv) differences in relative numbers of TNF-α secreting cells, or (v) differences in the rate of disappearance of TNF-α from the supernatants by binding to receptors on cells or degradation.

2.3.2 Stability of TNF-α Secretion by Males

Healthy young males were chosen as the study panel for TNF-α secretion because the cells would not be influenced by cyclical hormonal changes. It has been recently reported that TNF-α secretion in vitro by PBMC from pre-menopausal females has been shown to fluctuate when sampled randomly while TNF-α secreted by males does not (Jacob et al. 1990). The results of our analysis confirm those of Jacob et al. (1990) that TNF-α secreted by PBMC from males is stable but TNF-α levels from pre-menopausal females fluctuate when tested weekly throughout a 4 week period.

2.3.3 Possible HLA Associations with Secreted TNF-α Levels

Associations between varying levels of secreted TNF-α and HLA alleles would suggest a polymorphism or polymorphisms in the TNF-α gene or promoter regions that affects TNF-α expression. Because the TNF-α gene is found within the MHC region one might expect these polymorphisms to be inherited along with

the particular HLA allele. If this were the case most individuals with the same HLA alleles might be expected to secrete approximately the same amount of TNF-α if there were no other factors influencing TNF-α secretion.

The lack of association between HLA-DR2 and low TNF-α is contradictory to some previous reports. Jacob et al. (1990), Brendtzen et al. (1988) and Molvig et al. (1988) all report of finding an association between HLA-DR2 and low TNF-α secretion in males. High TNF-α secretion was also associated with HLA-DR3 and DR4 in one of the reports. However, a second paper by Molvig et al. (1990) did not find any associations with HLA-DR alleles and TNF-α secretion when purified monocytes instead of PBMC were used and Sachs et al. (1990) did not find any associations when PBMC were used. Molvig did suggest that the lack of association with HLA-DR2 may be due to the small number of DR2 positive individuals in that study (n=5). On the other hand they say that the DR2 and DR4 homozygous individuals had very similar TNF-α and IL-1β secretory levels and the DR2,4 heterozygous individuals had the lowest secretion levels. This finding also contradicts the Jacob data. They suggest that DR3 and DR4 are high secretors and are dominant. In the Jacob data most DR2,3 and DR2,4 heterozygous individuals were high secretors.

No studies, including this one, have demonstrated associations between HLA-B and TNF-α secretion. This lack of association of HLA-B alleles with TNF-α secretion is interesting because the HLA-B locus is closer to the TNF-α locus than

is HLA-DR. One might expect a greater degree of linkage disequilibrium between HLA-B alleles and putative TNF-α gene variants than between HLA-DR and TNF-α. One possibility, so far without evidence, is that another gene or regulatory region near the HLA-DR gene is involved in the regulation of the TNF-α gene.

If particular HLA-DR alleles are associate with putative high/low TNF-α expression, one might expect to find markers at or near the TNF locus. There is in fact a NcoI RFLP at the TNF locus that is diallelic, a 5.5 kb allele (TNFB*1) and a 10.5 kb aliele (TNFB*2) (Pociot et al. 1991; Fugger et al. 1989). Pociot and collegues have shown that TNF-α secreted from LPS stimulated monocytes is lowest in TNFB*1 homozygous individuals and highest in TNFB*2 homozygous individuals. The heterozygous individuals had intermediate secretion levels. Both Jacob and Pociot however, could find no association when unfractioned PBMC were stimulated. An interesting point about the NcoI RFLP is that the high secretor TNFB*2 allele is associated with HLA-DR2 and TNFB*1 with HLA-DR3.

More recently, microsatellite mapping has allowed for the detection of a greater number of variants in the TNF genomic region. Three polymorphic microsatellite regions have been identified : TNFa and TNFb 3.5 kb towards HLA-B from TNF-β and TNFc in the first intron of TNF-β (Nedospasov et al. 1991). Associations have been reported between alleles of the TNFa region and TNF-α secretion. In a study where monocytes were stimulated with 250 pg/ml LPS for 20 hours the strongest associations were between the TNFa2 allele and high TNF-α

secretion and between the TNFa6 allele and low secretion (Pociot et al. 1993).

TNFa data are available for the male study group (Michelle Simms, personal communication). Eight of the ten DR3 individuals are TNFa2, the "high secretor" associated allele in the Pociot study. However, TNFa2 also occurs in 16/39 of the DR3-negative individuals tested.

The use of cells from individuals homozygous for both the DR and microsatellite alleles would probably provide a better understanding of associations with TNF-α expression. Using heterozygous individuals is problematic because if there are linkages of TNF-α gene expression polymorphisms to HLA alleles and microsatellite variants, it is difficult to ascertain the contribution that each TNF-α gene variant may make to the total amount of TNF-α secreted. A low responder gene may be masked by a high responder gene.

The associations between TNF-α secretability and genetic markers near the TNF-α gene may not be a simple matter of polymorphisms of the TNF-α gene. From cell stimulation to the time the secreted protein is measured there are many steps that can affect the amount of protein that is detected in the supernatant. After LPS stimulation, a receptor is first activated and a signal sent to the nucleus to activate the TNF-α gene to transcribe mRNA. This is followed by production of a polypeptide which is transported to the membrane, cleaved and secreted. Genes located in or near MHC genes could control some of these events. The association of HLA-DR alleles and lack of association with HLA-B alleles with TNF-α secretion

might, in fact, point to factors other than a variant of the TNF-α gene.

Another factor that cannot be overlooked, especially in long term cultures of PBMC, is the rate of TNF-α degradaticn. TNF-α is degraded or removed from the supernatants by binding to TNF receptors. The amount of TNF-α detected in the supernatants at a given time is a function of both secretion and removal.

One potentially important observation about the published investigations is that no two experimental systems were exactly alike. There were differences in the type of cells used, choice of stimulants and incubation times. The different incubation times and the use of different stimulants may contribute to the different levels of TNF-α secretion observed. We do know for example, that LPS and PMA activate cells in different ways to produce TNF-α. LPS requires a receptor while PMA does not. It is possible that the kinetics of secretion varies from individual to individual and likely that the kinetics of secretion is affected by the type and quantity of stimulant used to induce TNF-α secretion. Experiments in the next section were designed to address some of these questions.

Chapter 3

Kinetics of TNF-α Secretion

PBMC from five individuals with different TNF-α secretion levels as defined using the experimental protocol in Chapter 2 were selected for further study. The patterns of TNF-α secretion over time were investigated using three different stimulation protocols; 20 μg/ml LPS, 1 ng/ml LPS and 1μg/ml Concanavalin A plus 2 ng/ml PMA.

3.1 Methods and Materials

3.1.1 Selection of Subjects

Five male subjects were chosen from the study group in chapter 2. They were chosen to represent high, medium and low TNF-α secretors based on the 2 hour, 20 μg/ml LPS stimulation of PBMC as described.

3.1.2 Preparation of Cells

PBMC were isolated from fresh blood as described in chapter 2.

3.1.3 Induction of TNF-α Secretion

Stimulation of the PBMC was the same as described in section 2.1.3 with the following changes. Three different stimulants were used to induce of TNF-α secretion. LPS was used at 20 μg/ml and at 1 ng/ml and a mixture of 1 μg/ml Concanavalin A (Sigma, St. Louis, MO) with 2 ng/ml PMA (Gibco/BRL, Burlington,

ON). The cells were stimulated under these conditions for 3, 6, 9, 24, and 53 hours.

3.1.4 Determination of Secreted TNF-α Concentration

TNF-α was measured in the supernatant of each experimental and control sample using the TNF-α ELISA described in section 2.1.2.

3.2 Results

3.2.1 Kinetics of TNF-α Secretion for 3 Different Stimulation Conditions

The kinetics of TNF-α secretion differ for each of the stimulation protocols used. Stimulation with 20 μg/ml LPS resulted in peak secreted TNF-α at 3 to 6 hours and declined thereafter until the end of the experiment at 53 hours (Figure 3.1). For 3 subjects, at 53 hours the TNF-α levels had dropped to <100 pg/ml, near the sensitivity limit of the ELISA. Stimulation with 1 ng/ml LPS resulted in a peak TNF-α level at 3 hours which had started to decline by 6 hours (Figure 3.2). By 53 hours all subjects were secreting <100 pg/ml. Stimulation with ConA/PMA shows a contrasting pattern of TNF-α secretion to that of LPS. TNF-α levels were lowest at 3 hours and continued to increase until the end of the experiment at 53 hours (Figure 3.3).

Although the secretion patterns of TNF-α are similar when TNF-α secretion is induced by 20 μg/ml and 1 ng/ml LPS, there are several differences. At 1 ng/ml

LPS, the maximum amount secreted is less than with 20 μg/ml LPS. The maximum with 1 ng/ml LPS was 1050 pg/ml TNF-α but 4900 pg/ml TNF-α with 20 μg/ml LPS. Also, the highest is at 3 hours or earlier with 1 ng/ml LPS but at 3 to 6 hours for stimulation with 20 μg/ml LPS.

3.2.2 Differences in Classification of "high" and "low" TNF-α Secretors under Different Stimulation Conditions

Although all 5 subjects have very similar TNF-α secretion patterns within each of the stimulation conditions, they differ in the relative amounts of secreted TNF-α under different stimulation conditions. With 20 μg/ml LPS for 3 hours, LS secretes approximately 3 times more TNF-α than SP or DB (Figure 3.4). However at 1 ng/ml LPS for 3 hours, LS secretes less than half the amount of TNF-α as SP and DB. LS has an 87% reduction in the amount of TNF-α secreted when the lower LPS concentration is used. DB however, has no reduction and SP drops by 30%.

A similar situation is seen when 20 μg/ml LPS for 3 hours is compared with ConA/PMA stimulation for 53 hours (Figure 3.5). With 20 μg/ml LPS, LS secretes 3 times more TNF-α than SP or DB. With ConA/PMA however, both SP and DB secrete more TNF-α than LS. SP and DB have a 4-fold increase in the amount of secreted TNF-α with ConA/PMA stimulation compared with 20 μg/ml LPS stimulation but there is no increase for LS.

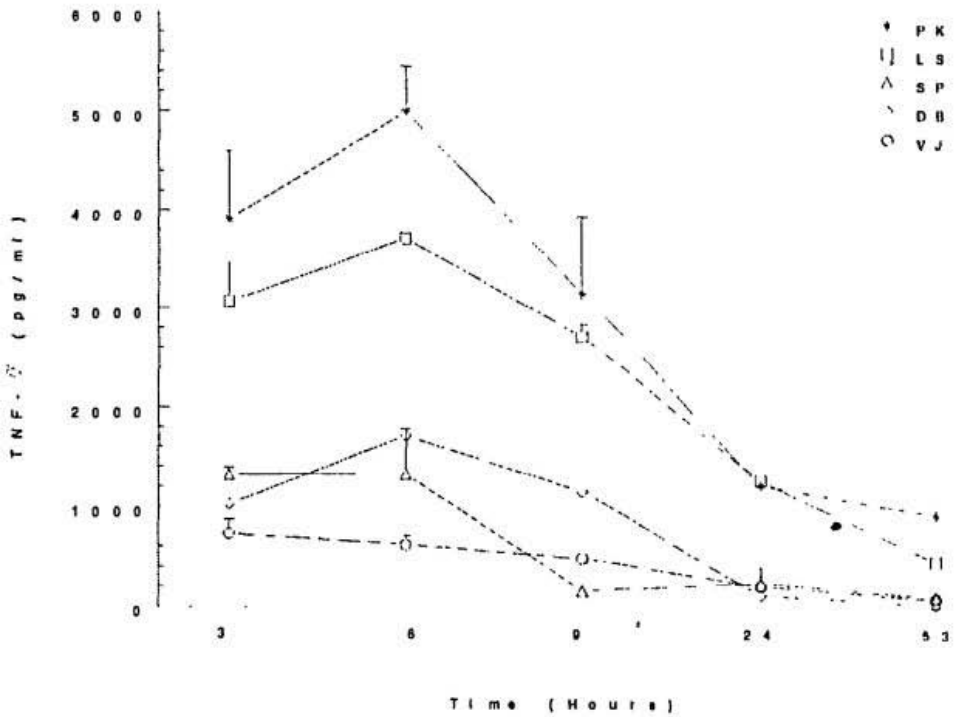


Figure 3.1 : Kinetics of TNF-α Secretion by PBMC from 5 Healthy Males. Cells were stimulated with 20 μg/ml LPS

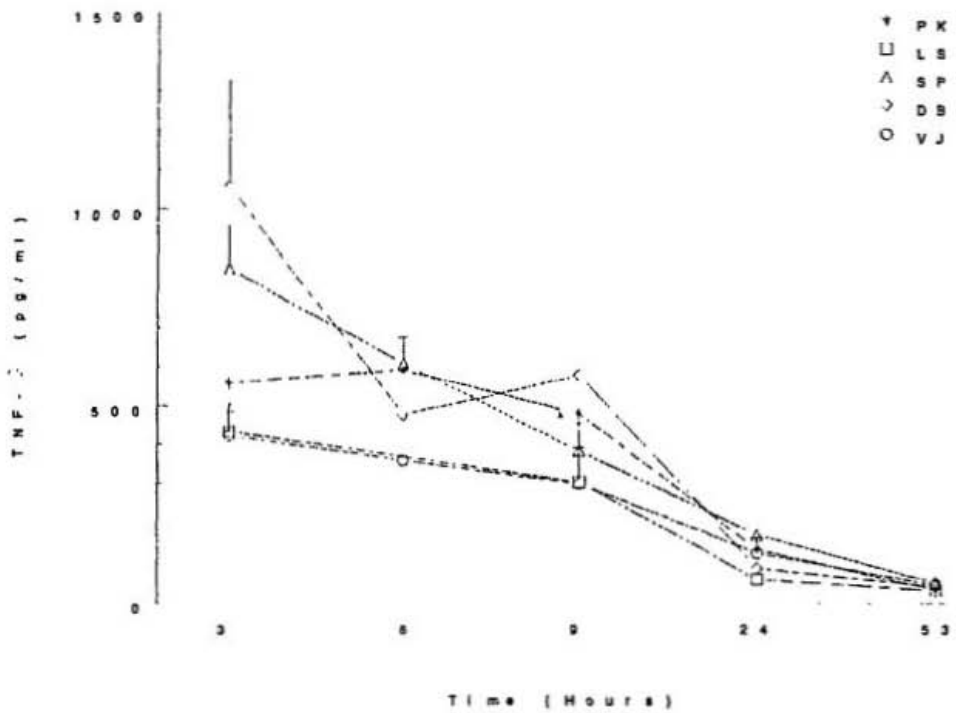


Figure 3.2 : Kinetics of TNF-α secretion by PBMC from 5 Healthy Males. Cells were stimulated with 1 ng/ml LPS

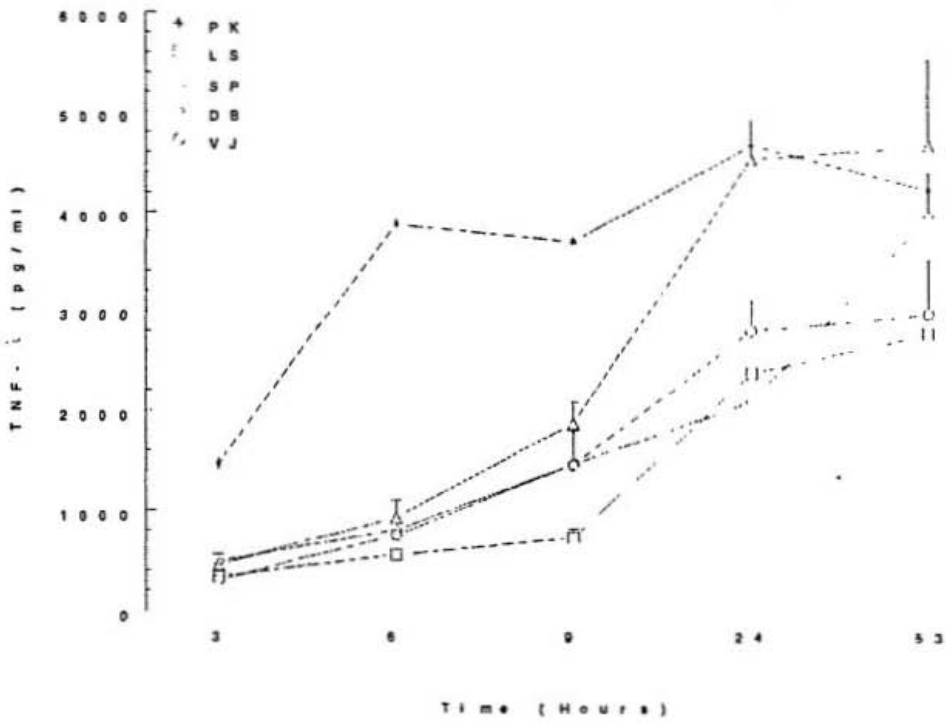


Figure 3.3 : Kinetics of TNF-α Secretion by PBMC from 5 Healthy Males. cells were stimulated with 1 μg/ml Con A + 2 ng/ml PMA

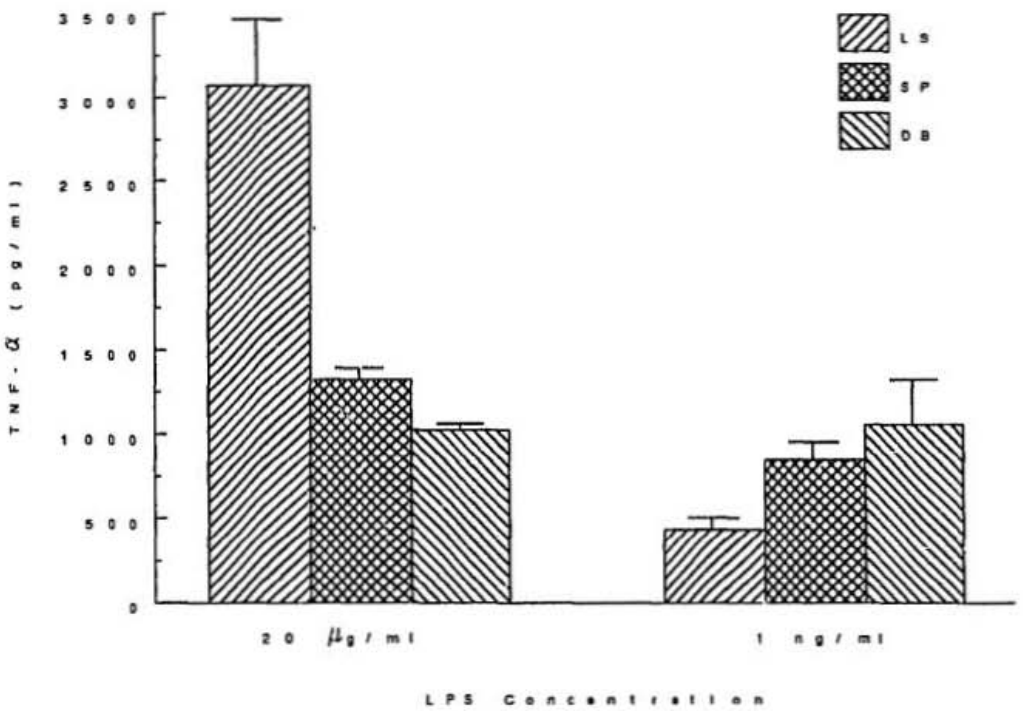


Figure 3.4 : Comparison of the effects of different concentrations of LPS on TNF-α secretion by PBMC from 3 males. Stimulation was for 3 hours.

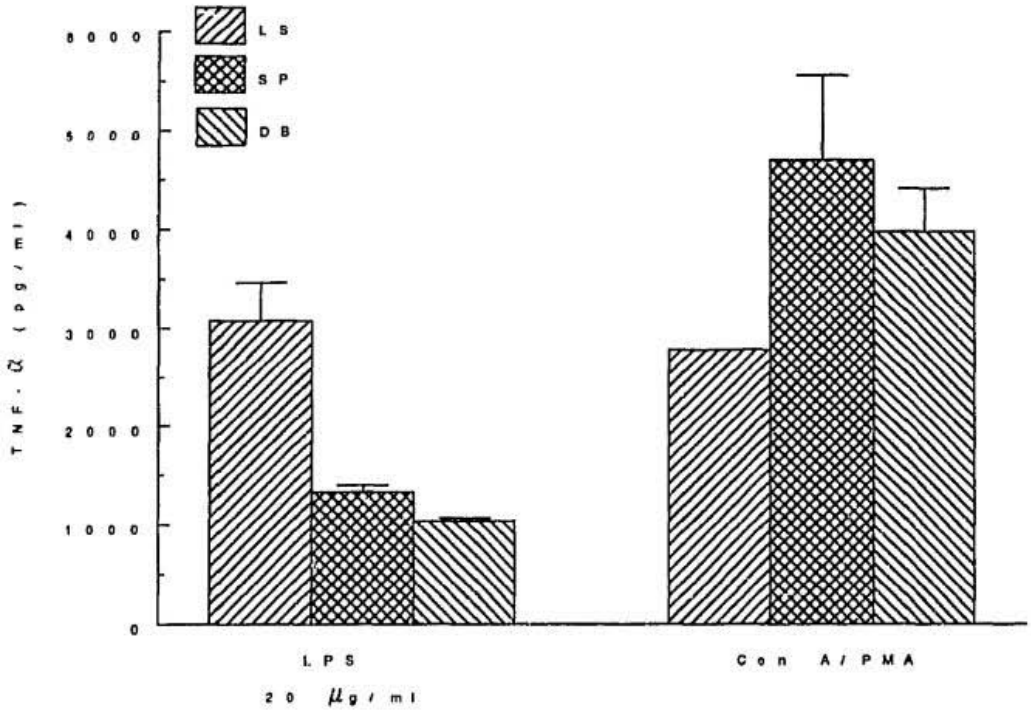


Figure 3.5 : Relative differences in levels of TNF-α secretion by PBMC from 3 males when stimulated with 20 μg/ml LPS for 3 hours and ConA/PMA for 53 hours

3.3 Discussion

3.3.1 Kinetic Differences

The kinetics of TNF-α secretion were investigated for several reasons. First, to determine, if under a given stimulation protocol, cells from all individuals reached peak TNF-α secretion levels at approximately the same time. This would answer the question as to whether "high" or "low" secretor individuals actually produce the same amount of TNF-α but reached their maximum levels at different times. Secondly, LPS and PMA have been used by many investigators to induce TNF-α secretion by different cell types. Jacob et al. (1991) used Con A/PMA to stimulate TNF-α secretion and to define associations between TNF-α secretion levels and HLA-DR alleles. Differences in experimental conditions may explain the conflicting results obtained by others as discussed in Chapter 2. Thirdly, there has been some criticism in the literature about the use of high concentrations of LPS because levels as high as 20 μg/ml are unlikely to be reached in vivo even in pathological conditions. The kinetics of two concentrations of LPS were compared to determine if LPS dose affected individual differences in TNF-α expression.

It is quite clear that the kinetics of TNF-α secretion are very similar among the 5 subjects for each the 3 stimulation protocols. The amounts of TNF-α secreted by different subjects differ but the pattern of secretion during the 53 hours is the same. For a given stimulation protocol they all peak at the same time or decline at the same time.

It is important to note that, although these experiments were designed to monitor the kinetics of TNF-α secretion, and this language is used throughout this Chapter, the ELISA measures TNF-α detectable in the cells' supernatants after stimulation. As described in Chapters 1 and 2, the amount detectable at any time represents a balance between TNF-α secreted and TNF-α degraded or removed by TNF-α receptors on the cells or in solution. Thus "kinetics of TNF-α secretion" is a convenient, but not totally accurate, expression of what is actually happening in the experiment.

The highest level of TNF-α using 20 μg/ml LPS was found at 3 to 6 hours and using 1 ng/ml LPS it was at 3 hours for all 5 subjects tested. Lonnemann et al. (1989) also reported peak LPS induced TNF-α secretion at 3 - 6 hours but for purified monocytes, not PBMC. This would suggest that the primary source of TNF-α in our LPS experiments is monocytes. There are no reports of TNF-α secretion by T- or B-lymphocytes stimulated with LPS. One can only speculate as to the role the lymphocytes may play in the accumulation of TNF-α in the supernatants. Lymphocytes are known to express TNF receptors and probably contribute in the removal of TNF-α from the supernatants.

The kinetics of TNF-α secretion are very similar for both concentrations of LPS tested. The main difference is the amount of TNF-α secreted. With 20 μg/ml LPS the highest levels found are more than four times the highest levels with 1 ng/ml LPS. Molvig et al. (1988), using LPS concentrations from 12.5 pg/ml to

0.25 μg/ml to stimulate monocytes and harvesting supernatants at 20 hours, did not observe any increase in secreted TNF-α levels with LPS concentrations over 500 pg/ml. Lynn et al. (1993) on the other hand, using the monocyte cell line THP-1, reported a continued increase in secreted TNF-α levels with increasing LPS concentrations as high as 10 μg/ml, which was the highest concentration tested. Because our data are for only two LPS concentrations we do not know if some concentration lower than 20 μg/ml LPS, but higher than 1 ng/ml would stimulate maximum TNF-α secretion. However, it should be noted that our lowest concentration of LPS was two times higher than the highest above which Molvig did not observe an increase in secreted TNF-α levels.

It was somewhat surprising to find the 20 hour LPS stimulations in the literature that describe TNF-α variation and HLA associations in light of our kinetics data as well as that published by Lonneman et al. (1989). At 20 hours, particularly for low LPS concentrations, most of the TNF-α has diminished to the point where there is very little variation among the individuals tested.

LPS activates monocytes to secrete TNF-α by binding to specific receptors on the cell surface. The differences in amounts of TNF-α secreted under stimulation with different concentrations of LPS may be due to differences in the number or type of receptors being bound by LPS. For maximum TNF-α secretion, receptors may have to be saturated with LPS.

The difference in kinetics between LPS stimulation, regardless of

concentration, and ConA/PMA stimulation is quite dramatic. LPS stimulations result in rapid TNF-α release with rapid disappearance from the supernatants. ConA/PMA stimulations result in slow TNF-α release at 3 hours which increases to the end of the experiment at 53 hours.

LPS binds LPS-receptors and these receptors are found on monocytes. PMA does not bind receptors but instead initiates cellular activity by activating protein kinase C directly and therefore can potentially induce TNF-α secretion in a broad range of cells. Thus, TNF-α induction by LPS and PMA may be two different phenomena affecting different populations of cells. We do know that both monocytes (Hass et al. 1991) and lymphocytes (von Fliedner et al. 1992) can be induced by PMA to secrete TNF-α and the secretion can be sustained for 48 - 72 hours.

There may even be different regulatory factors in different cells that may account for the differences in TNFα secretion between LPS and ConA/PMA. A recent review (Rao 1994) discussed a transcription factor, NF-AT, that is activated by increase calcium fluxes. This factor is found in T cells and activates the TNF-α gene as well as other cytokine genes. Crosslinking TCR/CD3 and Fcγ receptors leads to calcium mobilization in lymphocytes. LPS activation of monocytes through CD14 however, is mediated through protein kinase C, not calcium fluxes. NF-AT is also found in monocytes but its existence opens up the possibility of the existence of a number of regulatory factors that may influence TNF-α secretion

differently depending on the individual and the stimulant.

The addition of Con A to the culture with PMA results in increased TNF-α secretion over PMA alone (Jacob et al. 1990). This adds to the likelihood that lymphocytes are also producing TNF-α or contributing in some way to the production of TNF-α. Con A is a known T-cell mitogen, stimulating the proliferation of T-cells. If T-cells are proliferating in the culture the increased TNF-α could be due to an increase in the number of cells secreting TNF-α.

The kinetic differences between LPS and ConA/PMA induced TNF-α secretion may also be attributed to the involvement of LPS receptors in another way. LPS receptors may become unresponsive after prolonged LPS exposure. This would mean that when LPS first binds the receptor there is a signal generated to produce TNF-α. After prolonged exposure the cell becomes LPS tolerant in that the signal transduction pathway shuts down and TNF-α production stops even with continued exposure to LPS. PMA however, by by-passing the receptor and activating PKC directly can generate the signal to continually produce TNF-α. In fact, it has been shown that peripheral blood monocytes become unresponsive to LPS after prolonged exposure but these LPS-unresponsive cells are not unresponsive to PMA (Matic & Simon 1991).

PMA also downregulates the expression of TNF receptors on cell surfaces (Gatanaga et al. 1991). These receptors bind TNF-α to the cell or can be shed into the medium and bind TNF-α there. The result in either case is a loss of TNF-α

from the supernatants. If the number of receptors that can bind and remove TNF-α from the supernatants is reduced, there would be an expected increase in the amount of TNF-α accumulating in the supernatants.

We do not know if the antibodies used in the ELISA to detect TNF-α can bind TNF-α-receptor complexes. The disappearance of TNF-α from the supernatants from 6 - 53 hours suggest they do not because if the antibodies do bind TNF-α-receptor complexes then shed receptors would have no affect on the detection of TNF-α in the supernatants. This can be tested because TNF-α receptors, known as TNF-α binding protein, are now available commercially. TNF-α-receptor complexes could be prepared and tested in the TNF-α ELISA.

3.3.2 Differences in relative amounts of TNF-α secreted among individuals

The differences in amounts of TNF-α secreted under different stimulation conditions by different individuals relative to each other was quite surprising. It was assumed that if an individual was a "high" secretor relative to another using a given inducing agent, it would also be true with a different inducing agent. The "high" and "low" secretors in the work by Jacob et al. (1991) and Molvig et al. (1988) may very well only exist under those conditions.

The change in the amount of secreted TNF-α by LS relative to SP and DB at the lower LPS concentration resulted in LS becoming a low secretor relative to SP and DB. There is very little change for SP and DB. LS also becomes a lower

secretor than SP and DB when ConA/PMA for 53 hours is used to induce TNF-α secretion. In this case LS does not change compared with the 20 μg/ml LPS stimulation for 3 hours but instead SP and DB have increased levels. It should be noted that the ConA/PMA 53-hour protocol is similar to the one used by Jacob et al. (1991).

The reason why one individual may be a "high" secretor with one LPS concentration but be a "low" secretor relative to the other individuals when stimulated with a different LPS concentration may be due to individual differences in the types and numbers of LPS receptors. CD14 appears to be the primary LPS monocyte receptor that binds LPS under low LPS concentrations to induce TNF-α secretion. Under high LPS concentrations other LPS receptors may play an important role in TNF-α induction (Lynn et al. 1993). These receptors may be low affinity receptors and require higher LPS concentrations. Thus, at high LPS concentrations, both CD14 and other, low affinity, receptors may be activated. One might speculate that LS, for example, who is a relatively high producer at a high a LPS concentration but low at a low LPS concentration has lower levels of CD14 expression and/or higher expression of other low affinity receptors than some of the other individuals in the sample.

The differences that exist when comparing LPS and ConA/PMA stimulations may occur for a number of reasons. One is the initial proportion and the Con A-induced proliferation of T-cells in the 53 hour ConA/PMA cultures. The PBMC of

SP and DB may contain a relatively higher proportion of TNF-α-producing T-cells than LS. Another possibility could be a greater degree of downregulation of TNF receptors by PMA for SP and DB. If the cells were expressing fewer receptors less TNF-α would be removed from the supernatants allowing for a greater accumulation. The experiments in Chapter 4 were designed to further investigate the first of these possibilities, that differences in levels of TNF-α accumulating in the PBMC supernatants arise from differences in the relative numbers of different sub-populations of cells.

Chapter 4

Cell Phenotypes and TNF-α Secretion

This chapter describes two sets of data collected by flow cytometry. The first describes a double labelling flow cytometry protocol, and the development of the protocol to determine cytoplasmic TNF-α and cell phenotype simultaneously. The second is differential PBMC phenotype counts of the male study group to determine if there were any correlations between the number of cells of a specific phenotype and the levels of TNF-α induced by PBMC stimulations.

4.1 Methods and Materials

4.1.1 Reagents

4.1.1A Fixatives

(1) 0.5 % Paraformaldehyde

500 mg paraformaldehyde (BDH, Toronto)
100 mls PBS
Paraformaldehyde is dissolved by heating the solution

(2) 4 % Paraformaldehyde, pH 7.2-7.4

4 g paraformaldehyde
100 mls PBS
Dissolve paraformaldehyde by heating
Adjust pH to 7.2 - 7.4 with sodium hyroxide
Store at $4^{0}C$

4.1.1B Antibodies

(1) Purified Normal Goat Ig (Cedarlane, Mississauga)

(2) Polyclonal Rabbit anti-keyhole limpet hemocyanin (Sigma, St. Louis)

(3) Polyclonal rabbit anti-TNF-α (serum) (Gift from Dr. Steve Foster, ICI Parmaceuticals, Cheshire, England)
Purification described in section 4.1.2

(4) Purified Monoclonal anti-TNF-α conjugated with FITC (R & D Systems, Minneapolis)

(5) Purified Polyclonal Rabbit anti-TNF-α (Calbiochem, Palo Alto)

(6) Purified Polyclonal Rabbit anti-TNF-α (Bio-Design, Maine)

(7) Anti-CD14 (AMAC, Mississauga)
Binds CD14, a surface molecule on monocytes

(8) Anti-CD8 (AMAC, Mississauga)
Binds CD8, a surface molecule on cytotoxic T-cells

(9) Anti-CD4 (AMAC, Mississauga)
Binds CD4, a surface molecule on helper T-cells

(10) FITC conjugates of anti-CD14, anti-CD4, anti-CD8, anti-CD3 (T cells) and anti-CD45RO (Memory Helper T-cells) were purchased from Sigma, St. Louis. These antibodies were used for differential cell counts.

(11) Phycoerythrin-conjugated goat anti-rabbit Ig (Southern Biotechnology, Alabama)

(12) Phycoerythrin-conjugated goat anti-mouse Ig (Southern Biotechnology, Alabama)

4.1.1C Other Reagents

Monensin was purchased from Sigma, St. Louis. Saponin was purchased from ICN, Mississauga.

4.1.2 Purification of Rabbit anti-TNF-α

The IgG component of the serum provided by Dr. S. Foster was purified with the Affi-Gel Protein A MAPS II kit (Bio-Rad, Mississauga, ON). The protocol followed was as outlined in the instruction leaflet provided.

A 1 cm x 10 cm Econo-Column chromatography column (Bio-Rad) was packed with 1 ml of the Affi-Gel Protein A agarose beads. One millilitre of bead solution with 2-3 ml of binding buffer (provided as part of kit) was added to the column with the flow turned off until the beads settled to the bottom. To wash and equilibrate the beads, the flow was turned on and more binding buffer was added to the column with a Pasteur pipet to maintain a constant flow rate and to prevent the beads from drying out. The pH of the effluent was monitored until it reached the pH of the binding buffer (pH 9.0). Approximately 5-6 ml of binding buffer had been added.

A 1 ml aliquot of the serum sample was diluted with 2 ml of binding buffer and applied to the column. The effluent was collected in a tube and reapplied twice more to the column. After sample loading, the column was washed with binding buffer, approximately 15 ml, until the protein concentration reached baseline. The protein concentration in the effluent was monitored with a Bio-Rad Econo-System UV monitor (260 nm) and chart recorder.

Bound IgG was eluded using the elution buffer provided with the kit. Elution buffer was added slowly to the column and the protein content in the effluent

carefully monitored. At the moment protein was detected in the effluent it was collected in a separate tube until the protein content started falling to baseline. Approximately 4 ml were collected. The pH of the elution buffer was very low (pH 3.0) so the sample was collected in 0.5 mls of 1M Tris HCl at pH 9.0 to neutralize the eluate and minimize the time the antibody would be exposed to extremely low pH.

To remove the elution buffer, the sample was dialysed overnight at 4°C against 2 litres of PBS. The PBS was changed 3 times.

The purified antibody was filter sterilized with a 0.22 micron Millipore filter (Fisher Scientific, Montreal) and the concentration measured with the Bio-Rad Protein Assay Kit (Bio-Rad, Mississauga, ON). The antibody was aliquoted at 0.5 mg/ml and stored at -70°C.

4.1.3 Double Staining for Cytoplasmic TNF-α and Cell Phenotype

The choice of subjects was based on levels of secreted TNF-α determined previously. Two high, one moderate and one low TNF-α secretor were selected based on their willingness to frequently donate blood samples.

4.1.3A Induction of TNF-α

One 10 ml vacutainer tube (Becton Dickinson, New Jersey) with acetate-citrate-dextrose (ACD) as anti-coagulant was used to collect approximately 10 mls

of blood by venipuncture. Peripheral blood mononuclear cells were purified as described in chapter 2 and resuspended at 1 x 10^6/ml in culture medium.

The cells were put into wells of a sterile, 6-well flat bottom Falcon tissue culture plate (Fisher Scientific, Montreal). All the cells for each stimulation protocol were put into a single well. For example, if cells stimulated with LPS were to be labelled with four different combinations of antibodies for surface markers and cytoplasmic TNF-α, then 2 million cells were put into one well to be stimulated with LPS. Two million would have been required because the labelling and subsequent analysis were done at 500 000 cells per tube. The plates were then incubated at overnight at 37°C in a CO_2-incubator.

The following day the cells were transferred to 5 ml or 15 ml sterile centrifuge tubes and centrifuged at 800 x g for 10 minutes. Fresh culture medium with 3 μM monensin was prepared both with LPS at 20 μg/ml and without LPS. A stock solution of 3 mM monensin was first prepared by dissolving 10.3 mg monensin in 5 ml absolute methanol and then diluted to 3 μM by adding 10 μl of the stock to 10 ml of culture medium. The old supernatants were discarded by inverting the tubes and the cells were resuspended in the fresh medium, put back into the same wells of the 6-well plate and incubated at 37°C for 1 hour.

Using a rubber policeman sterilized in 95 % ethanol, the wells were scraped to dislodge adherent cells. It was very important to do this because monocytes, the primary TNF-α producers, are adherent to plastic in culture. The cells were then

transferred to sterile 5 ml centrifuge tubes and washed twice with sterile PBS. Washing was done by centrifuging the cells at 800 x g and resuspending in several millilitres of PBS.

In addition, cells were stimulated as described in Chapter 2 and secreted TNF-α measured by ELISA to determine if the cells were producing TNF-α.

4.1.3B Fixation and Permeabilization

After the final wash, the cells were centrifuged and 4 % paraformaldehyde in PBS was added to fix the cells at a concentration of 1 x 10^7/ml on ice for 10 minutes. The paraformaldehyde had been previously chilled to 4°C before use. All subsequent incubations were on ice with pre-cooled reagents and centrifugation in a 4°C refrigerated centrifuge. The cells were washed 3 times with cold PBS.

The cells were permeabilized by incubating the cells for 30 minutes on ice in 1 % saponin in PBS at 1 x 10^7cells/ml with 15 % normal human serum to block any non-specific protein binding sites. Normal human serum (NHS) was derived from several healthy donors, inactivated at 56°C for 30 minutes, pooled and stored at -20°C. The cells were washed 3 times with 0.1% saponin-PBS.

For the last wash, the cells were resuspended in 0.1% saponin-PBS at 1 x 10^6/ml and transferred to a round bottomed 96-well plate. They were transferred at 500 µl/well, each well receiving 500 000 cells. For example, 2 million LPS stimulated cells that would be labelled four different ways would now be equally

distributed among four wells of the plate. The plate was then centrifuged at 800 x g and the supernatant removed by inverting the plate.

Fc receptors (FcR) and any further non-specific binding sites that could bind immunoglobulin were blocked with normal goat immunoglobulin. The cells were incubated with 50 µl of 100 µg/ml goat Ig in 0.1% saponin-15% NHS-PBS for 15 minutes on ice. The cells were washed 3x with 0.1% saponin-PBS by adding 200 µl to each well and incubating for 10 minutes on ice before centrifugation.

4.1.3C Detection of Cytoplasmic TNF-α

Cytoplasmic TNF-α was detected by an indirect method. The cells were first incubated with 100 µl of primary antibody at 15 µg/ml in 0.1% saponin-15% NHS-PBS on ice for 30 minutes. The primary antibodies were polyclonal rabbit anti-TNF-α and polyclonal rabbit anti-keyhole limpet hemocyanin (anti-KLH). The polyclonal rabbit anti-TNF-α was a gift from Dr. Steve J. Foster at ICI Pharmaceuticals, Cheshire, England, and purified as described in section 4.1.2. The anti-KLH antibody was a negative control antibody of the same isotype as the anti-TNF-α antibody and was included for each of the different stimulations, including non-stimulated cells. The cells were washed 3 times, 10 minutes each wash, with 0.1% saponin-PBS.

100 µl of phycoerythrin-conjugated goat anti-rabbit Ig diluted to 1:10 in 0.1% saponin-15% NHS-PBS were added to each well and incubated for 30 minutes on

ice. The cells were washed as before.

4.1.3D Detection of Surface CD14 on Monocytes

After washing with 0.1% saponin-PBS, the cells were washed 3 times with PBS alone on ice. Any residual saponin would keep the cells permeabilized and the antibodies to determine cell phenotype would enter the cytoplasm. Therefore, to ensure that the antibodies only bound surface markers, the saponin was removed and all further incubations with antibodies were done in saponin-free buffer.

Anti-CD14 was used diluted 1:10 in 100 µl of PBS and the cells incubated on ice for 20 minutes. After washing 3 times with PBS, 100 µl of the secondary antibody, FITC conjugated $F(ab)_2$ goat anti-mouse, was added to each well and incubated on ice in the dark for 20 minutes. After an additional 3 washes with PBS, the cells were transferred from the wells to 500 µl PBS with 0.02% sodium azide in 5 ml polystyrene tubes.

Analysis was done on a Becton-Dickinson FACStar^{Plus} Flow Cytometer with an argon laser operating at 50 mW and with filter settings for FITC (530 nm) and phycoerythrin (585 nm). 10 000 cells were run per sample and the data were collected in list mode and later analyzed with Lysys II software. Ernie Stapleton and Leslie Daye were the flow cytometer operators.

4.1.4 Development of Double Staining Flow Cytometry Protocol

At the onset of this study there was no published method for the simultaneous labelling of cell associated (cytoplasmic) TNF-α and surface phenotype marker for a mixed population of PBMC. In 1989 Hofsli et al. described a method for labelling cytoplasmic TNF-α in purified monocytes for flow cytometry and immunofluorescent microscopic study. Simultaneous labelling of TNF-α and surface antigen to determine phenotype in a mixed mononuclear population excludes the need to manipulate the cells to purify a particular subset, which can affect cell activation. A labelling protocol, therefore, would have to be one that not only fixed and permeabilized the cells but also maintained the integrity of the surface antigens and the forward and side scatter properties of the different subsets. Maintaining forward and side scatter properties of the cells is important in helping to gate certain subsets such as monocytes. This section describes the various methods tested in developing the method of simultaneous labelling of surface phenotype and cell associated TNF-α that was described in section 4.1.2.

4.1.4A Stimulation of Cells for TNF-α Production

In the initial experiments testing the various methods, PBMC were stimulated with 20 μg/ml LPS for 1, 2, and 3 hours. These times were chosen

because it was unknown when peak cytoplasmic TNF-α occurred, and earlier experiments (Chapter 3) showed peak secreted levels at approximately 3 hours after stimulation.

4.1.4B Fixation, Permeabilization and Labelling Methods Tested

The following is a list of methods tested for labelling cytoplasmic TNF-α:

(1) Schmid et al. (1991) described a method of fixation and permeabilization using paraformaldehyde and Tween 20 detergent for staining intracellular antigens and DNA. The stimulated cells were fixed with 0.5 % paraformaldehyde at 4°C for 1 hour then permeabilized with 0.2 % Tween 20 in PBS for 15 minutes. We used the same polyclonal rabbit anti-TNF-α that was used in the TNF-α ELISA (Chapter 2) diluted in PBS followed by goat anti-rabbit-phycoerythrin to label cytoplasmic TNF-α.

(2) Pollice et al. (1992) described a sequential paraformaldehyde and methanol fixation/permeabilization method for flow cytometric analysis of intracellular proteins. We used the same antibodies described above for labelling cytoplasmic TNF-α. In addition, some of the cells were labelled with a mouse monoclonal anti-tubulin (Cedarlane, Hornby, ON) followed by goat anti-mouse-phycoerythrin to test their permeability.

(3) Absolute methanol at -70 C both fixes and permeabilizes cells at the same time (Dr. Alex Szalai, pers. communication). The polyclonal rabbit anti-TNF-

α with GAR-PE as described above and a FITC conjugated monoclonal anti-TNF-α (R & D Systems, Minneapolis) were used to label cytoplasmic TNF-α. The anti-tubulin control was also included.

(4) Late in 1992, de Caestecker et al. (1992) published a two-color immunofluorescence flow cytometric assay for the detection of cytoplasmic IL-1 and TNF-α and surface CD14 expression in whole blood. Instead of using whole blood, we used LPS stimulated PBMC and fixed with 4 % paraformaldehyde and permeabilized with 1 % saponin as described in the paper. The antibodies described above were used.

Dr. de Caestecker suggested (personal communication) trying a number of different polyclonal anti-TNF-α antibodies. Polyclonal rabbit anti-TNF-α antibodies from Calbiochem (Palo Alto, CA), Bio-Design (Maine) and the same antibody Dr. de Caestecker used, which was a gift from Dr. S. Foster, were tested. The antibody from Dr. Foster gave a weak signal (see Results, section 4.2.1) and this method was chosen for further work.

To determine the point at which TNF-α reached its peak in the cytoplasm, PBMC were stimulated with LPS for 30 minutes, 1, 2, 3, 12 and 24 hours and with ConA/PMA for 1, 12, and 53 hours. Cells stimulated with ConA/PMA for longer than 24 hours ruptured either during treatment or during the subsequent washings and therefore could not be stained. Peak TNF-α in the cytoplasm of LPS stimulated cells was at 1 hour but the increase in fluorescence above background

was very low.

To increase the fluorescent signal in weakly fluorescent cells, monensin was added to the culture medium during stimulation (Jung et al. 1993). Monensin interrupts intracellular transport allowing an accumulation of cytokine in the cell.

One disadvantage of monensin was its eventual cytotoxicity. Using propidium iodide (PI) uptake as an indicator of cell death, there was increased cytotoxicity after incubation for 16 hours in monensin and this cytotoxicity increased further with longer incubation periods (see Results, section 4.2.1). This limited any experiments with monensin to less than 16 hours.

4.1.4C Surface Antigen Staining

Surface antigens were stained using the amount of antibody specified by the manufacturer. However, instead of adding the undiluted antibody as the instructions indicated, better staining was achieved by diluting the antibody to a final volume of 100 µl in PBS.

It was important to remove all saponin by washing with PBS before staining surface antigens. When stained in the presence of saponin, background FITC fluorescence was high, making it difficult to gate on cells expressing the surface antigen.

4.1.5 Surface Antigen Staining for Differential Counts of CD14-, CD4-, CD8-,

and CD45RO-positive Cells

To determine the relative numbers of cell subsets for each individual in the entire male study group, PBMC were stained using standard protocols and manufacturers' instructions for each antibody. Briefly, 100 000 cells/test were stained with anti-CD14-FITC, anti-CD4-FITC, anti-CD8-FITC, and anti-CD45RO-FITC for 20 minutes in 5 ml polystyrene tubes (Fisher Scientific, Montreal) in the dark. After 20 minutes the cells were washed with PBS and fixed with 0.5 % paraformaldehyde.

4.1.6 Statistical Methods

To test for correlations between secreted TNF-α levels and the number of cells in specific PBMC subsets, the data were analyzed by least squares linear regression using the statistical software InPlot (GraphPad, San Diego). To allow for comparisons among the different PBMC subsets, only those subjects with data points for each of the PBMC subsets to be tested were included in the analysis. That is, if a person did not have a data point for all four subsets, CD14, CD4, CD8 and CD45RO, then that person was excluded from the analysis.

4.2 Results

4.2.1 Flow Cytometry Methodology

In the initial experiments cells were only stained for cytoplasmic TNF-α. To

ensure that we were not missing a weak signal in the monocyte subset, the cells were analyzed by setting a gate based on size to display the monocyte subset. A "gate" is a term used to mean selecting a particular group of cells and only displaying the data for those cells.

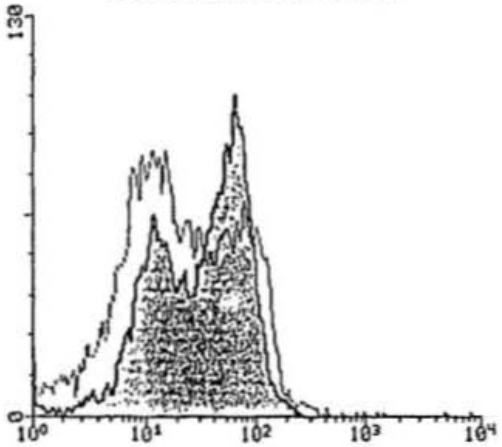
When the cells were stained for cytoplasmic TNF-α using either of the four fixation and permeabilization protocols, there were no fluorescent signals above background (Figure 4.1). However, the cells were permeable to the antibodies in all methods 2, 3 and 4 because they stained positive for cytoplasmic tubulin (Figure 4.2). Method 1, 0.5 % paraformaldehyde + Tween was not tested with anti-tubulin.

Only by using both the fixation method and polyclonal anti-TNF-α that Dr. de Caestecker had used did we get an increase in fluorescence over background. Incubation of the cells with monensin during stimulation increased the fluorescent signal (Figure 4.3). Monensin, however was cytotoxic after incubation for 16 hours and the cytotoxicity increased with longer incubations (Figure 4.4).

Peak cytoplasmic TNF-α was at 1 hour after incubation when 20 μg/ml LPS was used to induce production. Figure 4.5 shows that after 1 hour the number of cells staining positive for TNF-α diminishes. This experiment was repeated once with the same results. No cytoplasmic TNF-α data are available for ConA/PMA stimulations because for incubations longer than 12 hours the cells ruptured during the fixation and permeabilization steps.

Figure 4.1 : Examples of flow cytometry analysis of cytoplasmic staining of TNF-α in monocytes using the fixation/permeabilization techniques that were unsuccessful. A. 0.5 % paraformaldehyde + Tween 20, B. 0.5 % paraformaldehyde + 22.5 % methanol, C. absolute methanol, and D. 4 % paraformaldehyde + saponin. The shaded areas represent cells not stimulated and the open areas represent cells stimulated with 20 μg/ml LPS for 1 hour. The x-axis shows fluorescence and the y-axis shows the number of cells

A.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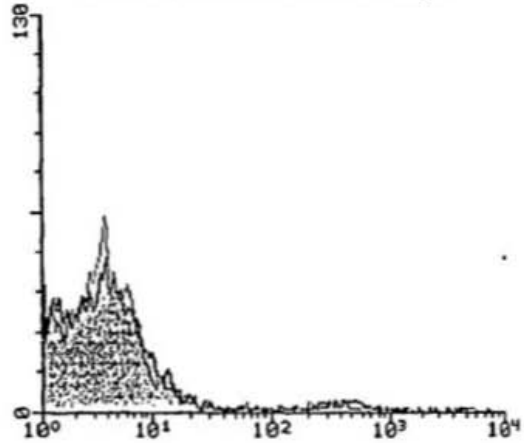
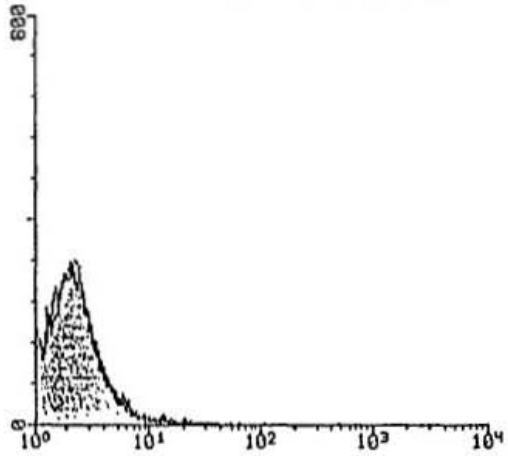
130
0
10^0
10^1
10^2
10^3
10^4

B.


V3:270592A006\FL2-H\FL2-Height
130
0
10^0
10^1
10^2
10^3
10^4

C.


V3:110992A010\FL1-H\FL1-Height
600
0
10^0
10^1
10^2
10^3
10^4

D.

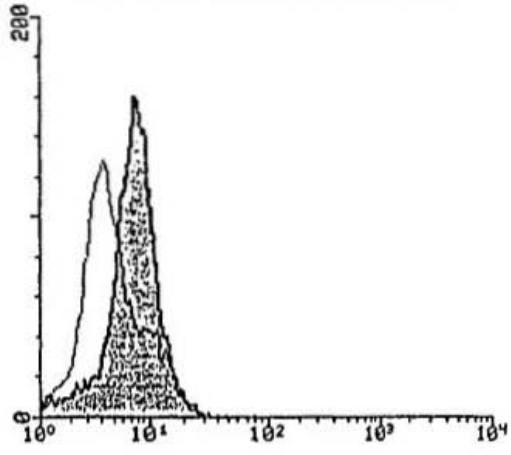
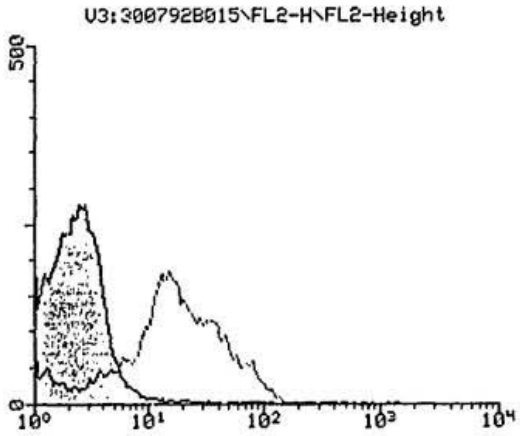

V3:040393A002\FL2-H\FL2-Height
200
0
10^0
10^1
10^2
10^3
10^4

Figure 4.2 : Cytoplasmic tubulin staining in PBMC after fixation and permeabilization with A. 0.5 % paraformaldehyde + 22.5% methanol, B. absolute methanol and C. 4 % paraformaldehyde + saponin. The shaded areas represent staining with isotype control antibody and the open areas represent cells stained with anti-tubulin. The x-axis shows fluorescence intensity and the y-axis show cell number.

A.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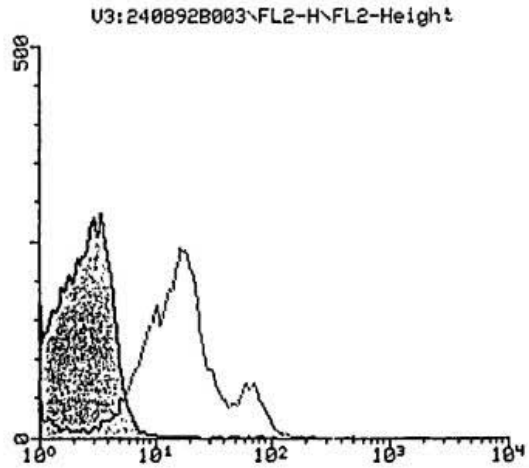
0
10⁰
10¹
10²
10³
10⁴

B.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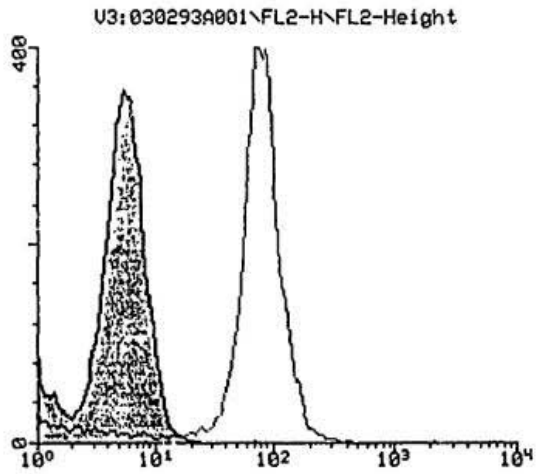
C.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10⁰
10¹
10²
10³
10⁴

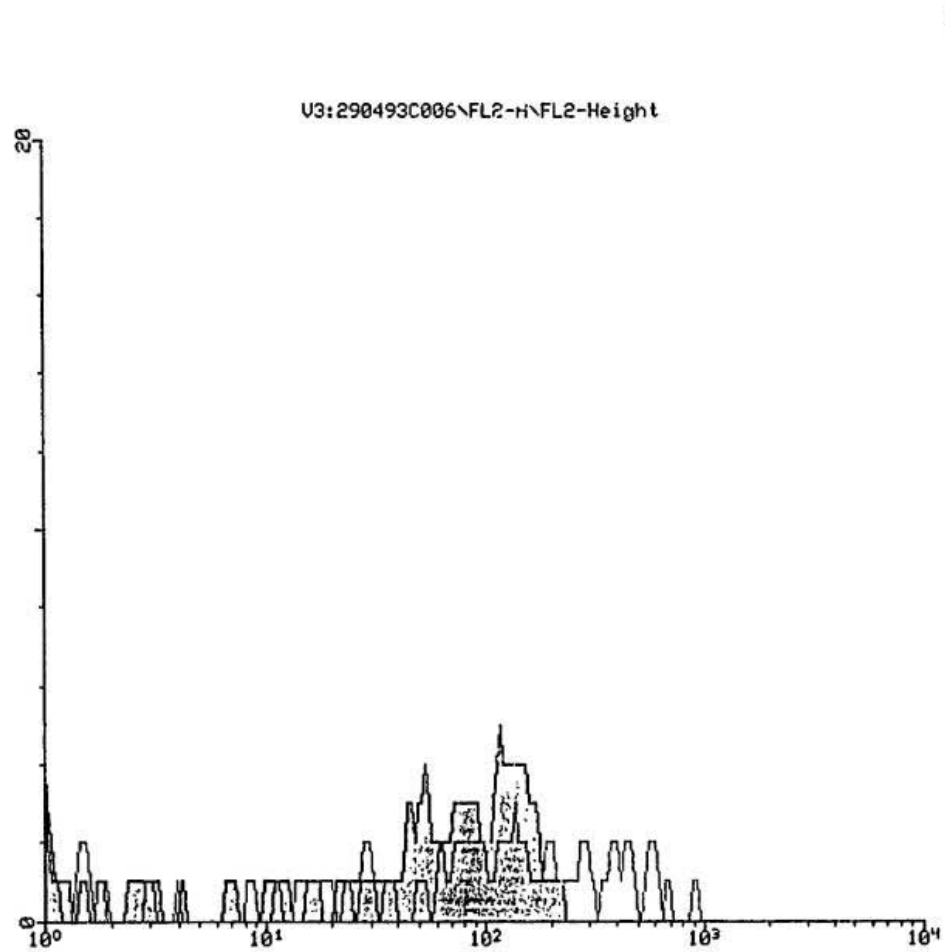


Figure 4.3 : The effect of incubating cells with monensin during TNF-α induction on the level of TNF-α that can be labelled in the cytoplasm of CD14 monocytes. The shaded area represents cells stimulated for 1 hour with LPS in the absence of monensin and the open area represents cells stimulated for 1 hour with LPS in the presence of monensin.

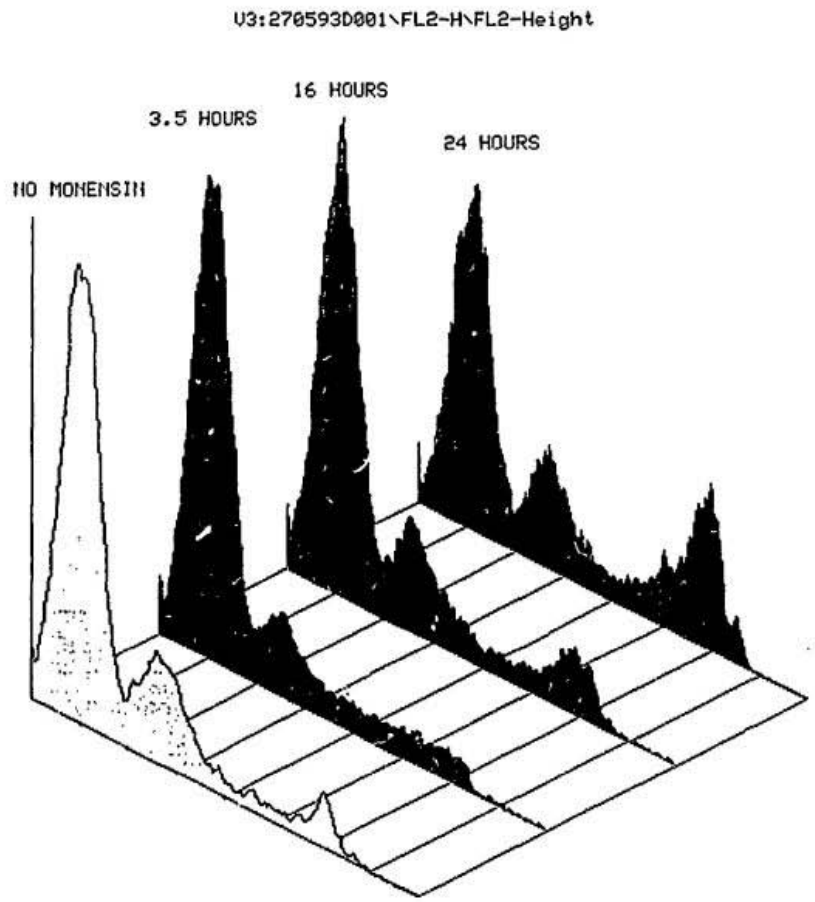
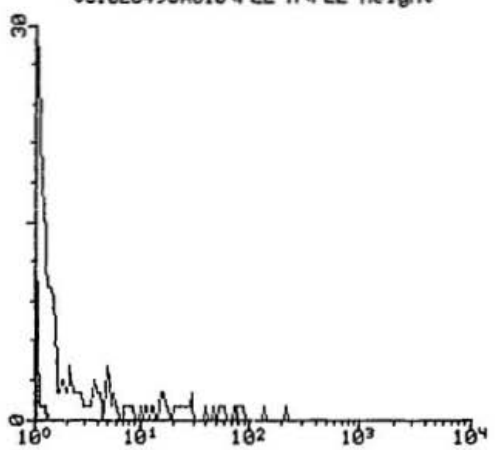


Figure 4.4 : Cytotoxicity of monensin to PBMC after incubation for 16 hours and longer. Cell death was determined by uptake of propidium iodide. The x-axis shows fluorescence and the y-axis shows cell number. Increased fluorescence is indicative of increased PI uptake.

Figure 4.5 : Flow cytometry analysis of kinetic studies to determine when the maximum number of CD14 monocytes are expressioning cytoplasmic TNF-α after stimulation with 20 μg/ml LPS. A. 30 minutes incubation. B. 1 hour incubation. C. 2 hours incubation. D. 3 hours incubation. The shaded areas represent non-stimulated cells and the open areas represent cells stimulated with LPS. The x-axis shows fluorescence and the y-axis shows cell number.

Rgn

A.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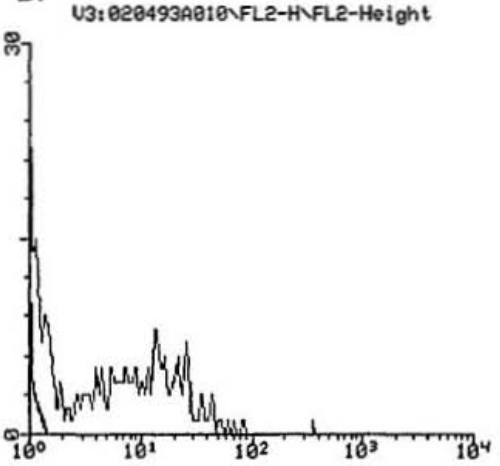
10¹
10²
10³
10⁴

B.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0
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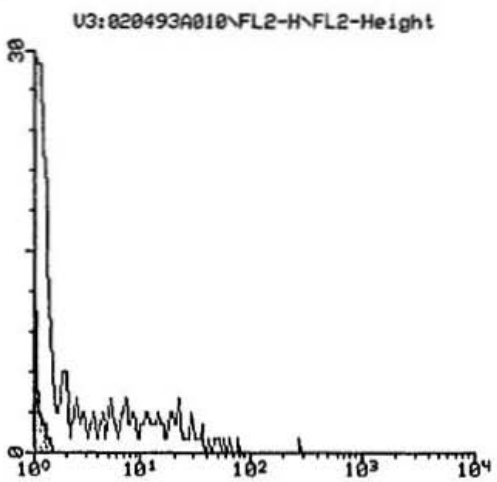
10¹
10²
10³
10⁴

C.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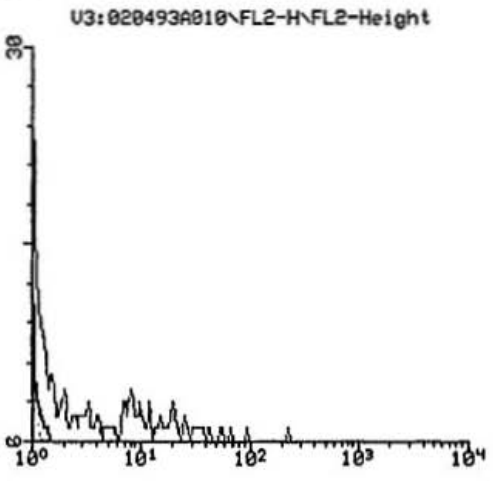
0
10⁰
10¹
10²
10³
10⁴

D.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30
0
10⁰
10¹
10²
10³
10⁴

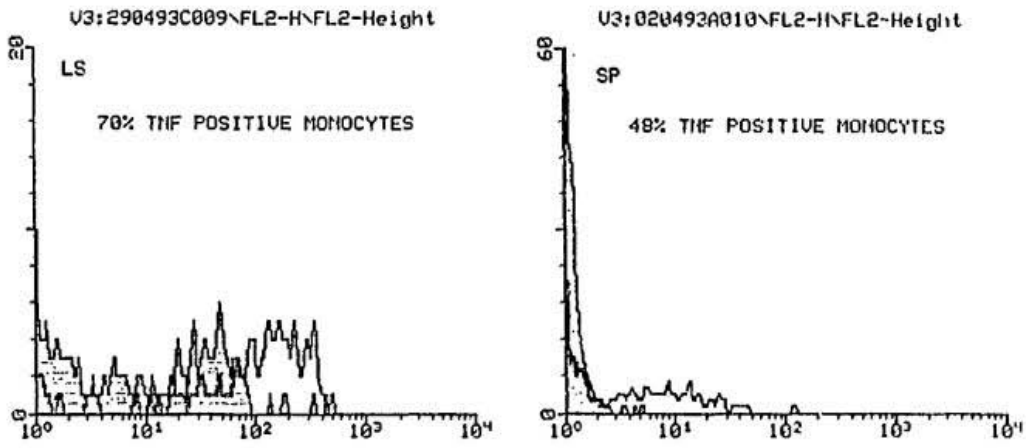
4.2.2 TNF-α Producing CD14$^+$ Monocytes

Because the cell sample was PBMC, a gate was set to display only the data for CD14$^+$ monocytes. CD14 was detected with antibodies conjugated to the green fluorescent molecule FITC, therefore the gate was set to display the data for the "green" cells. Cytoplasmic TNF-α was detected with the red fluorescent label phcoerythrin, therefore, using the computer software Lysis II, the number of "green" cells that were also "red" could be counted. Any cells that stained for cytoplasmic TNF-α but not for CD14 would not be included in the analysis.

There was no consistent relationship between the number of TNF-α-producing CD14$^+$ monocytes and the amount of secreted TNF-α for 2 subjects tested. Although the amount of secreted TNF-α was relatively consistent for different experiments, the number of TNF-α producing CD14$^+$ monocytes was not. In one experiment shown in figure 4.6A, of the CD14$^+$ monocyte subset, subject LS has 70% TNF-α producing monocytes while subject SP has 48%. In other words, for LS, of the cells that stained positive for CD14, 70 % also stained positive for cytoplasmic TNF-α. In a second experiment shown in figure 4.6B, both subjects have 10% TNF-α producing monocytes despite levels of secreted TNF-α consistent with those from the experiment shown in figure 4.6A. No correlation could therefore be made between secreted TNF-α levels and the number of cells producing TNF-α in the samples tested.

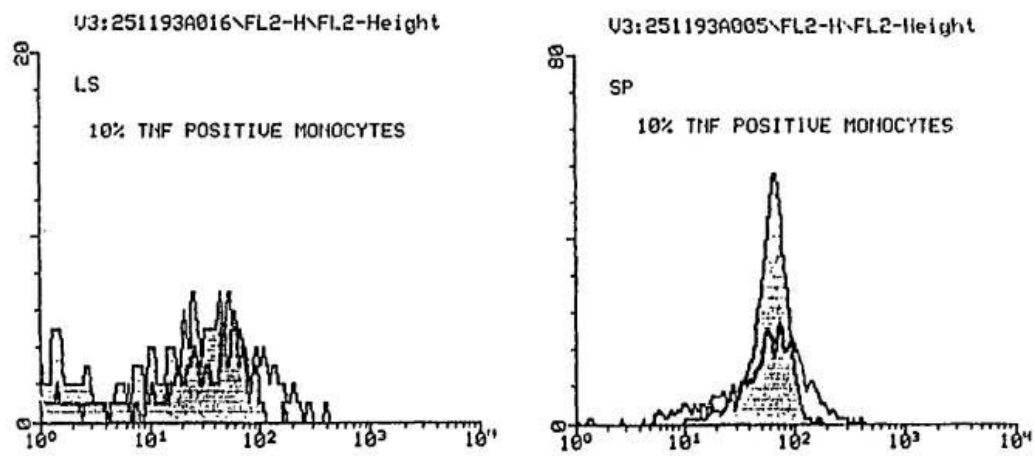
Figure 4.6 : Flow cytometry analysis of CD14 monocytes stained for cytoplasmic TNF-α for LS and SP from two experiments (A and B). The grey shaded area represents unstimulated cells. The open area designated by the black line represents cells stimulated with 20 μg/ml LPS for 1 hour

A.



LS : Secreted TNF-α at 3 hours = 3358.5 pg/ml
SP : Secreted TNF-α at 3 hours = 1173 pg/ml

B.



LS : Secreted TNF-α at 3 hours = 3567.5 pg/ml
SP : Secreted TNF-α at 3 hours = 1500 pg/ml

4.2.3 Relationship Between PBMC Subsets and Secreted TNF-α

To investigate whether there was any correlation between secreted TNF-α levels and numbers of cells in specific PBMC subsets, secreted TNF-α levels for the male subjects in chapter 3 and the per cent of cells in PBMC subsets were analyzed by least squares linear regression. There was no correlation between TNF-α secretion based on a 3 hour, 20 μg/ml LPS stimulation and numbers of $CD14^+$ monocytes, $CD4^+$ T cells, $CD8^+$ T cells and $CD45RO^+$ T cells. Figures 4.7 -4.9 show the regression lines for the relationship between the number of cells and TNF-α secretion. The slopes of the regression lines are not statistically different than zero (Table 6).

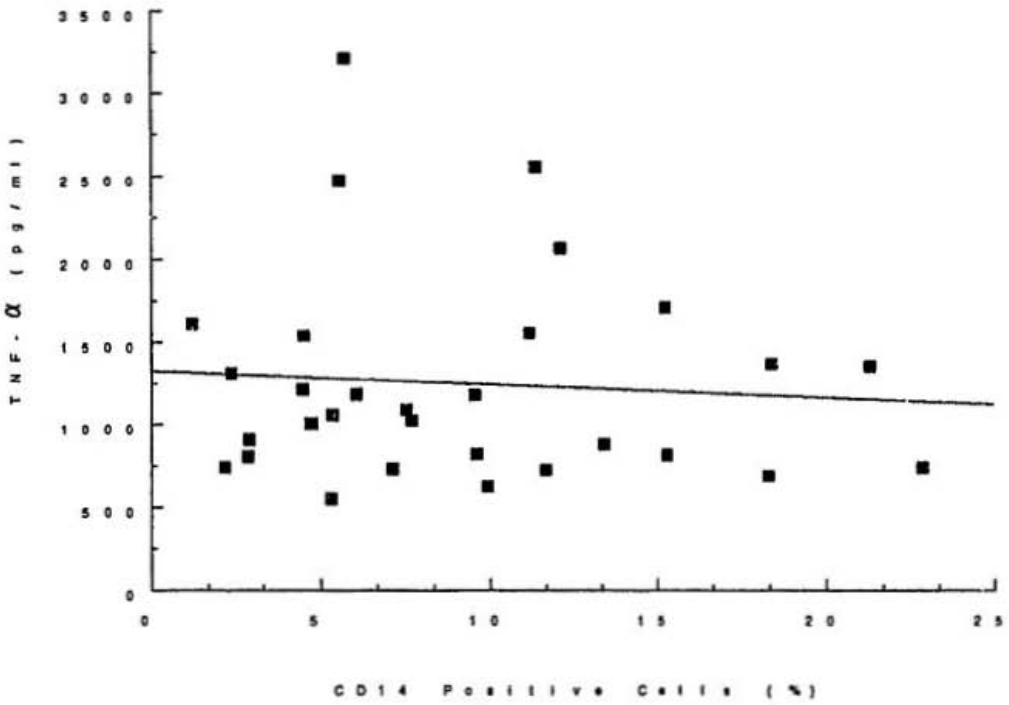
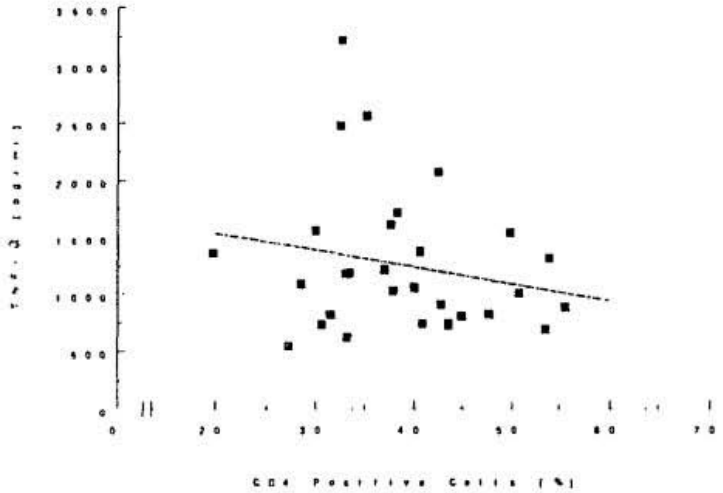


Figure 4.7 : Best-fitting regression line relating levels of TNF-α secretion to number of $CD14^+$ monocytes in a sample of 10 000 PBMC

A.



B.

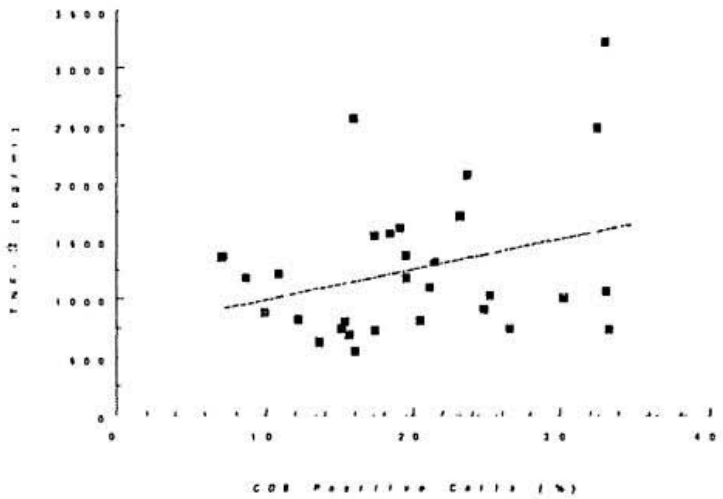


Figure 4.8 : Best-fitting regression line relating levels of TNF-α secretion to (A) number of $CD4^+$ T cells and (B) number of $CD8^+$ T cells in a sample of 10 000 PBMC

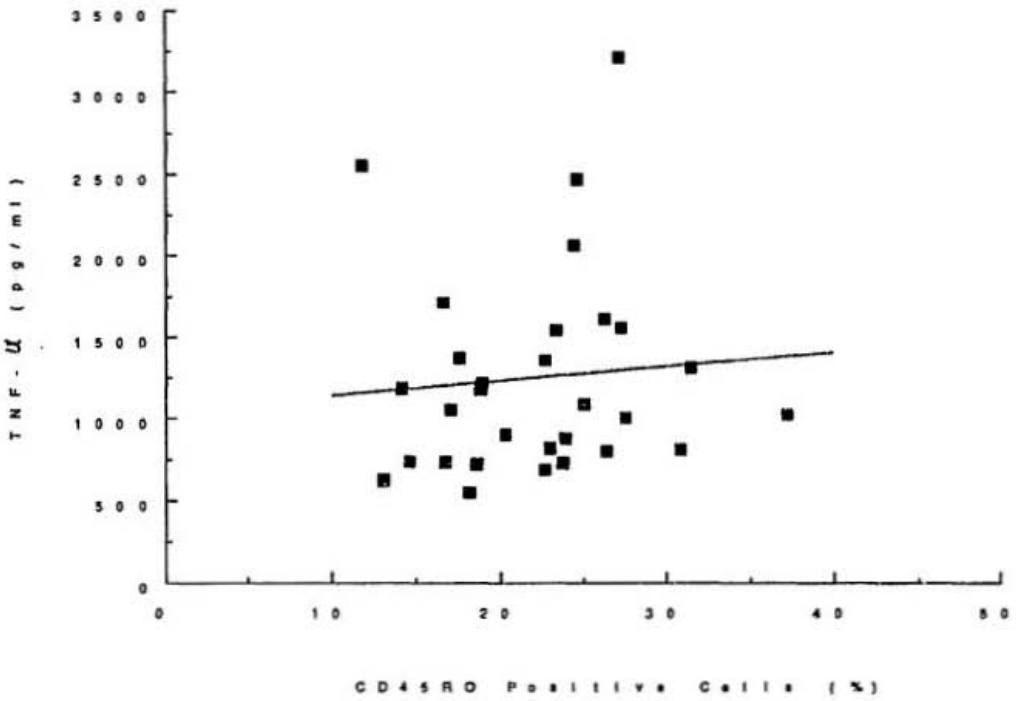


Figure 4.9 : Best-fitting regression line relating levels of TNF-α secretion and number of CD45RO$^+$ T cells in a sample of 10 000 PBMC

Table 6 : Correlation coefficients (Pearson r) and significance levels (P value) for the regression lines shown in Figures 4.7 - 4.9

Cell Type	N	Pearson r	P value
CD14	30	-0.074	0.696
CD45RO	30	0.082	0.665
CD4	30	-0.203	0.281
CD8	30	0.310	0.095

4.3 Discussion

4.3.1 Double Fluorescence Flow Cytometry

With the double-staining flow cytometry protocol developed, we had hoped to determine which cells in a sample of PBMC, and the proportion of cells of a particular phenotype that were producing TNF-α after stimulation with 20 μg/ml LPS, 1 ng/ml LPS and ConA/PMA. The response of PBMC from different individuals to the same kind and dose of TNF-α inducer might answer the question of whether individual differences in TNF-α secretion arise from individual differences in the number of cells producing TNF-α. Comparing the kinetics of responses to different inducers in this way might allow us to determine which particular subset(s) were producing TNF-α under each of the three experimental conditions and whether individuals differed in the quantity of their responses.

Because of the problem with cells disintegrating during fixation and permeabilization after stimulation with ConA/PMA for more than 24 hours, no experiments could be done with this method to determine which cells were producing TNF-α in 53 hour ConA/PMA cultures. For this reason no experiments were done whereby phenotypic markers for $CD4^+$, $CD8^+$, and $CD45RO^+$ subsets of T-cells were labelled. In addition, the requirement of monensin during stimulation to boost the cytoplasmic TNF-α signal limited the experiments to less than 16 hours. Therefore, the only experiments done were those to determine the number of $CD14^+$ monocytes that were producing TNF-α after LPS stimulation

Subjects LS and SP were chosen for the determination of the number of CD14 monocytes producing TNF-α because PBMC from LS secreted 3 - 4 times more TNF-α than SP when stimulated with 20 μg/ml LPS for 3 hours. If the level of TNF-α detectable in the supernatants is a function of the number of cells producing TNF-α, and $CD14^+$ monocytes are the major producers of TNF-α, then LS might be expected to have more $CD14^+$ monocytes producing TNF-α. The results, however, were inconsistent because in one experiment both LS and SP had the same number of monocytes producing TNF-α while in another LS had 22% more monocytes producing TNF-α. Because of these inconsistencies no conclusions could be made and no further experiments were done to examine the differences when PBMC were stimulated with 1 ng/ml LPS.

The inconsistencies are probably a result of problems with the method. Given that TNF-α secretion from both SP and LS is relatively stable over time, the output of TNF-α on a per cell basis would have to change dramatically to account for the difference in the number of cells producing TNF-α. This is unlikely because it would mean that at any given time there is a set level of TNF-α to be secreted regardless of the number of cells producing it.

Although this method as it stands was not useful in determining the number of cells producing TNF-α, it was useful in determining the types of cells producing TNF-α. In our case it was clear that LPS stimulated monocytes were producing TNF-α.

The reason or reasons why the cells disintegrated after stimulation with ConA/PMA are unknown. The use of gentler fixative or a gentler permeabilization technique may reduce the problem but de Caestecker et al. (1992) did point out that they had tested a number of different fixatives and permeabilization techniques and the 4 % paraformaldehyde followed by 1 % saponin was the best method for detection of intracellular TNF-α. Their stimulations, however, were only with LPS.

The problem of monensin limiting the use of the double-labelling protocol to 16 hours and less could be overcome if monensin could be added to the cultures in the last several hours of incubation instead at the beginning. For example, to determine which cells were producing TNF-α at 24 hours after stimulation with ConA/PMA, one may be able to add monensin to the cultures at 22 hours so that any TNF-α being produced would accumulate in the cells during the last two hours of stimulation.

4.3.2 Numbers of Cells and Secreted TNF-α

The lack of correlation between TNF-α and the number of cells of the PBMC subsets would mean that the amount of TNF-α secreted by PBMC stimulated with LPS is not dependant on the number of cells of any given phenotype tested. This suggests that the level of TNF-α detectable in the PBMC supernatants is a function of the rate of transcription/secretion and/or removal from

the supernatants, not the number of cells. Adding to this argument and assuming CD14 monocytes produce most of the TNF-α after LPS stimulation, is the finding that LS consistently had about half the number of CD14 monocytes as SP yet secreted more TNF-α. The work by Molvig et al. (1988) suggests the same phenomenon because they showed interindividual differences when fixed quantities of monocytes were used. If the level of TNF-α detected was due to individual differences in the proportion of TNF-α-secreting cells then, in homogeneous cultures with the same numbers of cells, you would expect all individuals to manifest approximately the same amount of TNF-α.

Inherent differences in rates of TNF-α secretion per cell may account for interindividual differences with LPS stimulations because in these cultures TNF-α peaks early and is produced primarily by monocytes. It may not be the only reason for the different response to ConA/PMA. We know that PMA induces T-cells to secrete TNF-α and Con A has a proliferative effect on T-cells. The amount of TNF-α secreted might therefore be partially dependant on the amount of T-cell proliferation. Thus "high"/"low" differences detected in the supernatants from ConA/PMA culture could be affected by both the difference in rate of transcription by one or all populations of cells activated by PMA and, in long-term culture, by increasing numbers of T-cells. If there are constitutive differences in rate of TNF-α secretion per cell then, all things being equal, the quantitatively greater difference should be in the ConA/PMA culture but consistent, ie. in the same direction, as the

LPS cultures. This was not the case in our experiments , suggesting that the TNF-α in the supernatants after LPS and ConA/PMA stimulations result from a different series of events.

The lack of any correlation with lymphocyte numbers and TNF-α induced by LPS for 3 hours is not surprising given that LPS is not known to stimulate lymphocytes to produce TNF-α. However, lymphocytes express TNF receptors and could possibly influence TNF-α in several ways. First, they can contribute to the removal of TNF-α from the cultures by binding TNF-α and promoting its degradation. Secondly, TNF-α can act as an autocrine hormone and stimulate its own production. T-cells might, therefore secrete some TNF-α after stimulation with TNF-α secreted by the monocytes.

Chapter 5

General Conclusions and Future Direction

A number of conclusions can be drawn from the results of these studies.

(1) There is considerable interindividual variation in LPS induced TNF-α secretion by PBMC from healthy, young males which appears, from the limited data, to be moderately stable. Thus, three studies, this one plus those by Molvig et al. (1988) and Jacob et al. (1990), using different methods to induce TNF-α secretion have shown stable, interindividual variation in levels of TNF-α secretion. In contrast, PBMC from pre-menopausal females show wide fluctuation in levels of LPS-induced TNF-α secretion when tested over a four week period.

(2) The association between low TNF-α secretion and HLA-DR2 reported by Molvig et al. (1988) and Jacob et al. (1990) was not confirmed in this study. Interestingly, our data has demonstrated an association between HLA-DR3 and low TNF-α secretion. The association with DR3 was not highly significant and the number of HLA-DR3 individuals was relatively small. However, the levels of TNF-α secretion for the $DR3^+$ individuals are within a much narrower range with a lower standard deviation and less variability that the TNF-α levels from DR2 and DR4 individuals.

The confounding factor in these types of studies is the use of individuals heterozygous for HLA-DR alleles. Many of the individuals in our study and possibly in Jacob's, although no mention is given to the number of homozygous individuals,

were heterozygous at the DR locus. Therefore, the need exists for a properly controlled study. The appropriate study here is a family study. If high and low TNF-α secretion is inherited and associates with HLA-DR, then one would expect that this could be demonstrated by family studies of HLA-DR alleles and TNF-α secretion. The ideal families would contain at least one homozygous DR and homozygous high/low TNF-α secreter. A sample of individuals homozygous at the DR locus would also be informative. In the analysis of the secreted TNF-α levels one would not have to consider the possible contribution of the putative high or low TNF-α expressing gene associated with the different HLA allele on the other chromosome.

In view of the kinetics results, the possibility exists that cells stimulated under different conditions show different associations. This should be tested in a population study in which all individuals are typed for HLA antigens and TNF-α secretion induced by the three stimulation protocols described in chapter 3. It would also be informative to compare the kinetics of TNF-α secretion from PBMC using different stimulation protocols for $DR3^+$ and $DR3^-$ individuals and $DR2^+$ and $DR2^-$ individuals.

(3) The kinetics of TNF-α secretion by PBMC is similar for cells of different individuals under the same stimulation conditions. Thus, results from a single time-sample for several individuals are likely to be a fairly accurate reflection of individual differences in the population.

(4) The kinetics of TNF-α secretion by PBMC stimulated with LPS are dramatically different from the kinetics of TNF-α secretion obtained with ConA/PMA stimulation. There are a number of mechanisms which might contribute to these differences, such as different signalling pathways of LPS and PMA, LPS-receptor downregulation by LPS, TNF-α receptor downregulation by PMA and T cell proliferation by Con A.

This observation was important because, for the individuals tested, the rank order based on levels of TNF-α detected in the supernatants was dependent on the type of TNF-α inducer and the concentration. One individual identified as a "high" TNF-α secretor under one stimulation protocol could be a "low" secretor in another. This has implications for studies on the genetics of TNF-α expression because it would imply that the differences in the amounts of TNF-α secreted are not entirely determined by the TNF-α gene, or polymorphisms at the TNF locus. This may also explain the conflicting data of Jacob et al. (1990), Molvig et al. (1988) and that presented in this study with respect to associations between TNF-α secretion and other genes in the MHC.

The possibility of LPS-receptor downregulation by LPS, PMA downregulation of TNF-α receptors and T cell proliferation as reasons for the differences in the kinetics of TNF-α secretion can all be investigated by flow cytometry. In kinetic experiments similar to those described in chapter 3, cells could also be removed at each time supernatants are harvested. The LPS receptor CD14 and TNF-α

receptors on the cell surfaces could be labelled with fluorescent antibodies and analyzed by flow cytometry. The strength of the fluorescent signal would be proportional to the number of receptors expressed on the cell surface. T cell proliferation could be determined by doing differential counts as described in chapter 4.

(5) The level of TNF-α detected in the supernatants of PBMC stimulated with LPS for 3 hours seems to be unrelated to the number of $CD14^+$ cells in the sample tested. In our experiments however, the number of CD14 cells in the PBMC sample was determined the day before the cells were stimulated. A better way to determine if the number of CD14 cells in a PBMC sample is related to the amount of TNF-α detected in the supernatants is to count the number of CD14 cells in the sample by flow cytometry at the time the supernatants are harvested. Alternatively, a better method might be to measure the transcription rates of the cells from different individuals with different stimulation conditions using nuclear run-on assays. The advantage of nuclear run-on assays in measuring transcription rates is that they measure nascent mRNA, unlike Northern Blot analysis where cytoplasmic mRNA is also measured.

Together, these experiments could tell us if there are differences in proliferation rates between individuals when different stimulants are used, if there are different rates of TNF-α receptor up- or down-regulation, and if TNF-α mRNA transcription rates differ between individuals when PBMC are stimulated by

different TNF-α inducers for different time periods.

The problem of monensin limiting the use of the double-labelling protocol to 16 hours and less could be overcome if monensin could be added to the cultures in the last several hours of incubation instead at the beginning. For example, to determine which cells were producing TNF-α at 24 hours after stimulation with ConA/PMA, one may be able to add monensin to the cultures at 22 hours so that any TNF-α being produced would accumulate in the cells during the last two hours of stimulation.

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Appendix A

Secreted TNF-α and HLA Types for the Male Study Panel

Subject	TNF-α pg/ml	HLA-A	HLA-B	HLA-DR
780	879.5	1	8,12	-----
050	1415	3,29	12,35	2,4
270	1561	11	15,40	2,4
370	868.5	1	57,63	1,7
650	802.7	----	-----	-----
720	1357	2,11	44	5,6
730	741.1	2	21	7
190	906.2	1,29	8,44	7
240	696.4	2,11	51,57	6,7
810	1070.5	30,32	44,13	3,7
380	1484.5	3,29	7,44	6
560	1056.7	26	8,35	3,11
330	1213.5	1,3	7,8	15,6
550	818.8	1,31	8,13	1,3
390	2587.5	24,25	8,44	11,6
420	1794.5	11,31	8,40	1,6
280	963.9	1,2	7,27	4,6
290	750.5	1,3	8,27	3,8
300	788	2	14,15	11,7
320	1368.5	9,10	51,27	5,6
310	2345.5	9	8,40	4,5
340	1585.5	1	17,40	2,6
350	1088.7	1,30	38,40	4,6
360	548.9	1,2	------	------
410	1026.6	31	8,14	3
400	3215.5	9,32	17,44	6,7
430	1183.5	3,26	7,44	2,4
440	2475.5	9	17,40	2
450	904.45	2,29	45	4,7
460	1189	1,2	17,18	2,5
470	1370.5	3,24	7	2
480	1713	1,28	8,70	4,9

490	813.2	11,30	51,44	4
500	625.55	3,32	40,62	------
510	731.6	1	44	1,7
520	1557.5	1,30	38,35	------
530	739.05	11	51,63	------
540	1005.4	2	7,27	1,2
600	844.35	1,24	35	5,10
590	844.4	1,3	8,44	3,5
580	1099	1,3	51,57	4,7
570	1185	3,24	7,62	------
620	725.05	1,24	7,8	1,3
640	2557.5	2,26	62,39	2,8
630	1393.5	2,26	51,44	4,6
610	1983	3	7,35	1
660	2068.5	1	7,57	------
670	1541.5	2	14,35	2
680	1611	2,3	7,50	5,7
690	1312	3,11	7,18	2,5
700	802.3	31,32	18,40	3,4
710	3900	1,2	8,44	------
770	689.2	3	8,67	------
820	898.8	------	-------	------
830	1295	1	8	3,6
840	1233.5	------	------	------
850	428.25	2,3	18,44	------
860	157.7	2	48,57	6,7
870	1309	------	------	------
960	1326.5	3	7,35	------
970	494.05	2,3	7	------
980	1051.5	------	------	------
990	617.25	1,2	8,44	------
1000	513.35	------	------	------
1010	516	------	------	------
1020	1033.4	24,28	7	------
1030	1430.5	2,24	44	------
1040	930.4	1,11	------	------
1050	888.5	1	62,8	3,6
1060	1014.5	1,2	7,8	2,6
1070	650.5	1	35	2,6

Appendix B

Secreted TNF-α and Differential PBMC Subset Counts for the Male Study Panel

Subject	TNF-α pg/ml	CD14	CD4	CD8	CD45RO
780	879.5	13.4	55.48	9.88	23.95
050	1415	4.25	38.6	21.09	-----
270	1561	3	23.64	18.48	-----
170	2508	7.33	19.61	26.99	-----
370	868.5	-----	45.98	20.61	23.31
650	802.7	-----	-----	-----	-----
720	1357	21.29	19.77	7.08	22.79
730	741.1	22.87	40.9	15.09	14.64
190	906.2	3.64			
240	696.4	1.67	14.84	6.75	-----
810	1070.5	-----	40.43	29.19	-----
380	1484.5	-----	20.99	30.73	25.56
560	1056.7	5.34	40.09	33.11	17.1
330	1213.5	4.5	37.03	10.85	18.89
550	818.8	9.62	47.72	12.17	23.04
390	2587.5	-----	40.36	24.12	23.06
420	1794.5	11.2	42.1	19.04	31.74
280	963.9	1.9	35.57	18.85	-----
290	750.5	5.04	54.74	22.28	-----
300	788	3.17	35.37	20.48	-----
320	1368.5	10.62	45.46	17.1	-----
310	2345.5	7.83	37.36	23.71	-----
340	1585.5	-----	33.19	22.06	19.63
350	1088.7	7.55	28.55	21.13	25.07
360	548.9	5.3	27.29	16.01	18.16
410	1026.6	7.7	38.91	25.17	37.32
400	3215.5	5.71	32.74	33.04	27.22
430	1183.5	6.05	33.51	8.62	14.16
440	2475.5	5.55	32.53	32.49	24.68
450	904.45	2.91	42.84	24.77	20.31
460	1189	9.56	33.1	19.52	18.82
470	1370.5	18.41	40.65	19.46	17.61
480	1713	15.25	38.29	23.18	16.68

490	813.2	15.31	31.54	20.47	30.85
500	625.55	9.93	33.25	13.58	13.08
510	731.6	7.13	30.66	33.28	23.79
520	1557.5	11.19	30.04	18.4	27.29
530	739.05	2.71	43.6	26.52	16.78
540	1005.4	4.74	50.73	30.21	27.55
600	844.35	-----	34.03	14.47	15.36
590	844.4	-----	42.16	23.02	15.57
580	1099	-----	14.71	27.12	18.47
570	1185	-----	51.15	16.28	24.12
620	725.05	11.67	43.58	17.32	18.59
640	2557.5	11.39	35.24	15.94	11.89
630	1393.5	-----	31.42	19.74	25.71
610	1983	-----	44.88	22.89	13.39
660	2068.5	12.11	42.52	23.66	25.41
670	1541.5	4.53	49.85	17.3	23.44
680	1611	1.18	37.62	19.08	26.27
690	1312	2.37	53.92	21.44	31.48
700	802.3	2.87	44.95	15.3	26.36
710	3900	-----	18.21	5.7	17.6
770	689.2	18.31	53.51	15.6	22.75
820	898.8	8.01	43.66	16.59	-----
830	1295	6.06	59.24	25.84	-----
840	1233.5	10.31	38.56	32.89	-----
850	428.25	3.39	36.17	47.5	-----
860	157.7	3.31	57.69	22.75	-----
870	1309	12.72	48.65	34.15	-----
960	1326.5	7.35	-----	-----	-----
970	494.05	16.76	-----	-----	-----
980	1051.5	14.66	-----	-----	-----
990	617.25	10.16	-----	-----	-----
1000	513.35	9.08	-----	-----	-----
1010	516	5.25	-----	-----	-----
1020	1033.4	13.16	-----	-----	-----
1030	1430.5	11.23	-----	-----	-----
1040	930.4	9.91	-----	-----	-----
1050	888.5	13.25	-----	-----	-----
1060	1014.5	7.64	-----	-----	-----
1070	650.5	-----	-----	-----	-----