T lymphocyte cyclooxygenase isotypes and the role of T lymphocytes in modulating monocyte and synoviocyte cyclooxygenase expression

By

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ABSTRACT

Rheumatoid arthritis is a common inflammatory condition associated with joint destruction and disability. T lymphocytes, monocytes, and synoviocytes present within the inflamed joint all contribute to the inflammatory response by production of lipid mediators (eicosanoids) and peptide mediators (cytokines).

Interleukin-17 is a relatively recently described pro-inflammatory T cell derived cytokine which has been shown to induce monocyte and synoviocyte cytokine production. In comparison to other T cell cytokines, such as interferon-γ, it is found in abundant levels in rheumatoid synovial tissue and fluid. T cells have also been shown to induce monocyte and synoviocyte cytokine production through direct cell-cell contact. Through these mechanisms T cells may contribute to the perpetuation of inflammation in rheumatoid arthritis.

The eicosanoids, prostaglandin E_2 and thromboxane A_2, which are formed via the cyclooxygenase pathway, also have a role in promoting inflammation. To date most work has concentrated on monocyte eicosanoid production and the effects of monocyte derived prostaglandin E_2 on T cell functions. Furthermore, there has been a view that T cells do not contain cyclooxygenase and are therefore unable to produce eicosanoids. The recognition that cyclooxygenase exists in both a constitutive form (cyclooxygenase-1) and an inducible form (cyclooxygenase-2) has renewed interest in cyclooxygenase in all cell types.

The aim of this thesis was to examine cyclooxygenase isotypes in T cells and to characterize eicosanoid production by each isotype. The ability of T cells to contribute to the inflammatory response via up-regulation of cyclooxygenase-2 in other cell types was also examined.

The data presented in this thesis indicate that T cells contain cyclooxygenase-1, which is able to produce small amounts of eicosanoids. Extensive analysis revealed no evidence for induction of cyclooxygenase-2 in T cells. Furthermore, activated T cells up-regulate expression of cyclooxygenase-2 in both monocytes and synoviocytes. Interleukin-17 was found to be an important mediator of cyclooxygenase-2 up-regulation both directly and through synergy with tumor necrosis factor-α and interleukin-1β. The therapeutic effects of tumor necrosis factor-α or interleukin-1β blockade may in part be mediated by inhibition of synergy with interleukin-17. Interleukin-17 should be considered as a therapeutic target in the management of rheumatoid arthritis.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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PUBLICATIONS AND ABSTRACTS ARISING FROM THIS THESIS

Stamp LK, James MJ and Cleland LG. Interleukin-17: The missing link between T cell accumulation and effector cell actions in rheumatoid arthritis. (Submitted)

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ABBREVIATIONS

The following abbreviations have been used throughout this thesis:

@	at
AA	arachidonic acid
Ab	antibody
ANOVA	analysis of variance
AP-1	activation protein-1
APC	antigen presenting cell
BSA	bovine serum albumin
^0C	degrees Celsius
CI	carboxyheptyl imidazole
ConA	concanavalin A
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
cpm	counts per minute
CsA	Cyclosporin A
CTLA8	cytotoxic T lymphocyte antigen 8
$cTXA_2$	carbocyclic thromboxane A_2
Da	Dalton
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
DP	prostaglandin D_2 receptor
DP IV	dipeptidyl peptidase IV
DPBS	Dulbecco's phosphate buffered saline
ECL	enhanced chemiluminescence

EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E_2 receptor
ETOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FIA	Freund's Incomplete Adjuvant
FITC	fluorescein isothiocyanate
gm	gram
g	gravity
GM-CSF	granulocyte macrophage colony-stimulating factor
h	hour (s)
HLA	human leukocyte antigen
H_2O	water
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HBSS	Hanks buffered salt solution
HEPES	N-2-hydroxyethylpiperazine-N-2 ethansulfonic acid
HRP	horse radish peroxidase
HUVEC	human umbilical vein endothelial cells
IC	intracellular
ICAM-1	intercellular adhesion molecule-1

IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1Ra	IL-1 receptor antagonist
IP	prostacyclin receptor
$iPLA_2$	intracellular calcium-independent PLA_2
k	kilo
L	litre
LFA-1	leukocyte function associated antigen-1
LPS	lipopolysaccharide
m	milli
M	molar
mAb	monoclonal antibody
MAP kinases	mitogen activated protein kinases
MFI	mean fluorescence intensity
mg	milligram
µg	microgram
MHC	major histocompatibility complex
min	minute(s)
ml	millilitre
µL	microlitre
µM	micromolar
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase

MNC	mononuclear cell
mRNA	messenger ribonucleic acid
MS	mass spectrometry
ng	nanogram
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
NFWC	nylon fibre wool column
NHS	normal human serum
NK cells	natural killer cells
NRS	normal rat serum
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OPG	osteoprotegerin
p	pico
pAb	polyclonal antibody
PBS	phosphate buffered saline
PE	phycoerythrin
PG	prostaglandin
PGES	prostaglandin E synthase
PHA	phytohemagglutinin
PLA_2	phospholipase A_2
PMA	phorbol myristate acetate
$pTXA_2$	pinane thromboxane A_2
r	recombinant
R	receptor

RA	rheumatoid arthritis
RANK	receptor activator of NF-κB
RANKL	RANK ligand
RIA	radioimmunoassay
rpm	revolutions per minute
s	seconds
SD	standard deviation
SDS	sodium dodecyl sulphate
SEB	Staphylococcal enterotoxin B
SEM	standard error of the mean
SpA	spondyloarthropathy
$sPLA_2$	secretory phospholipase A_2
TBS	tris buffered saline
TCR	T cell receptor
TDL	thoracic duct lymph
Th cell	T helper cell
TIMP	tissue inhibitors of matrix metalloproteinase
TLC	thin layer chromatography
TNF	tumour necrosis factor
TP	thromboxane receptor
TPA	tetradecanoylphorbol-13 acetate
TX	thromboxane
U	unit
VCAM	vascular cell adhesion molecule
VLA	very late antigen

Chapter 1

Literature Review

"All this will not be finished in the first 100 days. Nor will it be finished in the first 1,000 days, nor in the life of this Administration, nor even perhaps in our lifetime on this planet. But let us begin."

J. F. Kennedy 1961

1.1 Introduction

Rheumatoid arthritis is a chronic inflammatory condition affecting approximately 1% of the general population. It is associated with significant morbidity and mortality and bears a high cost to both the affected individual and the community. While there are a number of pharmacological therapies available, side effects and partial or non-response are common.

While the aetiology and pathogenesis of rheumatoid arthritis (RA) remain unclear, a number of the inflammatory mediators have been defined. The eicosanoids prostaglandin E_2 (PGE_2), and thromboxane A_2 (TXA_2), which are synthesised via cyclooxygenase (COX)-1 and/or COX–2, have a range of biological activities which are important in mediating the inflammatory response. The cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α have been shown to be important in the tissue destruction seen in RA. With the recognition of the importance of both the eicosanoids and various cytokines, new therapies targeting these molecules have been developed. Specific COX-2 inhibitors, which reduce production of the eicosanoids, are in

widespread clinical use. Biological agents targeting both TNF-α and IL-1β are available and while they are certainly effective in some, like the more traditional agents, there are those who have an unsatisfactory response. This may at least in part, reflect the importance of other pro-inflammatory cytokines present within the rheumatoid joint.

The eicosanoids are a family of biologically active lipids whose synthesis involves the oxidation of C20 fatty acids, most commonly arachidonic acid (AA). When an inflammatory stimulus interacts with a cell, phospholipase A_2 (PLA_2) is activated, resulting in the release of AA from cell membrane phospholipid. AA is metabolised by COX to PGH_2. The eicosanoids, PGE_2, PGD_2, prostacyclin (PGI_2), and TXA_2 are produced from PGH_2 by their respective terminal synthases.

Most work has concentrated on production of PGE_2 by monocytes and its role in monocyte and lymphocyte function. Until recently there has been a view that T cells do not contain COX and are incapable of producing COX derived eicosanoids (Goldyne 1989). With the recognition that COX has two isoforms, COX-1 and COX-2, there has been renewed interest in the presence of COX in all cell types, including T cells. While a number of recent studies have claimed to demonstrate the expression of COX-2 in stimulated human peripheral blood T cells (Iniguez et al 1999; Pablos et al 1999), the ability of T cells to produce eicosanoids via COX-2 has not been well documented. With this in mind, the initial aim of this thesis was to examine the expression of COX isozymes and their relative importance in the production of eicosanoids by human peripheral blood T cells (Chapter 4).

The importance of the eicosanoids as inflammatory mediators lies partly in their ability to affect the production of cytokines such as IL-1β and TNF-α. The effect of PGE_2 on monocyte and T cell function has been well documented. However, the effects of other eicosanoids, such as TXA_2, have received little attention. The effects of TXA_2 on T cell function will be examined in Chapter 5.

The role of T cells in the pathogenesis and/or maintenance of the rheumatoid disease process has been the subject of considerable debate. While 30-50% of rheumatoid synovial tissue cells are $CD4^+$ T helper (Th) cells, there are relatively low levels of the prototypic Th1 lymphokine interferon (IFN)-γ, present within the joint. However, higher levels of the lymphokine, IL-17, have been reported recently (Chabaud et al 1999; Kotake et al 1999; Ziolkowska et al 2000). IFN-γ is generally thought to be pro-inflammatory in its actions. However, it is also capable of down-regulating the immune system and therefore low levels may have a negative effect in RA. While T cells can act directly as effectors of inflammation, they can also influence the activities of other effector cells. T cells, monocytes, and synoviocytes lie in close proximity within joints. Therefore there is potential for direct cell-cell interactions as well as interaction via soluble mediators i.e. paracrine effects. These interactions between T cells and other cells within the joint may, at least in part, explain the apparent paradox between T cell number and cytokine levels in RA. Interactions between T cells and monocytes or synoviocytes (through both soluble mediators and direct cell contact), with respect to the expression of COX-2 and production of eicosanoids and cytokines, are examined in Chapters 6 and 7 respectively. Particular attention has been paid to the role of IFN-γ and IL-17 in mediating these effects.

This thesis deals in large part with the production of eicosanoids via cyclooxygenase. Therefore, it is important to consider how eicosanoids are produced. A review of the important steps in eicosanoid production follows.

1.2 Eicosanoid biosynthetic pathway

There are two major classes of eicosanoids, the prostanoids (prostaglandins (PG) and thromboxanes (TX)) and the leukotrienes. The prostanoids are regulators of a variety of cellular functions including enhancement and attenuation of inflammatory cellular events. At a tissue or organ level they provide homeostatic functions such as gastric cytoprotection and renal vascular tone.

There are three steps in eicosanoid production. Firstly, AA is released from cell membrane phospholipid by PLA_2; secondly, COX sequentially metabolizes AA to PGG_2 and PGH_2; and thirdly, reduction or isomerization of PGH_2 by the terminal prostaglandin or thromboxane synthases produces the definitive eicosanoids (Figure 1.1).

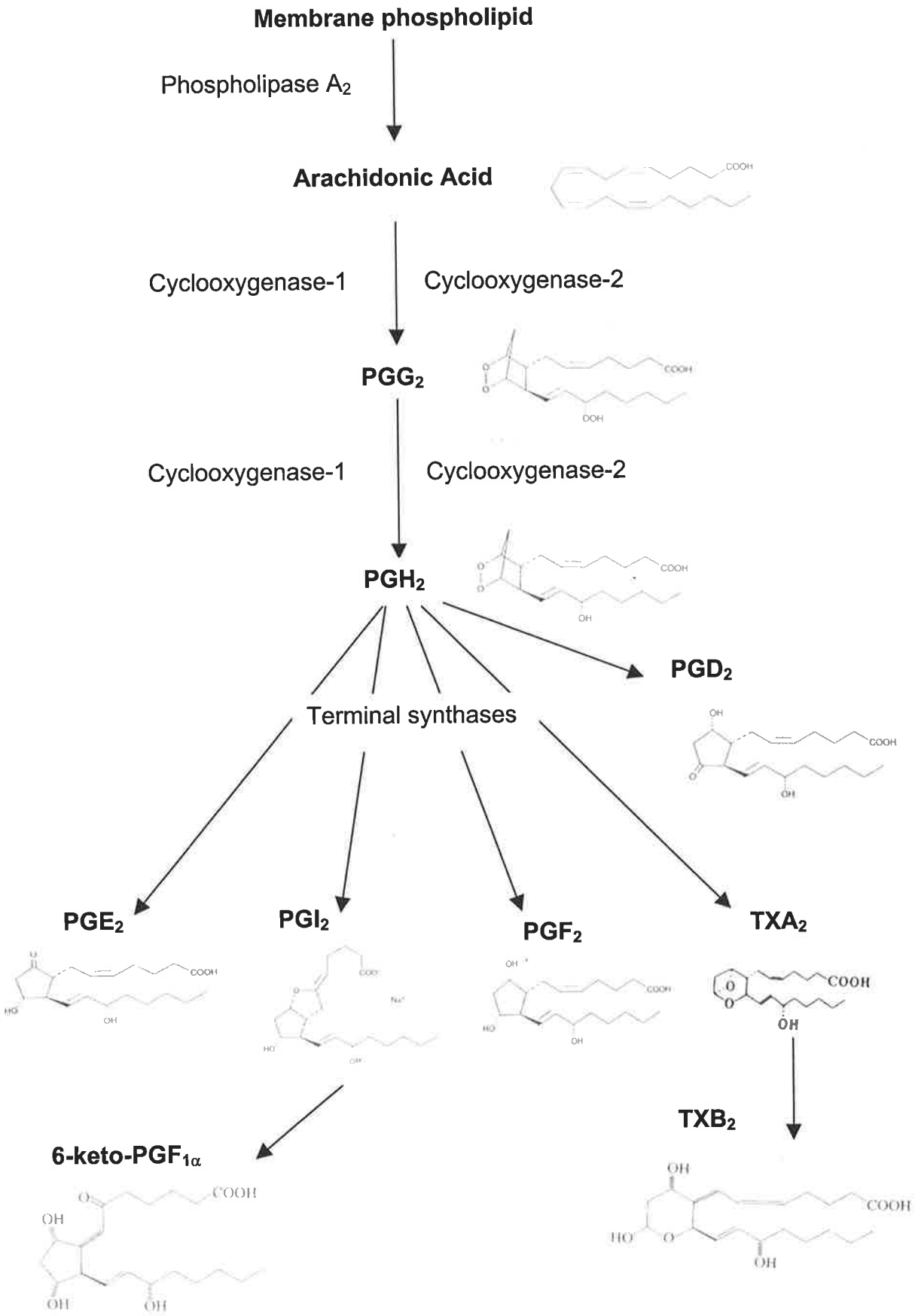


Figure 1.1: Biosynthetic pathway of the eicosanoids

1.2.1 Phospholipase A_2

When an inflammatory stimulus interacts with a cell, PLA_2 is activated and catalyses the hydrolysis of the *sn-2* fatty acid ester bond of cell membrane phospholipids, resulting in the release of free fatty acid and lysophospholipid (Figure 1.2)(Dennis 2000).

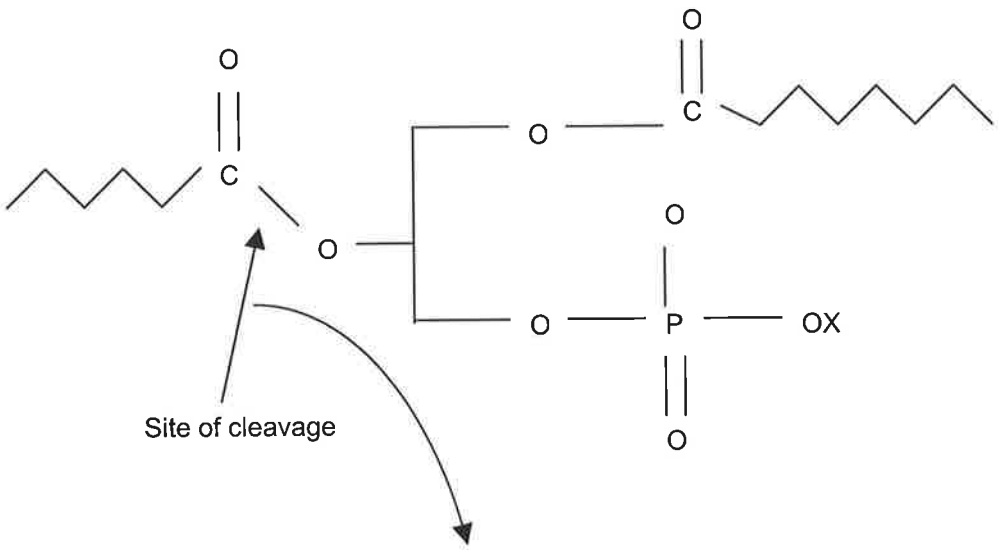


Figure 1.2: Structure of phospholipid and site of PLA_2 cleavage

PLA_2 enzymes exist in a variety of forms, each with different properties such as structure, sequence, location, calcium dependence, and function (Table 1.1). From a functional perspective PLA_2 enzymes can be divided into three main groups: secretory (s)PLA_2, cytosolic (c)PLA_2, and intracellular calcium-independent (i)PLA_2. All three types may be involved in AA mobilization and turnover, with the relative contribution dependent on the stimulus and cell type. This discussion will focus on the three main groups of PLA_2.

Table 1.1: Phospholipase A_2 enzymes

Type	Source	Size (kDa)	Calcium	Location
I A	Cobra venom	13-15	mM	Secretory
I B	Porcine/human pancreas	13-15	mM	Secretory
II A	Human synovial fluid, platelets, snakes	13-15	mM	Secretory
II B	Snakes	13-15	mM	Secretory
III	Bee, lizards	16-18	mM	Secretory
IV	Human U397 cells, platelets	85	<μM	Cytosolic
V	Human/rat heart, lung, P388D1 macrophages	14	mM	Secretory
VI	P388D1 macrophages	80-85	0	Cytosolic
VII	Plasma	45	0	Secretory
VIII	Bovine brain	29	0	Cytosolic
IX	Marine snail	14	mM	Secretory
X	Human leukocytes	14	mM	Secretory

The $sPLA_2$ enzymes are of low molecular weight (13-15kDa), have a high number of disulphide bonds, require millimolar concentrations of calcium for activity, and have little fatty acid selectivity in vitro (Tischfield 1997). Multiple $sPLA_2$ enzymes have been identified. Type I is found mainly in the pancreas and is the digestive enzyme for dietary phospholipids. Type II $sPLA_2$, which has been found in rheumatoid synovial fluid (Seilhamer et al 1989; Bomalaski and Clark 1993), can be induced by inflammatory stimuli in many cells. Although $sPLA_2$ enzymes do not have a predilection for arachidonate-containing phospholipids, they are thought to have a role in the inflammatory response (Murakami et al 1998).

$cPLA_2$ has a higher molecular weight (85kDa) and a preference for arachidonate containing phospholipid (Dennis 1994). There is no sequence homology with other PLA_2 enzymes. $cPLA_2$ is found in most cell types including thymocytes and immature B cells, however mature T and B cells do not contain $cPLA_2$ (Gilbert et al 1996). The activity and synthesis of $cPLA_2$ is increased by a variety of cytokines such as TNF-α, IL-1β, and IFN-γ (Leslie 1997). In contrast to $sPLA_2$, which acts in the extracellular environment and has a high calcium requirement, calcium does not appear to be necessary for the catalytic action of $cPLA_2$ (Leslie 1997). However, calcium is important for translocation of the enzyme from the cytosol to the perinuclear and endoplasmic reticulum membranes, an event necessary for activity (Murakami et al 1998).

$iPLA_2$ appears to regulate the main pathway through which cells incorporate AA and other unsaturated fatty acids into membrane phospholipid. The rate of AA incorporation into membrane phospholipid determines the amount of free fatty acid available for eicosanoid synthesis. Therefore, $iPLA_2$ may have a role in regulation of prostaglandin synthesis by resting cells (Balsinde and Dennis 1997).

All three types of PLA_2 have an important role in determining free AA levels in cells and their external environment. $cPLA_2$, but not $sPLA_2$, is thought to regulate immediate AA release in response to stimuli that mobilize intracellular calcium. (Balsinde and Dennis 1996; Marshall et al 1997; Naraba et al 1998). In the delayed response, both $cPLA_2$ and $sPLA_2$ appear important. It has been suggested that $cPLA_2$ activates $sPLA_2$ (Balsinde and Dennis 1996; Murakami et al 1998; Naraba et al 1998) and vice versa (Reddy et al 1997; Murakami et al 1998; Naraba et al 1998). Once

active, either $sPLA_2$ or $cPLA_2$ mediate the release of AA, which can be captured by surrounding cells for use in eicosanoid production. $iPLA_2$ incorporates the unused AA back into cell membrane phospholipid.

1.2.2 Cyclooxygenase

Cyclooxygenase is a critical enzyme in the biosynthesis of the prostanoids. It catalyses the sequential conversion of AA to PGG_2 and then PGH_2. Prior to 1991 only one form of COX was known. In the early 1990s, glucocorticoid inhibition of mitogen-induced prostaglandin production and COX activity led to the suggestion that there was an inducible form of COX (Fu et al 1990; Masferrer et al 1990; Kujubu et al 1993). It subsequently became apparent that there was a constitutive form of COX (COX-1) and an inducible form of COX (COX-2). Both enzymes catalyse the production of PGH_2 but are distinct proteins with independent regulation, expression, and subcellular locations (Table 1.2).

Table 1.2: Features of COX-1 and COX-2

Feature	COX-1	COX-2
Size	65.5kDa	72kDa
Regulation	Constitutive	Inducible
Chromosomal location	9q32-q33.3	1q25.2-q25.3
Subcellular localization	Endoplasmic reticulum	Cytoplasm, nuclear envelope and endoplasmic reticulum
Tissue localization	Probably all cells and tissues except red blood cells	Present in kidney and brain and can be induced in most (but not all) other tissues/cells with appropriate stimuli
Amino acid sequence homology	60% identical, 75% homologous with conservation of the heme binding sites, active site, glycosylation site, and epidermal growth factor domain	
Steroid effect	No effect	Inhibition of synthesis
Inducing stimuli	Nil	Inflammatory stimuli such as IL-1β, lipopolysaccharide, and TNF-α
Functions	Constitutive and homeostatic - protection gastric mucosa - platelet activation - renal functions - macrophage differentiation	Responsive - pathologic (pain, fever, inflammation, dysregulated proliferation) - tissue repair - physiological (reproductive, renal functions) - development (thymocytes, kidney)

1.2.2.1 Structure of cyclooxygenase protein and catalytic reactions

The structure of the two COX isozymes is similar in a number of important ways with conservation of key residues in the active sites and almost identical binding constants and Vmax values (Smith et al 1996). The active site of COX is a channel lined with hydrophobic residues, which protrudes towards the centre of the major globular domain of the enzyme. The hydrophobic environment favours the entry of fatty acid substrate. Within the active site the substitution of valine in COX-2, for isoleucine in COX-1, at position 523 allows molecular access to a side pocket in COX-2 which has been exploited in the production of specific COX-2 inhibitors (Kurumbail et al 1996).

After binding, AA is bis-oxygenated, with the addition of the oxygen atoms at C11 and C15, to form PGG_2. This reaction has an absolute requirement for hydroperoxide, which oxidizes the heme group. The oxidized heme subsequently oxidizes Tyr-385 located in the COX active site and the resulting tyrosyl radical abstracts the 13-S hydrogen from AA. The peroxidase activity of COX reduces the 15-hydroperoxide group of PGG_2 to an alcohol, resulting in the formation of PGH_2. The heme prosthetic group of COX is essential for this 2-electron reduction reaction. These two activities of COX, oxygenation and peroxidase activity, are associated with spatially distinct, but interactive sites within the enzyme (Smith and Marnett 1991).

1.2.2.2 Subcellular location of cyclooxygenase-1 and cyclooxygenase-2

Both COX enzymes are integral membrane proteins, which interact with the lipid bilayer through a unique membrane-binding domain. Using immunofluorescence and confocal microscopy COX-1 has been shown to be located in the endoplasmic reticulum and the nuclear envelope, while COX-2 is found preferentially in the nuclear

envelope (Morita et al 1995). More recently this has been questioned with reports that by immunoelectron microscopy, COX-1 and COX-2 appear equally distributed in the endoplasmic reticulum and nuclear envelop of human monocytes and human umbilical vein endothelial cells (HUVECs)(Spencer et al 1998).

1.2.2.3 Regulation and expression of cyclooxygenase-1 and cyclooxygenase-2

Whilst COX–1 and COX–2 share a number of important features with regards structure and function, their regulation and expression is different. In most cells, COX-1 is constitutively expressed and in general has a constant level of expression. In comparison, COX-2 is undetectable under normal conditions in most cells, with the exception of kidney, brain, and reproductive cells (Vane et al 1998). However, COX-2 can be rapidly induced in a variety of cell types, including monocytes, endothelial cells, synoviocytes, osteoblasts, mast cells, and renal mesangial cells in response to inflammatory stimuli (Herschmann 1996). The up-regulation of COX-2 occurs rapidly with detectable levels of COX-2 protein after 4-6 hours and similarly after removal of the stimulus, there is a relatively rapid decline in COX-2 levels (half life ~5 hours) (Barrios-Rodiles et al 1999). The decline in COX-2 is due to instability of both COX-2 mRNA and protein (Otto and Smith 1995).

While pro-inflammatory stimuli induce COX-2 expression, anti-inflammatory stimuli will inhibit its expression. Corticosteroids (DeWitt 1991; Kujubu and Herschman 1992), as well as the anti-inflammatory cytokines IL-4, IL-10, and IL-13 (Niiro et al 1995; Onoe et al 1996; Niiro et al 1997; Berg et al 2001) all reduce the expression of COX-2.

There is ~60% sequence homology between COX-1 and COX-2 (Kosaka et al 1994) and while key residues at the active site are conserved, regions regulating gene expression have little similarity. The promoter region of the COX-1 gene has the characteristics of a "housekeeping" gene in that it is continuously transcribed and its expression is stable. The typical TATA and CAAT elements of inducible genes are absent in the COX-1 gene (Otto and Smith 1995). In comparison, promoter and enhancer regions present in the COX-2 gene explain its inducibility. TATA and CAAT elements, as well as binding sites for transcription factors commonly activated in response to inflammatory and mitogenic stimuli such as IL-1, TNF-α, lipopolysaccharide (LPS), and phorbol myristate acetate (PMA) are present (Maier et al 1990; Jones et al 1993).

A variety of transcription factors have been implicated in the regulation of COX-2 gene expression. These include Nuclear Factor (NF)-κB (human vascular endothelial cells (Schmedtje et al 1997), murine osteoblastic cell line (Yamamoto et al 1995)) and NF-IL6 and a cyclic AMP response element in the human COX-2 gene (Inoue et al 1995). COX-2 is also regulated post-transcriptionally with LPS and IL-1β increasing COX-2 mRNA stability (Ristimaki et al 1994).

1.2.2.4 Cyclooxygenase and T cells

During the 1970s and early 1980s, investigators examined the presence of COX in T cells and the production of eicosanoids via COX (summarized in Table 1.3 and 1.4). The data was interpreted as showing that T cells did not contain COX and were incapable of producing COX derived eicosanoids over and above levels expected from COX expressing contaminating cells (monocytes and platelets)(Goldyne 1989). With the recognition of the second COX isoform in the early 1990s, there has been renewed interest in the presence and role of COX in T cells.

It has recently been reported that like many other cells, T cells constitutively express COX-1, and COX-2 can be up-regulated during T cell activation (Iniguez et al 1999; Pablos et al 1999; Tanaka et al 2000; Bosticardo et al 2001; Paccani et al 2002).

In Chapter 4 I have addressed in more detail the issue of the presence of COX-1 and COX-2 in human T cells and the production of eicosanoids, while in Chapter 6 data are presented to account for the apparent discrepancy in results between my findings and previous studies.

Table 1.3: Studies reporting prostaglandin production by T cells

Reference	Source of cells	% T cells	Stimuli used	Prostaglandins produced	Method PG detection
Rapoport et al 1977	Human peripheral blood MNC	Not stated	Cultured with thyroid cells	PGE_2, $PGF_{2\alpha}$	Paper chromatography
Webb and Nowowiejski 1978	Murine spleen cells	>95% T cells	Phytohaemagglutinin (PHA)	Yes	Radioimmunoassay (RIA)
Bauminger 1978	Mouse thymocytes	Not stated		PGE_2	RIA
Parker et al 1979b	Human peripheral blood MNC	90-97% nucleated cells lymphocytes	PHA	TXB_2	Thin layer chromatography (TLC)
Morley et al 1979	Thoracic duct lymphocytes	63%	Nil	TXB_2	Gas chromatography, Mass spectrometry (MS)
Abraham et al 1986	HT-2 T cell line		Melittin (stimulator of AA release), poly-lysine, A23187	$PGF_{2\alpha}$, PGE_2, PGD_2 with melittin but not A23187	High performance liquid chromatography (HPLC)
Aussel et al 1987	Jurkat human T cell line		Lectins, anti-CD3 mAb,12-O-tetradecanoylphorbol-13 acetate (TPA)	PGE_2, PGB_2	TLC RIA
Iniguez et al 1999	Jurkat human T cell line		PMA, A23187	Yes – kit did not identify individual PG	Prostaglandin screen colorimetric kit (Cayman Chemicals)
	Human peripheral blood T cells	>95% $CD3^+$ T cells on flow cytometry	anti-CD3 + anti-CD28 mAbs		
Tanaka et al 2000	Th1 and Th2 cells generated from human peripheral blood mononuclear cells	>95% $CD4^+$ T cells	anti-CD3 + anti-CD28 mAbs	PGD_2 produced by Th2 cells	Prostaglandin-D_2-MOX enzyme immunoassay (EIA) kit (Cayman Chemicals)

Table 1.4: Studies reporting *absence of detected* prostaglandin production by T cells

Reference	Source of cells	% T cells	Stimuli used	Prostaglandins detected	Method PG detection
Ferraris and DeRubertis 1974	Murine spleen and lymph node	85%±5% MNC	PHA, Concanavalin A (ConA), Staphylococcal enterotoxin B (SEB)	PGE_2	MS RIA
Ferraris and DeRubertis 1974	Mouse thymocytes		ConA SEB	None	MS RIA
Ferraris and DeRubertis 1974	Human peripheral blood MNC	98-99% lymphocytes	ConA Staphylococcal enterotoxin B	None	MS RIA
Kennedy et al 1980	Human peripheral blood MNC CCRF human T cell line GM-130 human B cell line	98% T cells	PHA	None	TLC RIA
Dy et al 1980	Murine T cells isolated from spleen and lymph node	Pure	Mixed lymphocyte culture	None	RIA MS
Goldyne and Stobo 1982	Human peripheral blood MNC	98% T cells	PHA	None	RIA TLC
Hoffman et al 1987	Human peripheral blood MNC	T cells contained <1% monocytes	TPA A23187	None	RIA HPLC
Pablos et al 1999	Jurkat human T cell line	Pure	anti-CD3 mAb	None	EIA

1.2.2.5 Differential production of eicosanoids by COX-1 and COX-2

It appears that the two COX isotypes are associated with the production of varying ratios of particular eicosanoids. In rat peritoneal macrophages, COX-1 is associated with predominantly TXA_2, PGD_2, and PGI_2 synthesis, while the induction of COX-2 results in a shift to preferential synthesis of PGE_2 and PGI_2 (Naraba et al 1998; Brock et al 1999). In human monocytes there are similar findings with COX-1 favouring the production of TXA_2 over PGE_2 and COX-2 altering this balance with greater production of PGE_2 than TXA_2 (Penglis et al 2000).

A number of potential explanations have been given for this apparent coupling of COX-1/TXA_2 and COX-2/PGE_2. Brock et al suggest that the subcellular location of the COX isozymes and terminal synthases is important in determining which eicosanoids are produced. They postulated that co-localization of COX-2 and PGE synthase around the nuclear membrane results in metabolism of COX-2 derived PGH_2 by PGE synthase, whereas the more diffuse distribution of COX-1 results in a less selective delivery of PGH_2 to the terminal eicosanoid synthases (Brock et al 1999). However, the difference in enzyme location has been questioned (Spencer et al 1998) and therefore does not satisfactorily explain the differences in eicosanoid production.

Changes in the level of expression of the terminal eicosanoid synthases may also account for changes in eicosanoid synthesis. Matsumoto et al reported that the addition of exogenous PGH_2 to LPS-stimulated rat peritoneal macrophages resulted in increased PGE_2, but not TXA_2 or PGD_2 production suggesting up-regulation of PGE synthase (Matsumoto et al 1997). Similar induction of PGE synthase in response to IL-1β, along with induction of COX-2, has been reported in a lung adenocarcinoma cell

line (Thoren and Jakobsson 2000). More recently it has been reported that IL-1β up-regulates both COX-2 and PGE synthase in human synovial fibroblasts (Stichtenoth et al 2001; Kojima et al 2002). In addition both enzymes were localized in the perinuclear region (Kojima et al 2002). The addition of exogenous PGH_2 resulted in an increase in PGE_2 production that correlated with PGE synthase expression. The addition of exogenous AA resulted in an initial rapid increase in PGE_2 production followed by a sustained increase in production over the 48 hour incubation period, suggesting that the coordinated up-regulation of both COX-2 and PGE synthase may be important in maintaining PGE_2 production (Kojima et al 2002).

In the rat there are two forms of PGE synthase, a constitutively expressed cytosolic form (cPGES) and an inducible membrane associated form (mPGES) (Murakami et al 2000; Tanioka et al 2000). Furthermore COX-1 appears to be functionally linked to cytosolic PGE synthase, while COX-2 is linked to the inducible membrane associated PGE synthase (Murakami et al 2000).

Functional coupling between various PLA enzymes and the different COX isoforms has also been suggested with different PLA_2 enzymes providing AA for the different COX isotypes (Balsinde et al 1998; Murakami et al 1999).

Finally, a difference in enzyme kinetics between TX synthase and PGE synthase offers an alternative explanation for the apparent coupling between COX-1 or COX-2 and the terminal synthases. Using human peripheral blood monocytes, Penglis et al reported that COX-1 activity results in production of $TXA_2 >> PGE_2$, whereas with induction of COX-2, the ratio of eicosanoids produced is reversed. By adding

increasing amounts of exogenous PGH_2 they demonstrated that TX synthase has a higher rate constant than PGE synthase and that TX synthase is saturated at lower levels of substrate than PGE synthase. Overall these finding suggest that differences in the terminal synthase enzyme kinetics make a significant contribution to determining the relative amounts of TXA_2 and PGE_2 produced by monocytes (Penglis et al 2000).

1.2.2.6 Cyclooxygenase and inflammation

The face value concept was that COX-1 regulates homeostatic functions, while the induction of COX-2 by inflammatory stimuli, with the resulting increase in pro-inflammatory eicosanoids, mediates inflammatory functions (Figure 1.3). However, it is becoming clear that COX-2 has a variety of homeostatic functions and that the initial concept represents an oversimplification of a complex interaction and/or overlap between COX-1 and COX-2 with respect to inflammatory and physiological responses.

In carrageenin-induced pleurisy in rats, it has been reported that COX-2 has pro-inflammatory properties early in the disease process. However, it also has anti-inflammatory properties that were important in resolution of the inflammatory process (Gilroy et al 1999). In addition, using the same animal model, COX-1 inhibition reduced the inflammatory cell infiltrate while COX-2 inhibition had little effect (Gilroy et al 1998).

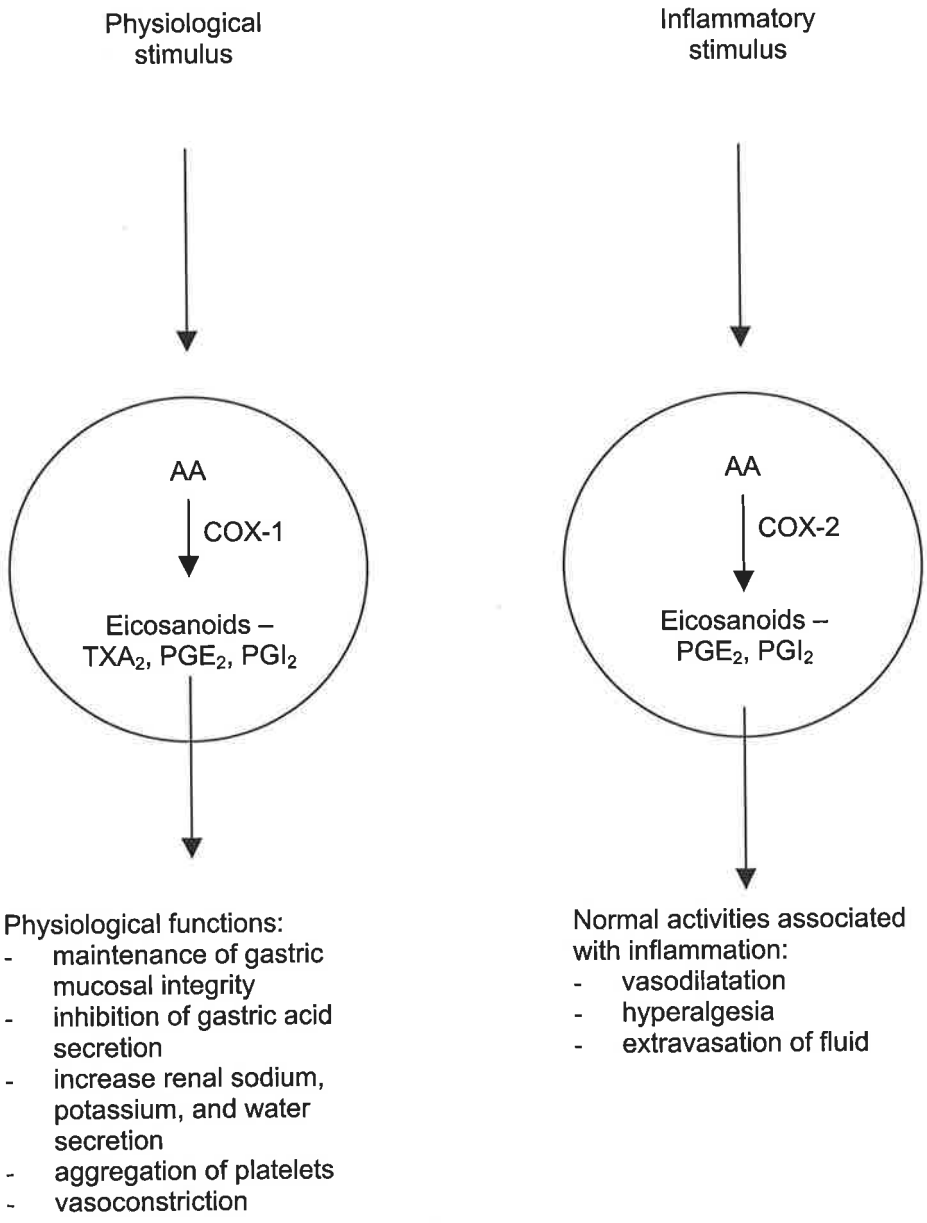


Figure 1.3: Initial concepts of the role of COX-1 and COX-2

COX-2 knockout mice have been reported to develop severe nephropathy and to have normal inflammatory responses to stimulation with TPA or AA (Morham et al 1995). Interestingly COX-1 knockout mice had increased inflammatory responses to the same stimuli (Langenbach et al 1995). In addition, there was no gastric pathology in the COX-1 knockout mice. While allowances must be made in knockout models for the potential impact on development of the deleted gene, these results suggest that COX-1 is important in regulating this type of inflammatory response in which COX-2 has little effect, and that COX-2 has a role in normal renal development.

There is also a complex interaction between COX-1 and COX-2 within the vascular system. TXA_2 is a vasoconstrictor and platelet agonist while PGI_2 is a vasodilator and inhibitor of platelet function. It has been reported that COX-2 is chronically up regulated by laminar shear stress in HUVECs (Topper et al 1996). Using the same source of cells, Caughey et al reported that TXA_2 was the predominant eicosanoid produced when only COX-1 was detected, while PGI_2 was the dominant eicosanoid when COX-2 was up-regulated (Caughey et al 2001b). In addition, platelet derived TXA_2 up-regulated endothelial COX-2 expression and PGI_2 synthesis (Caughey et al 2001a). Furthermore, in mice with genetic deletion of the PGI_2 receptor, platelet activation and injury-induced vascular proliferation were both augmented, while in mice treated with TXA_2 receptor antagonists or in which the TXA_2 receptor had been genetically deleted the opposite effects occurred. Deletion of both PGI_2 and TXA_2 receptors attenuated the enhanced response to vascular injury. The authors conclude that PGI_2 may limit vascular endothelial response to TXA_2 (Cheng et al 2002). Thus, the balance between COX-1 derived TXA_2 and COX-2 derived PGI_2 contributes to the maintenance of vascular and platelet homeostasis. Selective inhibition of COX-2 may

alter this balance, resulting in a tendency toward a pro-thrombotic state. It is interesting to note that there are case reports of thrombotic events occurring in patients taking selective COX-2 inhibitors (Crofford et al 2000; Cleland et al 2001). In addition, a recent study of rofecoxib in patients with RA revealed a four-fold increase in myocardial infarctions in the group assigned to rofecoxib, compared to those on the more traditional NSAID, naproxen (Bombardier et al 2000).

1.2.2.7 Cyclooxygenase and arthritis

COX-2 has been demonstrated to be elevated in synovial tissue from patients with inflammatory arthritis (RA, ankylosing spondylitis, or psoriatic arthritis) compared to patients with osteoarthritis (OA) (Siegle et al 1998). In addition, COX-2 in rheumatoid synovial explant cultures and cultured rheumatoid synovial fibroblasts is induced by IL-1β and PMA and inhibited by dexamethasone (Crofford et al 1994), suggesting a role for COX-2 in regulating inflammation in RA. In rat adjuvant-induced arthritis, selective inhibition of COX-2 has been shown to reduce paw oedema as well as to reduce the level of PGE_2 and COX-2 mRNA and protein expression in paw tissue. These data suggest an important role for COX-2 in mediating adjuvant-induced arthritis in the rat (Anderson et al 1996).

A recent report describes a reduced incidence and severity of collagen-induced arthritis in COX-2 knockout mice, compared to wild-type and COX-1 knockout mice. In addition the COX-2 knockout mice had reduced anti-CII IgG antibody levels indicating a decreased humoral immune response to the arthritogen. Importantly, arthritis could not be transferred passively to naïve COX-2 knockout mice with

monoclonal antibodies against type II collagen, suggesting a role for COX-2 in the effector phase of the disease (Myers et al 2000).

Given the apparent importance of synovial COX-2 in mediating the inflammatory process within joints, the regulation of COX-2 expression is of interest. The role of T cells in regulating COX-2 expression in monocytes and cultured human fibroblast-like-synoviocytes will be examined in Chapters 6 and 7 respectively.

1.2.2.8 Pharmacological cyclooxygenase inhibitors

In 1971 Vane recognized the pivotal role of cyclooxygenase in the production of the inflammatory mediators, PGE_2 and TXA_2, and its inhibition by aspirin (Vane 1971). There are three broad groups of COX inhibitors: non-selective non-steroidal anti-inflammatory drugs (NSAIDs), aspirin, and selective COX-2 inhibitors.

The NSAIDs and aspirin are relatively non-selective inhibitors of both COX-1 and COX-2. The NSAIDs compete with AA for binding at the COX active site thereby producing reversible COX inhibition (Rome and Lands 1975; Smith et al 1996). By comparison, aspirin acetylates serine 530 in the COX active site resulting in irreversible inhibition of COX activity (Roth et al 1975; Marnett et al 1999). In addition, aspirin has been shown to inhibit the activation of NF-κB (Kopp and Ghosh 1994), a process which may be mediated by binding of aspirin to the cellular kinase complex IKK-β (Yin et al 1998).

The NSAIDs and aspirin are in widespread clinical use and aspirin has been shown to have benefits in prevention of thrombosis, coronary artery disease (Antithrombotic

Trialists' Collaboration 2002), inflammatory disease (Seibert et al 1994), and colon cancer (Thun et al 1991).

While the NSAIDs and aspirin have an important role in modulating inflammation, they are associated with significant risk of gastrointestinal side effects (Day et al 1992; Henry et al 1996). A number of mechanisms are thought to be responsible for NSAID induced gastric ulceration, including inhibition of prostaglandin synthesis. The discovery of COX-2 and elucidation of structural differences between the two COX isoforms has allowed development of relatively selective COX-2 inhibitors. The aim of selective COX-2 inhibition was the reduction of gastric side effects associated with inhibition of COX-1, while retaining the anti-inflammatory effects associated with inhibition of COX-2. The COX-2 inhibitors take advantage of the side-pocket present in the COX-2, but not the COX-1 active site, allowing relative selectivity for COX-2 (Kurumbail et al 1996; Hawkey 1999).

A number of trials have reported reduced gastrointestinal side effects with the use of the COX-2 inhibitors, compared to standard NSAIDs (Langman et al 1999; Bombardier et al 2000; Goldstein et al 2000; Silverstein et al 2000). However, these agents do not abolish the risk of gastrointestinal side effects. This may, at least in part, be explained by data from animal models, which suggests that both COX-1 and COX-2 have a role in maintenance of the gastric mucosa. In COX-1 knockout mice there is less indomethacin-induced gastric ulceration but no spontaneous gastric ulceration (Langenbach et al 1995), while inhibition of COX-2, which is induced during the acute stages of gastric ulceration, slows gastric ulcer healing (Mizuno et al 1997).

Furthermore chronic gastric ulcer healing in rats may also be delayed by COX-2 inhibition (Berenguer et al 2002).

Another explanation for only partial suppression of upper gastrointestinal adverse events, particularly with celecoxib, is that it has only modest COX-2 selectivity (Patrono et al 2001). In fact, it has similar COX-1/COX-2 selectivity to that of diclofenac (Patrono et al 2001) and a large clinical trial (Silverstein et al 2000) showed that it had no gastrointestinal safety advantage over diclofenac (Juni et al 2002).

In addition to gastric ulceration NSAIDs are associated with a separate clinical entity known as NSAID enteropathy. Using knockout mice neither long-term COX-1 deficiency nor short-term pharmacological inhibition of COX-1 was associated with altered intestinal integrity or intestinal inflammation. However, long-term inhibition of COX-2 was associated with small intestinal damage despite the presence of relatively normal levels of PGE_2, suggesting that COX-2 may have a role in the maintenance of small intestinal mucosal integrity (Sigthorsson et al 2002).

1.2.3 Terminal eicosanoid synthases

PGH_2, formed from AA by COX, is the common intermediate precursor for the different eicosanoids, with the individual eicosanoid produced being determined by the terminal synthase present. For the purpose of this discussion the main focus will be on TXA_2, PGE_2, and PGD_2 and their respective terminal synthases.

1.2.3.1 Thromboxane synthase

TX synthase catalyses the conversion of PGH_2 to TXA_2. It has been purified from human platelets and shown to have a molecular weight of ~58kDa (Haurand and Ullrich 1985). The cDNA for TX synthase has been sequenced and cloned for human (Yokoyama et al 1991; Ohashi et al 1992), mouse (Zhang et al 1993), and rat (Tone et al 1994). The human TX synthase gene has been mapped to chromosome 7q33-34 (Miyata, Yokoyama et al. 1994).

TX synthase has been classified as a cytochrome P450 enzyme. However, unlike other P450 enzymes, it catalyses an isomerization reaction rather than a reduction reaction (Haurand and Ullrich 1985) and substrate binding is the rate limiting step of the reaction (Wang et al 2001). Thromboxane synthase undergoes "suicide inactivation" with a linear correlation between the amount of its metabolite TXB_2 produced and the loss of enzyme activity (Jones and Fitzpatrick 1990; Jones and Fitzpatrick 1991).

The tissue distribution of TX synthase is remarkably similar in human, mouse, and rat. TX synthase is present in small amounts in rat platelets and leukocytes, with higher levels in resident peritoneal cells, bone marrow, spleen, lung, thymus, liver, kidney, and uterus (Tone et al 1994). In the mouse, TX synthase is found in lung, spleen, and kidney (Zhang et al 1993). In humans, TX synthase is predominantly found in platelets and monocytes with lesser amounts in the spleen, lung, liver, kidney, placenta, and thymus (Miyata et al 1994). Using enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry, Nusing et al reported the presence of TX synthase in monocytes, endothelial cells, and dendritic cells but not in lymphocytes (Nusing et al 1990; Nusing and Ullrich 1990).

With recognition of the important role of TXA_2 in platelet function and thrombosis, specific TX synthase inhibitors have been developed. The carboxyalkylimidazoles are one class of TX synthase inhibitors, which have been shown to inhibit TX synthase specifically without affecting the other terminal eicosanoid synthases (Yoshimoto et al 1978; Kayama et al 1981).

1.2.3.2 Prostaglandin E Synthase

PGE synthase catalyses the isomerization of PGH_2 to PGE_2. As noted in section 1.2.2.5 there are two forms of PGE synthase, a constitutively expressed cytosolic form and an inducible membrane associated form (Murakami et al 2000; Tanioka et al 2000).

Cytosolic PGE synthase is expressed in a wide array of cells and is a 26kDa protein (Tanioka et al 2000). Microsomal PGE synthase is a member of the membrane-associated proteins of the eicosanoid and glutathione metabolism family of enzymes. It has been identified as a 15kDa protein that is induced by IL-1β and is dependent on glutathione for its activity (Jakobsson et al 1999).

1.2.3.3 Prostaglandin D synthase

PGD synthase catalyses the isomerization of PGH_2 to PGD_2. Two PGD synthases have been identified, the lipocalin-type (L-) PGD synthase and the haematopoietic-type (H-) PGD synthase. Both have a molecular weight of ~26kDa but have a number of important differences.

H-PGD synthase is dependent on glutathione for its activity. It is widely distributed in peripheral tissue, including lung, heart, lymph nodes, appendix, and bone marrow (Kanaoka et al 2000), as well as being localized in antigen presenting cells (APCs) and mast cells (Urade et al 1989). H-PGD synthase is expressed in megakaryocytes but not platelets, suggesting a role in megakaryocyte maturation (Mahmud et al 1997). Recently H-PGD synthase has been reported to be up-regulated in stimulated human Th2, but not Th1, cells (Tanaka et al 2000). The H-PGD synthase gene has been mapped to chromosome 4q21-22 (Kanaoka et al 2000).

L-PGD synthase is a member of the lipocalin superfamily and in contrast to H-PGD synthase, L-PGD synthase is glutathione-independent. It is highly expressed in brain and is also been found in male genital tissues and the heart (Eguchi et al 1997). The gene for L-PGD synthase has been mapped to chromosome 9 (White et al 1992).

1.3 Eicosanoid activities in monocytes and lymphocytes

Whether T cells produce eicosanoids or whether the eicosanoids are monocyte-derived, they appear to have an effect on T cell function. While the effects of PGE_2 on both monocyte and T cell function have received considerable attention, the influence of other eicosanoids such as TXA_2 and PGD_2 on both monocyte and T cell function has received little attention. The activities of PGE_2, TXA_2, PGD_2, and PGI_2, with particular reference to effects on T cells, are summarized in Table 1.5.

Table 1.5: Functions of eicosanoids in the immune system

PG	Function
PGE_2	Inhibition T cell proliferation Suppression Th1 activity (↓IL-2, IL-2 receptors, and IFN-γ) Promotion Th2 activity (little effect on IL-4, ?↑IL-5) Inhibition T cell TNF-α production Attenuation T cell migration Inhibition cell proliferation induced by monocytes Inhibition monocyte TNF-α and IL-1β production Inhibition monocyte accessory function including stimulation of T cells Promotes immunoglobulin isotype switching from IgM to IgG1 Suppression neutrophil superoxide generation & lysosomal enzyme release
TXA_2	Facilitates monocyte IL-1β and TNF-α production Facilitates endothelial adhesion molecule expression May be involved in negative selection of T cells within the thymus Platelet aggregation Vasoconstriction
PGD_2	Amplification Th2 responses Suppression leukocyte infiltration Vasodilatation Bronchoconstriction Inhibition platelet aggregation
PGI_2	Inhibition platelet aggregation Vasodilatation Increase heart rate secondary to hypotension Increase renal blood flow

1.3.1 Prostaglandin E_2

PGE_2 has a diverse array of biological actions, some of which can be considered pro-inflammatory while others can be considered anti-inflammatory.

1.3.1.1 PGE_2 and monocyte function

The primary PGE_2 producing cells in the immune system are monocytes and macrophages. PGE_2 can act in an autocrine manner to inhibit macrophage proliferation (Kurland et al 1978), cytolytic function (Taffet and Russell 1981), and major histocompatibility complex (MHC) class II expression (Snyder et al 1982; Figueiredo et al 1990). Exogenous PGE_2 has also been shown to inhibit monocyte/macrophage production of TNF-α and IL-1β (Kunkel et al 1986; Hart et al 1989) and to stimulate IL-6 production (Williams and Shacter 1997). PGE_2 can inhibit IL-12 production by LPS-stimulated monocytes, an effect that may be mediated by up-regulation of IL-10 production (Harizi et al 2002). Thus PGE_2 can inhibit macrophage/monocyte accessory function, which includes stimulation of T cells.

1.3.1.2 Effects of PGE_2 on T cells

The effects of PGE_2 on T cells are predominantly inhibitory. PGE_2 has been shown to have anti-proliferative effects on murine thymocytes (Rotondo et al 1994) and mature human T cells (Minakuchi et al 1990; Elliott et al 1992). While PGE_2 has been reported to mediate apoptosis in unstimulated thymocytes and resting human peripheral T cells (Suzuki et al 1991; Pica et al 1996), it has also been shown to have anti-apoptotic effects in peripheral T cells (Porter and Malek 1999).

PGE_2 may also have a role in thymic lymphocyte selection. Rocca et al report that COX-1 dependent PGE_2 facilitates the formation of $CD4^+CD8^+$ thymocytes from $CD4^-CD8^-$ thymocytes, while COX-2 dependent PGE_2 results in selection of $CD4^+$ lymphocytes (Rocca et al 1999b).

PGE_2 has actions that suppress Th1 while promoting Th2 activity. PGE_2 inhibits production of IL-2, IL-2 receptors (Rappaport and Dodge 1982; Chouaib et al 1985; Rincon et al 1988; Anastassiou et al 1992), and IFN-γ (Hasler et al 1983; Betz and Fox 1991). In addition, T cell production of TNF-α and lymphotoxin-β is inhibited (Ferreri et al 1992). At low concentrations, PGE_2 has little effect on IL-4 production and may up-regulate IL-5 production (Betz and Fox 1991; Snijdewint et al 1993). IL-12 up-regulates T cell IFN-γ production and therefore PGE_2 mediated inhibition of IL-12 production by antigen presenting cells may, at least in part, contribute to the tendency toward inhibition of Th1 and promotion of Th2 responses (Kalinski et al 1997; Kuroda et al 2000).

PGE_2 also effects B cells, promoting immunoglobulin (Ig) isotype switching from IgM to IgG_1 (Phipps et al 1991).

Inhibitory effects of PGE_2 are mediated at least in part, by its ability to increase intracellular cAMP (Rincon et al 1988; Minakuchi et al 1990; Anastassiou et al 1992), as well as inhibition of signal transduction through the tyrosine kinase JAK3 (Kolenko et al 1999).

1.3.2 Thromboxane A_2

The main cellular sources of TXA_2 are monocytes and platelets. TXA_2 has a half-life of ~30 seconds at 37^0C, being rapidly hydrolysed to the more stable, but biologically inactive TXB_2. Within the vascular system TXA_2 is a potent vasoconstrictor and inducer of platelet aggregation.

1.3.2.1 TXA_2 and monocyte function

In addition to its functions within the vascular system, TXA_2 can generally be considered to have pro-inflammatory actions. It has a role in monocyte leukotaxis and adhesion and facilitates endothelial cell adhesion molecule expression (Campbell and Tolson 1988; Ishizuka et al 1996). TXA_2 is also an important paracrine or autocrine facilitator of monocyte TNF-α and IL-1β production (Caughey et al 1997).

1.3.2.2 TXA_2 and T cell function

The finding of TX synthase in bone marrow, spleen, thymus, and peripheral blood leukocytes along with identification of the thromboxane receptor in spleen, thymus, and immature T cells suggests a role for TXA_2 in the human immune system. The effect of TXA_2 on T cell function will form the basis for Chapter 5.

1.3.3 Prostaglandin D_2

PGD_2 is the major prostaglandin produced by mast cells, although a recent report showed that some Th2 cells are capable of producing PGD_2 (Tanaka et al 2000).

Within the vascular system, PGD_2 acts as a vasodilator and inhibitor of platelet aggregation (Eguchi et al 1997). Within the kidney, PGD_2 increases renal blood flow and redistributes blood flow from the cortex to the juxtamedullary nephrons. The net result is increased salt and water excretion. In the central nervous system, PGD_2 induces sleep and may alter release of hypothalamic and pituitary hormones (Giles and Leff 1988). While most of the actions of PGD_2 are inhibitory in the nervous system, PGD_2 can be excitatory and induce hyperalgesia.

The actions of PGD_2 in the immune system are not fully understood. Inhibition of cell proliferation, as well as inhibition of DNA, RNA, and protein synthesis has been shown in murine and human cell lines (Giles and Leff 1988). In a model of allergic asthma using mice lacking the PGD_2 receptor, there was increased serum concentrations of IgE, reduced Th2 cytokines, increased Th1 cytokines, accumulation of eosinophils, and an absence of lymphocytes compared to wild type mice (Matsuoka et al 2000). The role of PGD_2 may therefore be important in amplification of Th2 mediated responses. PGD_2 has also been shown to suppress leukocyte infiltration in a rat model of pleurisy (Gilroy et al 1999).

1.3.4 Prostacyclin

Prostacyclin (PGI_2) is the main eicosanoid produced by vascular endothelium. It has an important role in vascular homeostasis through its actions as a vasodilator and inhibitor of platelet aggregation. It has been reported that endothelial derived PGI_2 is predominantly COX-2, rather than COX-1, derived (Caughey et al 2001b). Up-regulation of HUVEC PGI_2 synthesis by both lymphocytes and platelets has been reported. Direct cell-cell contact between lymphocytes and HUVECs results in activation of $cPLA_2$ and production of PGI_2 by HUVECs (Merhi-Soussi et al 2000). In contrast, the effects of platelets on HUVEC PGI_2 synthesis occurred in the absence of direct cell contact and appeared to be mediated by induction of COX-2 through TXA_2 (Caughey et al 2001a).

1.4 *Eicosanoid receptors*

The prostanoid receptors belong to the family of G-protein coupled receptors. The TXA_2 receptor (TP) was the first to be characterized, with receptors for PGD_2 (DP), PGE_2 (EP1-EP4), $PGF_{2\alpha}$ (FP), and PGI_2 (IP) following. Although there are a number of conserved sequences among the receptors, overall sequence homology, even among EP receptor subtypes, is limited to ~20-30%.

Whilst individual receptors for each prostanoid have been isolated, ligand binding is not specific. For example, PGD_2 can bind the FP receptor in addition to the DP receptor and in the mouse, two TP ligands can also bind the EP2 receptor (Narumiya et al 1999). Thus, there could be overlapping actions or even competitive inhibition between different eicosanoids. Table 1.6 summarizes the distribution and activities of prostanoid receptors in the immune system.

Prostanoid receptors have been grouped into three categories based on signal transduction and action:

1. Relaxant receptors – mediate an increase in cAMP and smooth muscle relaxation (IP, DP, EP2, EP4)
2. Contractile receptors – mediate an increase in Ca^{2+} and smooth muscle contraction (TP, FP, EP1)
3. Inhibitory receptors – mediate an increase in cAMP and inhibit smooth muscle relaxation (EP3).

Table 1.6: Summary of prostanoid receptor distribution and activities relevant to immune function

Receptor type	Distribution	Signal transduction	Activities relevant to immune system
EP1	Kidney, gastrointestinal tract, respiratory tract, vas deferens, uterus	↑increase intracellular calcium (IC) [Ca^{2+}]	
EP2	Thymus, smooth muscle	↑ adenylate cyclase activity, ↑cyclic AMP (cAMP)	Inhibition of T cell proliferation Regulation of APC function
EP3	Brain, uterus, kidney, adipocytes, thymus, adrenal gland, spleen, stomach, liver, heart, skeletal muscle	↑ IC [Ca^{2+}], inhibition of adenylate cyclase ↓cAMP	
EP4	Kidney, thymus, peripheral blood mononuclear cells	↑ adenylate cyclase activity, ↑cAMP	Regulation of APC function B lymphocyte class switching
TP	Platelets, monocytes, vascular smooth muscle, lungs, thymus, spleen, kidney, heart, uterus	Activation phospholipase C with ↑ inositol triphosphate (↑ IC [Ca^{2+}]) and diacyl glycerol (activates protein kinase C)	? negative selection of T cells within the thymus
DP	Platelets, brain, lung, vascular smooth muscle, uterus	↑ adenylate cyclase activity, ↑cAMP	Inhibition of cell proliferation Amplification Th2 responses
IP	Platelets, mature thymocytes and splenic lymphocytes, vascular smooth muscle	↑ adenylate cyclase activity, ↑cAMP	

1.4.1 Thromboxane receptor

The thromboxane receptor has been purified from human platelets and is ~55kDa (Ushikubi et al 1989; Hirata et al 1991; Kim et al 1992). After agonist binding thromboxane receptors rapidly undergo desensitization, which is associated with a reduction in the number of binding sites and a change in receptor affinity (Murray and FitzGerald 1989). Pharmacological and radioligand binding studies suggest the presence of various thromboxane receptor isoforms. However there is little molecular evidence to support this notion (for review see (Halushka 2000)).

1.4.1.1 Tissue distribution

In the mouse the highest level of expression of the thromboxane receptor has been shown to be the thymus, followed by the spleen and lung. Lower amounts are found in the kidney, uterus, brain, heart, liver, stomach, ileum, testis (Namba et al 1992), and thymocytes (Ushikubi et al 1993). In humans there is a similar tissue distribution (Miggin and Kinsella 1998). In addition the thromboxane receptor has been identified in platelets (Ushikubi et al 1989; Kim et al 1992) and monocytes (Allan and Halushka 1994).

1.4.1.2 Signal transduction pathways and second messengers

The thromboxane receptor is linked via a number of different G proteins (including Gαq, Gαi2, Gαs, Gα11, Gα12, Gα13, Gα15, Gα16, and Gh) (Shenker et al 1991; Knezevic et al 1993; Offermans et al 1994; Vezza et al 1999) to phospholipase C (Brass et al 1987). Activation of phospholipase C leads to an increase in inositol triphosphate and diacyl glycerol, which release intracellular calcium and activate

protein kinase C respectively. Mitogen activated protein (MAP) kinases can also be activated via phospholipase C dependent Gi coupling (Gao et al 2001).

1.4.2 Prostaglandin E_2 receptors

There are at least four different PGE_2 receptor subtypes (EP1-EP4), which have been identified based on their association with different intracellular signaling pathways. These receptors are widely distributed throughout the body and mediate diverse effects of PGE_2.

Binding to the EP1 receptor results in increases in intracellular calcium (Funk et al 1993) and smooth muscle contraction. It is found in a variety of tissues including kidney, gastrointestinal tract, respiratory tract, and vas deferens.

The effects mediated through EP2 are generally considered inhibitory and are due to stimulation of adenylate cyclase and an increase in intracellular cAMP (Coleman et al 1994; Fedyk and Phipps 1996). EP2 is highly expressed in the thymus, where PGE_2 inhibits the function and growth of T cells. Recently it has been shown in mice that PGE_2 regulates immune responses through distinct EP receptors on different cell populations with EP2 receptors directly inhibiting T cell proliferation, while both EP2 and EP4 receptors regulate antigen presenting cell function (Nataraj et al 2001).

EP3 mediated effects are the best characterized of the PGE_2 receptors and they include uterine contraction, inhibition of gastric acid release, modulation of neurotransmitter release, lipolysis, sodium and water resorption in the kidney, and catecholamine release from the adrenal gland.

Like EP2, EP4 results in stimulation of adenylate cyclase. Both EP2 and EP4 have a role in the immune system including B lymphocyte immunoglobulin isotype switching to IgE (Fedyk and Phipps 1996).

1.5 Cytokines

Cytokines are polypeptides that regulate the immune system and mediate inflammatory reactions. A single cytokine can act on a variety of cells, thereby mediating diverse biological functions; in addition several different cytokines can have the same biological effect. The actions of cytokines are usually local, although they can be systemic. Thus they can be autocrine, paracrine or occasionally endocrine. Cytokines exert their effects by binding specific membrane receptors on target cells. Binding is often of very high affinity and thus, only a relatively small number of molecules need occupy receptors to exert the biological effect. The binding of a cytokine to its receptor usually results in a change in gene expression within the target cell leading to expression of a new function by the cell and occasionally cellular proliferation. Two important exceptions to this are the chemokines, which lead to cell migration, and TNF-α, which can cause cell death, both of which can occur without new gene expression.

1.5.1 Cytokines in rheumatoid arthritis

There is a complex cytokine milieu at work in the rheumatoid joint. Both IL-1β and TNF-α, which are pre-dominantly monocyte/macrophage derived, can be detected in rheumatoid synovium and have been implicated in promoting the tissue damage seen in RA (Brennan et al 1992; Arend and Dayer 1995; Badolato and Oppenheim 1996).

In RA the majority of infiltrating cells are $CD4^+$ T cells that mediate T helper functions. $CD4^+$ T cells can be further characterized as Th0, Th1, or Th2, on the basis of their cytokine secretion profiles. Th0 cells, found in the early phase of the immune response, have a mixed lymphokine secretion pattern (Firestein et al 1989). Prototypic Th1 cells produce pro-inflammatory cytokines, including IL-2, IFN-γ, TNF-α, and lymphotoxin-β, while prototypic Th2 cells produce anti-inflammatory cytokines, including IL-4, IL-5, IL-10, and IL-13. There are two important features of these subsets. Firstly, they are autocrine amplifiers of cytokine production and differentiation; in other words the cytokines secreted from a particular subset induces further production of that cytokine, as well as differentiation of naïve T cells into that subset. Secondly, the subsets negatively regulate the activity of the alternative subset; i.e. Th1 cytokine production inhibits Th2 cytokine production and vice versa. While levels of most T cell cytokines are generally low in rheumatoid joints, the overall balance is in favour of pro-inflammatory Th1 cytokines over anti-inflammatory Th2 cytokines. Alteration of this balance, with reduction of Th1 cytokines and an increase in Th2 cytokines, such as IL-4 and IL-10, may help suppress disease activity (van Roon et al 1995; Horsfall et al 1997; Joosten et al 1997). Below is a more detailed discussion of the cytokines that have particular relevance to this thesis, namely IL-17, IFN-γ, TNF-α, and IL-1β.

1.5.2 Interleukin–17

In 1993, Rouvier et al cloned a rat cDNA sequence from an activated T cell hybridoma, which they termed cytotoxic T lymphocyte antigen (CTLA)-8. CTLA8 was found to be ~57% identical to an open reading frame found in a T-lymphotropic herpes virus *Herpesvirus saimiri*, HVS13 (Rouvier et al 1993). The suggestion that

CTLA8 may have cytokine like properties was based on the presence of an AU rich sequence, within the 3' untranslated region of CTLA8 mRNA, similar to mRNA sequences found in other cytokines and growth factors (Caput et al 1986; Shaw and Kamen 1986). Yao et al subsequently demonstrated that both CTLA8 and HVS13 had cytokine like properties including activation of NF-κB and induction of IL-6 secretion by fibroblasts. They proposed that CTLA8 and HVS13 be known as IL-17 (Yao et al 1995a). Human IL-17 was subsequently shown to be a 20-30kDa glycosylated homodimeric polypeptide of 155 amino acids (Yao et al 1995b; Fossiez et al 1996).

1.5.2.1 Conditions that stimulate IL-17 secretion

IL-17 is secreted by activated $CD4^+$ T cells with a memory/activation phenotype ($CD45RO^+$) (Yao et al 1995b; Fossiez et al 1996). The T cell mitogens PHA, PMA, ConA, and a combination of antibodies directed against CD3 and CD28 can all stimulate IL-17 production by $CD4^+$ T cells (Lenarczyk et al 2000). Reports of IL-17 production by $CD8^+$ T cells are conflicting (Shin et al 1998; Albanesi et al 1999).

A novel cytokine, IL-23, has been shown to induce a distinct T cell activation state characterized by secretion of IL-17 as the principal effector molecule (Aggarwal et al 2003). In addition, Infante-Duarte et al demonstrated that T cell receptor (TCR) transgenic Th cells primed with cognate peptide in the presence of microbial lipopeptides, expressed IL-17 mRNA at higher levels than cells primed with peptide and IL-12, which drives Th1 responses (Infante-Duarte et al 2000). Furthermore, T cells with a TCR transgene for a *Borrelia burgdorferi* peptide were found to co-ordinately secrete IL-17 and TNF-α (Infante-Duarte et al 2000). Peripheral blood mononuclear cells increase IL-17 expression when exposed to recall antigenic peptides

(Lenarczyk et al 2000). Stimulation of T cells with antibodies against CD3 and CD28, an experimental method that achieves broad non-antigen specific signaling, through the normally antigen-specifically activated TCR-CD3 signaling complex, is a potent stimulus for IL-17 synthesis and release. Thus stimulation of T cells can trigger production of IL-17.

1.5.2.2 IL-17: relationship to recognized Th subsets

While it is clear that activated $CD4^+$ T cells produce IL-17, classification of IL-17 with respect to the Th1 /Th2 paradigm has been problematic. Lenarczyk et al reported that production of IL-17 by stimulated human peripheral blood mononuclear cells did not correlate with production of other Th1 or Th2 cytokines (Lenarczyk et al 2000). Albanesi et al demonstrated that Th1, Th2, and Th0 nickel-specific T cell clones could all produce IL-17 (Albanesi et al 2000). In contrast, Aarvak and co-workers reported that a subset of Th1/Th0, but not Th2 cells produce IL-17 and suggested that IL-17 may define a new subset of T cells (Aarvak et al 1999).

Since $CD4^+CD45RO^+$ T cells are the major producers of IL-17 (Fossiez et al 1996), there may be a distinct subset of T cells previously activated by IL-17 inducing conditions. Having acquired the $CD45RO^+$ differentiated memory-state and capacity to subsequently secrete IL-17, these T cells can then produce IL-17 upon subsequent activation.

1.5.2.3 IL-17 receptor and signal transduction mechanisms

In contrast to the limited subset of T cells that appear to produce IL-17, the IL-17 receptor (IL-17R) is ubiquitously expressed (Yao et al 1997). Human IL-17R has close

sequence homology with murine IL-17R. However, neither human nor murine IL-17R contains sequences known in other cytokine receptor families, suggesting that they belong to a unique cytokine receptor family (Yao et al 1997).

IL-17 exerts its biological effects by engaging its receptor, IL-17R. IL-17 has been reported to activate NF-κB in human chondrocytes (Shalom-Barak et al 1998; Martel-Pelletier et al 1999), intestinal epithelial cells (Awane et al 1999; Andoh et al 2001), and colonic myofibroblasts (Hata et al 2002). In addition, IL-17 enhances activator protein (AP)-1 activity (Andoh et al 2001). The cellular signaling mechanisms resulting in NF-κB activation are complex and have been shown to include activation of MAP kinases p38, p44/42, and JNK (Shalom-Barak et al 1998; Awane et al 1999; Martel-Pelletier et al 1999). Furthermore, tumour necrosis factor-associated factor-6 is reported to be critical in IL-17 activation of NF-κB (Awane et al 1999; Schwandner et al 2000). It has also been suggested that the JAK/STAT pathway may be involved in IL-17 signal transduction (Subramaniam et al 1999).

1.5.2.4 IL-17 in other disease states

IL-17 has also been found in other inflammatory and autoimmune conditions including inflammatory bowel disease (Fujino et al 2003), psoriasis (Teunissen et al 1998), and ischaemic stroke (Kostulas et al 1999). IL-17 has been demonstrated to be increased in a rat model of renal allograft rejection, as well as in human renal allografts with subclinical rejection (Loong et al 2002).

1.5.2.5 IL-17 in rheumatoid arthritis

IL-17 has been found in abundance in rheumatoid synovium and synovial fluid (Kotake et al 1999; Ziolkowska et al 2000; Chabaud et al 2001a; Honorati et al 2001). Chabaud et al reported that 16/18 rheumatoid synovial explants, compared to only 2/12 osteoarthritic synovial explants, spontaneously produced biologically active IL-17. This production was inhibited by IL-4 and IL-13, but not IL-10. In addition IL-17mRNA was detected in RA synovium but not OA synovium (Chabaud et al 1999). Honorati and co-workers showed that IL-17 staining was present in synovial biopsies from patients with RA, but not patients with OA, spondyloarthropathy (SpA), or post-traumatic arthritis (Honorati et al 2001). If this is confirmed in a larger series, the presence of IL-17 producing cells in synovial tissue may provide a powerful means by which RA can be diagnosed and distinguished from other inflammatory arthropathies. Using immunohistochemistry, Kotake et al reported that IL-17 was detected within a subset of $CD4^+CD45RO^+$ T cells, but not $CD8^+$ T cells present in rheumatoid synovium (Kotake et al 1999). Notwithstanding the abundance of IL-17 in RA synovium, only ~1% of T cells present within the rheumatoid joint stained for IL-17 (Chabaud et al 1999). In experimentally induced delayed hypersensitivity responses, it has been demonstrated that only a few antigen-specific activated T cells are present (Modlin et al 1988). There are thus precedents for a minor population of T cells focussing the activities of non-specific effector cells. If IL-17 is the principal cytokine of a new subset of T cells, then finding the conditions that promote differentiation of naïve T cells into this subset may provide clues to the aetiology of RA. The possible correlation between antigen specificity and IL-17 production in RA is an unresolved and potentially important question. It is notable in this regard that IL-17 knockout mice have impaired antigen-specific immune responses to experimental challenge

(Nakae et al 2002). IL-17 has been reported to be released early in the T cell activation process (Yao et al 1995b). In addition, IL-17 is increased in inguinal lymph nodes in the induction phase of rat adjuvant-induced arthritis (Bush et al 2001). This increase in IL-17 during the induction phase of disease suggests IL-17 may play an important role in the pathogenesis of rat adjuvant-induced arthritis. Whether IL-17 is involved primarily in the initiation or perpetuation of RA, the presence of IL-17 within rheumatoid joints provides a likely mechanism for communication between T cells and other cells within the synovium.

1.5.2.5.1 IL-17 receptor in RA

While the production of IL-17 appears to be limited to synovium from patients with RA, rather than other forms of arthritis such as OA or SpA, the IL-17R has a wider disease distribution (Honorati et al 2001). Honorati et al reported that the IL-17R was present in synovial biopsies and chondrocytes from patients with RA, OA, and SpA. The synovial expression of IL-17R was most prominent in the vascular endothelial cells and was absent in synoviocytes. While found in samples from all conditions examined, expression in vascular endothelial cells was highest in patients with RA, followed by SpA and lowest in OA. Interestingly, expression of IL-17R in chondrocytes was highest in SpA and OA and lowest in RA (Honorati et al 2001). In contrast, Kehlen et al reported that IL-17R was more highly expressed by fibroblast-like synoviocytes from patients with RA compared to those with OA. Furthermore, treatment of synoviocytes with cyclosporin A (CsA), methotrexate, or dexamethasone was associated with enhanced IL-17R expression (Kehlen et al 2002). The differential expression of IL-17R in various arthropathies reported by Honorati et al (Honorati et al 2001) needs to be confirmed in larger series. If confirmed, differential expression of

IL-17R could lead to insights into pathophysiological processes that mediate the similarities and differences between these conditions.

1.5.2.5.2 Effects of IL-17 on inflammatory mediator production in RA

IL-17 is considered a pro-inflammatory cytokine and many of its actions are similar, although less potent, than those of IL-1β and TNF-α. IL-17 induces IL-6 and IL-8 release and adhesion molecule expression by human fibroblasts (Yao et al 1995b). It induces IL-6, IL-8, GM-CSF, and PGE_2 release by human epithelial cells, endothelial cells, and primary cultured synovial fibroblasts (Fossiez et al 1996). Human macrophages treated with IL-17 release IL-1β, TNFα, IL-6, PGE_2, IL-10, IL-12, and IL-1 receptor antagonist (IL-1Ra) (Jovanovic et al 1998).

In addition to its direct effects on monocytes and synoviocytes, IL-17 enhances many of the effects of IL-1β and TNF-α on these cells (Chabaud et al 1998; Chabaud et al 2001b; Katz et al 2001; LeGrand et al 2001). This will be discussed in more detail in Chapter 7.

1.5.2.5.3 Effects of IL-17 on cartilage degradation and joint destruction

IL-17 has been reported to have deleterious effects on cartilage, as well as amplifying the damaging effects of other cytokines, such as TNF-α, present within inflamed joints. A variety of mechanisms have been described through which IL-17 may mediate bone destruction including induction of matrix metalloproteinases (Koshy et al 2002), stimulation of differentiation of osteoclast precursors (Kotake et al 1999), and inhibition of proteoglycan synthesis (Chabaud et al 2001a).

The matrix metalloproteinases (MMPs) are a family of enzymes produced by both macrophages/monocytes and synoviocytes that are involved in degradation of extracellular matrix. Their activity is regulated in part by binding to tissue inhibitors of metalloproteinases (TIMPs). Monocyte/macrophage MMP-9 production is increased by IL-17 (Jovanovic et al 2000) and while IL-17 also induced TIMP-1 expression, the increase was less than that of MMP-9 resulting in an imbalance in favour of MMP-9 (Jovanovic et al 2001). In order to investigate the effects of endogenously produced IL-17, Chabaud et al cultured synovial explants in the presence of an IL-17 neutralizing antibody. This resulted in reduction of MMP-1 production, collagenase release, and collagen destruction but no effect on TIMP-1 (Chabaud et al 2000). In explants of bovine nasal cartilage, IL-17 treatment resulted in a dose dependent release of proteoglycan and type II collagen degradation products, which could be inhibited by exogenous TIMP-1 or a metalloproteinase inhibitor, BB-94. Furthermore, primary cultures of chondrocytes from bovine nasal cartilage treated with IL-17 alone expressed increased amounts of MMP-1, MMP-3, and MMP-13 mRNA compared to controls, while TIMP-1 levels were essentially unchanged. IL-17 also synergised with TNF-α, IL-1β, and IL-6 to enhance collagen degradation and MMP release (Koshy et al 2002). These observations suggest that IL-17 may result in an imbalance between MMPs and TIMPs in favour of MMPs, thereby promoting cartilage degradation.

Using an *ex vivo* murine cartilage model, IL-17 was associated with inhibition of proteoglycan synthesis and enhanced proteoglycan degradation (Chabaud et al 2001a). Using murine cartilage explants Lubberts et al reported that although IL-17 inhibited cartilage proteoglycan synthesis, the effect was smaller than that of IL-1β. The combination of IL-1β and IL-17 resulted in a greater effect than IL-β alone.

Furthermore pre-treatment of cartilage with IL-4, but not IL-10, could at least partially reverse IL-17 induced inhibition of proteoglycan synthesis (Lubberts et al 2000). Similar effects were observed on fetal mouse metatarsal cartilage where neither IL-17 nor TNF-α alone had significant effects, whereas the two cytokines in combination induced marked cartilage degradation associated with reduced proteoglycan content (Van Bezooijen et al 2002).

It has been proposed that nitric oxide is involved in the cartilage degradation associated with RA through inhibition of proteoglycan synthesis (Taskiran et al 1994), activation of MMPs (Murrell et al 1995), and inhibition of type II collagen production (Cao et al 1997) (for review see (Clancy et al 1998)). IL-17 has been reported both to increase and to have no effect on nitric oxide formation. In osteoarthritic knee cartilage explants and cultured chondrocytes from human osteoarthritic cartilage, IL-17 enhanced the production of nitric oxide (Attur et al 1997; Shalom-Barak et al 1998; Martel-Pelletier et al 1999). In explanted murine articular cartilage, the inhibition of proteoglycan synthesis by IL-β and IL-17 appeared to be mediated by nitric oxide (Lubberts et al 2000). In a rat model, IL-17 inhibited proteoglycan synthesis both *in vivo* and *in vitro*. While IL-17 treatment increased nitric oxide production in this study, IL-17 mediated inhibition of proteoglycan synthesis was only partially reversed by the presence of a nitric oxide synthase inhibitor, suggesting that nitric oxide was only partially involved in IL-17 induced inhibition of proteoglycan synthesis (Pacquelet et al 2002). The apparently divergent effects of IL-17 on nitric oxide production may be partly explained by the use of different experimental systems and cytokine combinations.

IL-17R mRNA has been shown to be expressed in a murine osteoblastic cell line and fetal mouse long bones. While IL-17 alone had no effect on basal or IL-1β stimulated osteoclastic bone resorption, it increased TNF-α stimulated bone resorption (Van Bezooijen et al 1999). Furthermore, while IL-17 in combination with TNF-α induced nitric oxide production in osteoblastic cells and fetal mouse metatarsals, inhibition of nitric oxide production by specific inhibitors did not alter osteoblastic bone resorption suggesting the process is independent of nitric oxide (Van Bezooijen et al 2001; Van Bezooijen et al 2002).

Interaction of Receptor Activator of NF-κB (RANK) with RANK ligand (RANKL) is an important pathway to osteoclastic bone resorption. This interaction can be inhibited through binding of RANK with a naturally occurring decoy protein, osteoprotegerin (OPG). Kotake et al proposed that IL-17 induced PGE_2 production by osteoblasts results in osteoblast RANK expression. Subsequent RANK/RANKL interaction lead to differentiation of osteoclast precursors into mature osteoclasts (Kotake et al 1999). Van Bezooijen et al reported that high dose OPG could only partially inhibit IL-17 and TNF-α stimulated osteoclastic bone resorption in fetal mouse metatarsals (Van Bezooijen et al 2001).

Taken together these observations suggest that a number of different pathways are involved in IL-17 mediated bone destruction. Through both direct effects and combined effects with IL-1β and TNF-α, IL-17 may have a significant role in bone and cartilage degradation in RA. Some of the mechanisms by which IL-17 contributes to bone loss remain to be delineated.

1.5.2.5.4 Effects of IL-17 in animal models of arthritis

Experimental animal models of arthritis illustrate the effects of IL-17 on cartilage. In mice repeated injection of IL-17 into the knee joint resulted in joint inflammation and cartilage proteoglycan depletion, through increased degradation rather than reduced proteoglycan synthesis (Dudler et al 2000). Intra-articular injection of IL-17 into the knee joint of mice during the early phase of collagen-induced arthritis resulted in enhanced erosion at day 5, with complete joint destruction by day 10. This outcome differed from damage in the control group (Lubberts et al 2002). Furthermore, the effects of IL-17 on inflammation and joint damage has been reported to be unaffected by the presence of IL-1β neutralizing antibody indicating an effect of IL-17 that is independent of IL-1β (Lubberts et al 2001; Lubberts et al 2002). Finally, rats with adjuvant-induced arthritis treated with an IL-17 receptor IgG1 Fc fusion protein had a dose-dependent reduction in paw swelling, and reduced radiographic and histology scores compared with rats treated with controls (Bush et al 2002a).

Thus the effects of IL-17 in combination with those of IL-1β and/or TNF-α may be important in both the production of inflammatory mediators and destruction of bone, cartilage and possibly ligamentous tissue in RA. Further evidence for this comes from *in vitro* models which suggest that the combination of IL-17, IL-1β, and TNF-α blockade has greater effect with respect to inhibition of IL-6 release and collagen degradation than inhibition of the individual cytokines alone (Chabaud and Miossec 2001; Chevrel et al 2002).

1.5.3 Interferon-γ

Interferon (IFN)-γ is a 34kDa glycosylated protein produced by T cells and natural killer (NK) cells. It is encoded by a single copy gene, which gives rise to a polypeptide of 166 residues (Boehm et al 1997). Production of IFN-γ by T cells occurs following engagement of the T cell receptor by peptide presented by MHC molecules. T cell IFN-γ release can be induced experimentally by treatment with antibodies directed against CD3 and CD28. Similarly production by NK cells is the result of interaction with target cells, such as virus-infected cells. IFN-γ release by NK cells can be achieved experimentally by treatment with IL-12.

IFN-γ interacts with specific receptors that are ubiquitously expressed on all nucleated cells and are most abundant on cells outside the lymphoid system. The IFN-γ receptor consists of two subunits: a 90kDa α-chain, which is responsible for ligand binding and a 314 amino acid β-chain that is primarily involved in signaling. Interaction of IFN-γ with its receptor results in activation of the JAK/STAT pathway leading to transcription of a number of genes.

1.5.3.1 Pro-inflammatory actions of IFN-γ

IFN-γ is generally considered a pro-inflammatory cytokine. It activates macrophages, thereby providing a means by which T cells and NK cells stimulate macrophage phagocytosis of microbes. IFN-γ induces expression of MHC class I and II molecules and co-stimulatory molecules on antigen presenting cells, thereby enhancing MHC associated antigen presentation, especially to $CD4^+$ T cells. IFN-γ enhances the expression of adhesion molecules on endothelial cells, promoting T cell adhesion and

extravasation to sites of infection. IFN-γ promotes the differentiation of naïve $CD4^+$ T cells into Th1 cells, while inhibiting the development of Th2 cells. In addition IFN-γ acts on B cells to promote IgG isotype switching, activates neutrophils, and stimulates the cytolytic activity of NK cells.

1.5.3.2 Anti-inflammatory actions of IFN-γ

The classification of IFN-γ as a Th1 cytokine and its pro-inflammatory effects are well known to the extent that it seems counter-intuitive that IFN-γ may have anti-inflammatory properties. IFN-γ has been reported to down-regulate IL-1 induced production of IL-1 in human peripheral blood mononuclear cells (Ghezzi and Dinarello 1988). Using antibodies directed against IFN-γ and mice lacking the IFN-γ receptor, IFN-γ has been shown to down-regulate murine autoimmune encephalomyelitis (Matthys et al 2000). It has been suggested that the down-regulatory effects of IFN-γ in this model are mediated via nitric oxide (Willenborg et al 1999). It has also been suggested that local effects of IFN-γ in the central nervous system have a pro-inflammatory effect, while at the systemic level IFN-γ may have an anti-inflammatory effect by inducing production of suppressor cells (Matthys et al 2000). Production of IFN-γ by B cells stimulated with LPS, PMA, or SDF-1 (a B cell chemoattractant) has also been shown to inhibit adhesion of B cells to the extracellular matrix protein fibronectin and thus inhibit B cell homing to lymph nodes (Flaishon et al 2000). *In vitro* studies with murine T cells have also shown that low dose IFN-γ can inhibit adhesion of T cells to fibronectin and thus inhibit migration of naïve T cells and Th2 cells in response to stimulation with PMA (Flaishon et al 2002).

1.5.3.3 IFN-γ in rheumatoid arthritis

IFN-γ has been reported to have both disease limiting and disease promoting effects in the murine model, collagen-induced arthritis, depending on the timing of administration. In the early phase of disease, administration of an IFN-γ neutralizing antibody decreased the severity of arthritis, while later administration either increased severity or had no effect (Boissier et al 1995). Similarly, administration of recombinant (r)IFN-γ after immunization, but before the onset of arthritis, resulted in increased severity of collagen-induced arthritis (Mauritz et al 1988). Furthermore in IFN-γ knockout mice severe collagen–induced arthritis develops and is associated with increased IL-1β expression (Guedez et al 2000). In mice lacking the IFN-γ receptor there is increased frequency and severity of collagen-induced arthritis following standard inoculation procedures (Manoury-Schwartz et al 1997; Vermeire et al 1997).

In the rat model, adjuvant-induced arthritis, timing of administration of IFN-γ also appears to be important. Administration of rIFN-γ 24 hours before inoculation with adjuvant, enhanced arthritis whereas suppression of arthritis was observed when IFN-γ was given 24-48 hours after the inoculation. In addition, administration of IFN-γ neutralizing antibody during days 4-8 of the procedure reduced disease severity, while between day 12-24 it enhanced disease severity (Jacob et al 1989). This suggests that IFN-γ may be an important pro-inflammatory molecule during the induction phase of disease. However, in the effector phase of disease it may have disease-limiting effects. Using an adoptive transfer model of rat adjuvant-induced arthritis, which allows examination of the effector phase of disease, independent of the inductive phase of disease, administration of IFN-γ neutralizing antibody at the time of transfer, increased

the severity of arthritis. However, if the administration of IFN-γ neutralizing antibody was delayed until 4 days after the transfer, there was little effect on disease severity, suggesting that IFN-γ plays a down-regulatory role early in the effector phase of disease (Brasted 2002).

In cultured rheumatoid synoviocytes, IFN-γ treatment inhibits cell growth and IL-1β induced collagenase production (Nakajima et al 1990). IFN-γ has also been shown to inhibit TNF-α but not IL-1β induced rheumatoid synoviocyte proliferation and collagenase production (Alvaro-Garcia et al 1990). These observations along with evidence that IFN-γ inhibits IL-1 induced monocyte production of IL-1 (Ghezzi and Dinarello 1988) led to several clinical trials of IFN-γ in rheumatoid arthritis. IFN-γ was beneficial in some, but not all patients (Veys et al 1988; Cannon et al 1993; Veys et al 1997).

1.5.4 Tumour Necrosis Factor-α

Tumour necrosis factor-α is an important mediator of inflammatory joint disease, including rheumatoid arthritis. The TNF superfamily is expanding with at least 19 known members including TNF-α and TNF-β (also known as lymphotoxin-β).

The major cellular source of TNF-α is activated monocytes/macrophages. However, T cells, mast cells, endothelial cells, and NK cells can also produce TNF-α. TNF-α is initially produced as a 233 amino acid, 26kDa protein that is expressed as a membrane bound protein. The membrane bound protein is subsequently cleaved in the extracellular portion by TNF-α converting enzyme to form the soluble 17kDa TNF-α.

Both the membrane bound and soluble forms of TNF-α are biologically active, with the membrane bound form mediating its effects through direct cell-cell contact. TNF-α is one of the most rapidly produced cytokines with detectable levels of mRNA within 15 minutes and equally rapid degradation (Dinarello and Moldawer 2000).

The biological responses to TNF-α are mediated by binding to two distinct receptors. The type I TNF-receptor is a 55kDa protein (p55 receptor), while the type II receptor is 75kDa (p75 receptor). Both receptors are present on most cell types, except red blood cells, and while the p55 receptor is constitutively expressed, the p75 receptor is inducible (Ware et al 1991). Members of the matrix metalloproteinase family can cleave the extracellular portion of both receptors, resulting in the release of soluble TNF receptors into the circulation. These soluble receptors retain biological activity, binding and inhibiting the activity of TNF-α.

TNF-α has multiple biological actions and is an important mediator of inflammatory responses. It induces expression of endothelial cell adhesion molecules, which interact with ligands on the surface of inflammatory cells resulting in recruitment of these cells into sites of inflammation. TNF-α is an autocrine and paracrine inducer of cytokine secretion from endothelial cells and macrophages including IL-1, IL-6, IL-8, and GM-CSF. In some situations TNF-α is produced in amounts that can have systemic effects including fever, synthesis of hepatic acute phase proteins, cachexia, and inhibition of myocardial contractility and vascular smooth muscle tone with resultant hypotension.

1.5.4.1 TNF-α in rheumatoid arthritis

TNF-α has been identified as an important cytokine in RA. It can be detected in rheumatoid synovium where it is primarily produced by macrophages/monocytes (Firestein et al 1990). TNF receptor expression is also up-regulated in rheumatoid tissues (Alsalameh et al 1999). In cultured human synovial fluid cells and synovial membrane cells, TNF-α antibodies inhibit IL-1 production (Brennan et al 1989).

Animal models also implicate TNF-α in arthritis. Collagen-induced arthritis in mice is associated with increased TNF-α expression in the synovial lining layer, which occurs contemporaneously with overt signs of joint inflammation (Marinova-Mutafchieva et al 1997; Mussener et al 1997). The disease is exacerbated by exogenous administration of TNF-α (Cooper et al 1992), while TNF-α inhibitors have been shown to prevent disease onset and to reduce the severity of established disease (Thorbecke et al 1992; Williams et al 1992). Interestingly in rat adjuvant-induced arthritis early treatment (day 0, 2, and 4 post-induction of arthritis) with PEGylated soluble TNF receptor type 1 did not suppress disease whereas treatment with an anti-TNF antibody did. The authors suggest that either a shorter half-life of the PEGylated soluble receptor or development of antibodies directed against the PEGylated receptor may account for their results (Bush et al 2002b). However, treatment with PEGylated soluble TNF receptor type 1 later in the disease course (day 8,10, and 12 post induction) reduced paw swelling, histology scores and synovial T cell numbers (Bush et al 2002c).

Recently biological therapies that aim to inhibit TNF-α have become available for the management of RA. Both TNF-α monoclonal antibodies and a soluble TNF-α

receptor fusion protein decoy receptor have been shown to reduce disease activity and joint damage in RA (Maini et al 1998; Moreland et al 1999; Weinblatt et al 1999).

1.5.5 Interleukin-1β

IL-1β has many actions similar to TNF-α in mediating inflammation. The main cellular sources of IL-1β are activated monocytes/macrophages. There are three primary members of the IL-1 gene family: the agonists IL-1α and IL-1β and the antagonist IL-1Ra. In addition there are at least four other members of the IL-1 family whose function has not yet been elucidated. For the purposes of this discussion I shall focus on IL-1β, which is the main circulating form of IL-1.

IL-1β is produced as a 33kDa propeptide that is cleaved by IL-1β converting enzyme to form the mature 17kDa IL-1β, which is secreted from the cell. In monocytes the production of IL-1β is stimulated by bacterial products, such as endotoxin, as well as by other cytokines such as TNF-α. IL-1β can also up-regulate its own expression.

There are two distinct IL-1 receptors, type I and II, which exist in both cell surface and soluble forms. Binding of IL-1β to the type I receptor results in the formation of a signal transduction complex, leading to activation of transcription factors such as NF-κB and AP-1. The type II receptor lacks an intracellular signaling domain and therefore does not result in signal transduction. It appears to function as a decoy receptor, competitively inhibiting IL-1β binding by the type I receptor. The soluble forms of both receptors also competitively bind IL-1β thereby reducing IL-1β mediated cell activation.

In small amounts IL-1β mediates inflammation locally by increasing endothelial cell adhesion molecule expression. When more abundant, IL-1β can have endocrine effects, sharing with TNF-α the ability to induce fever, acute phase proteins, and cachexia.

1.5.5.1 IL-1β in rheumatoid arthritis

IL-1β can be detected in synovial fluid from patients with RA (Kahle et al 1992) as well as in synovial tissue (Firestein et al 1990). The predominant source of IL-1β in rheumatoid synovium is macrophages/monocytes. In addition, plasma levels of IL-1β are elevated in patients with active RA (Eastgate et al 1988). In rats intra-articular injection of IL-1β results in a destructive arthritis (Chandrasekhar et al 1990) and chronic over expression of IL-1β in rabbits results in an arthritis not dissimilar from rheumatoid with synovial hyperplasia and bone erosion (Ghivizzani et al 1997). Antibodies directed against IL-1β have been shown to suppress proteoglycan degradation and prevent inflammation and cartilage destruction in murine collagen-induced arthritis (van den Berg et al 1994).

The IL-1 receptor antagonist, IL-1Ra, is also present in rheumatoid joints, although its levels may be too low to inhibit the effects of IL-1β (Malyak et al 1993; Firestein et al 1994). More recently recombinant human IL-1 receptor antagonists have been developed. The results of clinical trials suggest that IL-1Ra may reduce joint erosion in RA (Bresnihan et al 1998; Jiang et al 2000).

1.6 Rheumatoid arthritis

Rheumatoid arthritis is a common chronic systemic inflammatory disorder characterized by synovial inflammation which can lead to irreversible damage to periarticular tissues (bone, articular cartilage, supporting ligaments) and joint failure. The peak incidence is between the fourth and sixth decades. There is a 2:1 female predominance. While RA primarily affects joints it can affect other organ systems. The standardised mortality ratio is increased in RA (~2.26 compared to otherwise matched individuals without the disease) and has been linked to disease severity (Wolfe et al 1994). Excess mortality is due to an increase in infections, cardiovascular events, and malignancy as well as treatment-related events (Mitchell et al 1986; Myllykangas-Luosujarvi et al 1995).

1.6.1 Role of T cells in rheumatoid arthritis

While the cause of RA remains unknown it is has been proposed that exogenous and/or endogenous auto-antigen may activate T cells, which initiate an inflammatory cascade involving monocytes/macrophages and synoviocytes. Whether T cells and their secretory products are needed to sustain synovial macrophage activity in established RA remains controversial. While there is evidence to suggest T cells may have a central role, it has also been suggested that the macrophage is the most important cell in the pathogenesis of RA (Firestein and Zvaifler 1990). From an immunological perspective, the issue centres on whether rheumatoid disease emanates from and is maintained by acquired specific responsiveness to an endogenous or exogenous immunologic arthritogen or whether it is due dysregulation of innate immunity. The latter could result from a constitutive predisposition to loss of homeostatic control.

RA can be considered to have three separate but interrelated phases: disease initiation, disease maintenance or perpetuation, and end-stage tissue destruction. The potential role of T cells in each of these stages is outlined below.

1.6.1.1 Initiation of disease

Evidence for the importance of T cells in initiating RA has been based on several factors including the abundance of T cells within the synovium, animal models, genetic studies, and the response to T cell directed therapeutic manipulations.

1.6.1.1.1 T cells within the synovium

The synovial intima is the interface between the synovium and the intra-articular space. It is comprised of loosely associated macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes. The synovial intimal layer provides nutrients for articular cartilage and is an abundant source of the glycosaminoglycans in synovial fluid that lubricate the articular surfaces.

In RA, the synovium becomes hyperplastic and oedematous. It is infiltrated with inflammatory cells, including T cells, B cells, and macrophages. The population of both type A and B synoviocytes is expanded and there is increased new blood vessel formation (Figure 1.4). The lining undergoes villous hypertrophy. The inflammatory tissue that extends onto articular cartilage and erodes periarticular bone and ligaments is known as pannus.

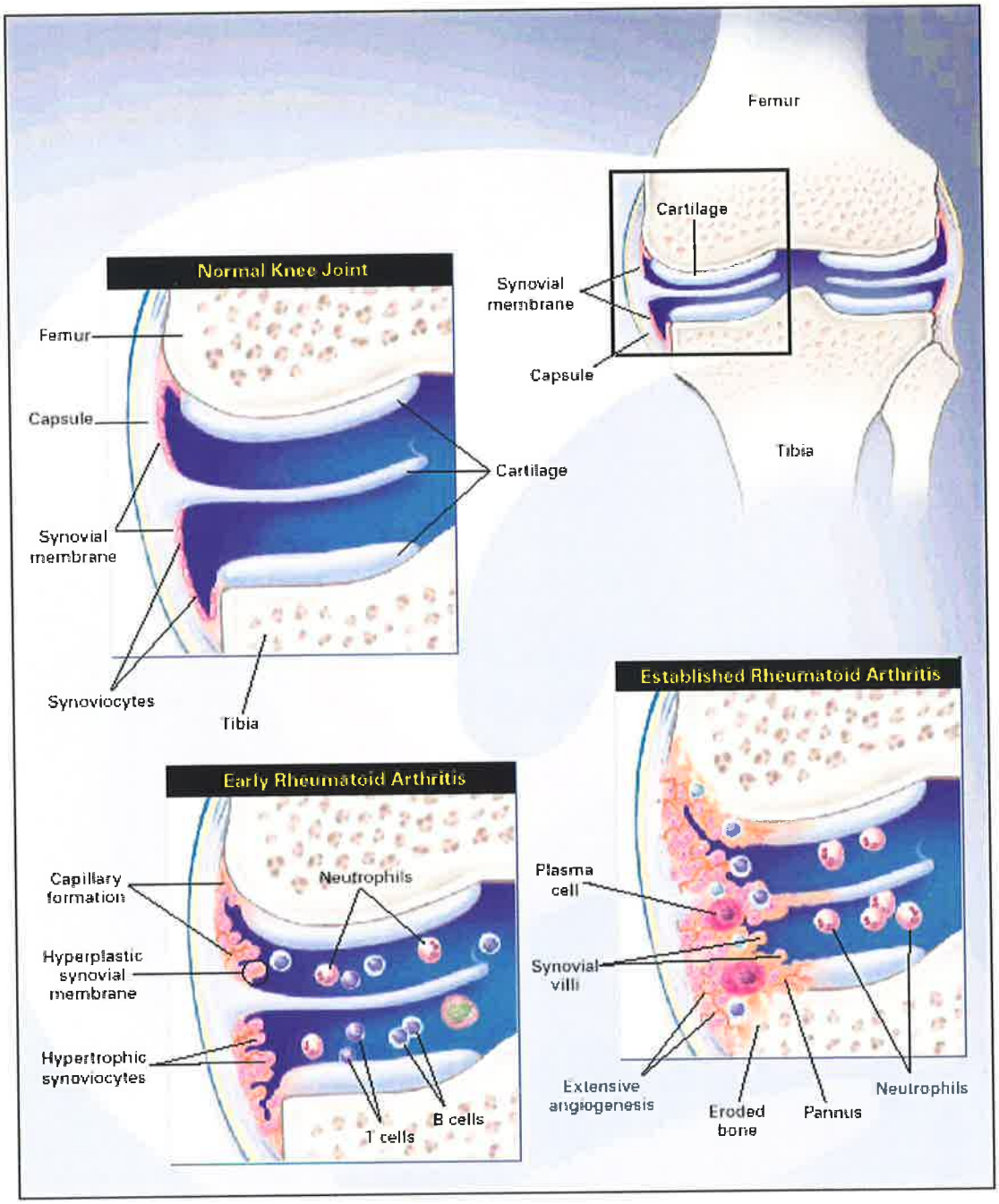


Figure 1.4: Schematic representation of a normal joint versus a rheumatoid joint (from Choy & Panayi 2001)

T cells account for approximately 30-50% of synovial tissue cells (Kinne et al 1997) and have been shown to aggregate around synovial capillaries (Kurosaka and Ziff 1983). The majority of synovial T cells are $CD4^+$ cells, which also express the low molecular weight isoform of CD45, CD45RO, characteristic of "memory" T cells (Kohem et al 1996).

Activation of T cells by antigen, presented in association with MHC class II molecules, results in clonal proliferation of T cells, which can stimulate effector cells such as monocytes. Activated T cells may also influence the functions of synoviocytes (Figure 1.5). While a number of triggering antigens have been proposed, it is unlikely that the same antigen triggers disease in all patients. Attempts to identify clones of T cells present within synovium, thus implicating T cells in the initiation of RA, have been equivocal (Sottini et al 1991; van Laar et al 1991; Jenkins et al 1993). However, in other inflammatory states it has been demonstrated that only a few antigen-specific activated T cells are required to orchestrate the immune response (Modlin et al 1988). Furthermore, there may be continued influx of T cells into the inflamed joint, through antigen independent mechanisms, which result in activation of the recruited cells and dilution of the antigen-specific T cells present.

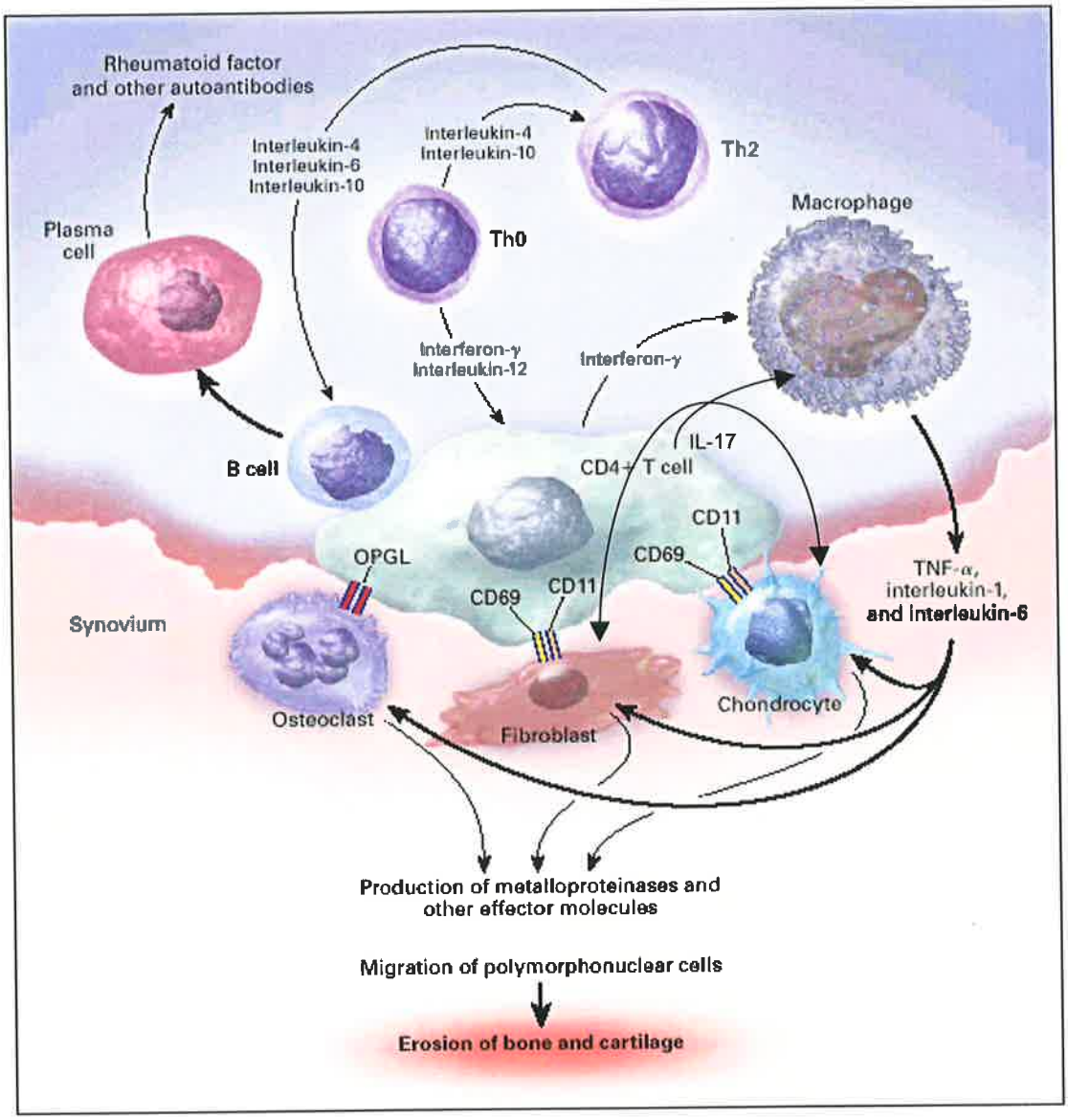


Figure 1.5: Cellular interactions and cytokines in rheumatoid arthritis (modified from Choy & Panayi 2001)

T cells present within the synovium are activated as evidenced by up-regulation of activation markers such as very late activation antigen (VLA)-1, CD69, human leukocyte antigen (HLA)-DR, and CD2R and down-regulation of CD3, CD2, and leukocyte function associated antigen (LFA)-1 (Panayi et al 1992). However, the IL-2 receptor, a T cell activation marker induced by TCR engagement, is absent (Pitzalis et al 1987; Afeltra et al 1993). T cell activation by antigen-independent mechanisms provides an alternative explanation for this specific activation of state of synovial T cells. Iannone et al demonstrated that *in vitro* T cells activated with PHA or antibodies directed against CD3 and CD28, sequentially express CD69, CD25, HLA-DR, and finally VLA-4. In contrast synovial fluid T cells expressed a combination of early (CD69) and late (HLA-DR and VLA-4) markers suggesting activation via antigen independent mechanisms. Furthermore they demonstrated that adhesion of T cells to HUVECs resulted in partial T cell activation with up-regulation of CD69 and HLA-DR but no change in CD25 expression. The authors suggest that pre-activated T cells may be preferentially recruited into joints and that CD69 and HLA-DR may be up-regulated during trans-endothelial migration (Iannone et al 1994). Interaction of VLA-5 receptor on $CD4^+$ T cells with fibronectin has also been shown to activate T cells and induce AP-1 transcription factor independent of TCR signaling (Yamada et al 1991). A further possible explanation, is that the function of dendritic cells, the professional antigen presenting cells of the immune system, which are abundant in RA synovium, may have altered or aberrant function. Dendritic cells, through their plethora of adhesive molecules and secretory capabilities may also be involved in stimulation of bystander T cells without relevant antigen specificity (Thomas and Quinn 1996; Pettit and Thomas 1999).

1.6.1.1.2 Animal models of arthritis

There are a number of animal models of arthritis and while no single model provides a definitive replica of human rheumatoid disease they can provide some insights in to disease pathogenesis in ways that are not feasible in human (clinical) studies. $CD4^+$ T cells have been shown to have an important role in the pathogenesis of animal models of polyarthritis (Banerjee et al 1992). Arthritis can be adoptively transferred in the absence of antigen. For example, in adjuvant-induced arthritis in the rat, it has been reported that $CD4^+$ T cells expressing the activation markers CD25, CD71, CD134, and MHC class II are fully competent arthritogenic effector cells (Spargo et al 2001).

1.6.1.1.3 Genetics

Twin studies indicate a genetic contribution to the development of RA (Aho et al 1986; Silman et al 1993). HLA-DR4 and /or HLA-DR1 are associated with RA and may be markers for more severe forms of disease (Arnett 1994). Within the DRβ1 alleles there is conserved region known as the "shared epitope". Several models have been suggested to explain how the presence of these MHC molecules contributes to RA. Most focus on the role of the HLA molecule in recognition and presentation of antigen to $CD4^+$ T cells. In this regard it is notable that the "shared epitope" is defined by neutral and positively charged amino acids that line the peptide binding grove of the HLA-DR variable chain (DRβ1) and make it suitable for presentation of candidate peptides that are negatively charged.

1.6.1.1.4 Treatment of rheumatoid arthritis

Despite recent advances, the management of RA remains unsatisfactory. The aim of therapy is to suppress synovial inflammation in quest of restoration and maintenance of joint structure and function. To this end there are a variety of agents available that modulate the immune system. With advances in the understanding of the role of cytokines in RA, biological agents targeting specific cytokines, such as TNF-α and IL-1β have become available. While these have proved of some benefit, their lack of or limited efficacy in some patients (Bresnihan et al 1998; Moreland et al 1999) highlights the fact that no single cytokine is responsible for the inflammatory process seen in RA.

Eicosanoids are also specifically targeted in the management of RA. Non-steroidal anti-inflammatory drugs inhibit eicosanoid production helping to reduce the pain and swelling associated with joint inflammation. Enhancement of dietary omega-three fatty acids, which provide an alternative substrate for COX, result in the production of eicosanoids, which in general, have lesser activities than their AA derived counterparts, have also been shown to be of benefit (Fortin et al 1995; James and Cleland 1997).

If T cells have an important role in RA one would predict that T cell directed therapies would be beneficial and indeed a number of T cell directed therapies have been used with some success. These include depletion of T cells by physical methods (total lymphoid irradiation, thoracic duct drainage, and lymphocytopheresis), chemical methods (cyclosporin A), and more recently biological therapies (monoclonal antibodies directed against T cell antigens including CD4, CD5, and CD7).

Of particular interest for the purpose of this thesis is cyclosporin A (CsA), an agent with potent immunosuppressive properties which inhibits of T cell function. CsA is a naturally occurring cyclic undecapeptide that was originally isolated from the fungus *Tolypocladium inflatum*.

CsA binds the intracellular receptor, cyclophilin, and the resulting CsA-cyclophilin complex inhibits calcineurin, a calcium and calmodulin dependent phosphatase, which is thought to be important in cytokine gene transcription. CsA inhibits production of a variety of cytokines, including IL-2, IL-4, and IFN-γ at the level of transcription (Kronke et al 1984; Russell et al 1992). Nuclear factor of activated T cells (NF-AT) is essential for the transcription of IL-2 and the inhibition of calcineurin by CsA, prevents the translocation across the nuclear membrane of a cytoplasmic subunit of NF-AT, thereby preventing the transcription of IL-2 (O'Keefe et al 1992; Schreiber and Crabtree 1992). The inhibition of IL-2 blocks proliferation of T cells and secondarily inhibits IL-4 and IFN-γ production.

1.6.1.2 Perpetuation of disease

Whether T cells are involved in the initiation of RA they are present within the joint and may have a role in perpetuation of the inflammatory state. Two important T cell related mechanisms may be involved: cytokine production, or lack thereof, and direct cell-cell contact between T cells and other cells present within the joint.

1.6.1.2.1 Cytokines

Despite the predominance of $CD4^+$ T cells within the synovium, there is a paucity of most T cell cytokines. Neither Th1 nor Th2 cytokines are abundant within rheumatoid joints. While the hallmark Th1 cytokine, IFN-γ, is detectable (Firestein et al 1990; Simon et al 1994; Ulfgren et al 1995; Dolhain et al 1996b; Sew Hoy et al 1997), the levels are low when compared to those in other Th1 mediated disease processes (Barnes et al 1990; Dolhain et al 1996a) or to the levels of IL-1β and TNF-α present. In addition there appears to be little Th2 activity, manifest by low levels of IL-4, IL-10, and IL-13 (Simon et al 1994; Sew Hoy et al 1997; Woods et al 1997; van Roon et al 2001). Th2 cytokines inhibit the production of pro-inflammatory cytokines and prostaglandins and thus low levels of Th2 cytokines may contribute to perpetuation of the inflammatory process in RA. Recently IL-17 has been found in high levels in rheumatoid synovium and synovial fluid (Chabaud et al 1999; Ziolkowska et al 2000). Overall the balance of cytokines present, rather than the absolute level of any one cytokine, is likely to be important in RA. The effects of IL-17, IL-1, TNF-α, and IL-1β have been discussed in section 1.5; the actions of other cytokines in RA are summarized in Table 1.7.

Table 1.7: Cytokines in rheumatoid arthritis

Cytokine	Pro-/anti-inflammatory	Cellular source	Level in RA	Role in rheumatoid arthritis
IL-1β	Pro-inflammatory	Monocytes/macrophages	High	↑TNF-α, ↑adhesion molecules, ↑IL-6, ↑COX-2, ↑PGE_2, ↑ bone resorption
IL-2	Pro-inflammatory	Th1 cells	Low	T cell growth factor, ↑IFN-γ, ↑TNF-α
IL-4	Anti-inflammatory	Th2 cells	Low	↓IL-1β, ↓TNF-α,↓IFN-γ
IL-6	Pro-/anti-inflammatory	Monocytes/macrophages, Th2 cells	High	↑acute phase proteins, ↓IL-1β, ↓TNF-α
IL-7	Pro-inflammatory	RA synovial tissue		↓lymphocyte cell death, ↑ adhesion molecules
IL-8	Pro-inflammatory	Monocytes/macrophages		↑angiogensis, ↑neutrophil activation
IL-10	Anti-inflammatory	Monocytes/macrophages, Th2 cells	Low	↑TNF receptors, ↓IL-2, ↓TNF-α, ↓IFN-γ, ↓GM-CSF, ↓IL-1β, ↓PGE_2, ↓adhesion molecules
IL-12	Pro-inflammatory	Monocytes/macrophages, Th1 cells	High	↑IL-1β, ↑TNF-α, with IL-18 ↑IFN-γ, induces T cells
IL-13	Anti-inflammatory	Th2 cells, monocytes	Low	↓IL-1β and TNF-α production by monocyte
IL-15	Pro-inflammatory	Monocytes, fibroblasts, endothelial cells	High	Induces T cells, induces NK cells, ↑IFN-γ,↓T cell death, activates neutrophils, ↑TNF-α
IL-16	Pro-/anti-inflammatory	Th2 cells, fibroblasts		↓IFN-γ, ↓↑IL-1β, ↓TNF-α
IL-17	Pro-inflammatory	Th1 cells	High	↑PGE_2, ↑MMP-9, enhances effects of IL-1β and TNF-α

Continued next page

Table 1.7 continued

Cytokine	Pro-/anti-inflammatory	Cellular source	Level in RA	Role in rheumatoid arthritis
IL-18	Pro-inflammatory	Monocytes/macrophages	High	↑IFN-γ, activates Th1 cells, ↑TNF-α, ↑IL-1β, ↑NK cells, enhances macrophage activation
TNF-α	Pro-inflammatory	Monocytes/macrophages, T cells	High	↑IL-1β, ↑adhesion molecules, ↑COX-2, ↑PGE_2,
IFN-γ	Pro-/anti-inflammatory	Th1 cells	Low	↑MHC II, activates monocytes/macrophages, ↑TNF-α, ↑/↓IL-1β
Transforming growth factor-β	Pro-/anti-inflammatory	Platelets, macrophages	High	↑PGE_2, ↓IL-1β, IL-2, TNF-α, ↑collagen synthesis
Macrophage inhibitory factor	Pro-/anti-inflammatory	Macrophages, T cells, pituitary cells	High	↑TNF-α, IL-1β, nitric oxide, ↑MMP-1 and MMP-3, ↑ by steroids, anti-proliferative

1.6.1.2.2 Direct cell-cell interactions in rheumatoid arthritis

In rheumatoid joints, T cells, monocytes, and synoviocytes lie in close proximity and interactions between the different cell types are known to occur. Recently it has been shown that activated T cells can induce monocyte/macrophage cytokine production (McInnes et al 1997; Parry et al 1997; Brennan et al 2002) and MMP release (Lacraz et al 1994). Furthermore, T cell enriched populations freshly isolated from RA synovial fluid, without further stimulation, induced TNF-α production by U937 monocytic cells (McInnes et al 1997). Activation of T cells with a combination of cytokines present within rheumatoid joints (IL-2, IL-6, TNF-α, and/or IL-15) induced monocyte TNF-α, but not IL-10 release. Addition of GM-CSF or IFN-γ enhanced monocyte TNF-α release even further (Sebbag et al 1997). The authors suggest cytokine activated T cells may thus contribute to the excess of TNF-α and paucity of IL-10 within inflamed joints.

Interactions between T cells and antigen presenting cells, including monocytes/macrophages, involve several co-stimulatory molecules including CD28/CD80-86 and CD40/CD40 ligand. CD40/CD40 ligand interactions have been reported to have a role in T cell contact dependent induction of monocyte IL-12 (Shu et al 1995) and IL-1β (Wagner et al 1994). Furthermore T cells derived from CD40 ligand knockout mice failed to induce macrophage TNF-α production (Stout et al 1996). However, it has also been reported that direct cell-cell interaction between THP-1 cells (monocyte cell line) and human Th1 cell clones resulted in preferential production of IL-1β, while Th2 cell clones induced IL-1Ra production (Chizzolini et al 1997). While IL-1β production was inhibited by blockade of the CD40/CD40 ligand with monoclonal antibodies, IL-1Ra production was unaffected (Ribbens et al 2000). Thus, although

CD40/CD40 ligand interactions may have a role in mediating activation of monocytes, via antigen-independent contact with T cells, other cell surface molecules must be involved.

Activation of T cells results in the expression of a number of cell surface molecules that may be involved in interactions with monocytes/macrophages. Incubation of T cells and monocytes with neutralizing antibodies directed against CD69, LFA-1, or intercellular adhesion molecule (ICAM)-1 have been shown to inhibit synovial T cell mediated cytokine production by macrophages (Isler et al 1993; McInnes et al 1997). Membrane bound TNF-α is a logical candidate for T cell-macrophage interaction and communication through T cell membrane bound TNF-α and monocyte TNF-α receptors. This mechanism has been shown, at least in part, to mediate T cell regulation of monocyte IL-10 (Parry et al 1997). In contrast, Lacraz et al reported that membrane forms of TNF-α were not involved in T cell contact mediated induction of monocyte MMP expression. In addition antibodies directed against CD11b, CD11c, and CD69 were able to partially inhibit IL-1β production but had no effect on MMP release (Lacraz et al 1994).

Direct cell-cell contact between T cells and fibroblast-like synoviocytes results in PGE_2 production (Burger et al 1998). In addition direct cell-cell contact between T cells and synoviocytes increases synoviocyte expression of vascular cell adhesion molecule (VCAM)-1 and ICAM-1, as well as increasing the amount of TNF-α, IFN-γ, and IL-6 in co-culture supernatants (Bombara et al 1993). The up-regulation of VCAM-1 and ICAM-1 and the production of cytokines was inhibited when direct contact between T cells and synoviocytes was prevented, but diffusion of soluble mediators was allowed (Bombara et al 1993). With regard to IL-17/IL-17R, contact between T cells and synoviocytes has been reported to increase T cell IL-17R mRNA expression. IL-17 mRNA was also increased

after co-incubation of T cells and fibroblasts. In addition supernatants from T cells co-incubated with synoviocytes enhanced the production of IL-6 and IL-8 by fibroblast-like synoviocytes (Thiele et al 2000). Furthermore fibroblast like synoviocytes activated by IL-1β and TNF-α produce cytokines, including IL-15 and IL-18 (McInnes et al 1997; Gracie et al 1999), which can in turn activate T cells resulting in the generation of positive feedback loops between synoviocytes and T cells.

These observations suggest that synovial T cells may have an important role in activating adjacent cells in the rheumatoid synovium. Figure 1.5 schematically represents some of the potential cellular interactions within the rheumatoid joint. Cell-cell interactions will be discussed further in relevant chapters.

The recognition of IL-17 as a pro-inflammatory lymphokine present within the rheumatoid joint and the interactions between T cells and monocytes/synoviocytes give new insights into the role of T cells in rheumatoid arthritis and may explain some of the paradoxical properties of T cells within the rheumatoid synovium. As part of this thesis I shall examine whether T cells can modulate COX-2 expression and eicosanoid synthesis by monocytes (Chapter 6) and synoviocytes (Chapter 7). The role of direct cell-cell contact and cytokines, in particular IL-17, IFN-γ, and TNF-α will be addressed.

1.6.1.3 End-stage destruction

Osteoporosis and bone and cartilage destruction are end-stage events in RA, which are thought to be related to chronic inflammation of the synovium. T cells may be directly involved in bone loss through production of IL-17 and expression of RANKL. The effects of IL-17 on cartilage destruction are discussed in section 1.5.2.5.3. Interaction between

RANKL expressed on T cells and RANK expressed on chondrocytes, osteoclast precursors, and mature osteoclasts results in the induction of osteoclastogenesis and bone loss (Kong et al 1999). Furthermore, activated peripheral T cells can induce osteoclastogenesis from adherent peripheral blood mononuclear cells *in vitro* (Kotake et al 2001).

T cells may also be involved in joint destruction through the induction of MMP production by monocytes and/or synoviocytes upon direct cell-cell contact with T cells (Lacraz et al 1994; Burger et al 1998).

1.7 Summary

Whether T cells are bystanders or important cells in the pathophysiology of RA, their presence affects the overall cytokine milieu within inflamed joints. T cell production (or lack of production) of cytokines and/or eicosanoids, as well as T cell contact dependent induction of cytokine and/or eicosanoid production by other cells may be important in the perpetuation of RA.

As a starting point, it is important to understand T cell COX isotype biology, including production of eicosanoids, which are potential mediators of inflammation. T cell derived eicosanoids may also affect T cell activity and T cell influences on neighboring heterotypic cells. Interactions between T cell and monocyte derived cytokines present in joints, may influence the expression of COX and the production of eicosanoids by T cells, monocytes and/or synoviocytes. Thus, in addition to examining T cells in isolation, interactions, via both soluble mediators and direct cell contact, between these different cell types that lie in close proximity in inflamed rheumatoid joints will be examined.

Aims

The aims of these studies were:

1. To determine a reliable and reproducible method for isolation of T cells with minimal platelet and monocyte contamination (Chapter 3).

2. To characterize the COX isotypes present in T cells and eicosanoid production by each isotype (Chapter 4).

3. To investigate the effects of TXA_2 on T cell function (Chapter 5).

4. To examine whether activated T cells could up-regulate monocyte COX-2 and if so identify candidate mediators (Chapter 6).

5. To examine interactions between T cells and synoviocytes with respect to COX-2 expression and eicosanoid production (Chapter 7).

Chapter 2

Materials and Methods

"Aristotle maintained that women have fewer teeth than men; although he was married twice, it never occurred to him to verify this statement by examining his wives' mouths."

Bertrand Russell 1952

2.1 T cell isolation

T cells were isolated from fresh human buffy coats by density gradient separation of mononuclear cells (MNC) followed by passage through nylon fibre wool columns (NFWC) (Werner et al 1977).

2.1.1 Preparation of mononuclear cells

Fresh buffy coats were obtained from the Red Cross Blood Center, Adelaide, South Australia. Buffy coats diluted 1:2 with phosphate buffered saline (PBS) were layered over 7mls of pyogen-free Lymphoprep^{TM} density separation medium (density 1.077 g/ml) (Nycomed Pharma, Oslo, Norway). Cells were centrifuged @ 160xg for 10 minutes and the platelet rich supernatant removed. The remaining cells were then centrifuged for 20 minutes @ 400xg to obtain a mononuclear cell enriched fraction. The mononuclear cells were removed and washed twice with PBS @ 160xg for 5 minutes. The mononuclear cells were counted using a haemocytometer and resuspended to a maximum of $1x10^8$/ml in warm RPMI 1640 medium supplemented with 10% heat-inactivated low LPS fetal calf

serum (FCS), L-Glutamine, HEPES, penicillin (100U/ml) and gentamicin in preparation for loading onto a NFWC.

2.1.2 Preparation of nylon fibre wool columns

Nylon fibre wool was obtained from Geneworks (Adelaide, South Australia). If not pre-sterilized, the wool was sterilized by boiling in 1% HCL for 5-10 minutes. After cooling, the wool was washed with RO water until the wash water was pH neutral and then dried at room temperature. 10 or 20ml nylon fibre wool columns were prepared using 10 or 20ml sterile syringes with the plunger removed. Wool was weighed (0.6gm wool for a 10ml column or 1.2gms wool for a 20ml column), combed until free of knots, folded, and placed into the syringe barrel. Columns were heat sealed in sterile bags and autoclaved at 121^0C for 15 minutes with a slow exhaust.

On the day of T cell isolation columns were opened in sterile conditions. A 21-gauge needle, attached to a short length of tubing, was attached to the syringe (Figure 2.1). RPMI 10%FCS, warmed to 37^0C, was placed in the column until running freely through the wool. The tubing was then clamped and more RPMI added. Air was removed from the column by pushing a pasteur pipette down the inside of the barrel. The clamp was removed to allow the RPMI to run through. Using the syringe plunger the wool was compressed to 5mls (10ml column) or 10mls (20ml column). After clamping the tubing 2mls of warm RPMI 10%FCS was added to prevent the column drying out. The column was sealed with cling film and incubated at 37^0C for at least 45 minutes.

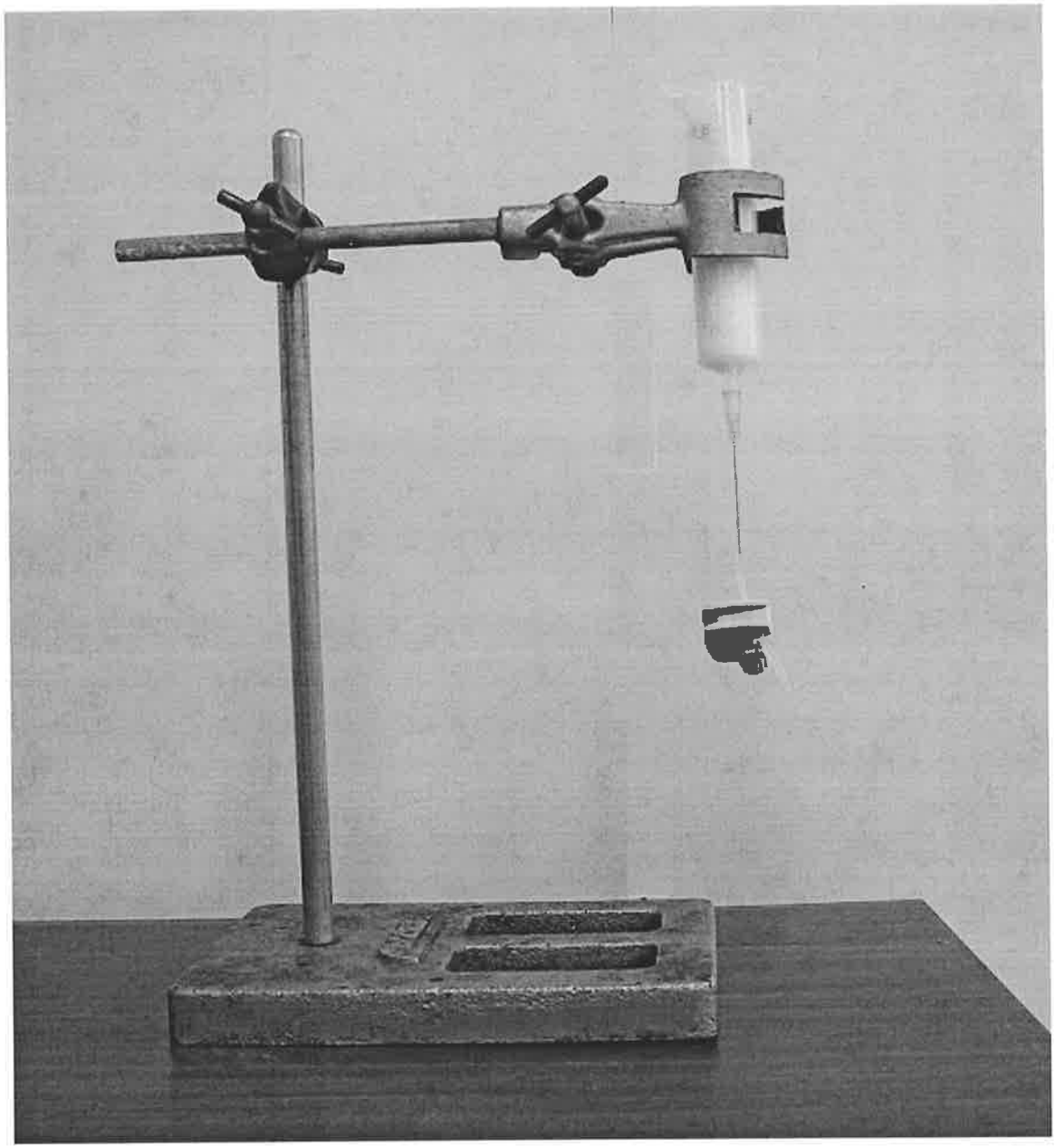


Figure 2.1: Nylon fibre wool column for T cell isolation

2.1.3 Isolation of T cells by passage through NFWC

The prepared mononuclear cells, suspended to a maximum of $1x10^8$/ml in warm RPMI 10%FCS, were dripped onto the prepared NFWC with the clamp open. A further 0.5-1.0ml of warm RPMI 10%FCS was added to ensure all cells had penetrated the column. The column was clamped, a further 1ml of warm RPMI added to a 10ml column (or 2ml for a 20ml column), and the column sealed with cling film. After incubation for one hour at 37^0C, 5%CO_2 non-adherent T cells were washed through the wool with 15mls of warm RPMI 10%FCS. Cells were centrifuged, counted, and resuspended at the desired concentration for experiments.

2.2 T cell stimulation

A variety of T cell stimuli were used (Table 2.1). The pharmacological agent phorbol 12-myristate 13-acetate (PMA) activates protein kinase C. It can be used alone or in combination with the calcium ionophore A23187, which acts to increase intracellular calcium levels. The lectin, concanavalin A (ConA), is also widely used for T cell activation and although its precise mechanism of action is unknown, it is thought to indirectly cross-link T cell receptors.

For stimuli other than monoclonal antibodies directed against CD3 and CD28, T cells were cultured in duplicate using a total volume of 1ml (or 2.5mls for Western immunoblot) in Minisorp non-adherent teflon tubes (Nunc, Copenhagen, Denmark). Cells were incubated for 18 hours at 37^0C, 5%CO_2, unless stated otherwise. Cell viability after the incubation period was assessed by trypan blue exclusion (>98%).

Table 2.1: T cell stimuli

Stimulus	Final concentration	Source
Phorbol 12-myristate 13-acetate (PMA)	15ng/ml	Sigma P-8139
Calcium ionophore A23187	1μM	Sigma C-7522
Concanavalin A (Con A) from *Canavalie ensiformis Type IV*	10μg/ml	Sigma C-2010
Immobilized anti-CD3	2μg/ml	BD Pharmingen
Soluble anti-CD28	250ng/ml	BD Pharmingen
Staphylococcal enterotoxin B (SEB)	5μg/ml	Sigma

2.2.1 T cell stimulation with immobilized anti-CD3 and soluble anti-CD28

This method of T cell stimulation involves the use of a monoclonal antibody directed against the T cell receptor signaling complex component, CD3, coupled with engagement by a second monoclonal antibody to the co-stimulatory molecule CD28. The antibody against CD3 was immobilized by adherence to the wells of microtitre plates whence it can cross-link CD3 resulting in cell activation.

Cell culture plates were prepared on the day of use. 30μL of anti-CD3 (2μg/ml in PBS) was added to each well of a 96-well round bottom plate (or 200μL per well in 24-well plate). Control wells were treated with PBS alone. The plates were covered and incubated for 90 minutes at 37^0C, 5%CO_2. The wells were washed three times by adding 200μL of cold PBS to each well and then inverting the plate to remove the PBS. 100μL of PBS was added to each well and the covered plates were stored at 4^0C until ready for use (not >24 hours). T cells were placed in the plate pre-coated with anti-CD3, to which the soluble

anti-CD28 (250ng/ml) was then added. Cells were incubated for 18 hours at 37^0C, 5%CO_2 unless otherwise stated. Cells were harvested by gently pipetting the supernatant in the well and then transferring the cells and supernatant into a non-adherent teflon tube. Cells and supernatants were then separated by centrifugation.

2.2.2 T cell stimulation with superantigen

Superantigens are microbial or viral protein toxins that are potent stimulators of T cells that bear particular Vβ components in their T cell receptor molecular rearrangement. Whilst peptide antigens activate ~0.01% of all T cells, superantigens, such as Staphylococcal Enterotoxin B (SEB), are capable of activating 20-50% of the T cell population. Superantigens bind MHC class II molecules, found on antigen presenting cells, and the variable region of the T cell receptor β-chain (Vβ). Unlike peptide antigens that require processing and presentation by an antigen presenting cell, superantigens require no processing. They bind outside the peptide-binding groove of the MHC cross-linking the MHC and T cell receptor Vβ-chain resulting in T cell activation. While superantigens can activate T cells in the absence of antigen presenting cells, the most profound activation of T cells occurs when superantigen is presented in association with MHC II present on antigen presenting cells.

Because optimal T cell activation requires the presence of antigen presenting cells, such as monocytes, mononuclear cells were isolated as described in section 2.1.1, resuspended at $2x10^6$/ml, and incubated for 18 hours at 37^0C, 5%CO_2 in non-adherent teflon pots with or without SEB (5μg/ml)(Sigma Chemicals Co., St. Louis, MO). T cells were then isolated

using nylon fibre wool columns as described above and monocytes were isolated by counter-current elutriation as described in section 2.4.

2.2.3 Inhibition of T cell activation by Cyclosporin A

As discussed in section 1.6.1.1.4 CsA is a potent immunosuppressive that inhibits the production of several T cell cytokines whose transcription is up-regulated on T cell activation. T cells were treated with CsA (100ng/ml)(Sigma Chemicals Co., St Louis, MO) for 30 minutes. Without washing cells were then transferred to wells pre-coated with anti-CD3 to which soluble anti-CD28 was added. Effective inhibition of T cell activation by CsA was demonstrated by measurement of [^{3}H]-thymidine incorporation and/or T cell cytokine production as shown in the relevant chapters.

2.3 T cell fixation

After stimulation T cells were collected and washed three times in RPMI 10%FCS. Cells were resuspended in 1ml of freshly prepared 2% paraformaldehyde for 5 minutes. Cells were washed a further three times in RPMI 10%FCS prior to resuspension in RPMI 10%FCS @ 2x10^6/ml. Cells were stored @ 4^{0}C until use (not longer than 12 hours).

2.4 *Monocyte isolation*

Monocytes were isolated from fresh human buffy coats by density gradient centrifugation followed by counter-current elutriation.

2.4.1 Counter-current elutriation

Counter-current elutriation separates cells on the basis of size and density. Cells suspended in buffer are loaded into a v shaped elutriation chamber housed within a centrifugal rotor. The flow pattern of the fluid within the chamber is counter to, or against the centrifugal force of the rotor (i.e. cells are carried by fluid that is pumped in towards the center of the rotor while the centrifugal force of the rotor drives the cells towards the outside of the rotor). At an appropriate rotor speed and flow rate the cells become dynamically suspended within the chamber in a zone where the sedimentation rate of the cell and the flow rate are balanced. Smaller cells will become located closer to the top of the chamber or the center of the centrifuge than larger cells and will exit the chamber earlier. Very small cells such as platelets that have a sedimentation rate too low to be balanced are washed out. Small cells, such as lymphocytes and red blood cells, are eluted out at low flow rates while larger cells such as monocytes can be retained within the chamber under conditions that elute lymphocytes. Thus cell populations can be isolated on the basis of size by using appropriate rotor speed and flow rates (Figure 2.2).

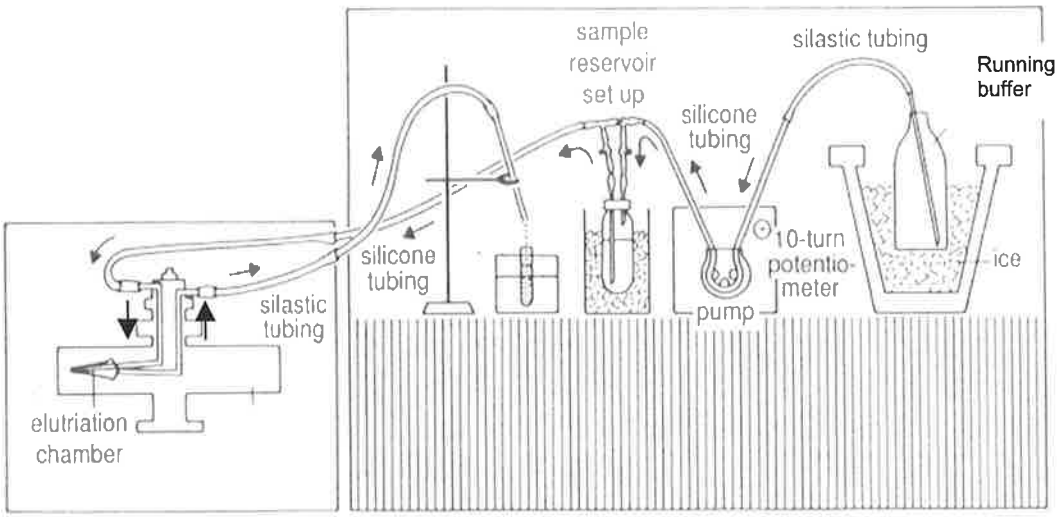


Figure 2.2: Schematic representation of counter-current elutriation set up (modified from Coligan 1991)

2.4.2 Monocyte isolation

Mononuclear cells were obtained from fresh buffy coats as described in section 2.1.1. Monocytes were then isolated by counter-current elutriation using a Beckman JE-5.0 elutriation rotor mounted in a Beckman J-6M/E series centrifuge (Beckman Instruments Inc. Fullarton, CA). A strobe light enabled viewing of the elutriation process.

Before each elutriation the machine was cleaned with 250mls of 1% E-toxa clean solution (Sigma Chemical Co., St Louis, MO). The machine was then flushed with 250mls of 70% ethanol solution and rinsed with 250mls of milliQ H_2O to remove any residue. Prior to elutriation of monocytes the machine was equilibrated with running buffer. The freshly prepared mononuclear cells, suspended in running buffer, were slowly injected into a sample reservoir upstream of the elutriation chamber. The flow rate was gradually increased over 10-15 minutes. The platelets and red blood cells were eluted first followed by lymphocytes while monocytes were retained within the elutriation chamber. After a total running time of 30 minutes the rotor was stopped and the flow rate increased to empty the remaining cells from within the elutriation chamber. The first 50mls of cell suspension was collected and the cells centrifuged @ 200xg. Cells were resuspended in RPMI 10%FCS and counted using a haemocytometer. Monocyte purity, determined by flow cytometry, was generally ≥85%.

2.5 Monocyte stimulation

Monocytes were resuspended in RPMI 10%FCS at $2x10^6$/ml, unless otherwise stated. Each experimental condition was undertaken in duplicate, using a total volume of 1ml (or 2.5mls for Western immunoblot), in Minisorp non-adherent teflon tubes for 18 hours at

37^0C, 5%CO_2 unless otherwise stated. Monocytes were stimulated with LPS (Sigma Chemical Co.) to a final concentration of 200ng/ml.

The effects of IL-17 and IFN-γ on monocytes were examined using recombinant human (r)IL-17 (R&D Systems) and rIFN-γ (Endogen). Each experimental condition was undertaken in duplicate, using $5x10^6$ cells in 2.5mls in Minisorp non-adherent teflon tubes. Recombinant cytokines were added at the start of the 18 hour incubation period of unless otherwise stated.

2.6 *Determination of cyclooxygenase activity*

The activity of COX, before or after cell stimulation, can be assessed using short-term incubations in the presence or absence of AA (Sapphire Biosciences, Sydney, Australia). After incubation with stimulus, cells were washed twice with FCS-free RPMI prior to the addition of AA (10μM) for 30 minutes @ 37^0C, 5%CO_2. The reaction was stopped by centrifugation of samples, followed by separation of cells and supernatants. Supernatants were stored at -20^0C until analysis was undertaken.

2.7 *Selective inhibition of COX-1 and COX-2*

COX-1 inhibition was achieved by treating cells with aspirin (55μM) (Roche) for 30 minutes. Cells were then resuspended in fresh medium for 10 minutes before washing to remove all aspirin prior to cell stimulation. The transient aspirin treatment followed by aspirin removal before cell stimulation, allows inhibition of basal constitutively expressed

COX-1 with no effect on COX-2, which if up-regulated by the cell stimulus, is not exposed to aspirin (Demasi et al 2000).

Specific COX-2 inhibition was achieved by treatment of cells with NS398 (0.5μM)(Cayman Chemicals, Ann Arbor, MI).

2.8 *Determination of the effects of TXA_2*

The effects of TXA_2 on T cells were examined using a specific TX synthase inhibitor (carboxyheptyl imidazole)(Biomol), thromboxane receptor antagonists (SQ29548 or pinane (p)TXA_2)(Cayman Chemicals), or a stable TXA_2 analogue that acts as a TX receptor agonist (carbocyclic (c)TXA_2)(Cayman Chemicals, Ann Arbor, MI). Carboxyheptyl imidazole (CI) has previously been shown to specifically inhibit TX synthase without affecting the other terminal eicosanoid synthases (Yoshimoto et al 1978; Kayama et al 1981).

T cells were treated with CI (10μM), SQ29548 (1μM or 10μM), or pTXA_2 (10μM) for 30 minutes. Without washing cells were transferred to wells pre-coated with anti-CD3 and soluble anti-CD28 was added. cTXA_2 was added to a final concentration of 0.01-10μg/ml immediately prior to stimulation. Vehicle controls were included for each experiment.

2.9 *T cell - monocyte co-culture*

$2.5x10^6$ T cells in 0.5ml RPMI 10%FCS were placed into 24-well plates pre-coated with antibody directed against CD3 and to which antibody directed against CD28 (250ng/ml) were added. A transwell (0.4μM, 10mm Nunc, Roskilde, Denmark) was inserted into each well and $2.5x10^6$ monocytes in 0.5ml RPMI 10%FCS, placed into the transwell. The transwell prevents direct heterotypic cell contact but allows migration of soluble mediators (Figure 2.3). Each experimental condition was undertaken in duplicate. After incubation for 18 hours at 37^0C, 5%CO_2 T cells and monocytes were collected separately. T cell and monocytes from duplicate wells were combined for protein extraction for Western immunoblot.

2.9.1 Neutralization of IL-17 and IFN-γ in the transwell system

Monoclonal neutralizing antibodies directed against IL-17 (R&D Systems) and IFN-γ (R&D Systems and Endogen) were used to examine the effect of these cytokines in the interaction between T cells and monocytes in the transwell system. T cells and monocytes were isolated as described above and resuspended at $5x10^6$/ml. Neutralizing antibodies directed against IL-17 (10μg/ml), IFN-γ (1μg/ml or 10μg/ml) or their respective isotype control antibodies (IgG2a (10μg/ml) or IgG1 (1μg/ml or 10μg/ml)) directed against an irrelevant specificity were added to both T cells and monocytes. T cells and monocytes were then placed in the transwells and stimulated as described above.

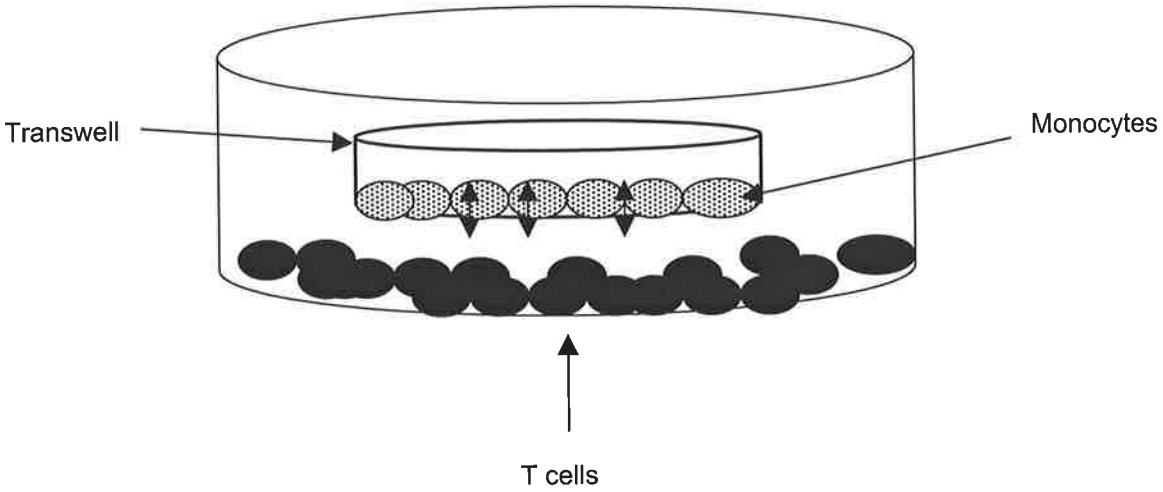


Figure 2.3: Transwell co-culture system for T cells and monocytes

2.10 *Culture of fibroblast-like synoviocytes*

Ethical approval was obtained from the Royal Adelaide Hospital Human Ethics Committee. Written informed consent for use of synovial fluid samples was obtained from patients with inflammatory arthritis undergoing joint aspiration. Synovial fluid was collected into a sterile syringe and transferred to a sterile 50ml conical tube. Cells were centrifuged @ 500xg for 10 minutes and the supernatant removed. Cells were resuspended in complete RPMI 20%FCS supplemented with 2μg/ml amphotericin and transferred to tissue culture flasks (Cellstar). Cells were incubated at 37^0C, 5%CO_2 for the duration of culture. Medium was changed until the cells were confluent.

To remove fibroblast-like synoviocytes from the plate, medium was removed and the cells washed once with warm sterile PBS. 0.05% Trypsin/0.53M EDTA was added and cells incubated @ 37°C until cells were released from the plate, usually 5-10 minutes. An equal volume of RPMI 10%FCS was added and the cells collected into a sterile 50ml tube. Each plate was washed once to complete removal of synoviocytes. Cells were centrifuged at 400xg for 5 minutes and sub-cultured in tissue culture flasks. Fibroblast-like synoviocytes were used between the 3^{rd} and 6^{th} passages. Flow cytometric analysis revealed no $CD14^+$ macrophage-like synoviocytes were present at the third passage.

2.10.1 Freezing synoviocytes

On some occasions fibroblast-like synoviocytes were frozen and stored. Synoviocytes were removed from the plate using trypsin-EDTA, centrifuged, counted, and resuspended at a concentration $\leq 2x10^7$/ml in RPMI 10%FCS. Immediately prior to freezing, an equal volume of freezing mix (20%FCS, 20%DMSO, 50%RPMI) was added drop wise over 5 minutes. Cells were aliquoted into 1ml ampoules and immediately subject to a controlled rate-freezing program (Planer Kryolo Series 2).

When required for use cells were rapidly thawed in a 37^0C water bath. Cells were transferred to a sterile tube and 1 ml of warm RPMI 10%FCS was added drop wise over 5 minutes. Cells were allowed to rest for 15 minutes prior to the addition of a further 2mls warm RPMI 10%FCS over 5 minutes. After a further 15 minute rest, cells were centrifuged and washed twice with RPMI 10%FCS. Cells were counted and viability assessed by Trypan Blue exclusion test (≥95%). Cells were resuspended in RPMI 20%FCS with 2μg/ml amphotericin and cultured as described above.

2.11 Synoviocyte stimulation

One day prior to the experiment, synoviocytes were trypsinised in the tissue culture flask and centrifuged @ 400xg. Cells were counted and resuspended in RPMI 20%FCS @ $2.5x10^5$/ml. $5x10^5$ cells, in 2mls, were added to each well of a 6-well plate (Nunc, Roskilde, Denmark). Cells were incubated at 37^0C, 5%CO_2 for 18 hours, the medium was then removed and the cells washed once with RPMI 10%FCS. 2mls of fresh RPMI 10%FCS was added to each well. Synoviocytes were stimulated with IL-1β at a final concentration of 2ng/ml (Boehringer Mannheim) and incubated for a further 18 hours at

37^0C, 5%CO_2 unless otherwise stated. Alternatively synoviocytes were stimulated with rIL-17, rIFN-γ, or rTNF-α at concentrations described in the relevant results sections.

2.12 Synoviocyte - T cell co-culture

One day prior to the experiment, synoviocytes were plated in 6-well tissue culture plates as described in section 2.11. Synoviocytes were incubated for 18 hours at 37^0C, 5% CO_2 and the medium was removed. Synoviocytes were washed once with RPMI 10%FCS and 1ml of fresh RPMI 10%FCS added to each well.

One day prior to the experiment, T cells were prepared as described in section 2.1. $1x10^6$ T cells in 1ml RPMI 10%FCS, were added to 24-well tissue culture plates pre-coated with anti-CD3 (2μg/ml) to which soluble anti-CD28 (250ng/ml) was added. After 18 hours incubation T cells and supernatants were collected and separated by centrifugation.

Fixed T cells resuspended in 1ml of fresh medium, or 1ml of supernatant from T cells were added to synoviocytes. One ml of fresh medium was added to control wells with no fixed cells or supernatants, so the total volume in each well was 2mls. Cells were incubated for 18 hours at 37^0C, 5%CO_2, unless otherwise stated The supernatants were then collected and stored at -20^0C until eicosanoid measurement. Synoviocytes were collected from the plate and protein was extracted for Western immunoblot.

2.12.1 Neutralization of IL-17 and TNF-α in T cell-synoviocyte co-culture

Monoclonal neutralizing antibodies directed against IL-17 and TNF-α (R&D Systems) were used to examine the effects of these T cell derived cytokines in T cell-synoviocyte co-culture experiments. T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 as described above and after 18 hours supernatants were collected. Neutralizing antibodies directed against IL-17 (10μg/ml), TNF-α (1μg/ml), or their respective isotype controls (IgG2a (10μg/ml) or IgG1 (1μg/ml)) were added to the supernatants. Supernatants were incubated at room temperature for 4 hours at which time the supernatants were added to synoviocytes. Synoviocytes were incubated for a further 18 hours at 37^0C, 5%CO_2 unless otherwise stated.

2.13 Measurement of TXB_2, PGE_2, & 6-keto-$PGF_{1\alpha}$ by RIA

PGE_2, TXB_2, and 6-keto-$PGF_{1\alpha}$ (a metabolite of PGI_2) were measured using RIA. TXA_2 has a half-life of ~30 seconds being rapidly hydrolyzed to the more stable metabolite TXB_2, which was measured. RIA relies on competitive binding of the anaylate to specific antibodies. PGE_2 and TXB_2 antibodies were raised in rabbit as previously described (James and Walsh 1988). PGE_2 anti-sera was used at a dilution of 1:1500 in RIA buffer (0.1%gelatin, 0.9 NaCl, 0.01M Trisbase pH 7.3). The working dilution of rabbit anti-sera against TXB_2 was 1:4000. 6-keto-$PGF_{1\alpha}$ anti-sera (Sigma Immunochemicals) was used according to the manufacturers instructions. Working dilutions of anti-sera were stored at -20^0C until use.

Samples were prepared in triplicate, mixing 100μL of RIA buffer with 100μL the appropriate anti-sera and 100μL sample or standard (Sapphire Biosciences). 100μL of [^{3}H]-PGE_2 (183Ci/mmol) in Na_2CO_3, [^{3}H]-TXB_2 (212Ci/mmol) or [^{3}H]-6-keto-$PGF_{1\alpha}$ (190Ci/mmol) (Amersham, Australia) in PBS was added, making a total volume of 400μL. Samples were incubated at 37^{0}C, 5%CO_2 for 2 hours and then 4^{0}C for at least 1 hour. Dextran charcoal (500μL) was added to capture unbound radionuclide-labelled probe and samples centrifuged at 800xg for 20 minutes @ 4^{0}C. 500μL of supernatant was added to 1.5mls of scintillation fluid (OptiPhase Hisafe 3, Perkin Elmar Life Sciences) and counted in a Wallac 1409 liquid scintillation counter (Wallac Oy Turku, Finland).

Data were analyzed using Graph Pad Prism and final results were taken as the average of each triplicate. In the case of TXB_2, where RPMI 10%FCS contains detectable levels of TXB_2, medium alone was assayed and the level of TXB_2 detected subtracted from that found in experimental incubations.

2.14 Measurement of PGD_2

PGD_2 was initially assayed using a PGD_2-MOX enzyme immunoassay kit (Cayman Chemicals) according to the manufacturers instructions. When T cells produced detectable levels of PGD_2, attempts were made to establish our own RIA as described below.

2.14.1 Production of PGD_2 antibody

2.14.1.1 Preparation of PGD_2 antigen

Methods were adapted from Caldwell et al (Caldwell et al 1971). 5mg of PGD_2 (Cayman Chemicals) was dissolved in 0.5ml dimethyl formamide (DMF). 16.2mg of bovine

thyroglobulin was dissolved in 2mls of milliQ H_2O in a separate beaker. This was stirred constantly until dissolved and pH adjusted to 5.2. 10mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC)(Sigma) was then added to the thyroglobulin solution and pH adjusted to 5.2. The PGD_2/DMF was added to the thyroglobulin/H_2O/EDAC and stirred constantly for 24 hours.

The solution was resuspended in 0.5ml Na_2CO_3 0.5M solution and placed in dialysis tubing. The solution was dialyzed with Na_2CO_3 0.5M at 4^0C for 2 hours, three times, then with phosphate buffer 0.03M (KH_2PO_4 0.03M and K_2HPO_4 0.03M, pH7.4) for 8 hours at 4^0C. Finally the solution was dialyzed overnight with milliQ H_2O at 4^0C. The PGD_2-thryoglobulin conjugate solution was removed from the dialysis tubing and 0.6mg aliquots were stored at -20^0C.

2.14.1.2 Polyclonal antibody production

The Institute of Medical and Veterinary Science, Adelaide undertook polyclonal antibody production. 0.6mg of the PGD_2 solution with Freund's Complete Adjuvant was injected subcutaneously into 1 rabbit. Booster injections of the same dose, conjugated with Freund's Incomplete Adjuvant (FIA) were given at 3 and 6 weeks. A test serum was obtained after a further 10 days.

The pre- and post-inoculation sera were tested for PGD_2 antibody using RIA as described above. No PGD_2 antibody could be detected therefore a further dose of 1.2mg of the PGD_2 solution with FIA was given. A further blood sample taken 10 days later revealed detectable PGD_2 antibody in the serum. Two further doses of 1.2mg of PGD_2 (one with and one without FIA) were given before the rabbit was exsanguinated and sacrificed. The

presence of the PGD_2 antibody was tested by RIA and the working dilution of the serum was determined to be 1:500.

2.14.1.3 PGD_2 Standard Curve and PGD_2 antibody working dilution

5mg of PGD_2 was dissolved in ethanol and a stock solution of 100ng/100μL prepared. Standards were prepared by diluting the stock solution in PBS to form a concentration range from10pg/100μL to 10ng/100μL. A typical standard curve is shown in Figure 2.4.

2.14.1.4 Cross-reactivity of PGD_2 with PGE_2 and TXB_2

Given the similarity between structure of the prostaglandins, there is potential for cross-reactivity between the PGD_2 antibody and other prostaglandins, such as PGE_2. The antibody was tested against TXB_2 and PGE_2 and found to cross react with PGE_2 by ~ 30% (Figure 2.5). It was therefore decided that we could not obtain reliable results using the prepared antibody. No further attempts were made to measure PGD_2.

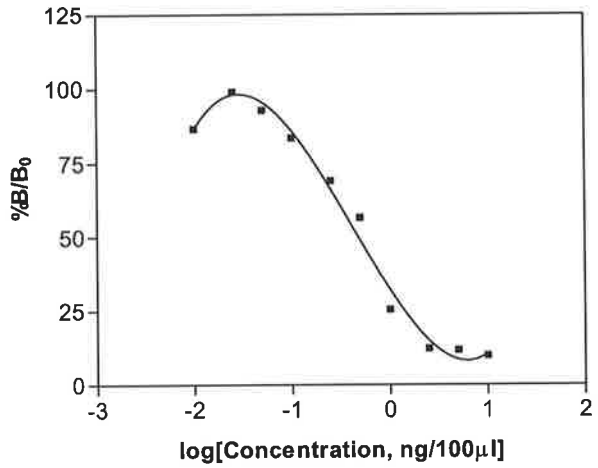


Figure 2.4: PGD_2 standard curve

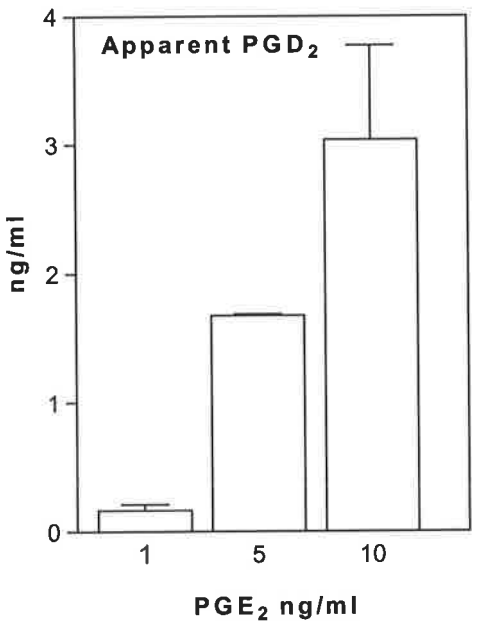


Figure 2.5: Cross-reactivity of PGE_2 with PGD_2 antibody

2.15 *Western immunoblot*

2.15.1 Protein extraction

5 x10^6 T cells or monocytes or 5x10^5 synoviocytes were pelleted after incubation with stimuli. Cells were washed twice with 1ml ice cold PBS. This was followed by the addition of 60μL of ice cold lysis buffer (HEPES-buffered HBSS, pH7.4, 0.5% Triton X-100, 10μg/ml leupeptin, 10μg/ml aprotonin) and 60μL of 2x sample buffer (0.125M Trizma base, pH6.8, 20% glycerol, 4% sodium dodecyl sulphate (SDS), 10% 2-mercaptoethanol). Samples were heated @ 95^{0}C for 7 minutes and then stored at –20^{0}C until use.

2.15.2 Protein separation and transfer

Samples were separated using a 9% acrylamide gel in a 15-well vertical gel unit (SE400, Hoeffer Scientific Instruments, San Francisco, California) (See appendix). 10μL of pre-stained broad range SDS-PAGE standard (BioRad Laboratories, CA) was loaded into the outside well and the samples into the remaining wells. Half of the protein obtained from T cells and monocytes was loaded while all of the protein from synoviocytes was loaded. Samples were allowed to run at 18mAmps for ~ one hour, followed by migration at 160 volts until the bottom of the gel had been reached (~5 hours). Proteins were transferred onto Trans-Blot® PVDF or nitrocellulose membrane at 4^{0}C for 16 hours at 300mAmps in transfer buffer.

2.15.3 Membrane probing for specific proteins

The membrane was soaked for 1 hour at room temperature in tris-buffered saline (TBS)-tween (TBS; 25mM Tris HCl, 0.2M NaCl, 0.15%Tween-20, pH7.6) containing 5% dried skim milk (w/v) to reduce non-specific binding. Membranes were then soaked in antibodies diluted in TBS-tween for 1 or 18 hours as required (Table 2.2). Membranes were soaked for 1 hour at room temperature, while those requiring 18 hours were soaked at 4^0C.

After exposure to primary antibody membranes were washed with TBS-tween for 1 hour and then exposed to secondary antibody, peroxidase-labeled goat anti-mouse or donkey anti-rabbit (1:20,000 dilution in TBS-Tween) for 1 hour. After a further wash with TBS-tween for 1 hour, antibody cross reactivity was revealed using enhanced chemiluminescence kit, (ECL^{TM})(Amersham), according to the manufacturers instructions. Membranes where then exposed to photographic film for 1-10 minutes as required.

Images were scanned using a Sharp JX-610 scanner and densitometry undertaken with ImageMaster 1D Elite software.

Table 2.2: Antibodies used for Western immunoblot

Target	Type	Concentration	Length exposure	Source
COX-1	Mouse monoclonal	10μg/ml	Overnight	Cayman Chemicals
COX-2 (human)	Rabbit polyclonal	10μg/ml (T cells) 5μg/ml (monocytes)	Overnight (T cells/synoviocytes) 1 hour (monocytes)	Cayman Chemicals
COX-2 (murine)	Rabbit polyclonal	1:1000	Overnight	Cayman Chemicals
TX synthase	Rabbit polyclonal	0.5μg/ml	Overnight	Cayman Chemicals
Thromboxane receptor	Rabbit polyclonal	1:1000	Overnight	Gift from Dr Halushka
PGE synthase	Rabbit polyclonal	2μg/ml	Overnight	Cayman Chemicals
H-PGD synthase	Rabbit polyclonal	2μg/ml	Overnight	Cayman Chemicals
Phospho-$cPLA_2$	Rabbit polyclonal	1:500	Overnight	Cell signaling
β-actin	Mouse monoclonal	0.5μg/ml	1 hour	Sigma

2.16 Fluorescence activated flow cytometry

Flow cytometry was used to assess cell purity and cell activation. It was also used to identify the presence of COX-1, COX-2, and TX synthase within cells. Antibodies used and their sources are listed in Table 2.3. The following directly conjugated monoclonal antibodies (mAbs) were obtained for use in 2-colour flow analysis: CD3-PE, CD3-FITC, CD25-PE (BD Pharmingen), COX-1-FITC and COX-2-PE (Cayman Chemicals). Polyclonal FITC-conjugated goat-anti-mouse or polyclonal FITC-conjugated goat-anti-rabbit antibody was used as the secondary antibody to reveal staining with indirectly conjugated primary antibodies. A minimum of 20,000 cells were analyzed using a COULTER® EPICS® XL-MCL flow cytometer and SYSTEM II™ v.3 software.

Table 2.3: Mouse anti-human and anti-rat monoclonal antibodies used for flow cytometric analysis

Antibody	Clone	Specificity	Distribution	Isotype	Form	Concentration	Source
CD3	UCHT1	ε-chain TCR	T cells	IgG1	purified	10μg/ml	BD Pharmingen
CD14	M5E2	Glycosylphosphatidylinositol-chain	Monocytes	IgG2a	purified	10μg/ml	BD Pharmingen
CD15	HI98	FAL/X hapten	Granulocytes	IgM	purified	10μg/ml	BD Pharmingen
CD16	3G8	FCγRIII	NK cells, granulocytes, and macrophages	IgG1	purified	10μg/ml	BD Pharmingen
CD19	HB19	Type 1 transmembrane glycoprotein	B cells (not plasma cells)	IgG1	purified	10μg/ml	BD Pharmingen
CD41a	HIP8	GIIBIIIA receptor	Platelets	IgG1	purified	neat	BD Pharmingen
CD25	M-A251	IL-2R	T cells	IgG1	purified	neat	BD Pharmingen
CD69	FN50	Early activation marker	Lymphocytes, monocytes	IgG1	purified	10μg/ml	BD Pharmingen
COX-1		COX-1	All cells	IgG2b	purified	100μg/ml	Cayman Chemicals
COX-2		COX-2	Inducible in some cells	IgG1	purified	40μg/ml	Cayman Chemicals
TX synthase		Thromboxane synthase	Monocytes, platelets	polyclonal	purified	10μg/ml	Cayman Chemicals

Continued next page

Table 2.3 continued

Antibody	Clone	Specificity	Distribution	Isotype	Form	Concentration	Source
Mouse IgG1-FITC		MOPC-21 mouse myeloma cell line	IgG1 control	IgG1	purified	neat	BD Pharmingen
Mouse IgG1-PE		MOPC-21 mouse myeloma cell line	IgG1 control	IgG1	purified	neat	BD Pharmingen
Mouse IgG2b-FITC		Hapten dansyl (5[dimethylamino] naphthalene-1-sulfonyl	IgG2b control	IgG2b	purified	neat	BD Pharmingen
	IB5	*Giardia* surface antigen	IgG1 control	IgG1	supernatant	neat	Hybridoma (L.Spargo)
	ID4.5	*Salmonella typhimurium*	IgG2a control	IgG2a	supernatant	neat	Hybridoma (L Ashmann)
	1A6.11		IgG2b control	IgG2b	supernatant	neat	Hybridoma (L Ashmann)
	IA6.12		IgM control	IgM	supernatant	neat	Hybridoma (L Ashmann)
Rabbit polyclonal	RPG	*Giardia*	Polyclonal control	Polyclonal	purified	10μg/ml	Prof. G Mayrhofer
	R73	Framework determinant of α/βT cell receptor	α/βT cells	IgG1	purified	2μg/ml	BD Pharmingen
	OX33	B cell specific isoform of CD45	B lymphocytes	IgG1	supernatant	neat	Hybridoma (L.Spargo)
	MARK-1	Anti-κ light chain	B lymphocytes	IgG1	supernatant	neat	Hybridoma (L.Spargo)

2.16.1 Single labeling

Primary antibodies were initially titrated using dilutions of 1:50, 1:250, 1:500, and 1:2500. For all subsequent analyses primary and secondary antibody dilutions of 1:50 were used.

In order to block non-specific binding through Fc receptors, antibodies were always used in the presence of 10% normal human serum (NHS) (or normal rat serum), which had been heat inactivated @ 56^0C for 40 minutes.

$1x10^6$ cells were suspended in 50µL of primary antibody, vortexed, and incubated on ice in the dark for 45 minutes. Cells were then washed twice in 3mls of immunofluoresence (IF) buffer (PBS, 2%FCS, 0.01M azide), resuspended in 50µl secondary antibody and incubated in the dark on ice for 45 minutes. Cells were again washed twice in 3mls IF buffer and resuspended in 0.5mls FACS fix (PBS, 1% formalin (v/v), 2% glucose (w/v), 0.02% azide). Cells were covered with aluminium foil and stored in the dark at 4^0C until flow cytometry could be performed (not longer than 3 days). Control incubations containing either no antibodies, isotype matched mouse-anti-rat antibodies against irrelevant specificities, or secondary antibody alone were undertaken with each analysis.

2.16.2 Double labeling

After first staining with the indirectly conjugated monoclonal antibody and its revealing antibody, 20μL of heat inactivated normal mouse serum (IMVS, Adelaide) was added and cells incubated for 15 minutes. Without washing, the cells were incubated on ice in the dark for 45 minutes with 50μL of the second directly conjugated antibody. Cells were washed twice with 3mls of IF buffer prior to the addition of 0.5mls of FACS fix. Cells were covered with aluminium foil and stored at 4^0C in the dark until flow cytometry could be undertaken.

2.16.3 Intracellular staining

COX-1, COX-2, and TX synthase were assessed by intracellular staining of permeabilised cells. $1x10^6$ cells were resuspended in 1ml of Dulbecco's (D)PBS 1%FCS and cell surface antibody (CD3 for T cells or CD14 for monocytes) applied as described above. Cells were then resuspended in 1ml DPBS (no FCS) and fixed with 1ml of 10% buffered formalin for 6 minutes. Cells were washed twice with 3mls of DPBS 1%FCS. A third wash was undertaken using 3mls of DPBS/1%FCS/0.1%saponin (Sigma Chemicals) to permeabilise the cells. Cells were then incubated with directly conjugated COX-1 or COX-2 antibody for 45 minutes on ice in the dark. Excess intracellular antibody was removed by washing cells with 3mls of DPBS/1%FCS/0.1%saponin buffer. Saponin was removed by washing twice with DPBS/1%FCS prior to the addition of 0.5ml of FACS fix. Cells were covered with aluminium foil and stored at 4^0C in the dark until flow cytometry could be undertaken. Since TX synthase was an indirectly conjugated antibody it was applied first and then followed by a second directly conjugated cell surface marker.

2.16.4 Preparation of normal human serum

30mls blood from health volunteers was collected into three 8ml Z serum sep. clot activator tubes. Blood was placed in a 37^0C water bath until clot retraction was complete (approximately 1-2 hours). Serum was separated by centrifugation @ 600xg for 10 minutes. Serum was heat inactivated @ 56^0C for 40 minutes, aliquoted, and stored at -20^0C until use.

2.17 T cell proliferation – [^{3}H]-thymidine incorporation

T cell proliferation was assessed by [^{3}H]-thymidine incorporation. $1x10^5$ T cells in 100μL RPMI 10%FCS were added to round bottom 96-well plates pre-coated with anti-CD3 to which soluble anti-CD28 was added. Cells were incubated for 72 hours at 37^0C, 5%CO_2 prior to addition of 1μCi [^{3}H]-thymidine (25Ci/mmol) (Amersham International, Little Chafont, England) per well. Cells were harvested after a further 18 hours incubation using a Skatron Combi 11025* cell harvester. Filter paper was pre-wet with milliQ H_2O, cells were harvested for 5 seconds followed by a 15 second wash and 10 second air dry. Filters were dried at room temperature and then each disc added to 1.5mls of scintillation fluid. The radioactivity was determined in a Wallac 1409 liquid scintillation counter. Each experimental treatment was undertaken in triplicate, the mean of which was used as the final result.

2.18 *Enzyme-linked immunosorbent assay*

IL-1β, IL-2, IL-17, IFN-γ, and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA). Antibodies used are detailed in Table 2.4. 96-well plates (Nunc) were coated with 100μL of primary capture antibody diluted in coating buffer (0.2M $NaHCO_3$, 0.2M Na_2CO_3, pH9.4-9.7) and refrigerated overnight at 4^0C. Excess coating antibody was removed by aspiration and 200μL of assay buffer (PBS, 1%bovine serum albumin (BSA)) added. Plates were incubated at 37^0C for at least one hour to reduce non-specific binding. After washing with wash buffer (PBS, 0.5% Tween-20) samples and standards were added. A standard curve (in duplicate) was created by adding 100μL of recombinant cytokine to each of two wells and serially diluting in PBS to create samples with a range of known concentrations of cytokine. Standard curve concentrations are listed in Table 2.4. 50μL of sample, at a dilution previously determined by ELISA in preliminary experiments, was added with each sample being assayed in triplicate. Samples were diluted as necessary in PBS. 50μL of secondary biotinylated antibody, diluted in assay buffer, was added to each well to achieve a final volume of 100μL per well. After incubation for two hours at room temperature, the plates were washed four times with wash buffer and blotted dry. Extravidin peroxidase (Sigma Chemical Co., St Louis, MO) was diluted 1:4000 in assay buffer and 100μL added to each well. Plates were incubated for 15 minutes at 37^0C, 5%CO_2 before being washed four times with wash buffer and blotted dry. Finally 100μL per well of substrate solution (phosphate citrate buffer, TMB, 0.1%H_20_2) was added to reveal the colour reaction. The reaction was stopped by adding 100μL of 2M H_2SO_4 per well. Absorbance was read on a microplate reader

(Model 450, Bio-Rad Laboratories, NSW, Australia) at 450nm wavelength. Final results were taken as the average of each triplicate.

Table 2.4: Antibodies used for ELISA

Cytokine	Source	Sample dilution	Concentration 1^0 antibody	Concentration 2^0 antibody	Standard curve concentration
IL-1β	Endogen	1:16	5μg/ml	50ng/ml	20ng/ml
IL-2	Endogen	1:16	5μg/ml	0.2μg/ml	2ng/ml
IL-17	R&D Systems	neat	4μg/ml	75ng/ml	20ng/ml
TNF-α	Endogen	1:4	5μg/ml	50ng/ml	20ng/ml
IFN-γ	Endogen	1:16	1μg/ml	0.5μg/ml	10ng/ml

2.19 [^{3}H]-arachidonic acid release

[^{3}H]-AA was used to establish whether AA was released from the cell membrane by a stimulus. Cells were incubated with [^{3}H]-AA (2μCi/ml) for 18 hours. Cells were washed three times with RPMI 10%FCS and stimulated. After the desired incubation period, cells and supernatants were separated and 100μL supernatant was added to 1.5mls scintillation fluid. Radioactivity was determined using the scintillation counter. Each experimental condition was assayed in triplicate with the final result taken as the average.

2.20 Rat thoracic duct lymphocytes

I am indebted to Mr. Llew Spargo and Professor Graham Mayrhofer who looked after the rats and performed thoracic duct cannulations to obtain thoracic duct lymphocytes. Ethical approval was obtained from the Royal Adelaide Hospital and University of Adelaide Animal Ethics Committees.

2.20.1 Animals and induction of arthritis

Six-week-old female dark agouti rats were obtained from the Gilles Plains Animal House Resource Centre, South Australia. Animals were housed in the Animal House of the Institute of Medical and Veterinary Science. Food and water were available *ad libitum*.

Arthritis was induced by subcutaneous injection of 100μL of Complete Freund's Adjuvant at the base of the tail.

2.20.2 Collection of thoracic duct lymph

T cells migrate through the thoracic duct in their transit from lymph nodes to the peripheral tissues via the blood stream. The lymphatic duct empties just before entering the right side of the heart, and these T cells can be collected by placing a catheter retrograde into the thoracic duct. Thoracic duct T cells were collected 9 days after inoculation of the donor rats with Complete Freund's Adjuvant.

Animals were anaesthetized using isoflurane, nitrous oxide, and oxygen. The ventral side of the abdomen was shaved and a subcostal incision made on the left-hand side.

The aorta was exposed by gently retracting the left kidney and fatty tissue with cotton buds. After gently dissecting the thoracic duct away from the aorta, a surgical suture was placed around the duct and tied. A 12-gauge needle was inserted through the dorsal body wall adjacent to the thoracic duct. A cannula was then passed through the needle. The needle was removed and the cannula filled with heparin (1u/ml) in PBS before being clamped at the external end.

A small incision was made into the thoracic duct anterior to the surgical thread and the cannula inserted into the thoracic duct. The cannula was secured in place both at the skin and thoracic duct. The retractors were removed and organs returned to their normal position. The incision was closed with silk sutures. The animals were housed in hooded Bollman cages with free access to food and water.

Thoracic duct lymph was collected overnight into 250ml tissue culture flasks (Nunc, Denmark) containing 5mls sterile PBS with 25u/ml heparin (Sigma, USA). Thoracic duct lymph was resuspended and filtered through cotton wool. Cells were then centrifuged @ 200xg for 10 minutes, washed twice in RPMI 10%FCS, counted, and resuspended at $2x10^6$/ml.

2.21 Statistics

Statistical analysis was undertaken using WINKS. Statistically significant differences between experimental treatments and controls were assessed using the Student's t-test with a level of significance of $p<0.05$. For multiple treatments within an experiment analysis was undertaken using one-way analysis of variance (ANOVA) followed by Neuman-Keuls multiple comparison analysis ($p<0.05$).

Chapter 3

Development of T cell isolation protocol and validation of T cell purity

" I am told it works even if you don't believe in it."

Neils Bohr

3.1 Introduction

While a number of investigators in the 1970s and 1980s reported the presence of COX in human peripheral blood T cells, justifiable concerns were raised about cell purity. In any case, the signal generated from lymphocytes was sufficiently modest that contaminating platelets and monocytes seemed a more likely source of COX products in preparations enriched for T cells (Goldyne 1989). While aspects of methodology for T cell isolation are broadly similar today, methods for assessing cell purity have become more accurate with the introduction of flow cytometry.

In our laboratory monocyte preparation has been routine for some years. By contrast, it was necessary for me to develop a method for isolating T cells with the requirement that it would be reliable, yield sufficient numbers of T cells, and would have a reasonable cost. The main contaminating cells of concern were platelets, which contain COX-1, and monocytes, which constitutively express COX-1 and express COX-2 when exposed to inflammatory stimuli.

3.2 *Results*

A number of potential methods for T cell isolation were considered. Positive or negative selection of T cells by biomagnetic beads yields relatively pure T cell populations. However, the cost involved was not sustainable. In addition positive selection would render the cells pre-activated by the presence of antibodies directed against CD3.

I therefore decided to evaluate the following methods (and various combinations thereof); platelet depletion by density gradient centrifugation, monocyte depletion by adherence, and T cell isolation by counter-current elutriation and passage of mononuclear cells through nylon fibre wool columns.

To determine the efficacy of monocyte, platelet, and B cell depletion, cell counts were undertaken before and after each technique using an automated Sysmex XE-2100 (Sysmex Corp., Japan) machine. This machine counts cells; determining cell type based on the Coulter principal and cell size. Alternatively, cell purity was assessed by flow cytometry.

3.2.1 Isolation of T cells by counter-current elutriation

As described in Chapter 2 counter-current elutriation is used in our laboratory to isolate monocytes, by depleting mononuclear cell preparations of platelets and lymphocytes. Both the platelets and lymphocytes can be collected at various points during the elutriation process. Early in the elutriation process, when the flow rate is low, platelets will be removed and as the flow rate increases, larger cells such as lymphocytes will be eluted and can be collected.

Elutriations were undertaken with mononuclear cell preparations from four different donors. Cells were collected at various times through the elutriation process. Purity of lymphocytes was determined by flow cytometry, based on cell size (forward scatter) and intracellular complexity (side scatter). Despite collecting cells within the same time period, it was not possible to obtain lymphocytes with a sufficiently reliable purity. The range of lymphocyte purity over the four different elutriations is shown in Table 3.1.

While there was the potential to obtain relatively pure lymphocyte populations between 16 and 29 minutes, there was considerable variability. In addition it was not possible to separate T cells and B cells using this method. I therefore decided that this method would not consistently yield relatively pure T cell populations and an alternative method was required.

Table 3.1: Purity of lymphocytes obtained by counter-current elutriation

Time of cell collection	Lymphocytes (%)
7-8 minutes	0
11-15 minutes	0 - 42
16-23 minutes	65 - 93
24-28 minutes	32 - 94
29-34 minutes	40 - 54

3.2.2 Isolation of T cells by nylon fibre wool columns

T cells can be isolated by passage of mononuclear cells through nylon fibre wool columns as described in Chapter 2. This method is reported to deplete mononuclear cell preparations of B cells, which adhere to the nylon wool, whereas T cells pass through the columns. While platelets and monocytes may also adhere to the wool, efforts were made to reduce the burden of platelet and monocyte numbers in the mononuclear cell preparations prior to their passage through the column.

3.2.2.1 Platelet depletion by density gradient centrifugation

Two protocols for platelet depletion were assessed. Buffy coats were obtained from the Red Cross Blood center, diluted 1:2 with PBS and layered over 7mls of Lymphoprep.

Protocol 1: Cells were centrifuged @ 140xg for 20 minutes. After removal of the platelet rich supernatant, cells were centrifuged for a further 20 minutes @ 400xg. The mononuclear rich fraction was removed and washed three times in PBS, 0.1%BSA.

Protocol 2: Cells were centrifuged @ 160xg for 10 minutes. After removal of the platelet rich supernatant, cells were centrifuged @ 400xg for 20 minutes. The mononuclear enriched cell fraction was collected and washed twice with PBS (no BSA).

Platelets present within the mononuclear cell enriched fractions prepared by both methods were assessed using the automated counter. Protocol 1 resulted in twice as many platelets in the mononuclear rich fraction as protocol 2; therefore protocol 2 was adopted for all further experiments.

3.2.2.2 Depletion of monocytes by adherence

In an effort to deplete the mononuclear cell preparations of monocytes, prior to passage through the nylon fibre wool columns, an adherence step was undertaken. Mononuclear cells, prepared by density gradient centrifugation, were counted and resuspended in RPMI 10%FCS at $2x10^6$/ml. 50mls of cell suspension was added to a $150cm^2$ tissue culture flask and incubated for 1 hour @ 37^0C, 5%CO_2. Non-adherent cells were removed and the plate washed once with RPMI 10%FCS. Cells were centrifuged for 10 minutes @ 300xg. The supernatant was discarded and cells resuspended in RPMI 10%FCS ready for passage through a NFWC.

3.2.3 Assessment of cell purity pre- and post-NFWC

Purity of the cells obtained after density gradient centrifugation and after passage through NFWC (with and without the adherence step) was assessed by flow cytometry. Cells were labeled with mAbs directed against CD3 (pan T cell), CD14 (monocytes), CD15 (granulocytes), CD16 (NK cells and granulocytes), or CD19 (pan B cells). Platelets counted using the automated Sysmex XE-2100 (Sysmex Corp., Japan) instrument comprised less than $1x10^6$/ml when nucleated cells were resuspended at $2x10^6$/ml. Table 3.2 represents the average cell purity from three separate experiments.

The adherence step did not contribute significantly to monocyte and granulocyte depletion ($p=0.234$ and $p=0.934$ respectively) and therefore, the adherence step was omitted for all remaining experiments. In any case, the adherence step is likely to activate monocytes, which could stimulate T cells indirectly.

Table 3.2: Cell purity as assessed by flow cytometry pre and post NFWC

	CD3 (%) T cells	**CD14 (%) Monocytes**	**CD15 (%) Granulocytes**	**CD16 (%) Granulocytes and NK cells**	**CD19 (%) B cells**
Post density gradient centrifugation	55.9	18.9	11.7	19.9	13.9
Post adherence	75.9	13.2	5.9	12.3	2.6
Post NFWC *no adherence*	86.7	0.5	0.8	9.4	0.6
Post NFWC *with adherence*	86	0.9	0.9	11.7	1.2

3.2.4 Final T cell isolation protocol

Based on the results above, the final protocol for T cell isolation was determined. Density gradient centrifugation was used to deplete platelets and isolate mononuclear cells, which were the passed through NFWC.

Using this method relatively pure T cells could be isolated reliably. The average cell purity over the period of experiments is shown in Table 3.3. The major contaminating cell population was NK cells.

Table 3.3: Average cell purity

Marker	% total ± SD (n=20)
CD3	84.0 ± 3.2
CD14	0.4 ± 0.2
CD15	0.87 ± 0.6
CD16	8.5 ± 3.0
CD19	0.8 ± 0.5
Platelets (10^6/ml)	0-1

3.2.5 Cell number and cell viability post NFWC

Two sizes of columns can be prepared 10ml (load $1x10^8$ mononuclear cells) or 20ml (load $2x10^8$ mononuclear cells). In general about 1/3 of the cells loaded onto a column were retrieved. Cell viability post column was consistently ≥99% as determined by trypan blue exclusion test.

3.3 Discussion

A reliable method for isolating T cells, relatively free of platelets and monocytes, was critical for this project. The use of NFWC for isolating T cells has been described previously (Werner et al 1977). While B cells and accessory cells adhere to the nylon wool, T cells do not. The underlying basis for this difference in adherence is not understood. In addition to producing relatively pure T cell preparations, this technique allowed me to isolate large numbers of T cells at little cost.

Using density gradient centrifugation followed by passage through NFWC, monocyte and platelet contamination was markedly reduced. On average $CD14^+$ monocytes accounted for 0.4% of cells. Thus, in an incubation of $2x10^6$ T cells one would expect $8x10^3$ monocytes. Platelets were consistently less than $1x10^6$/ml when cells were resuspended at $2x10^6$/ nucleated cells per ml. The major contaminating cell proved to be $CD16^+$ cells, most likely NK cells rather than neutrophils, given the small size of the cells and the differential staining with antibodies against CD15 and CD16. The low number of granulocytes, monocytes, and platelets in the T cell preparations allowed me to interpret more accurately data regarding the presence or absence of COX-1 and/or COX-2 in T cells, as well as eicosanoid production by T cells.

Chapter 4

Characterization of eicosanoid biosynthetic pathways in T lymphocytes

"If an elderly but distinguished scientist says that something is possible he is almost certainly right, but if he says that it is impossible he is very probably wrong."

Arthur C Clarke 'Relativity' 1923

4.1 Introduction

It is thought that COX is likely to be present in all nucleated cells and that all nucleated cells are capable of producing COX derived eicosanoids. Thus it follows that T cells, which are nucleated, contain COX and are capable of producing eicosanoids. However, the early studies in the 1970s and 1980s, when techniques for separating lymphocytes from monocytes and assessment of cell purity were less refined, led to perceptions that T cells did not produce eicosanoids in important quantities (Goldyne 1989), since the quantities of eicosanoids detected in T cell preparations were sufficiently modest that they were regarded as potentially arising from contaminating monocytes and/or platelets. It was timely to revisit this question in light of refinements in methods for identifying cells and the expression of genes within them.

The discovery of COX-2 has renewed interest in the expression of COX in all cell types including T cells. Several recent publications have demonstrated the presence of both COX-1 and COX-2 in human peripheral blood T cells and T cell lines (Iniguez et

al 1999; Pablos et al 1999; Tanaka et al 2000; Bosticardo et al 2001; de Gregorio et al 2001; Mori et al 2001), thymic cell lines (Rocca et al 1999a), and T cells within the small intestine (Kainulainen et al 2002). It has been suggested that COX-2 has a role in T cell development (Rocca et al 1999b) and T cell activation (Iniguez et al 1999; Iniguez et al 2000; Paccani et al 2002).

With regard to eicosanoid production in leukocytes, most work has concentrated on the production of PGE_2 by monocytes and its role in monocyte and lymphocyte function. Production of other eicosanoids such as TXA_2 has been relatively ignored. In the case of T cells, the production of eicosanoids other than PGE_2, such as TXA_2, and their effects on T cell function has received little attention. Recently Iniguez et al detected prostaglandin production by activated T cells. However, they used a general prostaglandin colorimetric screen kit (Cayman Chemicals) that did not allow identification of individual eicosanoids (Iniguez et al 1999).

The aims of these initial studies were to identify COX isotypes present within human peripheral blood T cells and to characterize the eicosanoids produced by each COX isotype. Having examined human peripheral blood T cells I then investigated whether the findings were similar in rat thoracic duct T cells.

4.2 Results

4.2.1 Effect of T cell stimulation on expression of CD69

T cell activation is accompanied by the sequential expression of cell surface antigens including CD69 and CD25 (IL-2 receptor). To assess whether T cell stimulation was adequate, flow cytometry was used to determine expression of these markers before and after stimulation. ConA (10μg/ml) and PMA (15ng/ml) resulted in activation of ~40-60% of $CD3^+$ T cells. The combination of PMA (15ng/ml) plus A23187 (1μM) appeared to be the most powerful stimulus with virtually all $CD3^+$ cells expressing CD69 (Figure 4.1).

T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 retained these antibodies on their cell surface, which then bound the secondary FITC-conjugated goat-anti-mouse polyclonal antibody. Interpretation of the expression of CD69 was therefore not possible using the indirectly conjugated CD69 antibody available. While expression of CD69 could not be determined in T cells stimulated with immobilized anti-CD3 and soluble anti-CD28, expression of CD25 could be assessed using a directly conjugated CD25 mAb. CD25 expression was up regulated in stimulated compared to unstimulated T cells using a variety of stimuli (Figure 4.2).

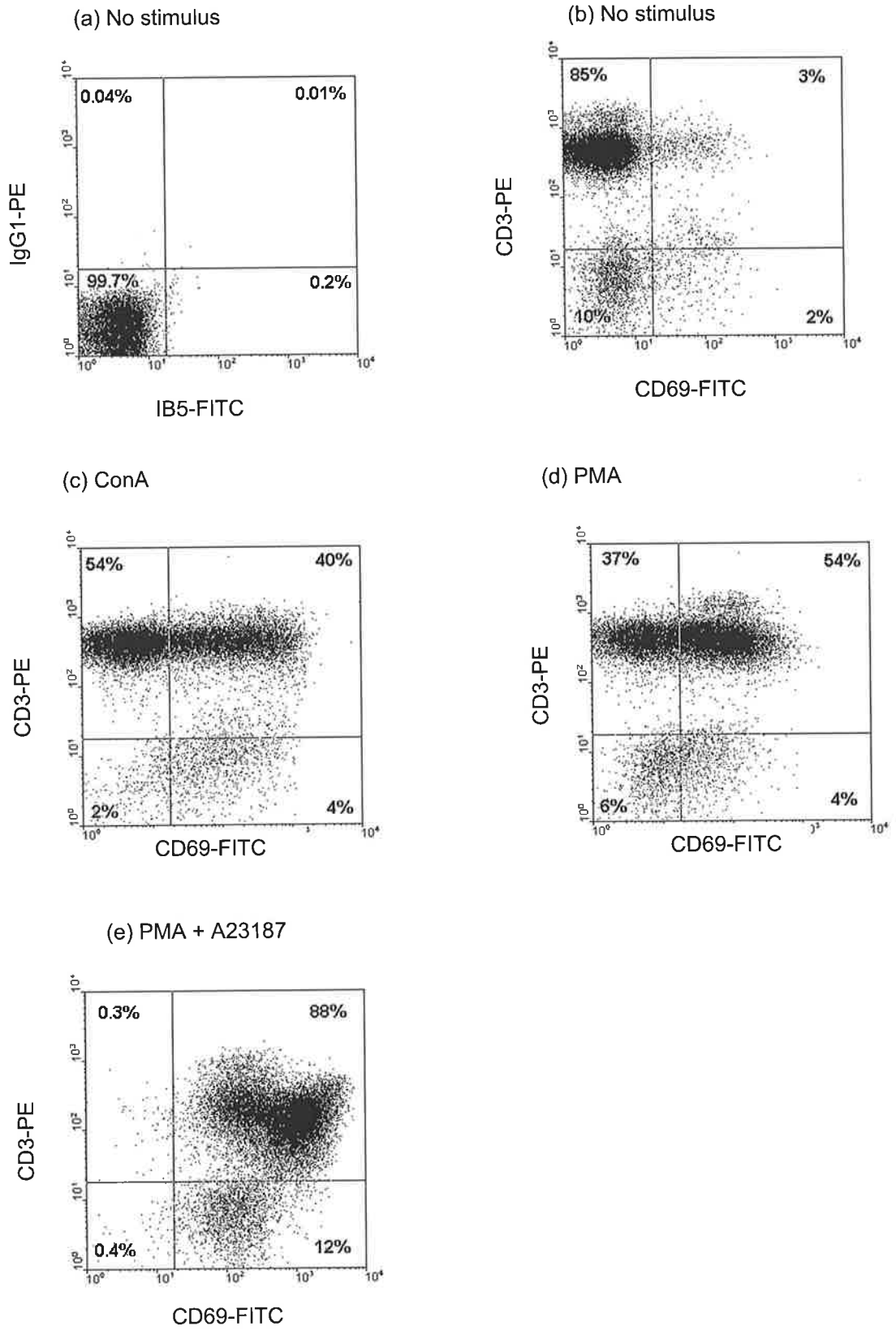
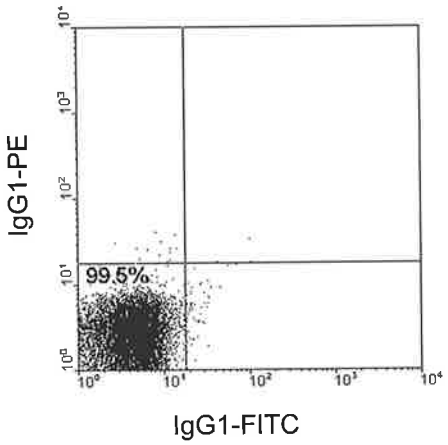
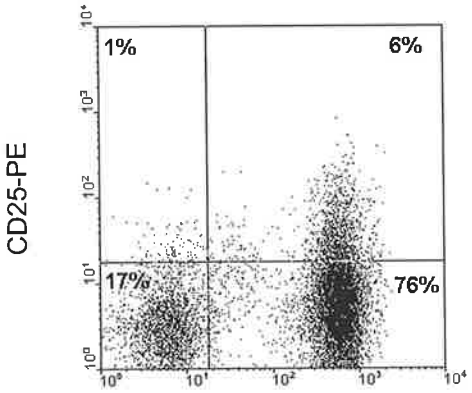


Figure 4.1: Expression of CD69 by T cells. T cells were incubated for 18 hours with (a) no stimulus, (b) no stimulus, (c) ConA (10μg/ml), (d) PMA (15ng/ml), and (e) PMA (15ng/ml) plus A23187 (1μM). Cells were processed for flow cytometry with indirectly conjugated mAbs directed against CD69 or IB5, or directly conjugated mAbs against CD3 or IgG1 isotype control. In part (a) there was no significant change in staining whatever the stimulus used, thus only one representative plot is shown.

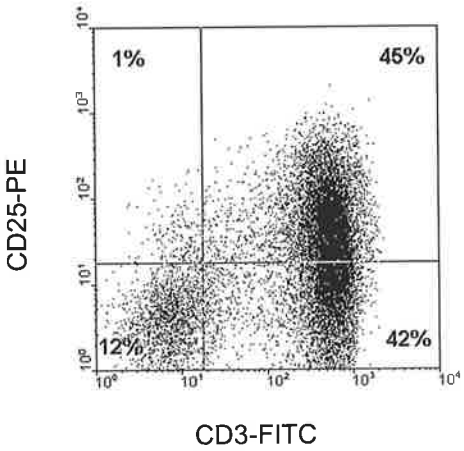
(a) No stimulus



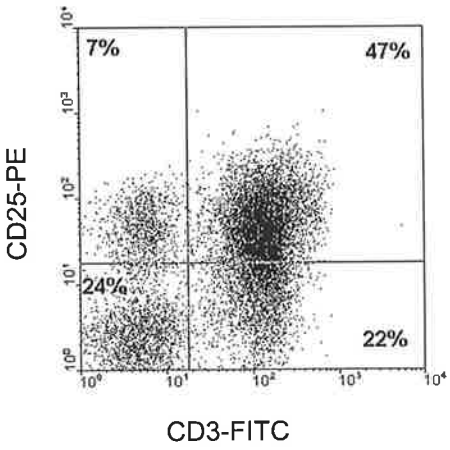
(b) No stimulus



(c) ConA



(d) PMA + A23187



(e) Anti-CD3 + anti-CD28

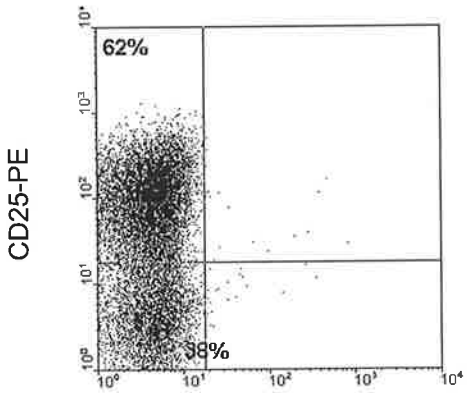


Figure 4.2: Expression of CD25 by T cells. T cells were incubated for 18 hours with (a) no stimulus, (b) no stimulus (c) ConA (10μg/ml), (d) PMA (15ng/ml) plus A23187 (1μM), or (e) immobilized anti-CD3 and soluble anti-CD28. Cells were processed for flow cytometry with directly conjugated mAbs directed against CD3, CD25, or IgG1 isotype control. In part (a) there was no significant change in staining whatever the stimulus used, thus only one representative plot is shown.

4.2.2 Expression of COX-1 and COX-2 in T cells

COX-1 was readily detected in fresh human peripheral blood T cells by Western immunoblot and the amount did not appear to change after stimulation with immobilized anti-CD3 and soluble anti-CD28 (Figure 4.3). Using serial dilutions of purified platelets, COX-1 was not detectable by Western immunoblot at the level of platelets expected in my T cell preparations, which was $\leq 1x10^6$ per $2x10^6$ nucleated cells (data not shown).

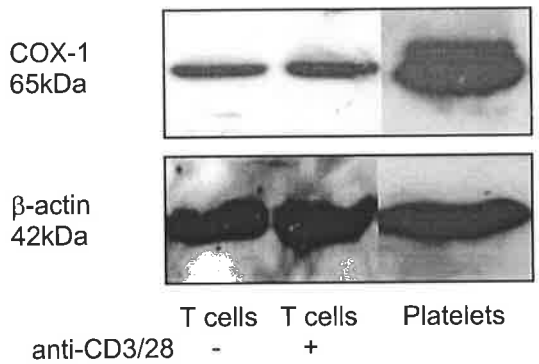


Figure 4.3: Presence of COX-1 in T cells and platelets. T cells were incubated in the absence or presence of immobilized anti-CD3 and soluble anti-CD28. After 18 hours cells were processed for Western immunoblot. Unstimulated platelets were used as a positive control for COX-1.

To confirm T cells as the source of COX-1 detected on Western immunoblot, two-colour flow cytometry was undertaken using intracellular staining with directly conjugated COX-1 mAb and cell surface staining with directly conjugated CD3 mAb. This confirmed the presence of COX-1 in $CD3^+$ T cells (Figure 4.4). COX-1 is ubiquitously expressed accounting for the $COX\text{-}1^+/CD3^-$ cells present. While a portion of $CD3^+$ cells appear to be $COX\text{-}1^-$ these could represent CD3 cells with low level expression of COX-1. Control incubations with non-fixed and/or non-permeabilised cells revealed no binding of the COX-1 antibody to the cell surface (data not shown).

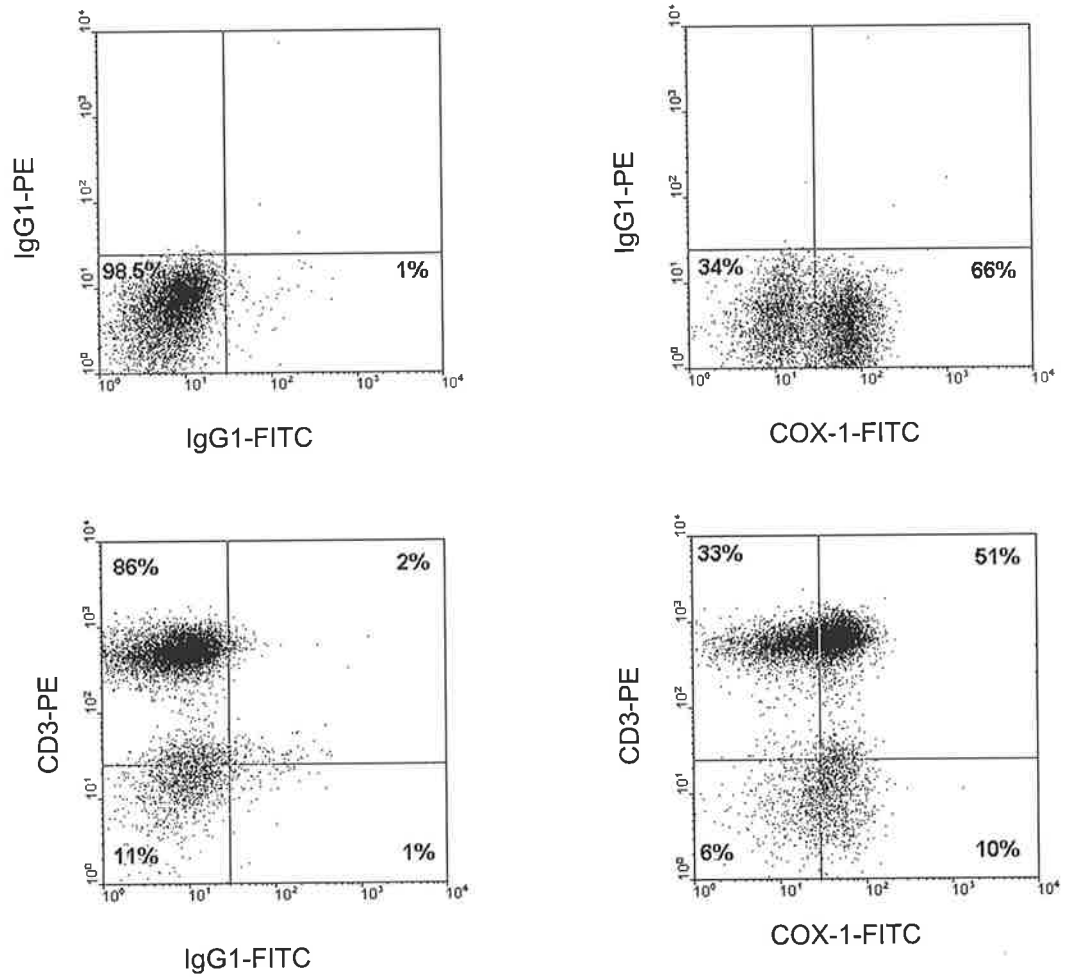


Figure 4.4: Expression of COX-1 in T cells by flow cytometry. T cells were isolated and immediately processed for flow cytometry. Cells were labeled using directly conjugated mAbs directed against COX-1, CD3, or IgG1 isotype control.

By contrast with COX-1, COX-2 could not be detected by Western immunoblot in unstimulated T cells or T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 (Figure 4.5). The same null result for COX-2 was observed in T cells stimulated with ConA or PMA plus A23187 (data not shown). COX-2 was detected by Western immunoblot in monocytes treated with LPS after ~6 hours and remained detectable for at least 18 hours (Figure 4.5 plus unshown data). However, COX-2 could not be detected in T cells stimulated for as little as 6 hours or as long as 72 hours (data not shown).

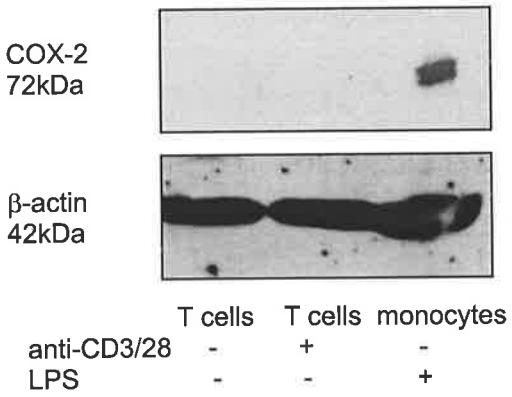


Figure 4.5: Expression of COX-2 in T cells and monocytes. T cells were incubated in the absence or presence of immobilized anti-CD3 and soluble anti-CD28. Monocytes incubated in the presence of LPS (200ng/ml) were used as a positive control for COX-2. After 18 hours cells were processed for Western immunoblot.

Using LPS–stimulated monocytes COX-2 could be detected by loading protein from $2.5x10^6$ monocytes and exposing the PVDF membrane to COX-2 polyclonal antibody (pAb) for 1 hour. When the same method failed to detect COX-2 in stimulated T cells, several changes were explored. Firstly protein from double the number of T cells (i.e. $5x10^6$) was loaded, secondly the membrane was exposed to COX-2 for 18 hours, thirdly the protein was transferred to nitrocellulose membrane, and finally the concentration of COX-2 pAb was increased from 1:1000 to 1:500 dilution. Despite these adjustments COX-2 could not be detected in stimulated T cells using Western immunoblot.

In a further attempt to identify COX-2 in T cells, flow cytometry was undertaken using intracellular staining with a directly conjugated COX-2 mAb. COX-2 was identified in LPS-stimulated $CD14^+$ monocytes using this technique. However, using T cells stimulated with PMA plus A32187, no COX-2 staining was observed in $CD3^+$ T cells despite up-regulation of the activation marker CD69 (Figure 4.6). A similar null result for COX-2 was observed when T cells were treated with immobilized anti-CD3 and soluble anti-CD28 (data not shown). Control incubations with non-fixed and/or non-permeabilised cells revealed no binding of the COX-2 antibody to the cell surface (data not shown).

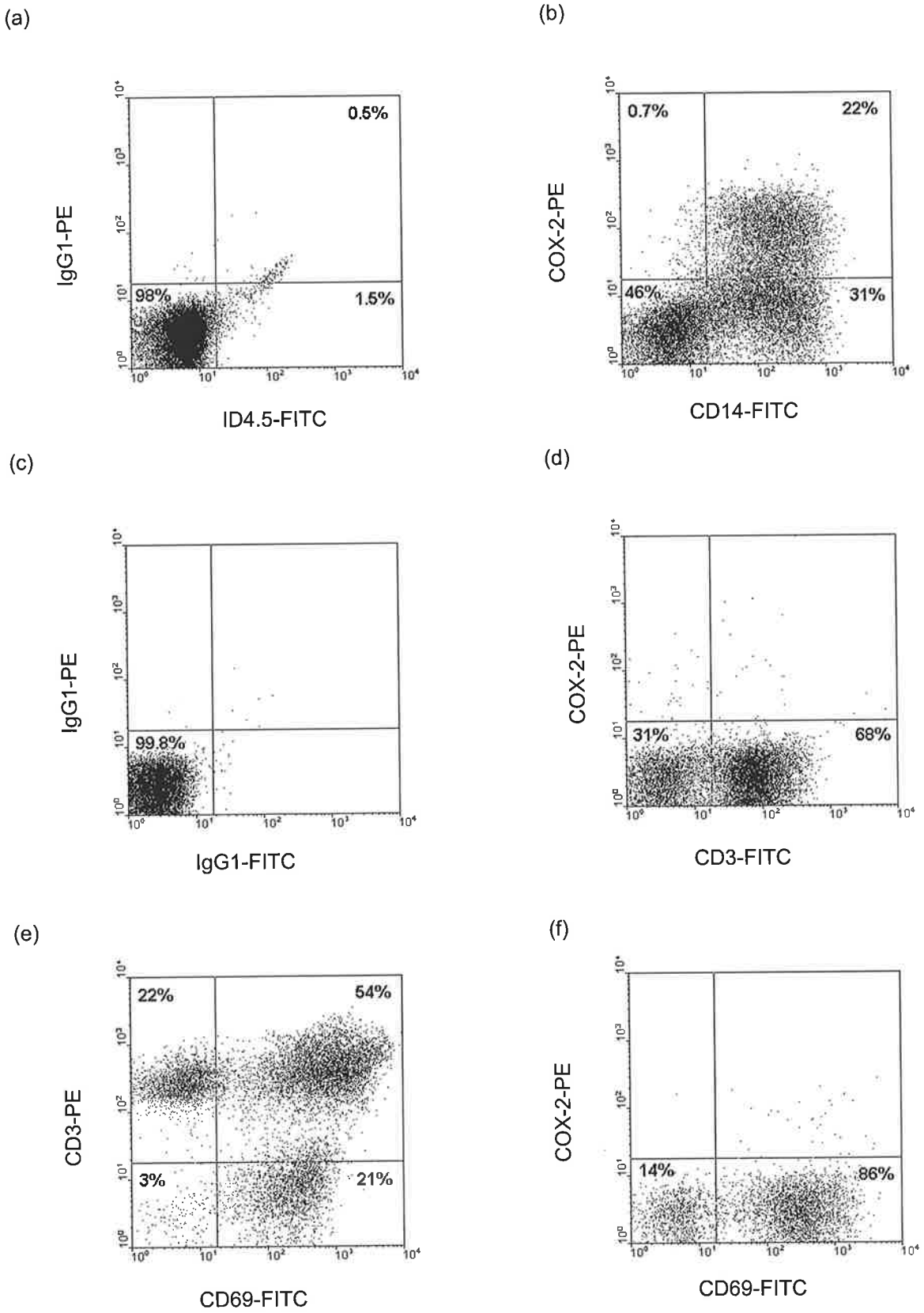


Figure 4.6: Expression of COX-2 in monocytes and T cells. (a-b) Monocytes were stimulated for 18 hours with LPS (200ng/ml). (c-f) T cells were stimulated for 18 hours with PMA (15ng/ml) plus A23187 (1μM). Cells were stained for flow cytometry using directly conjugated mAbs against CD3, CD69, COX-2, or IgG1 isotype control or indirectly conjugated mAbs against CD14 or ID4.5 (IgG2a isotype control) as shown.

4.2.3 Effect of superantigen on COX-1 and COX-2 expression in T cells

A final attempt to identify COX-2 in T cells was undertaken using the superantigen, Staphylococcal enterotoxin B (SEB) to stimulate T cells. While superantigens provide a powerful activating stimulus to T cells, they require the presence of antigen presenting cells. Thus peripheral blood mononuclear cells were obtained and incubated for 18 hours, with or without SEB (5μg/ml), prior to monocyte and T cell isolation by counter-current elutriation and passage through NFWC respectively.

Stimulation of T cells with SEB resulted in up-regulation of the activation marker CD69 (Figure 4.7). Despite this, COX-2 could not be detected by Western immunoblot (Figure 4.8). Surprisingly, COX-1 appeared to be down regulated in T cells stimulated with SEB (Figure 4.8).

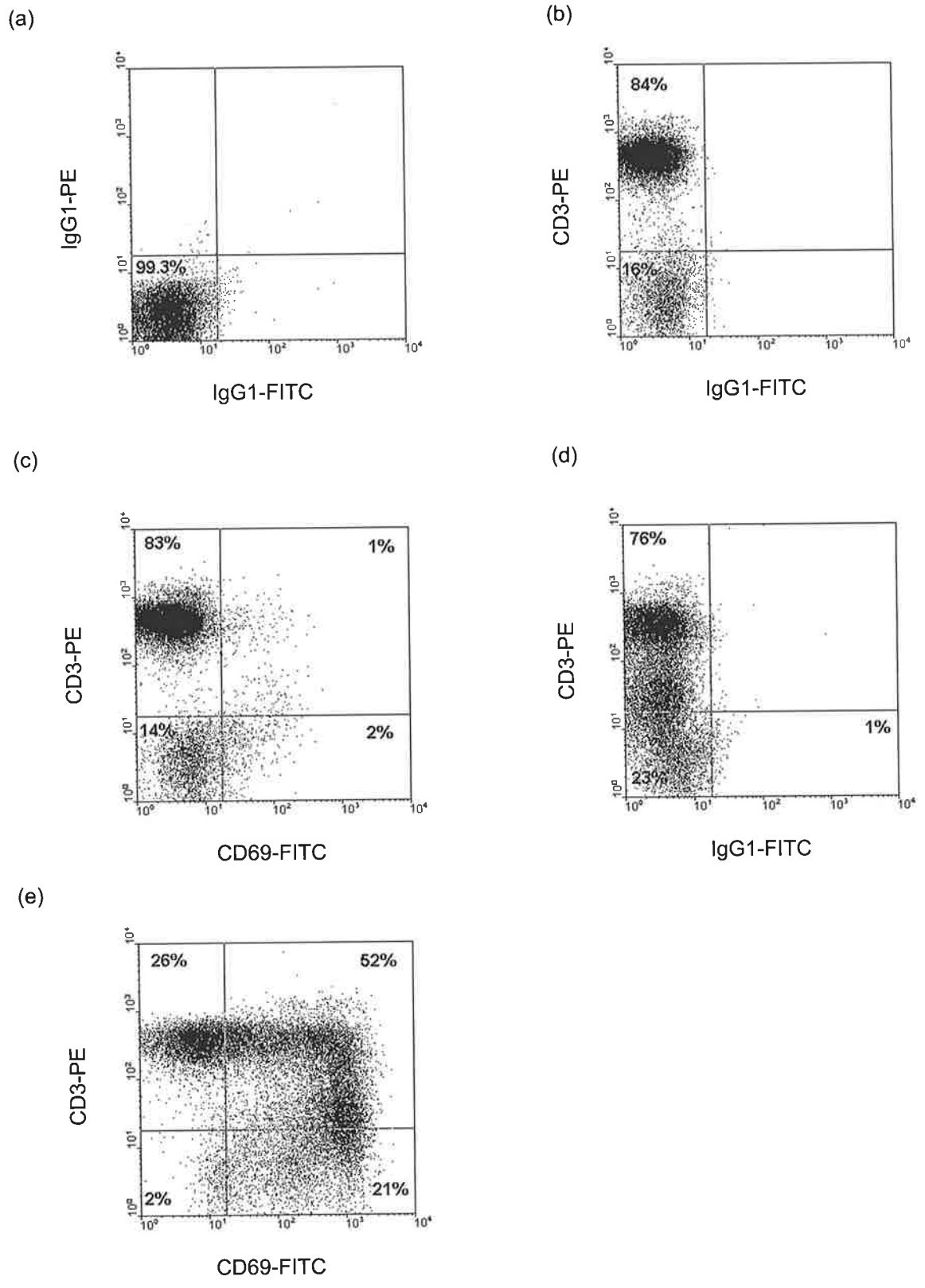


Figure 4.7: Expression of CD69 in T cells stimulated with Staphylococcal enterotoxin B (SEB). Mononuclear cells were incubated in the absence (a-c) or presence (d-e) of SEB (5μg/ml) for 18 hours prior to separation of T cells by passage of mononuclear cells through NFWC. T cells were then processed for flow cytometry using directly conjugated mAbs directed against CD3, CD69, and IgG1 (isotype control).

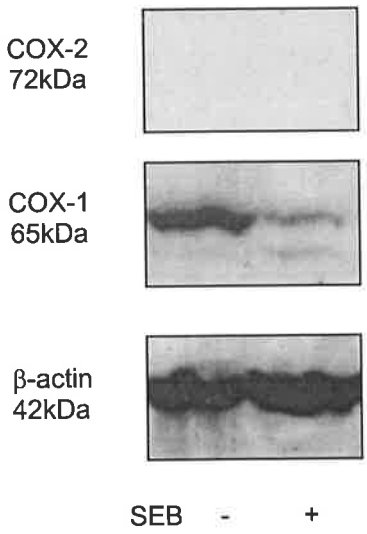
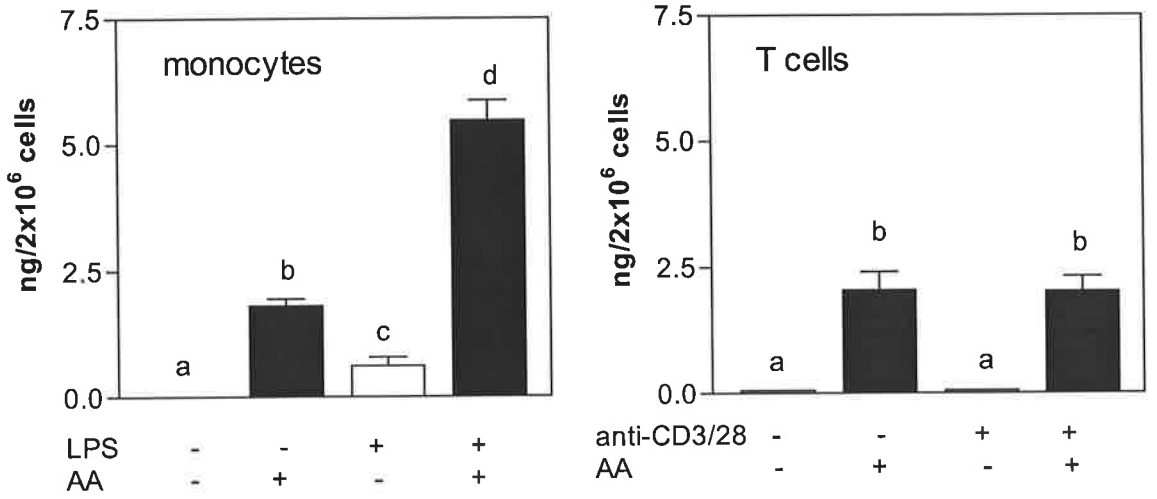


Figure 4.8: Expression of COX-1 and COX-2 in T cells stimulated with Staphylococcal enterotoxin B (SEB). Mononuclear cells ($300x10^6$ @ $2x10^6$/ml) were incubated in non-stick teflon pots in the absence or presence of SEB (5μg/ml) for 18 hours prior to isolation of T cells by passage through NFWC. T cells were then processed for Western immunoblot.

4.2.4 Production of eicosanoids by T cells: comparison with monocytes

To assess COX activity without dependence on endogenous AA release, cells were stimulated for 18 hours and after washing with FCS free RPMI cells were incubated with or without AA (10μM) for 30 minutes. Resting monocytes produced readily detectable TXB_2 and PGE_2 with the addition of AA, and this production was increased in cells that had been previously treated with LPS, which up-regulates COX-2. By comparison, resting T cells produced detectable levels of TXB_2 and PGE_2 with the addition of AA, but prior cell stimulation with immobilized anti-CD3 and soluble anti-CD28 had no effect on the rate of eicosanoid synthesis. Thus, there is no evidence from these results of up-regulation of COX-2 activity in stimulated T cells (Figure 4.9).

TXB_2



PGE_2

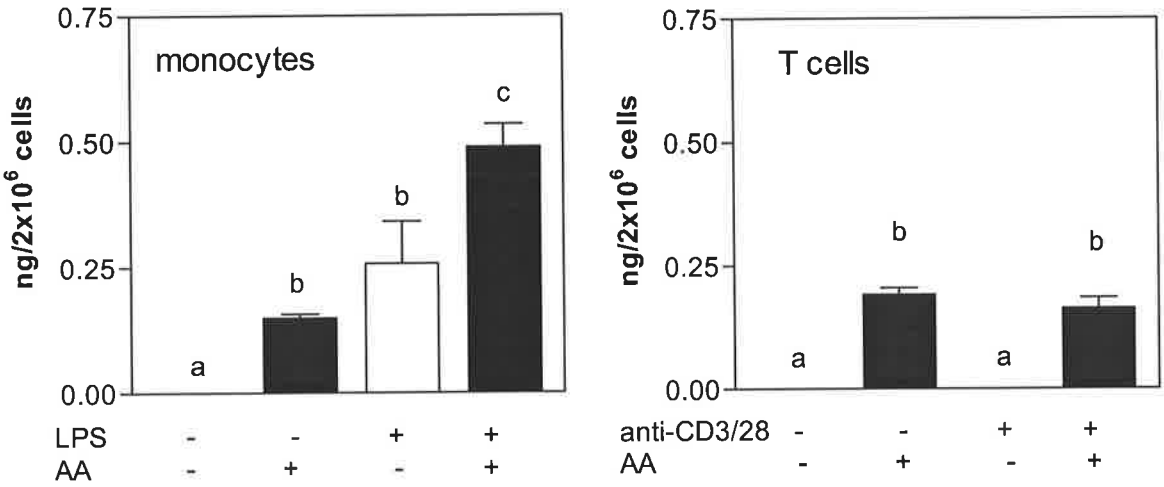


Figure 4.9: Production of TXB_2 and PGE_2 by monocytes and T cells. Monocytes were incubated without or with LPS (200ng/ml) and T cells were incubated without or with immobilized anti-CD3 and soluble anti-CD28 for 18 hours. After washing with FCS free RPMI cells were incubated without (clear bars) or with (solid bars) AA (10μM) for 30 minutes. Supernatants were then collected and assayed for eicosanoids. The results of one experiment, which is representative of three, are shown. Bars with the different letters are significantly different from each other; $p<0.05$, ANOVA followed by Newman-Keuls test for multiple comparisons.

Production of PGD_2 by T cells was assessed using a PGD_2-MOX enzyme immunoassay kit (Cayman Chemicals). Results were similar to those of TXA_2 and PGE_2 in that PGD_2 was not detected unless exogenous AA was added and levels did not alter with T cell stimulation (Figure 4.10). Unfortunately our attempts to develop a PGD_2 RIA were unsuccessful, as discussed in Chapter 2 and no further investigations were undertaken into the production of PGD_2.

Neither resting nor stimulated T cells produced detectable 6-keto-$PGF_{1\alpha}$, a metabolite of PGI_2, in the presence or absence of exogenous AA (data not shown).

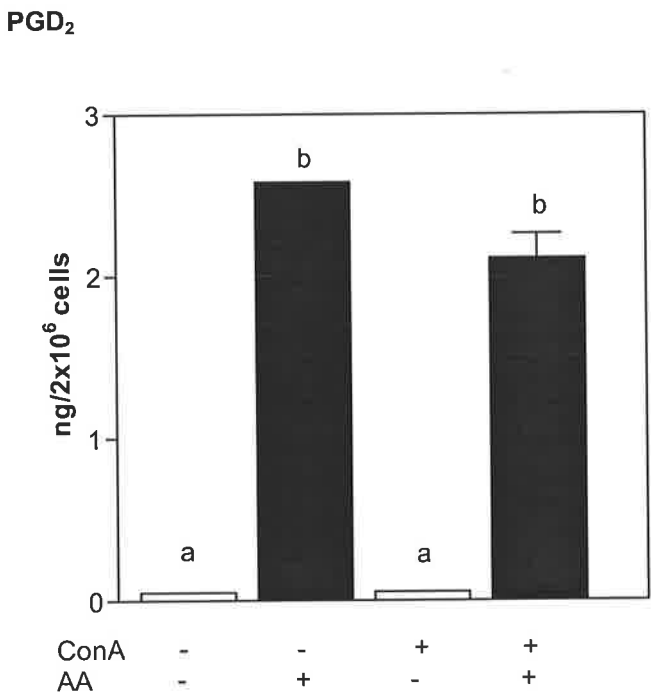


Figure 4.10: Production of PGD_2 by T cells. T cells were incubated without or with ConA (15ng/ml). After 18 hours cells were washed with FCS free RPMI, and incubated without (hollow bars) or with (solid bars) AA (10μM) for 30 minutes. Supernatants were assayed immediately for PGD_2 by PGD_2-MOX enzyme immunoassay kit (Cayman Chemicals). Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

4.2.5 Effect of selective COX-1 and COX-2 inhibition on T cell eicosanoid production

To examine further which COX isotypes are involved in T cell eicosanoid production, specific inhibitors of each isotype were used. Prior to overnight incubation cells were either treated briefly with aspirin (55μM), which inhibits COX-1 irreversibly or incubated in the presence of NS398 (0.5μM) to inhibit COX-2 selectively. Cells were stimulated for 18 hours and, after washing cells with FCS free RPMI, those cells already exposed to NS398 were re-treated with NS398. Cells were then incubated with or without AA (10μM) for 30 minutes. COX-1 inhibition significantly reduced the production of TXB_2 and PGE_2 by stimulated T cells while COX-2 inhibition had no effect, thereby implicating COX-1 in the production of eicosanoids by T cells (Figure 4.11). Interestingly, aspirin treatment appeared to be associated with more complete inhibition of TXA_2 production than PGE_2 although neither was inhibited by the selective COX-2 inhibitor NS398.

As noted, stimulation of T cells with SEB resulted in an apparent down-regulation of COX-1 on Western immunoblot. To investigate whether production of eicosanoids was also inhibited, mononuclear cells were stimulated with SEB (5μg/ml) for 18 hours. T cells were then isolated and washed with FCS free RPMI followed by incubation with or without AA (10μM) for 30 minutes. In the presence of AA, eicosanoid synthesis was detected. TXB_2 synthesis was significantly reduced by previous treatment of T cells with SEB. PGE_2 production was also reduced, but the reduction was not statistically significant (Figure 4.12).

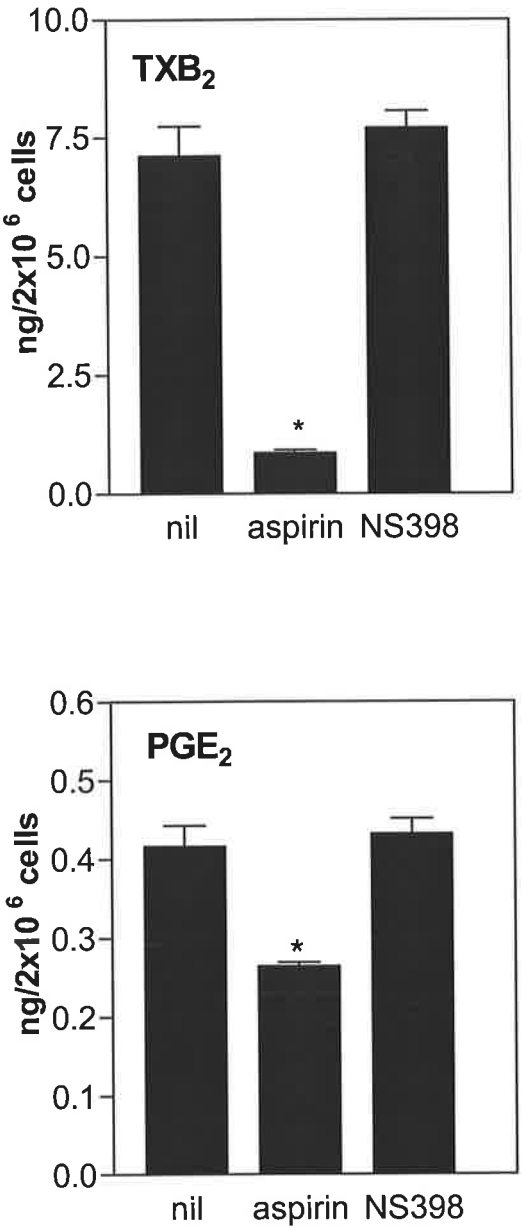


Figure 4.11: Effect of COX-1 and COX-2 inhibition on T cell TXB_2 and PGE_2 production. Cells were incubated with aspirin (55μM) for 30 minutes and washed, or alternatively NS398 (0.5μM) was added. Cells were then incubated with PMA (15ng/ml) plus A23187 (1μM). After 18 hours cells were washed with FCS free RPMI and those previously exposed to NS398 were retreated for 15 minutes prior to the addition of AA (10μM) for 30 minutes. *Significantly different from control; $p<0.05$. Similar results were observed when T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28. The results of one experiment, which are representative of three are shown.

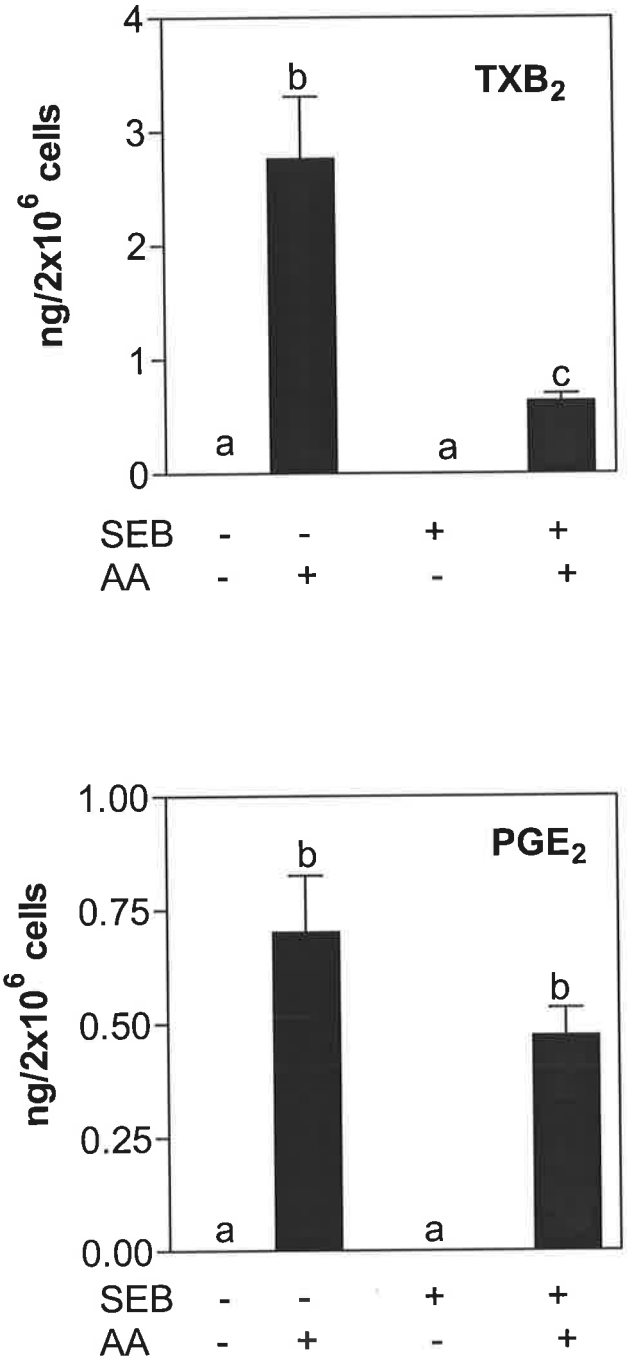


Figure 4.12: Production of TXB_2 and PGE_2 by T cells stimulated with Staphylococcal enterotoxin B (SEB). After incubation of mononuclear cells ($300x10^6$ @ $2x10^6$/ml) in non-stick teflon pots for 18 hours in the absence or presence of SEB (5μg/ml) T cells were isolated by passage of mononuclear cells through NFWC. $2x10^6$ cells were washed with FCS free RPMI and T cells incubated without (hollow bars) or with (solid bars) AA (10μM) for 30 minutes. Bars with different letters are significantly different from each other; $p<0.05$; ANOVA followed by Neuman-Keuls test for multiple comparisons.

4.2.6 Role of contaminating platelets and monocytes in eicosanoid production attributed to T cells

Despite T cell purification a small number of monocytes and platelets remained in the cell preparations. Flow cytometry indicated ~0.4% of cells were $CD14^+$ monocytes and therefore, in an incubation of $2x10^6$ cells enriched for T cells, there will be ~$8x10^3$ monocytes. Platelet concentrations were $\leq 1x10^6$ platelets/ml when nucleated cells were resuspended at $2x10^6$/ml. To evaluate the possible contribution of these contaminating cells to eicosanoids detected in the purified T cell preparations, serial dilutions of purified monocytes and platelets were incubated for 18 hours with immobilized anti-CD3 and soluble anti-CD28 or PMA plus A23187 and the supernatants assayed for PGE_2 and TXB_2. At no concentration of monocytes or platelets could PGE_2 or TXB_2 be detected. In addition, stimulation of T cells with LPS (200ng/ml) did not result in production of any detectable eicosanoids nor the up-regulation of COX-2 (data not shown). Incubation of increasing numbers of platelets with AA (10μM) for 30 minutes resulted in no detectable PGE_2 at any level. Between $1x10^6$ and $1x10^8$ platelets produced small amounts of TXB_2. However, the amount detected did not increase as platelet number increased and was not sufficient to account for the TXB_2 produced by enriched T cell preparations.

4.2.7 Expression of terminal eicosanoid synthases by T cells

Western immunoblot of the T cell enriched preparation demonstrated the presence of TX synthase (Figure 4.13).

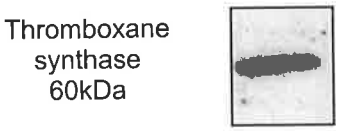


Figure 4.13: Presence of thromboxane synthase in T cells. T cells were obtained and immediately processed for Western immunoblot.

To confirm the cellular source of TX synthase, two-colour flow cytometry of fixed, permeabilised T cells was undertaken. Cells were labeled with antibodies directed against CD3, TX synthase, or isotype matched control antibodies. This demonstrated the presence of TX synthase in $CD3^+$ T cells (Figure 4.14). Control incubations with non-fixed and/or non-permeabilised cells revealed no binding of the TX synthase antibody to the cell surface (data not shown).

Using Western immunoblot neither PGD synthase nor PGE synthase was detected. Neither antibody was suitable for flow cytometry.

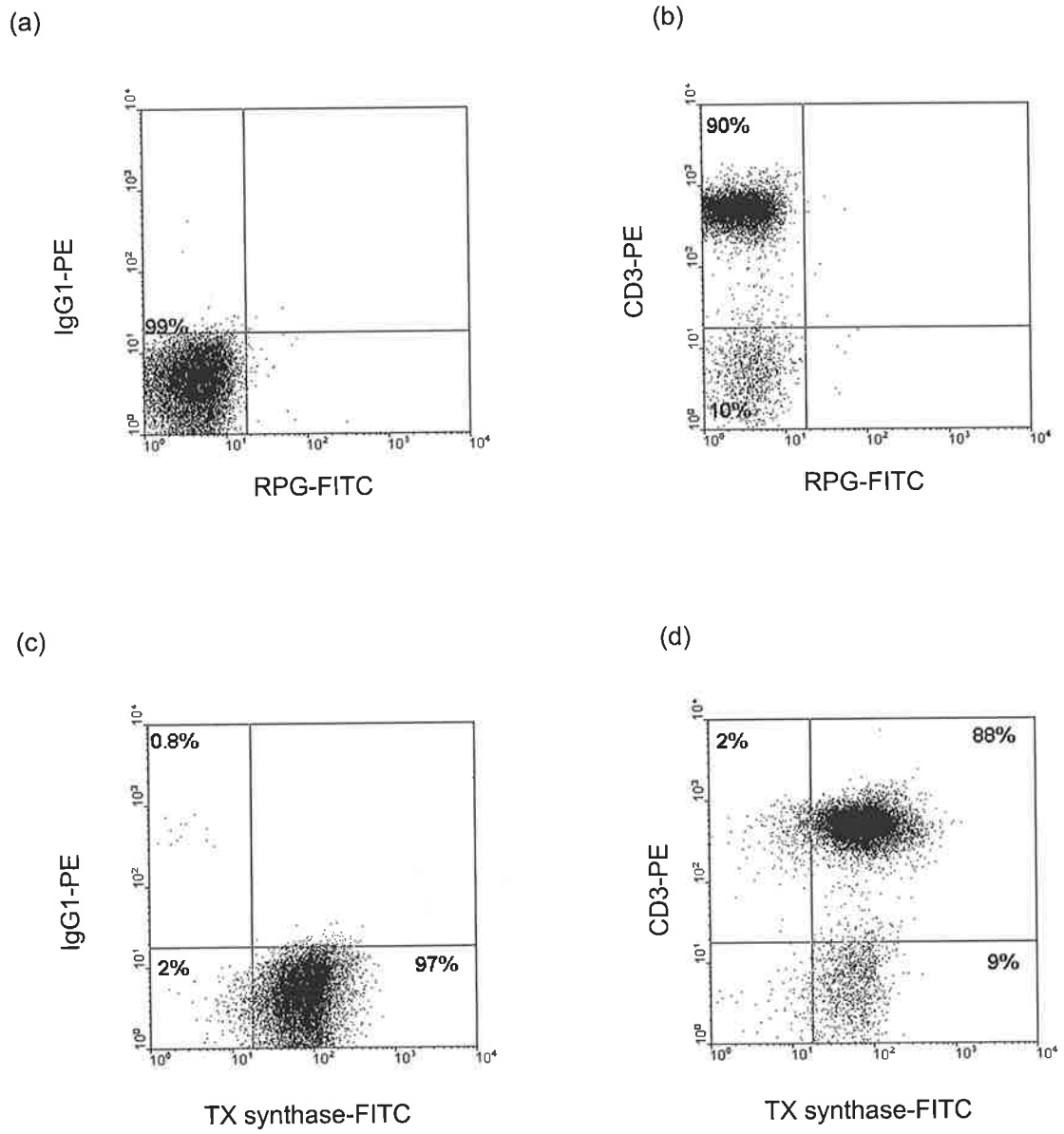


Figure 4.14: Presence of thromboxane synthase in T cells. T cells were obtained and immediately processed for flow cytometry. Cells were labeled with directly conjugated mAbs against CD3 or IgG1 isotype control, or indirectly conjugated pAbs directed against thromboxane synthase, or its isotype control, RPG (rabbit pAb directed against Giardia).

4.2.8 Expression of the thromboxane receptor by T cells

Western immunoblot demonstrated the presence of a thromboxane receptor in the T cell preparations (Figure 4.15). The thromboxane receptor antibody, PH4, was not suitable for flow cytometry due to low intensity of staining.

Figure 4.15: Presence of the thromboxane receptor in T cells. T cells were obtained and immediately processed for Western immunoblot.

4.2.9 Expression of COX-1 and COX-2 in rat thoracic duct lymphocytes

Having observed that human peripheral blood T cells contain COX-1, but not COX-2, and are capable of producing TXB_2 and PGE_2, I wished to investigate whether there were similar findings in rat thoracic duct lymphocytes. In addition I wished to assess whether there was any difference between normal and arthritic rats with respect to the expression of COX isotypes and production of eicosanoids.

Thoracic duct lymph (TDL) was obtained from rats with adjuvant-induced arthritis or normal control rats and processed for flow cytometry or Western immunoblot. COX-1 was detected in rat thoracic duct $\alpha\beta$ TCR^+ T cells and there was no difference between cells from normal rats and rats with adjuvant-induced arthritis (Figure 4.16).

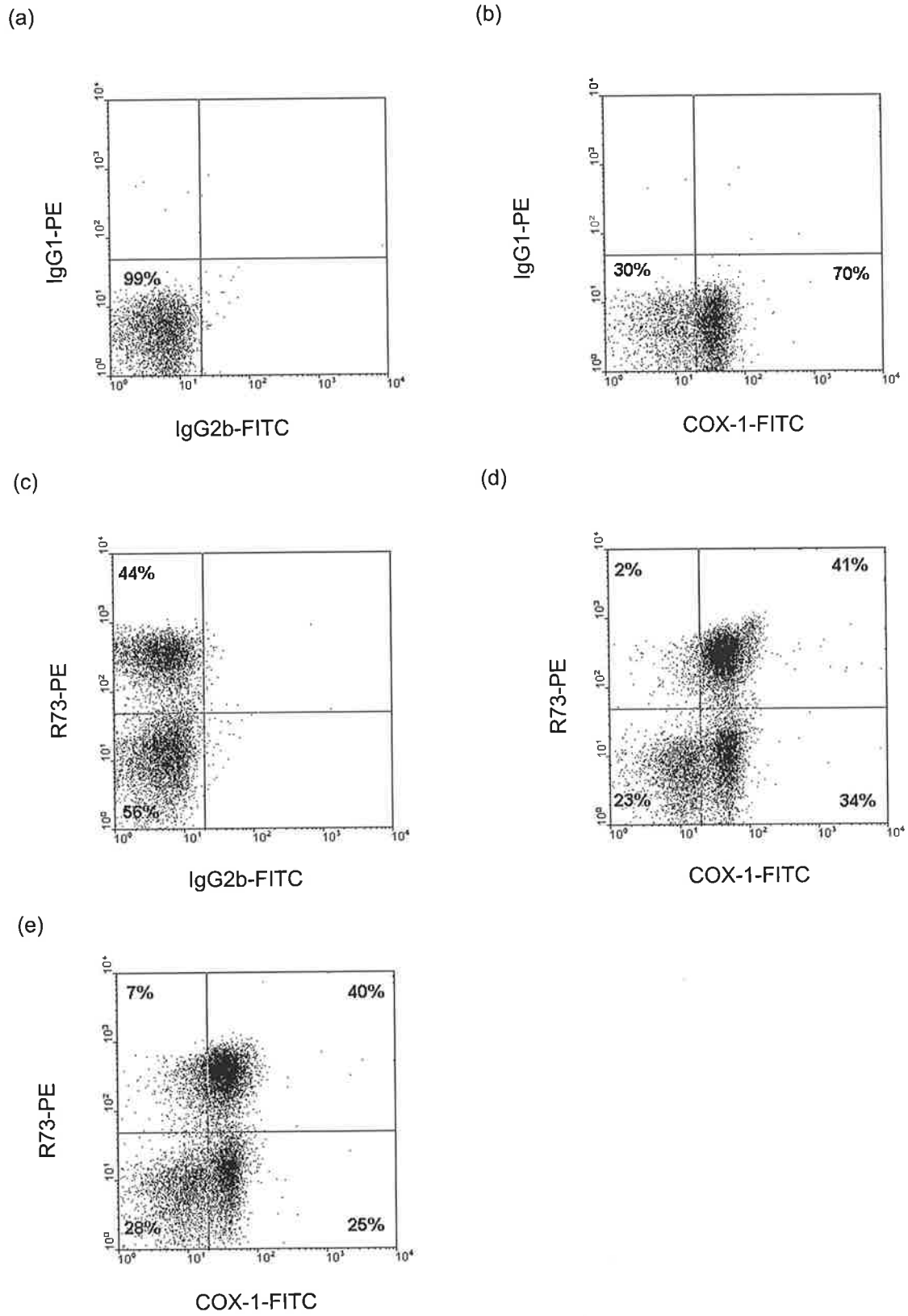


Figure 4.16: Expression of COX-1 in rat thoracic duct lymphocytes. Thoracic duct lymphocytes were obtained from (a-d) normal and (e) arthritic rats. Cells were stained with directly conjugated mAbs directed against COX-1, the αβ T cell receptor (R73), or their respective isotype matched control antibodies (IgG2b and IgG1). Incubations with isotype control antibodies were identical for normal and arthritic rats; therefore only those from the normal rat are shown.

According to the manufacturer the human COX-2 antibody for Western immunoblot cross-reacts with both human and rat COX-2. In thoracic duct T cells from both normal control rats and rats with adjuvant-induced arthritis, COX-2 was not detected by Western immunoblot. However, I was also unable to detect COX-2 in LPS-stimulated rat peritoneal macrophages used as a positive control. A second murine COX-2 pAb that the manufacturer also stated cross-reacted with rat COX-2 was obtained but gave similar results. Unfortunately the COX-2 antibody available for flow cytometry does not cross react with rat COX-2 and hence flow cytometry could not be performed. Therefore no firm conclusions could be made regarding the presence or absence of COX-2 in rat thoracic duct lymphocytes.

4.2.10 Expression of thromboxane synthase in thoracic duct lymphocytes from normal and arthritic rats

Thoracic duct lymphocytes from rats with adjuvant-induced arthritis and normal control rats were also examined for the presence of TX synthase. $\alpha\beta$ TCR^+ T cells displayed varying levels of staining for TX synthase (Figure 4.17). The mean fluorescence intensity (MFI) was somewhat increased in cells from rats with adjuvant-induced arthritis (MFI 2.41 vs. 1.65) with little change in the proportion of cells expressing TX synthase above threshold levels.

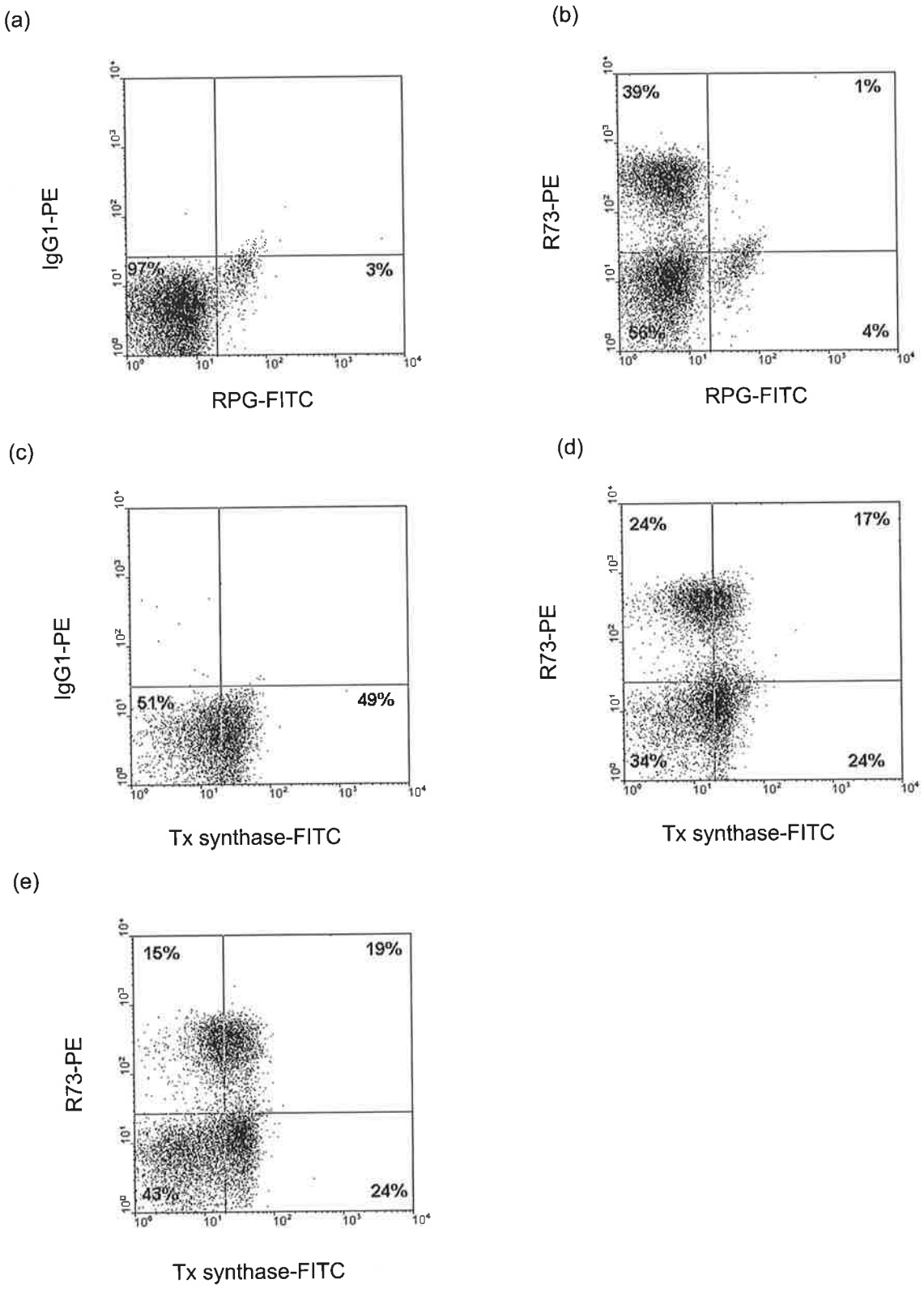


Figure 4.17: Expression of thromboxane synthase in (a-d) normal and (e) arthritic rat thoracic duct lymphocytes. Rat thoracic duct lymph was obtained and cells were stained with indirectly conjugated pAbs directed against thromboxane synthase or RPG (rabbit polyclonal control) or directly conjugated mAbs directed against the αβ T cell receptor (R73) or IgG1 (isotype control). Incubations with isotype matched control antibodies were similar between normal and arthritic rats; therefore only those from the normal rat are shown.

4.2.11 Eicosanoid production by thoracic duct lymphocytes from normal and arthritic rats

Thoracic duct lymphocytes were obtained from normal control rats and rats with adjuvant-induced arthritis. Lymphocytes were incubated, without further stimulation, in the presence or absence of AA (10μM) for 30 minutes. Like human peripheral blood T cells, lymphocytes from control normal rats produced TXA_2 and PGE_2 with the addition of exogenous AA (Figure 4.18). Interestingly lymphocytes from rats with adjuvant–induced arthritis produced significantly more TXB_2 and PGE_2 than lymphocytes from normal control rats (Figure 4.18).

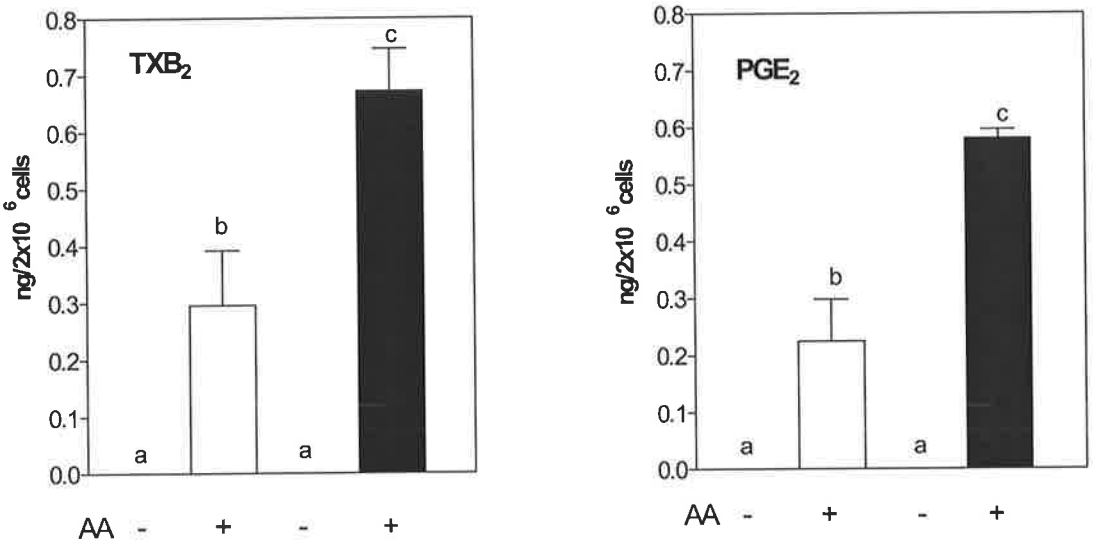


Figure 4.18: Production of TXB_2 and PGE_2 by thoracic duct lymphocytes from normal (hollow bars) and arthritic (solid bars) rats. Thoracic duct lymphocytes pooled from two rats with adjuvant-induced arthritis and two normal rats were incubated in the absence or presence of AA (10μM) for 30 minutes. Bars with different letters are significantly different from each other; $p<0.05$ ANOVA followed by Neuman-Keuls test for multiple comparisons.

4.3 Discussion

Recent reports describe the presence of COX-1 and COX-2 in human peripheral blood T cells and the Jurkat T cell line (Iniguez et al 1999; Pablos et al 1999; Tanaka et al 2000; Bosticardo et al 2001). While I have identified COX-1 in human peripheral blood T cells, I did not detect COX-2 by either Western immunoblot or flow cytometry after exposure to a variety of stimuli including immobilized anti-CD3 and soluble anti-CD28, PMA (with or without A23187), or ConA. By contrast, Iniguez et al reported the presence of COX-2 in stimulated Jurkat T cells and in anti-CD3/anti-CD28 stimulated human peripheral blood T cells using Western immunoblot. They reported that the up-regulation of COX-2 could be inhibited by cyclosporin A (Iniguez et al 1999). Pablos et al identified COX-2 in OKT3 (mAb directed against CD3) stimulated human T cells and Jurkat T cells by Western immunoblot (Pablos et al 1999). However, immunocytochemistry of peripheral blood mononuclear cells revealed COX-1 but no COX-2. Similarly immunocytochemical analysis of cytosmears failed to reveal COX-2 in $CD3^{+}CD4^{+}$ T cells from rheumatoid synovial fluid despite the up-regulation of the activation marker CD69 (Pablos et al 1999). Notwithstanding the negative immunocytochemistry findings, the authors argued that COX-2 was present in T cells because it was detectable in peripheral blood T cell preparations by Western immunoblot. Although their preparations contained some monocytes, they argued that because stimulation of these cells with LPS did not lead to an increase in COX-2, the COX-2 detected was more likely of lymphocyte than monocyte origin. They suggest that the lack of COX-2 in peripheral blood T cells on immunocytochemical analysis may have been due to low sensitivity of the antibody, and the lesser sensitivity of immunocytochemistry compared to Western immunoblot (Pablos et al 1999). However, an alternate explanation is that a small population of

contaminating cells, which may give rise to the positive COX-2 band on Western immunoblot, may be overlooked in immunocytochemical analysis, thereby explaining the negative result. The authors go on to suggest that the lack of COX-2 in synovial fluid T cells may be due to the particular activation status of these cells rather than lack of COX-2 in this cell type (Pablos et al 1999). Another possibility is that COX-2 is preferentially expressed in Th2 cells (Tanaka et al 2000), and that since Th1 cells predominate in RA, COX-2 is not detected. Finally, it is possible that COX-2 is poorly expressed in T cells. While there no longer seems any doubt the human T cells contain COX-1, I have not been able to confirm the presence of COX-2 using Western immunoblot or flow cytometry.

In my T cell isolates, there were on average 0.4% $CD14^+$ monocytes. Bosticardo et al report the presence of COX-2 in human peripheral blood T cells. However, their T cells were isolated by density gradient centrifugation only, a technique that is likely to result in significant monocyte contamination. They make no comment on T cell purity (Bosticardo et al 2001). Pablos et al report <2% $My4^+$ monocytes in their isolates (Pablos et al 1999). Monocytes express COX-2 when exposed to inflammatory stimuli; therefore it is possible that activated T cells may produce soluble mediators that are capable of up-regulating monocyte COX-2. Thus T cell preparations with even partial monocyte contamination may display monocyte COX-2 on Western immunoblot in response to specific T cell activation. However, COX-2 within monocytes should not be detected when T cells are examined specifically by flow cytometry for example. This proposition could explain the discordance between my findings and those reported previously (Iniguez et al 1999; Pablos et al 1999; Tanaka

et al 2000; Bosticardo et al 2001) and will be addressed in Chapter 6 where T cell-monocyte interactions are examined.

While some studies have demonstrated prostaglandin production by murine and human T cells (Rapoport et al 1977; Bauminger 1978; Webb and Nowowiejski 1978; Parker et al 1979b; Abraham et al 1986; Aussel et al 1987), others have not (Dy et al 1980; Kennedy et al 1980; Goldyne and Stobo 1982). The purity of T cell isolates has been questioned with synthesis by contaminating monocytes and platelets thought to account for prostaglandins detected (Goldyne 1989). More recently Iniguez et al used a non-specific prostaglandin colorimetric assay kit (Cayman Chemicals) and detected prostaglandin production by human peripheral blood T cells. However individual prostanoids were not assayed (Iniguez et al 1999). While Pablos et al did not detect prostaglandin production from unstimulated or stimulated Jurkat T cells incubated with AA for 30 minutes, they did not examine prostaglandin production in fresh human T cells (Pablos et al 1999). My studies have demonstrated that human T cells are capable of producing TXA_2, PGD_2, and small amounts of PGE_2. The production of TXA_2 was very sensitive to brief aspirin treatment, which inhibits COX-1, while the production of PGE_2 was only partially inhibited. The predominance of TXA_2 production over PGE_2 is consistent with data from previous studies, which suggest an apparent coupling between TXA_2/COX-1 and PGE_2/COX-2 in monocytes, at least (see section 1.2.2.5).

An important consideration in the production of eicosanoids is the availability of AA, the substrate for COX. The release of AA from cell membrane phospholipid after stimulation is mediated by phospholipases, especially $cPLA_2$. Recently it has been

demonstrated that mature T cells do not contain $cPLA_2$ but do contain $iPLA_2$ (Gilbert et al 1996; Roshak et al 2000). $cPLA_2$ is generally thought to be responsible for release of AA while $iPLA_2$ appears to regulate the main pathway through which cells incorporate AA and other unsaturated fatty acids into their membranes (Balsinde and Dennis 1997). The rate of AA incorporation into membrane phospholipid determines inversely the amount of free fatty acid potentially available for eicosanoid synthesis. $iPLA_2$ may therefore have a role in regulation of prostaglandin synthesis by resting cells (Balsinde and Dennis 1997). It has been reported that activation of T cells with ConA, PHA, or antibodies directed against CD3 does not result in increased release of AA from Jurkat T cells (Aussel et al 1987). Parker et al reported that human lymphocytes incubated with [^{3}H]-AA prior to simulation with PHA released <10% of incorporated [^{3}H]-AA and this occurred rapidly within the first 30 minutes after stimulation (Parker et al 1979a). Hoffman et al reported that T cells do release AA, although at a much lower level than monocytes (12.9% vs. 63.3% of incorporated AA released over 60 minutes) (Hoffman et al 1987). Thus, it may be that T cell activation does not result in release of substantial amounts of AA from the cell membrane and hence, there is little substrate available for COX. This may in part explain the lack of prostaglandins produced in previous studies of purified T cells. However, my studies demonstrate that T cells are capable of producing TXA_2, and this production is greatly enhanced by exogenous AA. In inflammatory arthritis, PLA_2 activity has been shown to be enhanced both in synovial fluid and peripheral blood monocytes (Bomalaski et al 1986; Bomalaski and Clark 1993) and extracellular release of AA from monocytes may provide substrate for T cell COX-1.

It is possible that contaminating monocytes are responsible for the detected eicosanoids. As expected, stimulation of purified monocytes, at the levels expected in the T cell preparations, with immobilized anti-CD3 and soluble anti-CD28 did not result in measurable production of eicosanoids by monocytes. However, this does not exclude a contribution of monocytes stimulated indirectly by T cells. Stimulation of T cell preparations with LPS did not result in expression of COX-2 or production of TXA_2 or PGE_2. This suggests that there are insufficient monocytes present to contribute to the eicosanoids observed. The addition of exogenous AA resulted in detectable levels of TXB_2 by purified platelets only at levels $\geq 1x10^6$/ml. Therefore, it is possible that contaminating platelets are responsible for the detected TXA_2 in T cell preparations. While platelets cannot be excluded as a source of some of the TXA_2, the T cell preparations that produced TXA_2 in the present studies contained $<1x10^6$ platelets. Furthermore, the presence of TX synthase and the production of small amounts of PGE_2 support the notion that T cells are capable of producing TXA_2 and PGE_2, albeit it to a much lesser degree than platelets or monocytes.

COX-2 is regarded as the inducible form of COX. COX-1 is considered to be constitutively expressed and non-inducible. There have been reports that the expression of COX-1 is to some extent regulated (Kaplan et al 1997; Taniura et al 2002). In my studies using the superantigen, Staphylococcal enterotoxin B as a stimulus, there appeared to be down-regulation of COX-1 expression. The mechanism and functional significance of COX-1 down-regulation is unclear. However, if COX-1 derived eicosanoids are important in T cell activation, the down-regulation of COX-1 may be a potential mechanism for limiting T cell activation and production of lymphokines that mediate the systemic effects of SEB. COX enzymes are subject to

activity dependent, oxygen free radical mediated, suicidal inactivation (Kulmacz et al 1994; Bambai and Kulmacz 2000). The structural changes accruing from this process may activate scavenger mechanisms that reduced the levels of the enzyme detected by immunoreactivity. Should removal of enzyme after a period of activity proceed at a rate exceeding new synthesis, a fall in enzyme level would occur. Such a scenario could explain the reduced COX-1 immunoreactive material seen following SEB stimulation of T cells depicted in Figure 4.8.

In the rat, TX synthase has been shown in to be present in small amounts in leukocytes and in larger amounts in lymphoid tissues, such as the thymus, spleen, and bone marrow (Tone et al 1994). Using immunohistochemistry Nusing et al reported that human T cells do not contain TX synthase. While the thymus and spleen stained positively for TX synthase, they reported the positive cells as macrophages and dendritic cells within these tissues, rather than T cells. The cells were identified on the basis of morphology without the use of additional cell surface markers to positively identify specific cell types (Nusing et al 1990). Using both Western immunoblot and flow cytometry, I have demonstrated that T cells contain TX synthase. It is possible that small numbers of contaminating cells, such as platelets, which express TX synthase, may give rise to a 'false positive' result by Western immunoblot. However, flow cytometric analysis located TX synthase within $CD3^+$ T cells. The expression of PGE synthase in T cells warrants further investigation.

Immature murine $CD4^-8^-$ and $CD4^+8^+$ thymocytes express very high levels of the thromboxane receptor. While receptor levels decrease during T cell maturation, peripheral murine T cells continue to express the thromboxane receptor (Ushikubi et al

1993). I have demonstrated that the thromboxane receptor is present in human peripheral blood T cells. While the role of PGE_2 in lymphocyte function has been well studied, the effect of other prostaglandins, such as TXA_2, has received relatively little attention. The presence of the thromboxane receptor suggests there may be a role for TXA_2, derived from either T cells or antigen presenting cells, in T cell function. Thromboxane synthase inhibitors and thromboxane receptor antagonists may therefore alter lymphocyte activities. The effects of TXA_2 on T cell function will be addressed in Chapter 5.

Like human T cells, rat thoracic duct T cells contain COX-1 and produce detectable levels of PGE_2 and TXB_2 in the presence of exogenous AA. It was unfortunate that both the COX-2 antibodies available for both Western immunoblot and flow cytometry did not cross-react with rat COX-2, so no comment can be made on the presence or absence of COX-2 in rat T cells. The presence of COX-2 in rat T cells warrants further investigation.

Interestingly lymphocytes from rats with adjuvant-induced arthritis produced larger quantities of TXB_2 and PGE_2 than normal control rats. Using a rodent model, adjuvant-induced arthritis can be adoptively transferred and the arthritogenic effector cells are $CD4^+$ T cells expressing the activation markers CD25, CD71 (transferrin receptor), and MHC II (Spargo et al 2001). The arthritogenic subpopulation comprises approximately 10-15% of the total lymphocytes in arthritic rat thoracic duct lymph and was not studied directly in my investigations. However, in the thoracic duct lymphocytes from rats with adjuvant-induced arthritis as an undifferentiated population, the increase in eicosanoid production may be due to up-regulation of

COX-2. An alternative explanation is that expression of the terminal eicosanoid synthases is increased. While I can make no comment on COX-2, the present results indicate that in rats with adjuvant-induced arthritis the intensity of expression of thromboxane synthase was increased. This may, at least in part, account for the observed increase in TXB_2.

Whether TXB_2 production by arthritogenic effector T cells has a role in the transfer of polyarthritis or maintenance of the inflammatory process, or whether inhibition of thromboxane synthase could prevent transfer or reduce the observed joint inflammation warrants further investigation.

In summary, T cells contain COX-1 but I did not find COX-2. T cells contain thromboxane synthase, are capable of producing TXB_2, PGD_2, and small amounts of PGE_2 via COX-1 with increased production in the presence of exogenous AA. Finally, T cells express the thromboxane receptor. Therefore it is possible that TXA_2 may effect T cell function. This is examined in the following chapter.

Chapter 5

Modulation of T lymphocyte function by thromboxane A_2

"It is much easier to make measurements than to know exactly what you are measuring."

J.W.N. Sullivan 1982

5.1 Introduction

In the previous chapter I observed that T cells contain thromboxane synthase and are capable of producing TXA_2 via COX-1. In addition, T cells express a thromboxane receptor. These findings indicate TXA_2 may have a role in T cell function.

While the role of PGE_2 in lymphocyte function has been well-studied (see section 1.3), the effect of TXA_2, in both monocytes and lymphocytes has received relatively little attention. TXA_2 has been reported to facilitate (Caughey et al 1997), while PGE_2 inhibits, monocyte TNF-α and IL-1β production (Kunkel et al 1986; Hart et al 1989). Therefore, it is possible that monocyte or lymphocyte derived TXA_2 may effect lymphocyte function indirectly via its ability to regulate cytokine production by monocytes. In addition, the presence of the thromboxane receptor on peripheral blood T cells suggests that TXA_2 can effect lymphocyte function directly. The aim of these studies was to assess the effect of TXA_2 on T cell function with respect to cell proliferation and cytokine production.

5.2 *Results*

5.2.1 Effect of T cell stimulation on [^{3}H]-thymidine incorporation

In response to certain stimuli T cells proliferate and the degree of proliferation can be measured by incorporation of [^{3}H]-thymidine into cells. It is claimed that, for measurement of [^{3}H]-thymidine incorporation T cells should be stimulated in round bottom, rather than flat bottom, 96-well plates for 48-72 hours prior to the addition of [^{3}H]-thymidine (Coligan et al 1991). To compare the difference in well shape, T cells were cultured in either round or flat bottom plates in the presence or absence of immobilized anti-CD3 and soluble anti-CD28. [^{3}H]-thymidine incorporation was substantially greater in round bottom plates (Figure 5.1), thus for all further experiments 96-well round bottom plates were used.

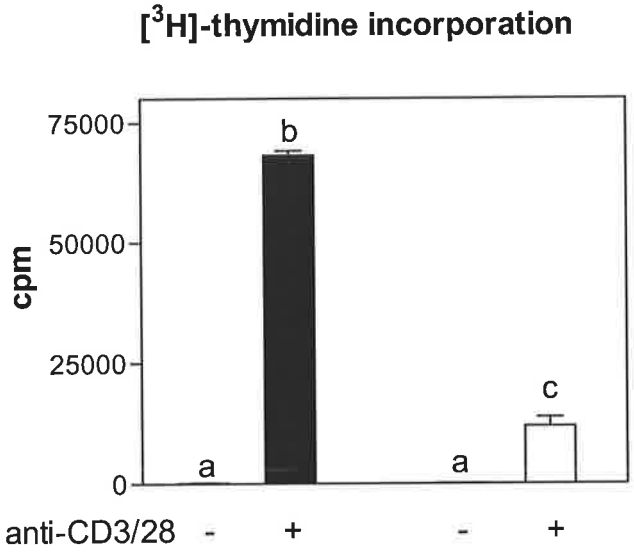


Figure 5.1: Effect of round (solid bars) or flat (hollow bars) bottom plates on T cell proliferation. T cells were incubated in the absence or presence of immobilized anti-CD3 and soluble anti-CD28 for 72 hours prior to the addition of [^{3}H]-thymidine. After a further 18 hour incubation [^{3}H]-thymidine incorporation was measured. Bars with different letters are significantly different; $p<0.05$ ANOVA followed by Neuman-Keuls test for multiple comparisons.

In a separate experiment, T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 for either 48 or 72 hours prior to the addition of [^{3}H]-thymidine. Cells were incubated a further 18 hours after addition of [^{3}H]-thymidine before incorporation of [^{3}H]-thymidine was assessed. T cells cultured for 72 hours prior to the addition of [^{3}H]-thymidine had a greater degree of [^{3}H]-thymidine incorporation than those cultured for 48 hours prior to addition of [^{3}H]-thymidine (Figure 5.2). Thus for all further experiments [^{3}H]-thymidine was added at 72 hours.

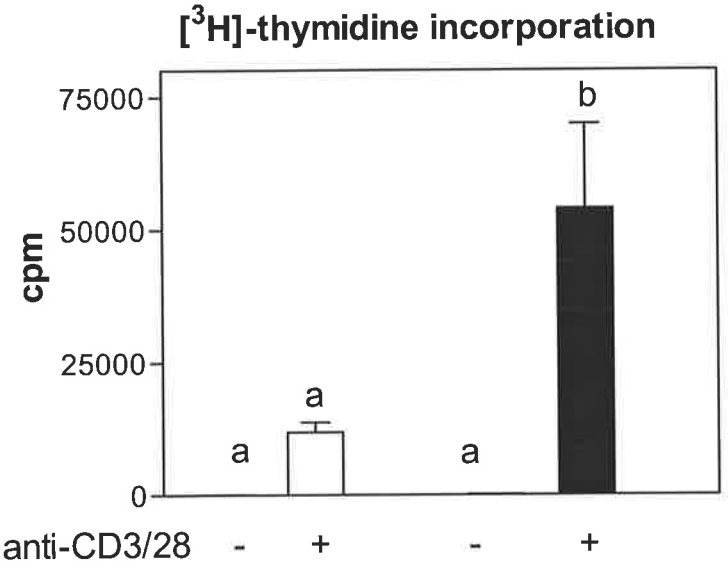


Figure 5.2: Effect of timing of [^{3}H]-thymidine addition on T cell proliferation. T cells were incubated in the absence or presence of immobilized anti-CD3 and soluble anti-CD28 for 48 hours (hollow bars) or 72 hours (solid bars) prior to the addition of [^{3}H]-thymidine. After a further 18 hour incubation [^{3}H]-thymidine incorporation was measured. Bars with different letters are significantly different; $p<0.05$ ANOVA followed by Neuman-Keuls test for multiple comparisons.

5.2.2 Effect of thromboxane synthase inhibition on TXA_2 and PGE_2 production by T cells

Carboxyheptyl imidazole (CI) is a TX synthase inhibitor. The carboxyalkylimidazoles have previously been shown to inhibit TX synthase specifically without affecting the other terminal eicosanoid synthases (Yoshimoto et al 1978; Kayama et al 1981). In the presence of exogenous AA (10μM), T cells produce larger amounts of TXA_2 and PGE_2 (see Chapter 4). Both TXA_2 and PGE_2 are derived from the same precursor PGH_2. Therefore, inhibition of TX synthase could result in larger amounts of PGH_2 being available for conversion to PGE_2. In both the absence and presence of exogenous AA, treatment of T cells with CI prior to stimulation inhibited production of TXB_2 (Figure 5.3). In the absence of exogenous AA, no PGE_2 was detected despite treatment of T cells with CI prior to stimulation. However, in the presence of exogenous AA (10μM), treatment of T cells with CI prior to stimulation resulted in a significant increase in PGE_2 (Figure 5.3). The findings suggest that TX synthase can achieve measurable conversion of basal amounts of PGH_2 to TXA_2 and that in the presence of AA and thromboxane synthase inhibition PGH_2 is utilized by PGE synthase.

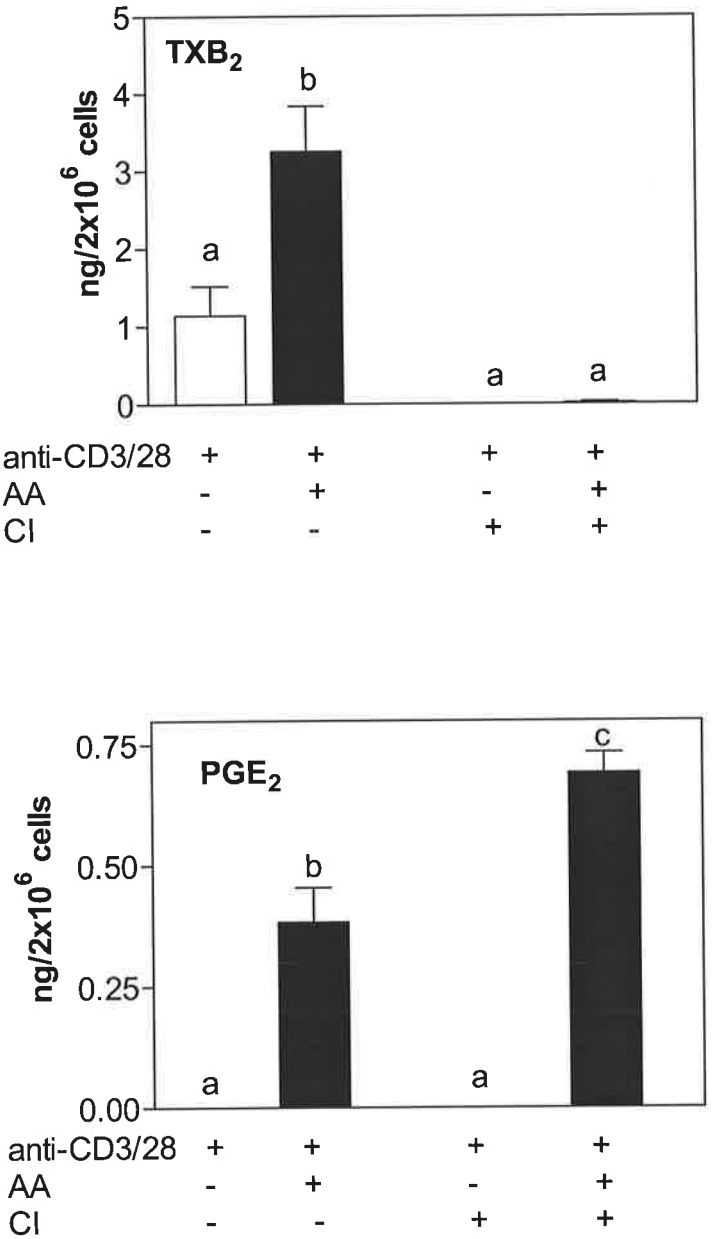


Figure 5.3: Effect of carboxyheptyl imidazole (CI) on TXB_2 and PGE_2 production by stimulated T cells. T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 in the absence (hollow bars) or presence (solid bars) of AA (10μM). Where indicated cells were treated with CI (10μM) for 30 minutes and without washing, cells were transferred to wells pre-coated with immobilized anti-CD3 and soluble anti-CD28 was added. After 24 hours supernatants were collected and eicosanoids assayed. Bars with different letters are significantly different from each other; $p<0.05$ ANOVA followed by Neuman-Keuls test for multiple comparisons.

5.2.3 Effect of TXA_2 on T cell proliferation

Inhibition of endogenous T cell derived TXA_2 by treatment of cells with CI (10μM) commencing 30 minutes prior to stimulation, resulted in inhibition of T cell proliferation as assessed by [^{3}H]-thymidine incorporation (Figure 5.4). As shown in section 5.2.2 there was no increase in PGE_2 in the presence of CI to account for the observed inhibition in cell proliferation. Incubation of T cells with the thromboxane receptor antagonists SQ29548 or pinane (p)TXA_2 commencing 30 minutes prior to stimulation also inhibited T cell proliferation (Figure 5.4).

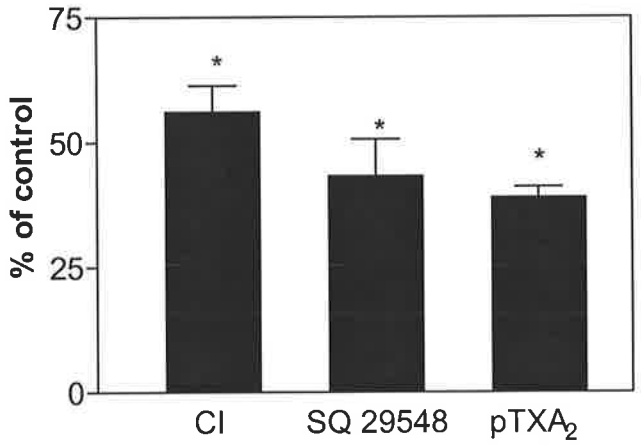


Figure 5.4: Effect of thromboxane synthase inhibition (CI) or thromboxane receptor antagonists (SQ 29548 or $pTXA_2$) on [^{3}H]-thymidine incorporation. T cells were treated with the TX synthase inhibitor, CI (10μM), or the thromboxane receptor antagonists SQ29548 (10μM) or $pTXA_2$ (10μM) for 30 minutes. Without washing cells were transferred to wells pre-coated with immobilized anti-CD3 to which soluble anti-CD28 was added. After 72 hours [^{3}H]-thymidine was added and after a further 18 hours incorporation of [^{3}H]-thymidine was measured. Control cells stimulated with immobilized anti-CD3 and soluble anti-CD28 had on average 42500cpm. (*Significantly different from control values $p<0.05$). The data shown are representative of 5 separate experiments.

The inhibition of T cell proliferation by CI could be overcome by low concentrations of thromboxane receptor agonist, carbocyclic TXA_2 ($cTXA_2$), while at higher concentrations $cTXA_2$ was inhibitory (Figure 5.5). When endogenous TXA_2 was present, i.e. without CI treatment, no stimulation of proliferation by $cTXA_2$ was seen (Figure 5.5). Cell viability and vehicle controls were no different among groups (data not shown).

Indomethacin is a non-selective COX inhibitor, which inhibits both TXA_2 and PGE_2 production. Treatment of T cells with indomethacin (10μM) prior to stimulation with immobilized anti-CD3 and soluble anti-CD28 resulted in a slight, but significant increase in T cell proliferation. Addition of the thromboxane receptor agonist, $cTXA_2$, in the presence of indomethacin had no effect on T cell proliferation at lower doses, but at higher doses was inhibitory (Figure 5.6).

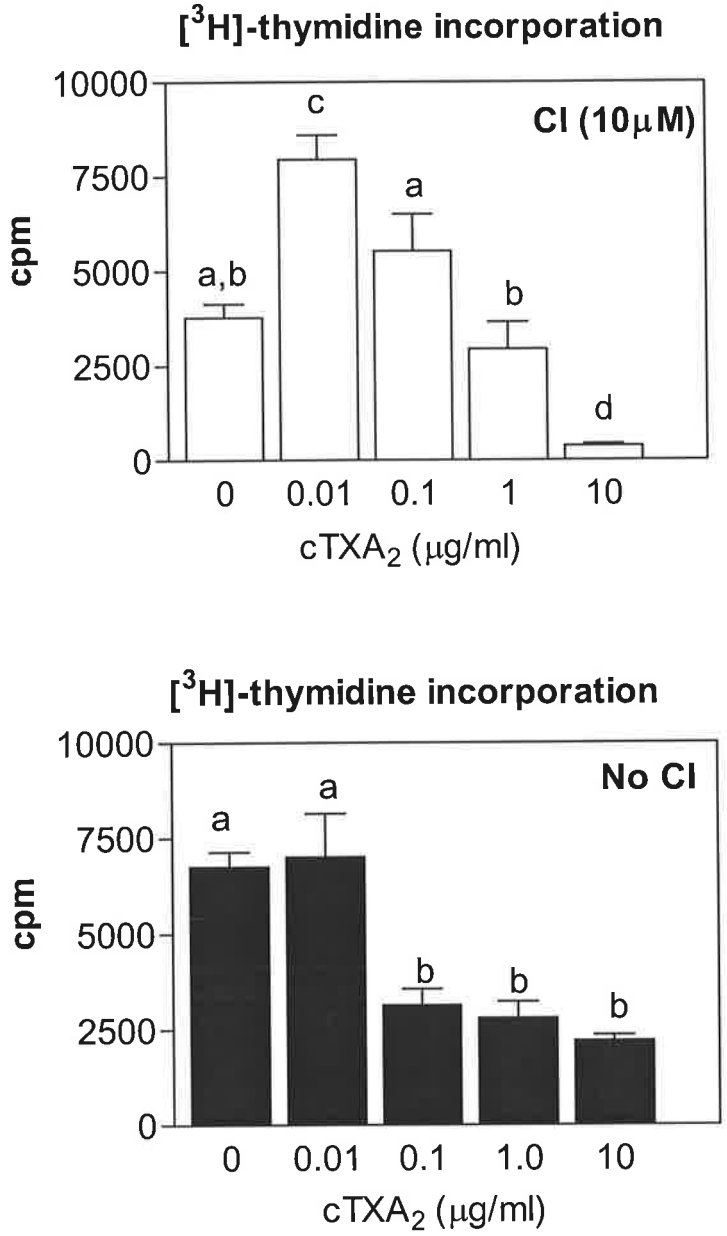


Figure 5.5: Effect of the TX receptor agonist, $cTXA_2$, on T cell proliferation in the absence or presence of endogenous TXA_2. T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 in the presence of $cTXA_2$ (0-10μg/ml). Where indicated cells were treated with Cl (10μM) for 30 minutes and without washing cells were transferred to wells pre-coated with immobilized anti-CD3 to which soluble anti-CD28 and $cTXA_2$ were added. After 72 hours [^{3}H]-thymidine was added and cells incubated for a further 18 hours before [^{3}H]-thymidine incorporation was measured. Results from this experiment are representative of four separate experiments. Bars with different letters are significantly different from each other; $p<0.05$; ANOVA followed by Neuman-Keuls test for multiple comparisons.

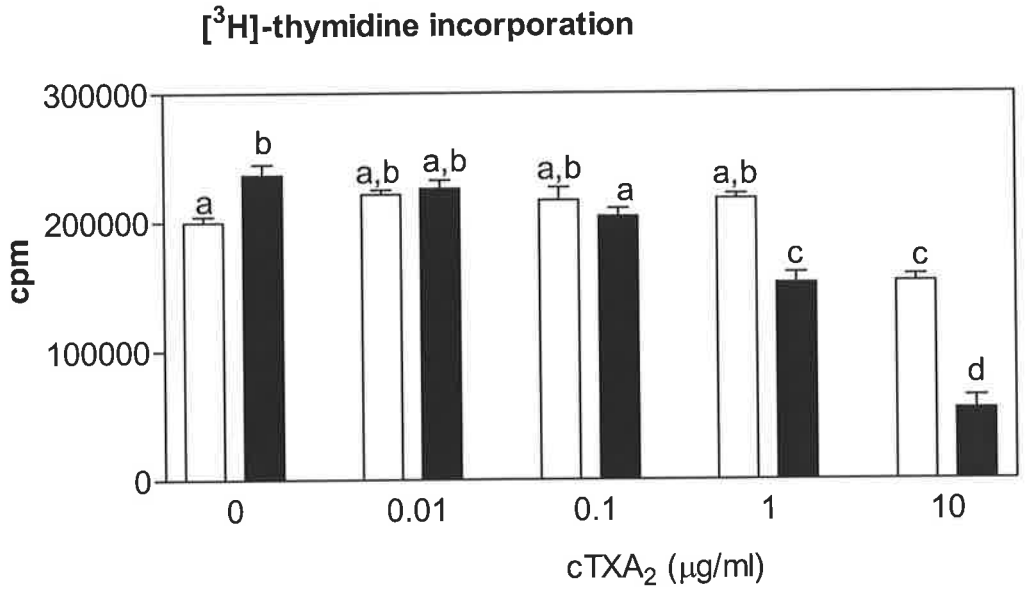
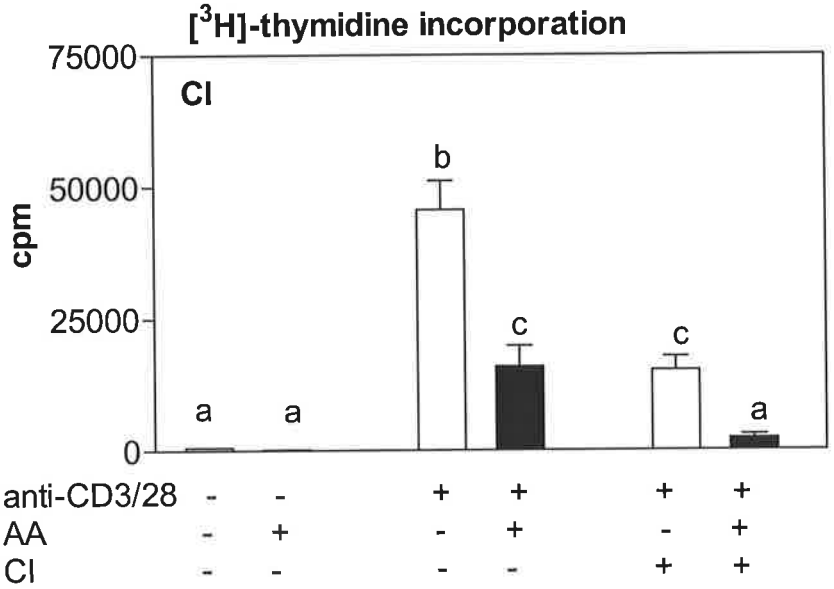


Figure 5.6: Effect of indomethacin on T cell proliferation in the presence or absence of the thromboxane receptor agonist, $cTXA_2$. T cells were treated without (hollow bars) or with (solid bars) indomethacin (10μM) for 30 minutes. Without washing cells were transferred to wells pre-coated with immobilized anti-CD3 to which soluble anti-CD28 and $cTXA_2$ (0-10μg/ml) were added. After 72 hours [^{3}H]-thymidine was added and cells incubated for a further 18 hours prior to measurement of [^{3}H]-thymidine incorporation. Bars with different letters are significantly different; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

5.2.4 Effect of exogenous arachidonic acid on T cell proliferation

The addition of AA (10μM) to T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 resulted in significant inhibition of [^{3}H]-thymidine incorporation (Figure 5.7). [^{3}H]-thymidine incorporation was again inhibited in cells treated with the thromboxane synthase inhibitor CI (10μM) prior to stimulation and the inhibitory effects of AA and CI were at least additive (Figure 5.7). By contrast, the presence of the thromboxane receptor antagonist SQ29548 did not further enhance the inhibition of T cell proliferation by exogenous AA (Figure 5.7).

(a)



(b)

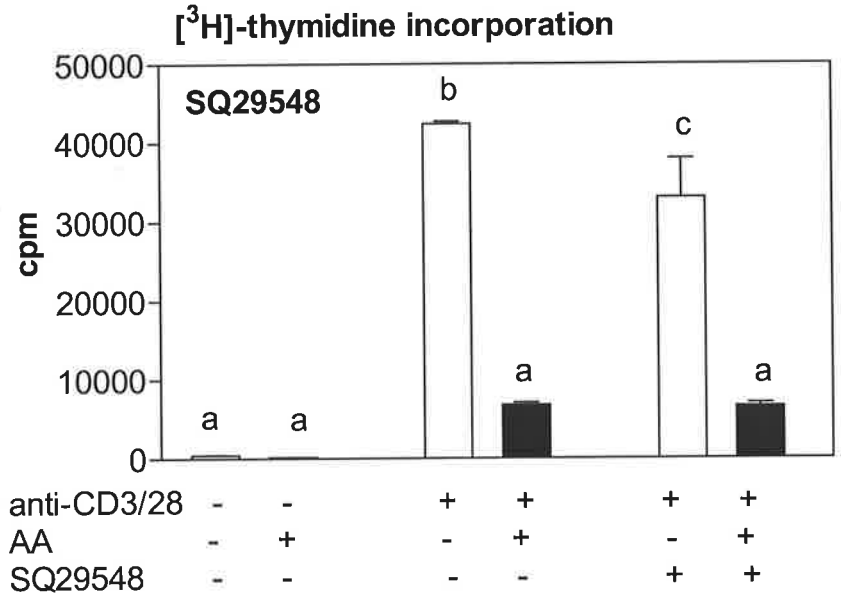


Figure 5.7: Effect of exogenous AA on [^{3}H]-thymidine incorporation by T cells in the presence of (a) thromboxane synthase inhibitor (CI) or (b) the thromboxane receptor antagonist SQ29548. T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 in the absence (hollow bars) or presence (solid bars) of AA (10μM). Where indicated cells were treated with CI (10μM) or SQ29548 (1μM) for 30 minutes before transfer, without washing, to wells pre-coated with immobilized anti-CD3 to which soluble anti-CD28 was added. After 72 hours [^{3}H]-thymidine was added and cells incubated for a further 18 hours before incorporation of [^{3}H]-thymidine was measured. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

5.2.5 Effect of TXA_2 on cytokine production by T cells

In response to stimulation T cells produce a variety of cytokines, including IFN-γ and IL-17. Production of IFN-γ and IL-17 was inhibited by treatment of T cells with the TX synthase inhibitor, CI (10μM) commencing 30 minutes prior to stimulation with immobilized anti-CD3 and soluble anti-CD28 (Figure 5.8).

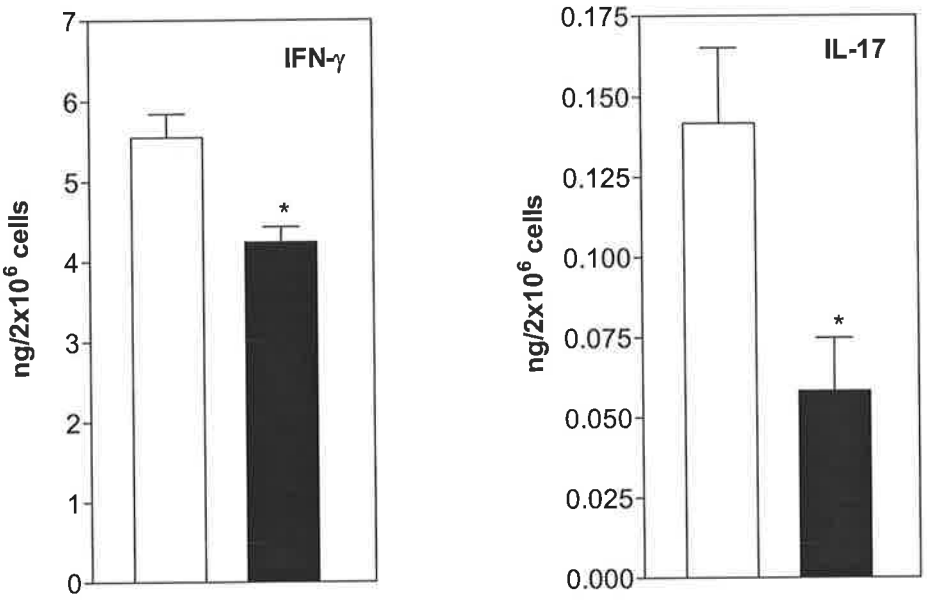


Figure 5.8: Effect of thromboxane synthase inhibition on T cell IFN-γ and IL-17 release. T cells were treated without (hollow bars) or with (solid bars) CI (10μM) for 30 minutes. Without washing cells were transferred to wells pre-coated with immobilized anti-CD3 and soluble anti-CD28 was added. After 24 hours supernatants were collected and cytokines measured by ELISA * $p<0.05$.

Production of IFN-γ was inhibited by the stable thromboxane receptor agonist, $cTXA_2$. This was not altered significantly by the presence of CI (Figure 5.9). While low and high concentration $cTXA_2$ resulted in a non-significant reduction in production of IL-17, moderate concentration $cTXA_2$ increased IL-17 production by stimulated T cells (Figure 5.9). This effect was also seen in the presence of CI (Figure 5.9).

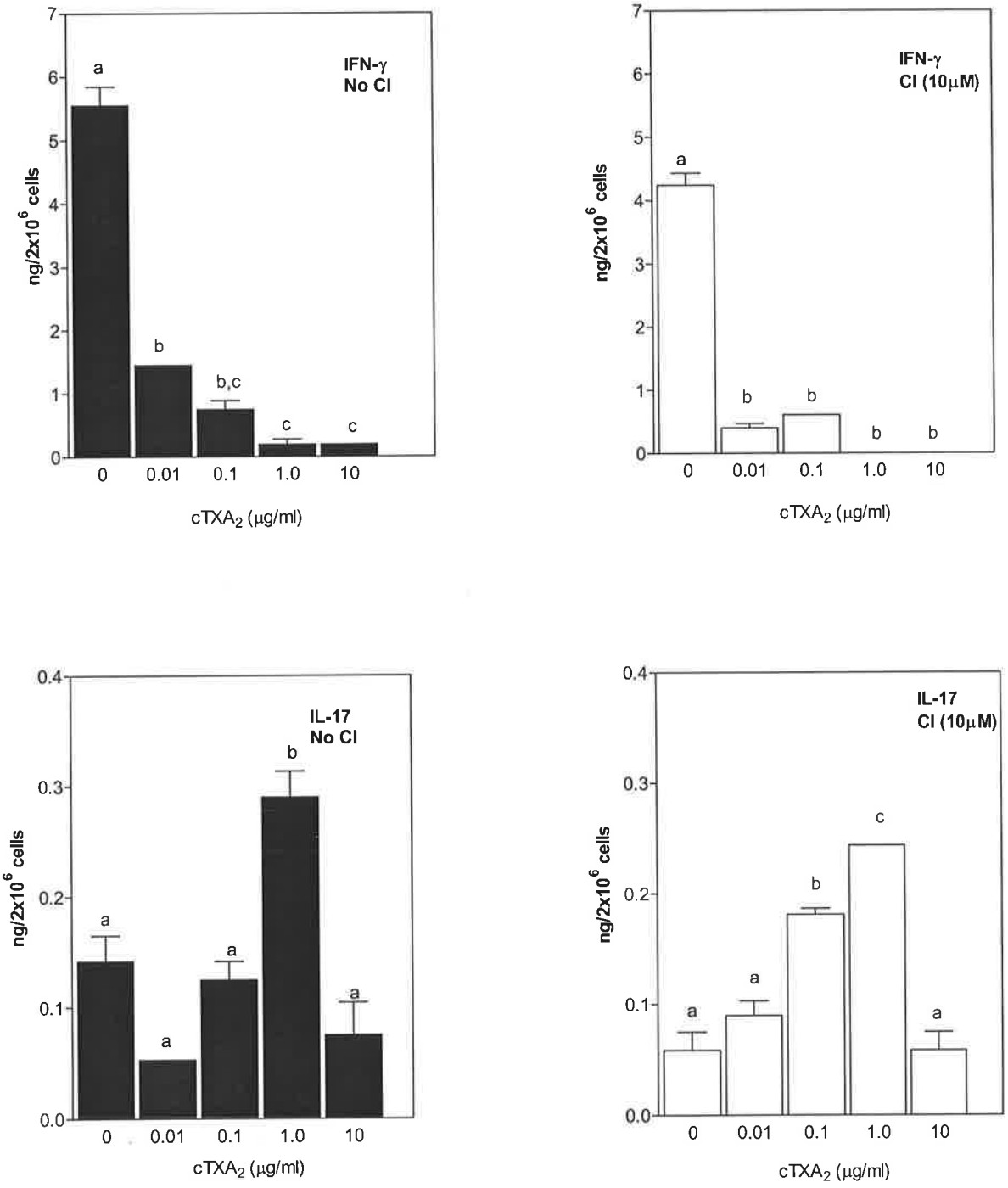


Figure 5.9: Effect of the thromboxane receptor agonist, $cTXA_2$, on T cell IFN-γ and IL-17 release in the absence or presence of endogenous TXA_2. T cells were treated without (solid bars) or with (hollow bars) CI (10μM) for 30 minutes. Without washing cells were transferred to wells pre-coated with immobilized anti-CD3 and soluble anti-CD28 and $cTXA_2$ (0-10μg/ml) were added. After 24 hours supernatants were collected. Bars with different letters are significantly different from each other; $p<0.05$; ANOVA followed by Neuman-Keuls test for multiple comparisons.

Production of IFN-γ was inhibited by treatment of T cells with the thromboxane receptor antagonists, SQ29548 or $pTXA_2$ prior to stimulation (Figure 5.10). Neither of the thromboxane receptor antagonists decreased production of IL-17 by stimulated T cells (Figure 5.10).

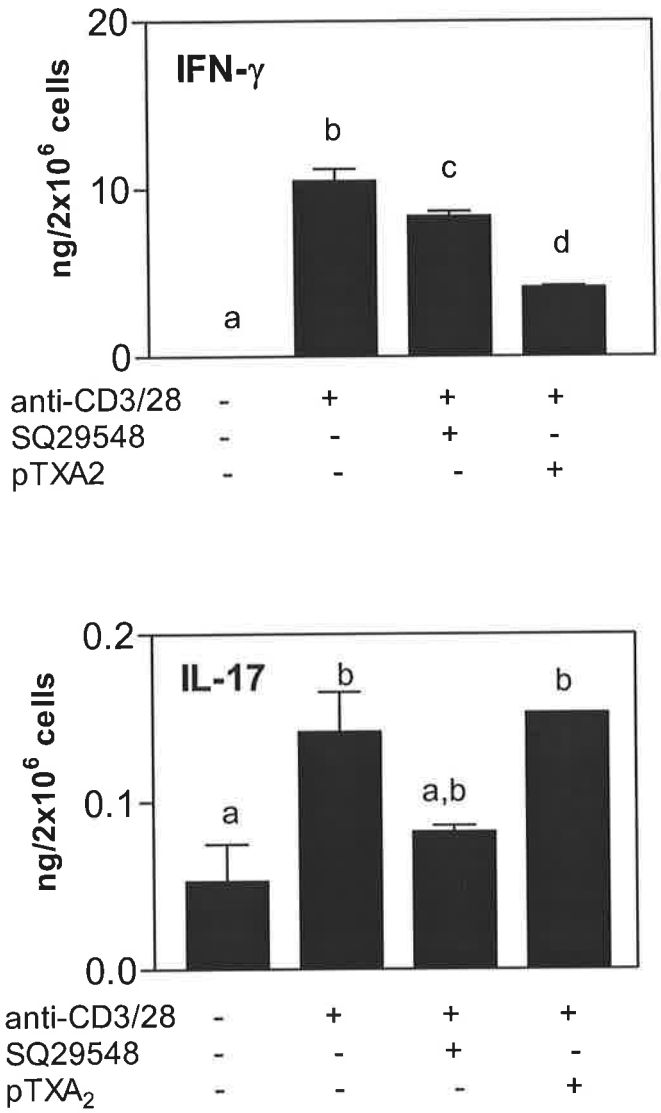


Figure 5.10: Production of IFN-γ and IL-17 by T cells in the presence of the thromboxane receptor antagonists SQ29548 or $pTXA_2$. T cells were treated with either SQ29548 (1μM) or $pTXA_2$ (1μM) for 30 minutes. Without washing cells were transferred to wells pre-coated with immobilized anti-CD3 and soluble anti-CD28 was added. After incubation for 18 hours supernatants were collected and IFN-γ and IL-17 measured by ELISA. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

IL-2 is a cytokine that is produced by T cells and which acts through IL-2 receptors (CD25) expressed on T cells, NK cells, and B cells. IL-2 acts in an autocrine manner to promote T cell survival and proliferation. While the other T cell derived cytokines (IL-17, TNF-α, and IFN-γ) could be readily and reproducibly measured by ELISA, the results for IL-2 were unsatisfactory. Results between separate experiments using cells from different donors produced results with no clear pattern. The problems were discussed with Prof. A. Ferrante (Women's and Children's Hospital, Adelaide, South Australia). He informed me that IL-2 was an unreliable index of T cell function because of great variation between different patients with respect to time of production and amount of IL-2 produced in response to *in vitro* T cell stimulation. Therefore no further attempts were made to measure IL-2 production.

5.2.6 Effect of TXA_2 on CD25 expression

The effect of TXA_2 on T cell expression of the IL-2 receptor (CD25) was examined by two-colour flow cytometry. Expression of CD25 by T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 was slightly enhanced by low concentration $cTXA_2$ (the TX receptor agonist), while at higher concentrations $cTXA_2$ was inhibitory (Figure 5.11). Treatment of T cells with the thromboxane receptor antagonists, SQ29548 or $pTXA_2$ prior to stimulation with immobilized anti-CD3 and soluble anti-CD28 inhibited expression of CD25 (Figure 5.12).

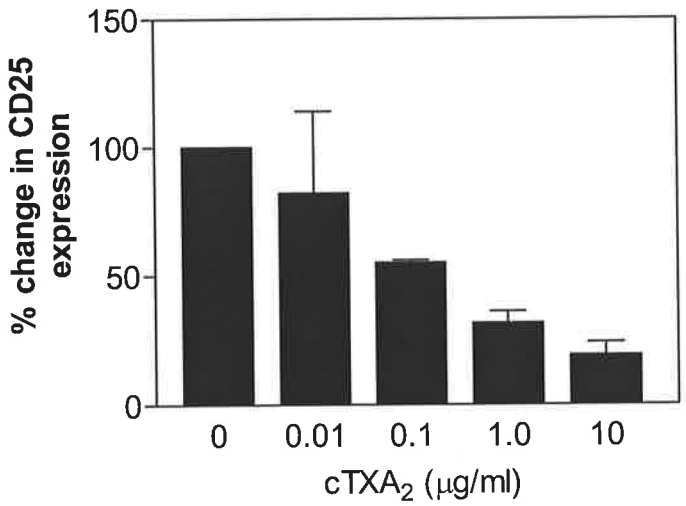


Figure 5.11: Expression of CD25 by T cells in the presence of $cTXA_2$. T cells were incubated for 18 hours in the presence of immobilized anti-CD3 and soluble anti-CD28 as well as $cTXA_2$ (0-10μg/ml). Cells were collected and processed for flow cytometry with directly conjugated mAbs directed against CD3, CD25, or IgG1. Shown is the percentage of $CD25^+CD3^+$ cells in the presence of $cTXA_2$ expressed as a percentage of those in the absence of $cTXA_2$. Data from one experiment representative of three is shown.

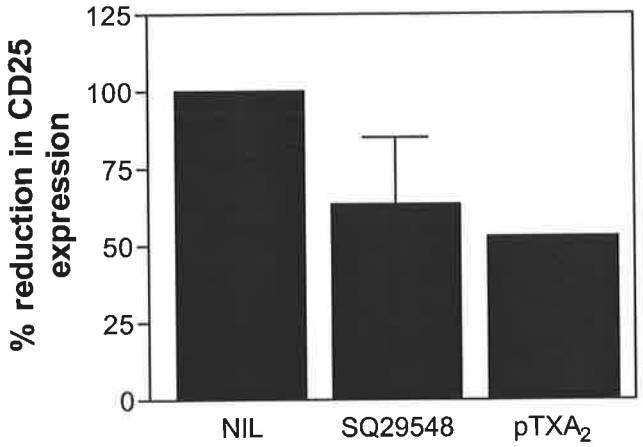


Figure 5.12: Expression of CD25 by T cells in the presence of the thromboxane receptor antagonists SQ29548 or $pTXA_2$. T cells were treated with SQ29548 (10μg/ml) or $pTXA_2$ (1μg/ml) for 30 minutes. Without washing cells were transferred to wells pre-coated with immobilized anti-CD3 and soluble anti-CD28 was added. After incubation for 18 hours cells were collected and processed for flow cytometry using directly conjugated mAbs against CD3, CD25, or IgG1. Shown is the percentage inhibition of CD25 expression by the $CD3^+$ cells. Data from one experiment representative of two is shown.

5.3 Discussion

Whether T cells produce TXA_2 or PGE_2 or whether these eicosanoids are monocyte-derived, they appear to have an effect on T cell function. The results presented in this chapter show that inhibition of TX synthase or blockade of the thromboxane receptor inhibits human peripheral blood T cell proliferation, IFN-γ production, and CD25 expression in response to stimulation with mAbs directed against CD3 and CD28. Endogenous T cell TXA_2 may therefore act in an autocrine manner to promote T cell proliferation. The inhibition of T cell proliferation by TX synthase inhibition could be overcome by low concentration of the TX receptor agonist $cTXA_2$, but at higher concentrations $cTXA_2$ is inhibitory. Therefore, it is possible that in a situation where T cells are activated, the small amount of TXA_2 produced by the T cell acts in an autocrine manner to facilitate activation and proliferation. However, in situations where T cells enter an inflammatory environment, such as an inflamed joint, larger concentrations of TXA_2 which could arise from monocytes, may act to prevent T cell activation and proliferation.

Previous studies have reported that TXA_2 potentiates T cell proliferation (Kelly et al 1979; Leung and Mihich 1980). Kelly et al reported that selective inhibition of TX synthase or combined TX synthase and COX inhibition resulted in reduced [^{3}H]-thymidine incorporation in purified human T cells. They concluded that inhibition of TXA_2, rather than an increase in PGE_2, was responsible for the inhibition of T cell proliferation. In addition, they suggested that monocyte derived TXA_2 may have a role in T cell activation (Kelly et al 1979). The data presented in this chapter is in accordance with the results of Kelly et al in as much as inhibition of TX synthase resulted in inhibition T cell proliferation. I have extended this to show that blockade of

the thromboxane receptor has similar effects. Furthermore my results suggest that small amounts of TXA_2 derived from T cells, may be important in promoting T cell activation, whereas larger amounts of TXA_2, which could be produced by monocytes for example, are inhibitory. On the other hand, caution needs to be exercised in the interpretation of actions of exogenous agents *in vitro* since high doses may reflect non-specific toxic effects rather than the selective actions of agents used at lower concentrations.

Increases in PGE_2, rather than a reduction in TXA_2, have been reported to be responsible for the inhibition of cell proliferation observed with TX synthase inhibitors. Ceuppens et al prepared human T cells by Ficoll-Hypaque density gradient centrifugation and reported that a TX synthase inhibitor could inhibit mitogen-induced cellular proliferation. This effect could be overcome by the addition of a TXA_2 analogue, U46619, at 10^{-7} M. However, while TXB_2 decreased in the presence of the TX synthase inhibitor, PGE_2 increased. In comparison, a thromboxane receptor antagonist did not affect cellular proliferation. When the TXA_2 analogue and indomethacin, which reduces production of both TXA_2 and PGE_2, were used together there was an increase in cell proliferation as assessed by [^{3}H]-thymidine incorporation. They concluded that it was the increase in PGE_2 rather than a reduction in TXA_2 that was responsible for inhibition of cellular proliferation (Ceuppens et al 1985). While the authors report their effects as being on lymphocytes, T cells were isolated by Ficoll-Hypaque density gradient centrifugation only. This technique produces a mononuclear cell enriched population, which contains large numbers of monocytes that are capable of producing PGE_2. Therefore, it is difficult to interpret their results with specific reference to TXA_2 and PGE_2 derived from T cells.

The results presented in this chapter show that despite TX synthase inhibition, there was no increase in PGE_2 synthesis unless exogenous AA was added. This suggests that lymphocyte derived TXA_2 can affect T cell mitogenesis. The addition of exogenous AA results in enhanced production of both TXA_2 and PGE_2 by T cells. Furthermore, exogenous AA inhibited T cell proliferation, which may be a result of increased PGE_2 and/or TXA_2. In the presence of exogenous AA, TX synthase inhibition led to an increase in PGE_2, over and above that seen with AA alone. This was associated with even more pronounced inhibition of T cell proliferation. In the presence of thromboxane receptor antagonists, the addition of AA resulted in no further reduction in cell proliferation over that with AA alone. These results suggest that the inhibitory effects of PGE_2, which is increased in the presence of AA, may override any role that TXA_2 has in facilitating cell activation. However, it follows that this effect would occur in the presence of exogenous AA. Alternatively the amount of TXA_2 produced with the addition of AA may reach a level where it acts to inhibit, rather than potentiate T cell proliferation. These results with exogenous AA must be interpreted with caution, as it has been reported that AA can inhibit IL-2 production in Jurkat T cells and mitogen-stimulated peripheral blood mononuclear cells independent of any effects on PGE_2 (Santoli and Zurier 1989). Also the concentration of AA used may have exerted non-specific membrane perturbing effects.

In the present studies indomethacin enhanced T cell proliferation in response to stimulation with mAbs directed against CD3 and CD28. However, the combination of the thromboxane receptor agonist, $cTXA_2$, and indomethacin inhibited T cell proliferation to a greater extent than that seen with $cTXA_2$ alone. In comparison Rivero et al reported that neither indomethacin nor a specific COX-2 inhibitor, DFU,

had any effect on CD3 or PHA stimulated T cell proliferation, expression of the activation markers CD69 and CD25 or intracellular calcium mobilization (Rivero et al 2002). Previous studies have demonstrated enhancement of T cell proliferation with NSAIDs (Ceuppens et al 1986; Haynes et al 1990) and two mechanisms have been proposed to be important. Firstly PGE_2, whose production is inhibited by NSAIDs, has inhibitory effects on cell proliferation. Secondly NSAIDs increase intracellular calcium, a necessary second messenger for T cell activation, thus priming T cells for enhanced response to mitogen (Flescher et al 1991).

The effects of indomethacin on T cell proliferation need to be interpreted with caution. It has long been suggested that inhibition of prostaglandin production by NSAIDs can not fully account for the therapeutic properties of these drugs. Aspirin and NSAIDs have been reported to block transcription factors required for expression of T cell inducible response genes. At a concentration of 0.1mM, aspirin inhibited AP-1 activity. While indomethacin increased AP-1 activity at lower concentrations (0.08-0.4μM), there was little effect at higher concentrations (2-10μM) (Dong et al 1997). Using the Jurkat T cell line, Paccani et al reported that ibuprofen (500-2000μM) inhibited NF-AT activity and selectively blocked p38 MAP kinase activity (Paccani et al 2002). These effects make interpretation of the data, with specific reference to eicosanoids, difficult.

In both human studies and rat models of renal transplantation, graft rejection is associated with increased TXA_2 (Foegh et al 1985; Gibbons et al 1987; Teraoka et al 1987b). Furthermore, graft survival can be prolonged by inhibition of TX synthase (Teraoka et al 1987a; Ruiz et al 1988). TXA_2 contributes to graft rejection by

promoting thrombosis and vasoconstriction (Coffman et al 1985). However, evidence derived from use of TX synthase inhibitors suggest TXA_2 may also act to enhance cytotoxic T cell mediated host anti-donor responses within the local renal microenvironment (Ruiz et al 1989), thus contributing to graft failure. Using both naïve murine lymphoid cells and primed alloreactive effector cells, TX synthase inhibition and thromboxane receptor antagonism reduced alloproliferative responses in the mixed lymphocyte reaction. The addition of TXA_2 had the opposite effect (Ruiz et al 1992), suggesting that TXA_2 potentiates the function of naïve and primed T cells.

Other studies have suggested that TXA_2 has no effect or inhibits T cell function. In a model of allergic dermatitis, knockout mice lacking the thromboxane receptor had increased ear thickness. Both Th1 and Th2 responses were enhanced suggesting that TXA_2 may act to down-regulate the immune system in this model (Kabashima et al 1999). Gordon et al studied the effects of six different imidazole compounds and concluded that endogenous TXA_2 had no effect on T cell mitogenesis. However, two patterns could be distinguished; firstly TX synthase inhibitors that increased PGE_2 in addition to reducing TXA_2 had little effect on cell proliferation and secondly TX synthase inhibitors that reduced TXA_2 with little effect on PGE_2 inhibited cell proliferation (Gordon et al 1981). It has also been suggested that TXA_2 exerts a regulatory role through negative feedback via its own receptor. Thus addition of TXA_2 results in inhibition of T cell cytotoxicity, whereas receptor blockade leads to at least partial enhancement (Rola-Pleszczynski et al 1985).

Monocyte contamination of T cell isolates may potentially confound the results of the published studies presented above. Dipeptidyl peptidase (DP) IV is a transmembrane

glycoprotein, identical to CD26, expressed on T cells, B cells, and NK cells. Experimental data using synthetic DP IV inhibitors suggest that active DP IV may act as an accessory molecule in T cell activation which leads to induction of T cell proliferation and cytokine production (Kahne et al 1999). It has recently been reported that the extracellular N-terminal portion of the TXA_2 receptor may inhibit T cell proliferation through interactions with DP IV resulting in suppression of T cell DNA synthesis and IL-2 production in tetanus toxoid-stimulated peripheral blood mononuclear cells (Wrenger et al 2000). The authors suggest that the monocyte TXA_2 receptor and T cell DP IV may interact during monocyte/T cell interactions acting as a negative feedback mechanism to down-regulate T cell activation (Wrenger et al 2000). Whether this mechanism has a role *in vivo* remains to be elucidated. In the T cell isolates used in my studies, $CD14^+$ monocytes account for ~0.4% of the cells. T cell proliferation was induced adequately through stimulation with immobilized anti-CD3 and soluble anti-CD28 and it seems unlikely that this potential inhibition of T cell activation through interaction of monocyte TXA_2 receptor with T cell DP IV had a significant effect on the results. In addition, thromboxane receptor antagonists would act on both T cell and monocyte TXA_2 receptors resulting in increased, rather than decreased, T cell proliferation, were the T cell DP IV/monocyte TXA_2 receptor interaction providing an inhibitory role in these experimental conditions.

Binding of TXA_2 to its receptor results in activation of protein kinase C and an increase in intracellular calcium. Both of these signals promote DNA fragmentation and cell death (apoptosis) in thymocytes (McConkey et al 1990). Apoptosis, which eliminates reactive T cell clones physically, and clonal anergy, which inactivates T cells functionally, are two important processes involved in T cell selection during cell

maturation. Using radioligand-binding studies the thromboxane receptor has been shown to be expressed on immature $CD4^-CD8^-$ and $CD4^+CD8^+$ murine thymocytes. In addition, a TXA_2 agonist induced DNA fragmentation and apoptosis in $CD4^+CD8^+$ thymocytes, an effect that could be inhibited by a specific TXA_2 antagonist. In comparison mature $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes were not affected (Ushikubi et al 1993). These results suggest that TXA_2 may be involved in negative selection of T cells in the thymus. While it is possible that TXA_2 may promote apoptosis in mature T cells via similar mechanisms, cell viability was similar despite the use of TX synthase inhibitors and thromboxane receptor agonists or antagonists suggesting that apoptosis was not contributing to the reduction in cellular proliferation which I observed.

The results from the present study suggest that the small amounts of TXA_2, produced by T cells via COX-1, may have a role in T cell activation. Paccini reported that both COX-1 and COX-2 are required sequentially for T cell signaling. Inhibition of COX-1 resulted in defective induction of COX-2 in response to T cell stimulation. The authors suggest that COX-1 derived PGE_2 may be involved in activation of p38 MAP kinase, which is required for induction of COX-2. They go on to suggest that COX-2 dependent prostaglandin production may contribute to the later stages of T cell activation. The effects of TXA_2 on this system were not examined (Paccani et al 2002). While I have not been able to identify COX-2 in my T cell isolates, the data presented in this chapter support the notion that T cell COX-1 derived prostaglandins have a role in T cell activation.

The effect of TXA_2, on cytokine production by T cells has not been investigated extensively. Della Bella et al reported that a TXA_2 agonist, U-46619, had no effect on PHA-stimulated T cell production of IL-2, IL-6, IFN-γ, or TNF-α (Della Bella et al 1997). Kumar and Das reported a concentration dependent effect of TXA_2 on T cell cytokine secretion with low concentration TXA_2 (1μg/ml) reducing IL-2 production and increasing IL-4 and TNF-α production. High concentration TXA_2 (5μg/ml) had the opposite effects (Kumar and Das 1994). However while the changes seen by Kumar and Das at higher concentrations were statistically different from controls, those at low concentration were not. In addition their T cells were isolated by density gradient centrifugation alone and they make no comment on cell purity. As suggested above, there is likely to be significant monocyte contamination with this method and this makes interpretation of the results difficult.

The results presented in this chapter show differing effects of TXA_2 on IFN-γ and IL-17. IFN-γ was inhibited at all concentrations of $cTXA_2$ while IL-17 release was enhanced by moderate concentrations of $cTXA_2$. In rheumatoid joints IFN-γ is only present in low amounts, while IL-17 has been found in higher amounts. Whether monocyte or T cell derived TXA_2 contributes to this pattern of lymphokine expression has not been determined but is an interesting possibility that warrants further investigation.

In summary endogenous T cell derived TXA_2 may have a permissive or enhancing effect on cell proliferation suggesting that thromboxane synthase inhibitors could be used to inhibit T cell mediated processes. However, larger amounts of TXA_2 that may be produced by accessory cells present within an inflammatory environment, may act

to inhibit T cell proliferation. This mechanism may have a regulatory role in limiting the T cell mediated inflammatory response.

Chapter 6

Up-regulation of monocyte COX-2 expression and cytokine secretion through interactions with T lymphocytes

"There is little doubt they are activated, as judged from several criteria, the mystery is the mechanism which brings this about."

G Panayi et al 1992

6.1 Introduction

I have detected COX-1 in human peripheral blood T cells. While others have identified COX-2 in these cells using Western immunoblot (Iniguez et al 1999; Pablos et al 1999; Bosticardo et al 2001), I did not detect COX-2 by either Western immunoblot or flow cytometry despite up-regulation of the T cell activation marker CD69 (Chapter 4). A possible resolution for these differing findings is that activated T cells may up-regulate monocyte COX-2. If this were the case, then contaminating monocytes in T cell preparations may account for any COX-2 detected by Western immunoblot.

It has been shown that T cells can activate monocytes/macrophages through direct cell-cell contact. Activated T cells induced monocyte TNF-α (Sebbag et al 1997), IL-1β (Isler et al 1993; Li et al 1995), IL-10 (Parry et al 1997), and metalloproteinase (Lacraz et al 1994) production in a cell contact dependent manner.

In addition to direct cell-cell contact, there is potential for soluble products of activated T cells to up-regulate monocyte COX-2. For example, IFN-γ is an important pro-inflammatory T cell cytokine. Furthermore IFN-γ, which is found in rheumatoid synovium, albeit only in small amounts, has been shown to enhance LPS-stimulated monocyte TNF-α production. More recently another T cell derived cytokine, IL-17, has been found in high levels in rheumatoid synovium and synovial fluid (Kotake et al 1999; Ziolkowska et al 2000; Chabaud et al 2001a; Honorati et al 2001). IL-17 is considered pro-inflammatory and has been shown to stimulate macrophage production of IL-1β, TNF-α, and PGE_2 (Jovanovic et al 1998).

Since T cells and monocytes lie in close proximity within rheumatoid joints interactions between these cell types is likely. The aim of these studies was to investigate whether activated T cells can up-regulate monocyte COX-2 and if so, to examine potential mechanisms involved. These results could provide insights into the activities of T cells in rheumatoid joints and could provide an explanation for the disparate findings on whether T cells can express COX-2.

6.2 Results

6.2.1 Effect of T cell stimulation on monocyte COX-2 expression and eicosanoid production

T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 up-regulated COX-2 in monocytes separated from direct cell contact with the T cells by transwells. The transwells allowed diffusion of soluble products through a 0.4μm pore membrane, but prevented cell contact between monocytes and T cells (see section 2.9). Treatment of T cells with CsA (100ng/ml) commencing 30 minutes prior to stimulation with immobilized anti-CD3 and soluble anti-CD28 inhibited the up-regulation of monocyte COX-2 (Figure 6.1). In control preparations with T cells or monocytes alone stimulated with immobilized anti-CD3 and soluble anti-CD28, neither monocyte nor T cell COX-2 was detected. In different control experiments, CsA had no effect on LPS-induced monocyte COX-2 expression (data not shown).

There was a 'dose-dependent' increase in monocyte COX-2 expression when the number of T cells was increased (Figure 6.2).

While monocyte COX-2 was up-regulated by co-incubation with T cells stimulated with immobilized anti-CD3 and soluble anti-CD28, no PGE_2 or TXB_2 was detected in supernatants from the transwells (data not shown). Since eicosanoid production requires release of arachidonate by cytosolic phospholipase A_2 ($cPLA_2$), we investigated whether the phosphorylated (activated) form of this enzyme was present in monocytes stimulated in transwells using Western immunoblot. While no activated $cPLA_2$ was detected, the meaning of the findings is uncertain since the antibody was found to stain variably in LPS stimulated monocytes.

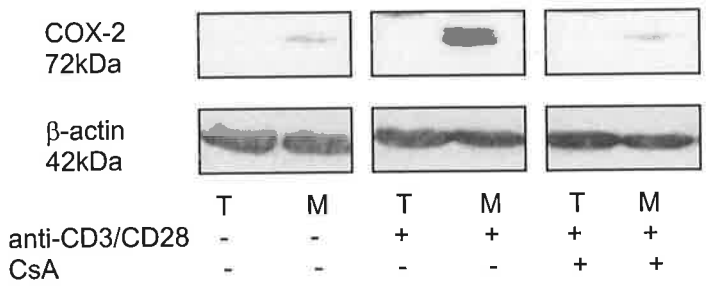


Figure 6.1: Expression of COX-2 by T cells and monocytes co-incubated in transwells. T cells ($2.5x10^6$ in 0.5ml) were placed in the bottom of a 24-well plate pre-coated with immobilized anti-CD3 to which soluble anti-CD28 was added. A transwell containing monocytes ($2.5x10^6$ in 0.5ml) was placed into each well. Where indicated CsA (100ng/ml) was added commencing 30 minutes prior to T cell stimulation. After 18 hours, T cells (T) and monocytes (M) were collected separately and processed for Western immunoblot.

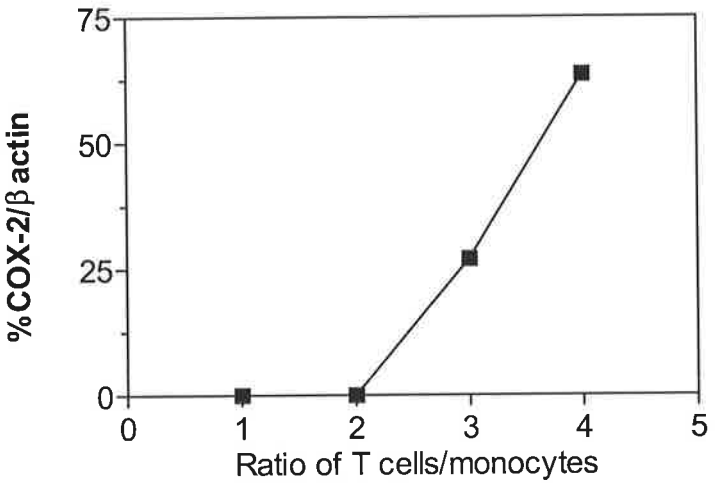


Figure 6.2: Effect of T cell number on monocyte COX-2 expression. The x-axis represents the ratio of T cells to monocytes, with the number of monocytes held constant at $2.5x10^6$. Shown is the density of the COX-2 band on Western immunoblot, expressed as a percentage of the density of the 'housekeeping' protein β-actin.

6.2.2 Effect of T cell supernatants and fixed T cells on monocyte COX-2 expression

T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 for 18 hours followed by separation of cells and supernatants by centrifugation. T cells, fixed using 2% paraformaldehyde as described in Section 2.3, or supernatants were added to freshly isolated human peripheral blood monocytes, which were then incubated for 18 hours. Supernatants from T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 up-regulated monocyte COX-2. Fixed activated T cells had no effect on monocyte COX-2 expression (Figure 6.3).

These findings suggest that soluble mediator(s) produced by activated T cells are responsible for the up-regulation of monocyte COX-2 by stimulated T cells.

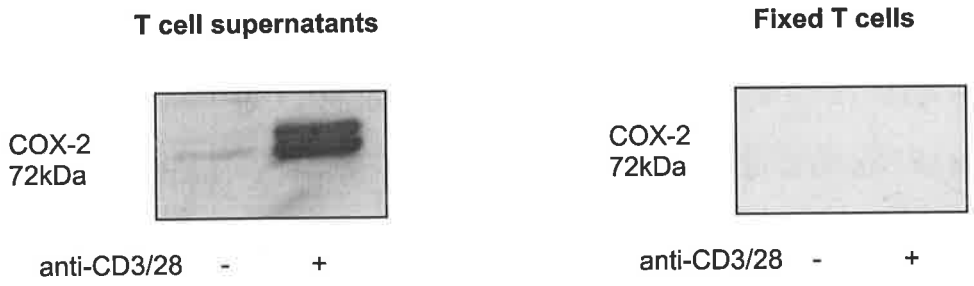


Figure 6.3: Expression of COX-2 by monocytes incubated with T cell supernatants or fixed T cells. T cells were prepared and incubated in the absence or presence of immobilized anti-CD3 and soluble anti-CD28. After 18 hours T cell supernatants or fixed T cells were added to freshly isolated monocytes. After a further 18 hour incubation, cells were collected and processed for Western immunoblot.

6.2.3 Production of potential soluble mediators by T cells

T cells produce a number of cytokines that are potential mediators in the up-regulation of monocyte COX-2. I have demonstrated that human peripheral blood T cells can produce TXA_2 (Chapter 4). In addition, T cells stimulated by immobilized anti-CD3 and soluble anti-CD28 produced IL-17, IFN-γ, and TNF-α in a time dependent manner (Figure 6.4). Production of these cytokines was inhibited by treatment of T cells with CsA commencing 30 minutes prior to stimulation (Figure 6.4). IL-1β was not detected in supernatants from T cells stimulated with immobilized anti-CD3 and soluble anti-CD28.

6.2.4 Effect of TXA_2 on monocyte COX-2 expression

It has been reported that the stable TXA_2 analogue, $cTXA_2$, which acts as a thromboxane receptor agonist, can up-regulate COX-2 in human umbilical vein endothelial cells (Caughey et al 2001a). Since T cells produce TXA_2 it is potentially a mediator in the up-regulation of monocyte COX-2. However, as shown in Chapter 4, T cells produce TXA_2 via COX-1 and levels do not change significantly with T cell stimulation. This observation diminishes the likelihood that TXA_2 is the T cell mediator of monocyte COX-2 induction. Furthermore, the addition of $cTXA_2$ (0.01μg/ml-10μg/ml) to freshly prepared human peripheral blood monocytes did not induce monocyte COX-2 expression (data not shown).

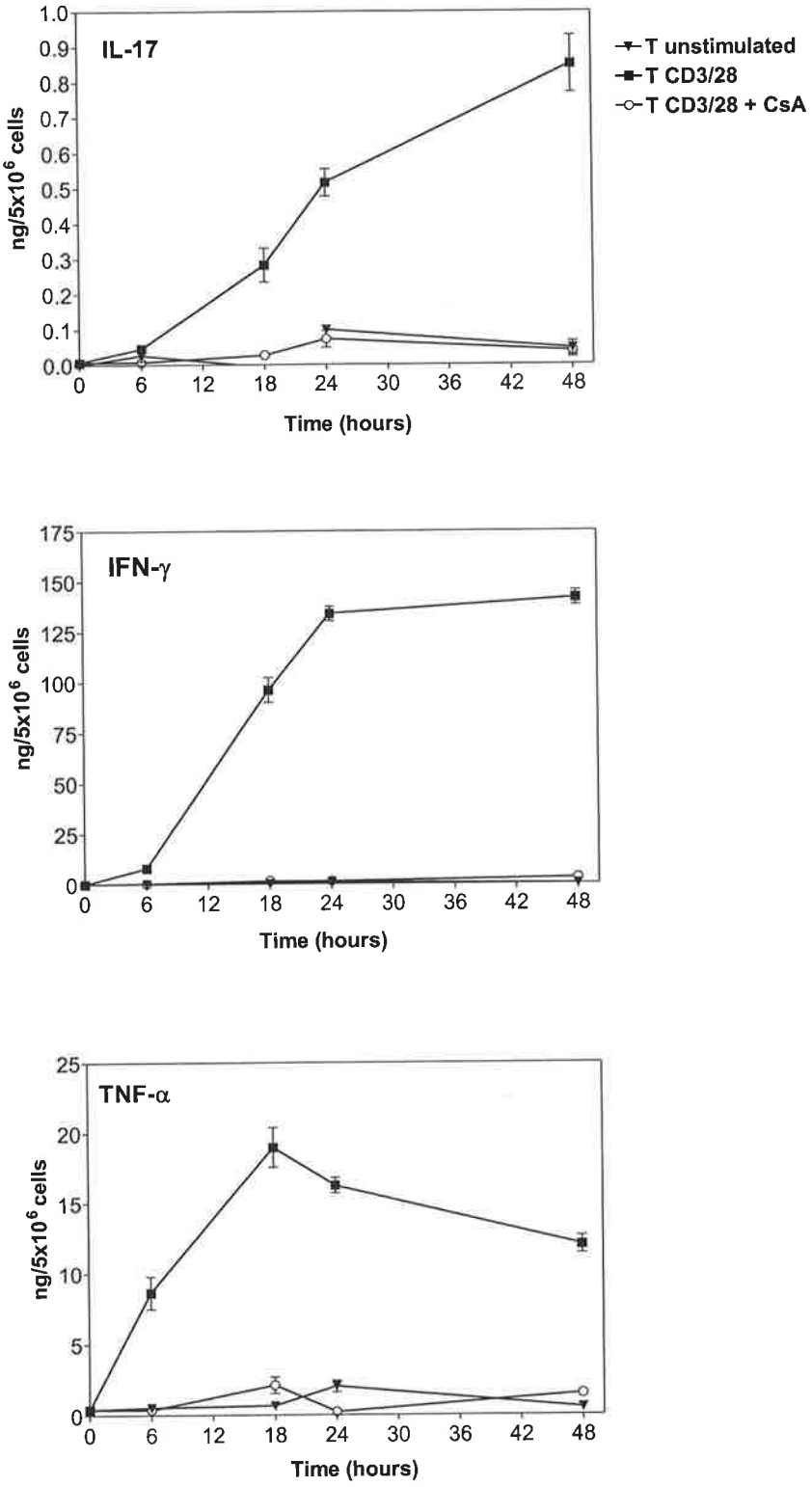
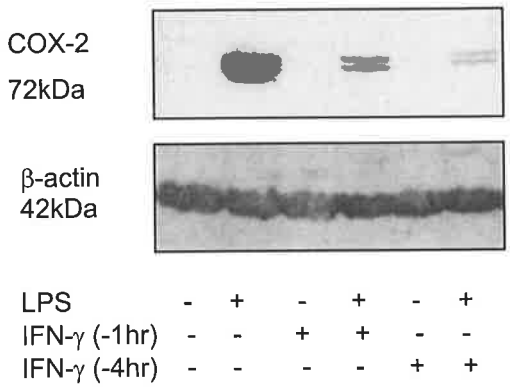


Figure 6.4: Production of IL-17, IFN-γ, and TNF-α by T cells over 48 hours. T cells were incubated with no stimulus (T unstimulated), immobilized anti-CD3 and soluble anti-CD28 (T CD3/28) or CsA (100ng/ml) commencing 30 minutes prior to transfer to wells pre-coated with immobilized anti-CD3 to which soluble anti-CD28 was added (T CD3/28+CsA). Supernatants were collected at 0, 6, 18, 24, and 48 hours and cytokines assayed by ELISA.

6.2.5 Effect of IFN-γ on monocyte COX-2 expression and eicosanoid production

IFN-γ is generally considered a pro-inflammatory cytokine and therefore a potential mediator in the up-regulation of monocyte COX-2 by stimulated T cells. Addition of recombinant (r)IFN-γ (150ng/ml) to freshly isolated human peripheral blood monocytes did not induce monocyte COX-2 expression or eicosanoid production. However, treatment of monocytes with rIFN-γ (150ng/ml) commencing 1 or 4 hours prior to the addition of LPS (200ng/ml) resulted in down-regulation of LPS-induced monocyte COX-2 expression. Furthermore there was reduced production of TXA_2 and abrogation of PGE_2 release (Figure 6.5).



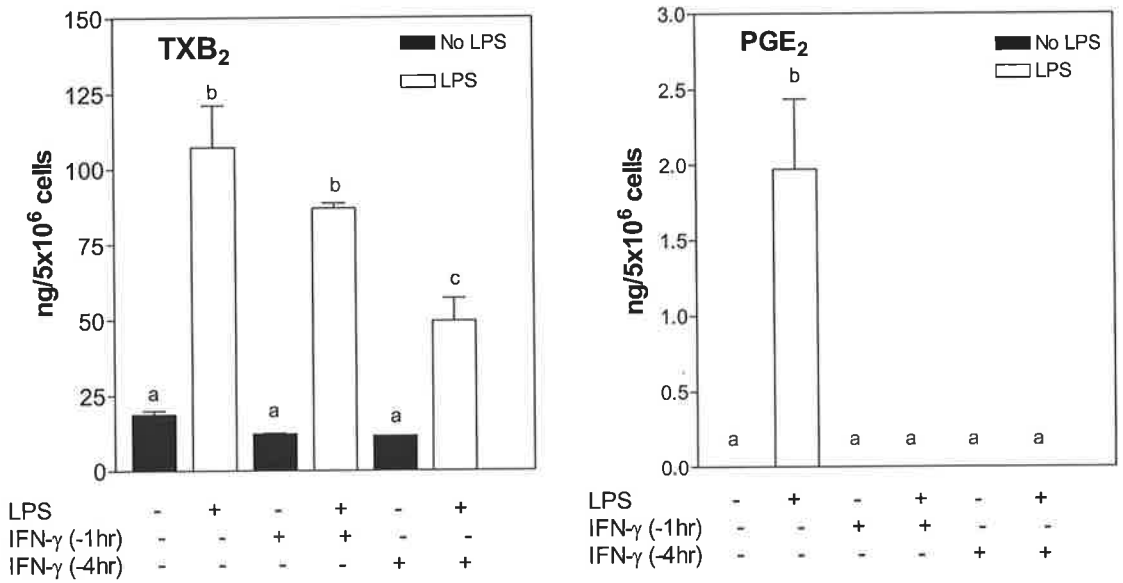


Figure 6.5: Effect of IFN-γ on monocyte COX-2 expression and eicosanoid synthesis. Monocytes were incubated with IFN-γ (150ng/ml) commencing 1 or 4 hours prior to the addition of LPS (200ng/ml). After further incubation for 18 hours cell pellets were processed for Western immunoblot, while supernatants were used to measure TXB_2 and PGE_2 release. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

To examine whether T cell derived IFN-γ had a paracrine role in regulating monocyte COX-2, co-incubation of T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 and monocytes was undertaken using the transwell system in the presence or absence of neutralizing antibody directed against IFN-γ. Addition of IFN-γ neutralizing antibody (1μg/ml) led to an increase in monocyte COX-2 expression in four separate experiments with T cells and monocytes from different donors (Figure 6.6). Incubation with IB5, an isotype matched control antibody using the transwell system or with monocytes alone did not result in any significant change in monocyte COX-2 expression (data not shown).

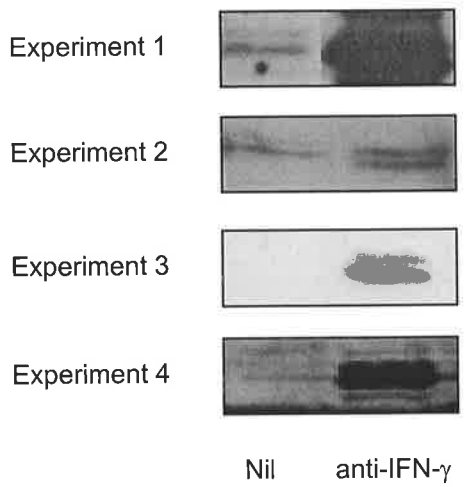
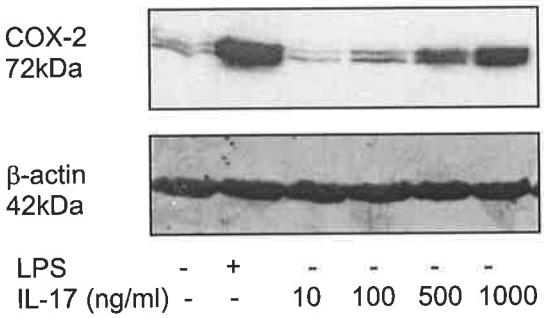


Figure 6.6: Effect of IFN-γ neutralizing antibody on up-regulation of monocyte COX-2 by T cells stimulated by immobilized anti-CD3 and soluble anti-CD28 in four replicate experiments. T cells and monocytes were co-incubated in 24-well plates, with separation by transwells in the absence (nil) or presence (anti-IFN-γ) of IFN-γ neutralizing antibody (1μg/ml). After incubation for 18 hours monocytes were collected and processed for Western immunoblot. Data from four separate experiments using four different donors is shown.

6.2.6 Effect of IL-17 on monocyte COX-2 expression and eicosanoid production

To investigate the role of IL-17 in monocyte COX-2 up-regulation, rIL-17 was added to freshly prepared human peripheral blood monocytes and incubated for 18 hours. Recombinant IL-17 up-regulated, in a dose dependent manner, monocyte COX-2 expression and TXA_2 and PGE_2 production (Figure 6.7). While IL-17 was capable of up-regulating monocyte COX-2 expression, the doses required were far in excess of those produced by activated T cells.



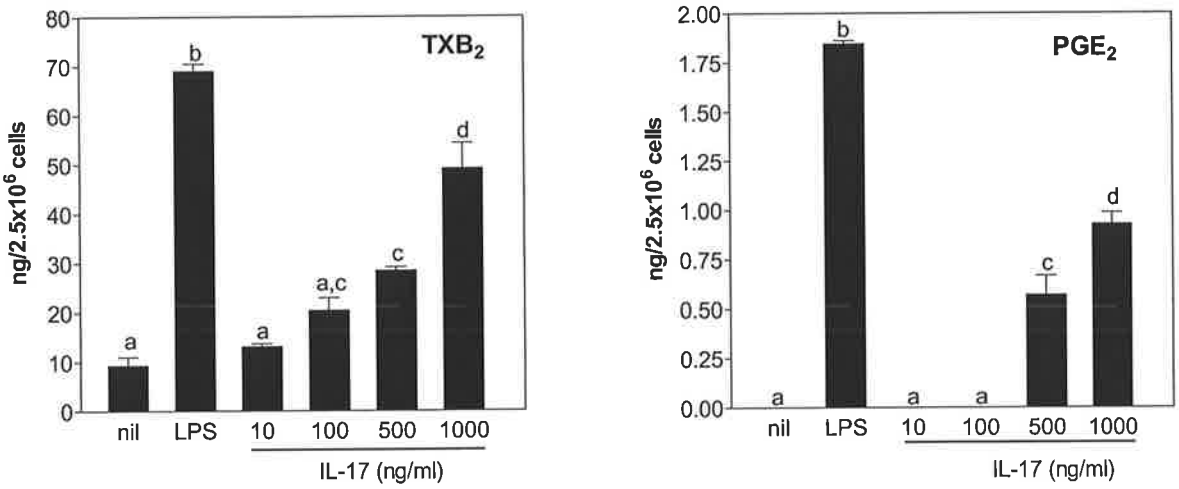


Figure 6.7: Effect of IL-17 on monocyte COX-2 expression and eicosanoid synthesis. Monocytes were incubated with LPS (200ng/ml) or rIL-17 (0-1000ng/ml) for 18 hours. Cell pellets were then processed for Western immunoblot while supernatants were used to measure TXB_2 and PGE_2 release. The data shown is representative of four separate experiments. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

To examine whether T cell derived IL-17 had a paracrine role in up-regulation of monocyte COX-2, T cells, stimulated with immobilized anti-CD3 and soluble anti-CD28, and monocytes were co-incubated using transwells in the presence or absence of IL-17 neutralizing antibody. Addition of IL-17 neutralizing antibody (1μg/ml) resulted in partial inhibition of anti-CD3/anti-CD28 T cell stimulated monocyte COX-2 expression (Figure 6.8). ID4.5, an IgG2a isotype control antibody, resulted in no significant change (data not shown).

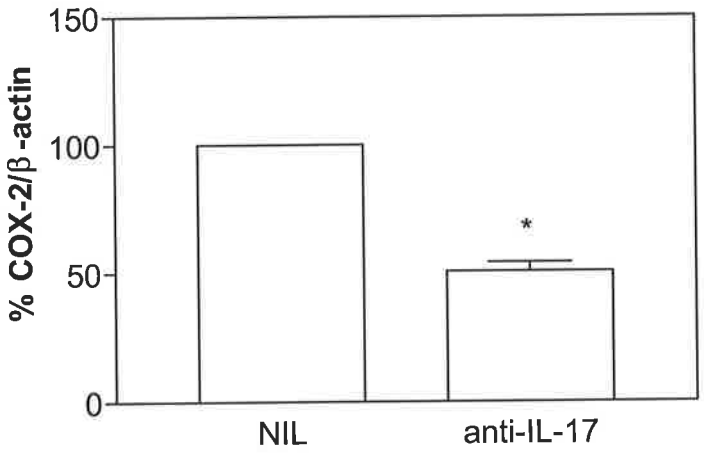
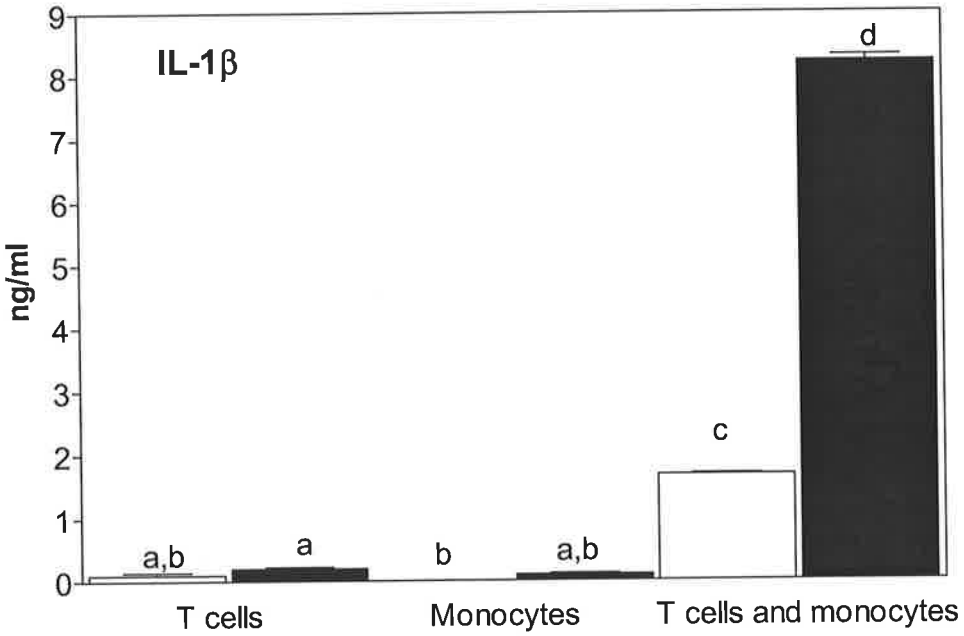


Figure 6.8: Effect of IL-17 neutralizing antibody on up-regulation of monocyte COX-2 by T cells stimulated by immobilized anti-CD3 and soluble anti-CD28. T cells and monocytes were co-cultured using transwells in the absence (Nil) or presence (anti-IL-17) of IL-17 neutralizing antibody (1μg/ml). Cells were incubated for 18 hours. The cells were then pelleted and processed for Western immunoblot. Shown is the density of COX-2/β-actin expressed as a percentage of the control. (*$p<0.05$). Two experiments were undertaken using cells from different donors and the data pooled.

6.2.7 Effect of T cell stimulation on monocyte cytokine production

While monocyte COX-2 expression was the primary focus of these studies, cytokine release was used as another measure of monocyte activation. Since the transwell system allows diffusion of soluble mediators (such as TNF-α and IL-1β), it is not possible to distinguish between soluble products released from T cells and monocytes. In an attempt to exclude T cells as a source of detected cytokines, T cells and monocytes were incubated separately, as well as in the transwell system, in the presence or absence of immobilized anti-CD3 and soluble anti-CD28. Little IL-1β was detected in supernatants from either T cells or monocytes incubated separately even in the presence of immobilized anti-CD3 and soluble anti-CD28 (Figure 6.9). However, co-incubation of monocytes with anti-CD3/anti-CD28 stimulated T cells using the transwell system resulted in an increase in IL-1β (Figure 6.9). This effect was inhibited by treatment of T cells with CsA (100ng/ml) commencing 30 minutes prior to stimulation with immobilized anti-CD3 and soluble anti-CD28 (Figure 6.9).

(a)



(b)

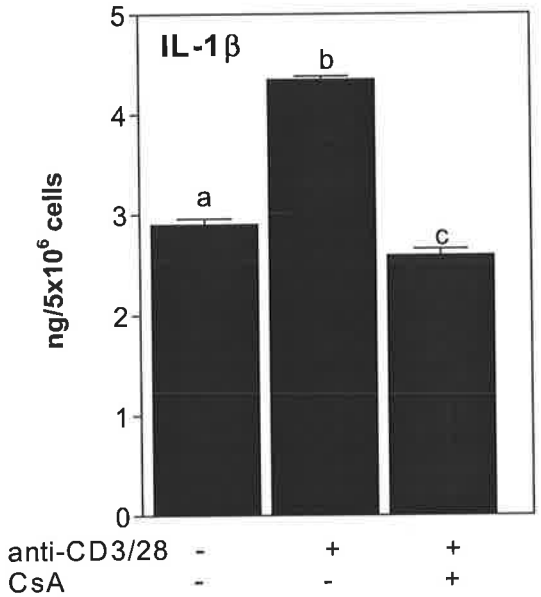
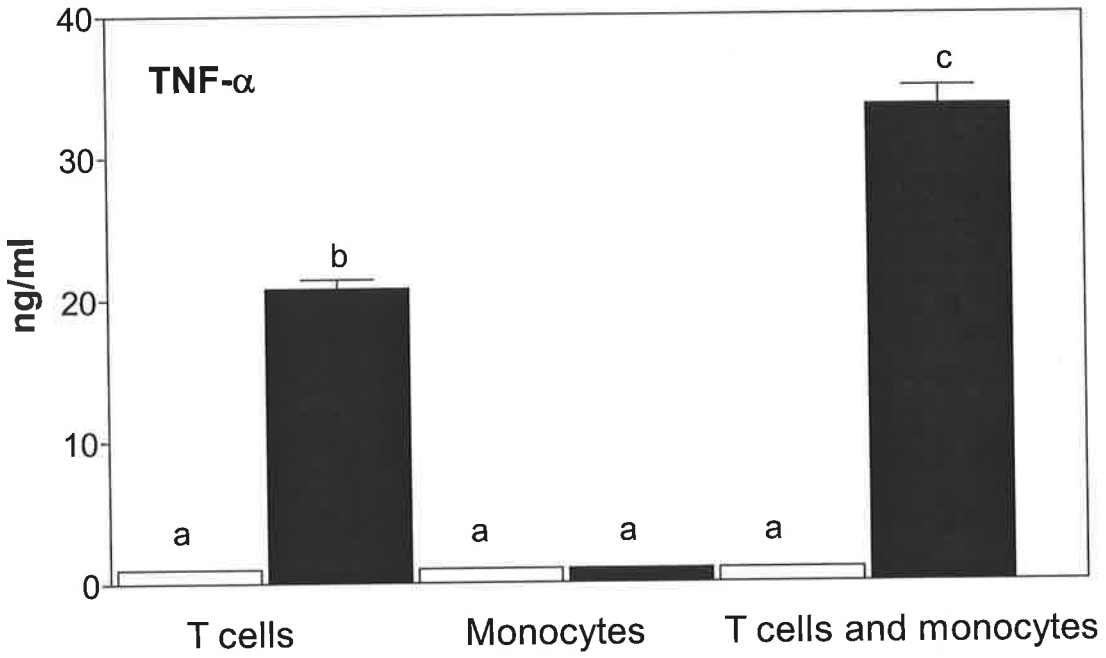


Figure 6.9: Production of IL-1β by monocytes and T cells alone or co-cultured using the transwell system. (a) T cells and monocytes were incubated separately or together using the transwell system, in the absence (hollow bars) or presence (solid bars) of immobilized anti-CD3 and soluble anti-CD28. After 18 hours supernatants were collected. (b) T cells ($2.5x10^6$ in 0.5ml) were placed in the bottom of a 24-well plate pre-coated with anti-CD3 to which soluble anti-CD28 was added. Transwells containing monocytes ($2.5x10^6$ in 0.5ml) were placed into the each well. Where indicated, CsA (100ng/ml) was added 30 minutes prior to T cell stimulation. After 18 hours supernatants were collected and assayed for IL-1β. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

In contrast to IL-1β, TNF-α is produced in measurable amounts by stimulated T cells. In an attempt to distinguish the cellular source of TNF-α, T cells and monocytes were incubated separately, as well as in the transwell system in the presence or absence of immobilized anti-CD3 and soluble anti-CD28. T cells, but not monocytes, incubated separately with immobilized anti-CD3 and soluble anti-CD28 produced TNF-α. Co-incubation of T cells and monocytes in the presence of immobilized anti-CD3 and soluble anti-CD28 resulted in enhanced TNF-α production over and above that seen by the equivalent number of T cells alone (Figure 6.10). In addition, T cells and monocytes were incubated using the transwell system in the presence of LPS (200ng/ml). Production of TNF-α was ~2.5 times greater when T cells and monocytes were co-cultured using immobilized anti-CD3 and soluble anti-CD28 stimulation than with LPS. Furthermore, the effect of immobilized anti-CD3 and soluble anti-CD28 was inhibited by treatment of T cells with CsA commencing 30 minutes prior to stimulation (Figure 6.10). In contrast, treatment of monocytes with CsA commencing 30 minutes prior to stimulation with LPS did not affect monocyte production of TNF-α (data not shown). The enhanced production of TNF-α by monocytes incubated with anti-CD3/anti-CD28 treated T cells, suggests T cells contribute to at least a portion of the TNF-α detected but in addition, a T cell derived product enhances monocyte TNF-α production.

(a)



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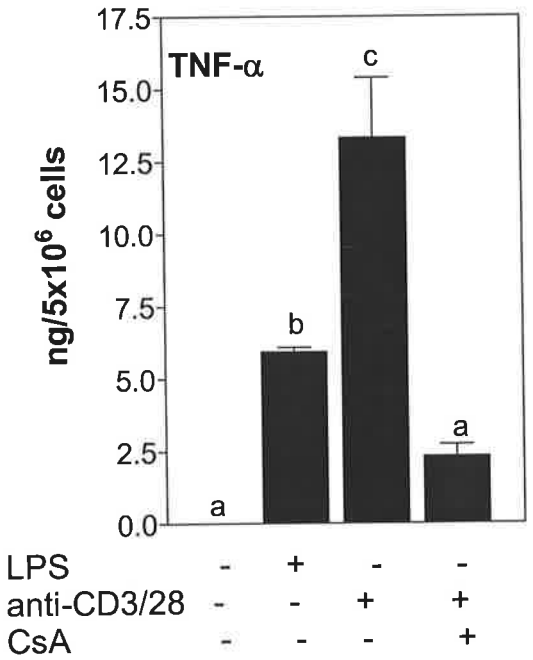


Figure 6.10: Production of TNF-α by T cells and monocytes alone or co-cultured using transwells. (a) T cells and monocytes were incubated separately or together using the transwell system, in the absence (hollow bars) or presence (solid bars) of immobilized anti-CD3 and soluble anti-CD28. After 18 hours supernatants were collected. (b) T cells ($2.5x10^6$ in 0.5ml) were placed in the bottom of a 24-well plate and transwells containing monocytes ($2.5x10^6$ in 0.5ml) were placed into the each well. Cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 or LPS (200ng/ml). Where indicated CsA (100ng/ml) was added 30 minutes prior to T cell stimulation. After 18 hours supernatants were collected and assayed for TNF-α. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

6.2.8 Effect of IFN-γ on monocyte TNF-α production: role of eicosanoids

Addition of rIFN-γ (150ng/ml) to freshly isolated human monocytes resulted in inconsistent production of small amounts of TNF-α. However, treatment of monocytes with rIFN-γ (150ng/ml) commencing 1 or 4 hours prior to stimulation with LPS resulted in marked enhancement of monocyte TNF-α. By contrast, IL-1β production by monocytes was inhibited by IFN-γ treatment (Figure 6.11).

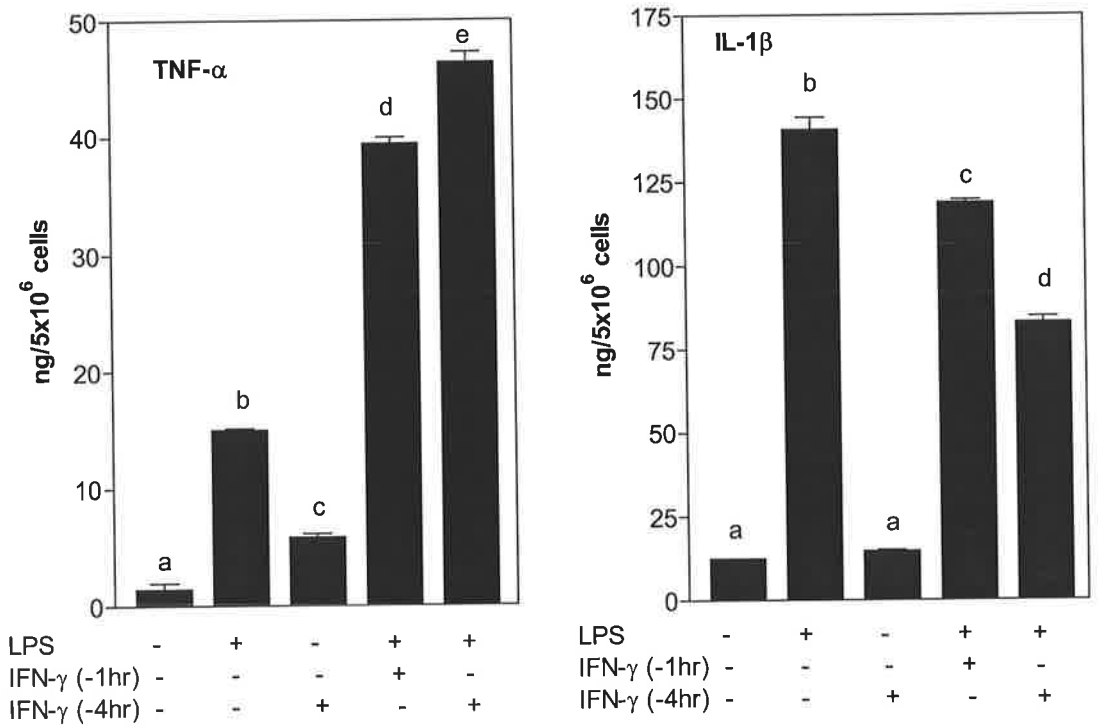


Figure 6.11: Effect of IFN-γ treatment on production of TNF-α and IL-1β by monocytes. Monocytes ($5x10^6$ in 2.5mls) were treated with IFN-γ (150ng/ml) commencing 1 or 4 hours prior to stimulation with LPS (200ng/ml). Alternatively monocytes were treated with IFN-γ or LPS alone. 18 hours after the addition of LPS supernatants were collected and assayed for TNF-α or IL-1β. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

PGE_2 and TXB_2 have been reported to have a role in determining monocyte TNF-α production, with TXA_2 promoting TNF-α synthesis and PGE_2 having an inhibitory effect (Caughey et al 1997). Treatment of monocytes with IFN-γ prior to LPS stimulation resulted in greater inhibition of PGE_2 than TXA_2 (Figure 6.5). Therefore, I hypothesized that reduction in the ratio of PGE_2 and TXB_2 may contribute to the enhancement of TNF-α production by monocytes treated with IFN-γ prior to LPS-stimulation. Monocytes were incubated with the thromboxane receptor antagonists, SQ29548 or $pTXA_2$, commencing 30 minutes prior to addition of IFN-γ (150ng/ml). Four hours after the addition of IFN-γ, LPS (200ng/ml) was added. Presence of the thromboxane receptor antagonists resulted in a small reduction in monocyte TNF-α production (Figure 6.12). Control incubations with SQ29548 or $pTXA_2$ alone or in the presence of LPS did not result in any significant change in TNF-α, TXB_2, or PGE_2 (data not shown).

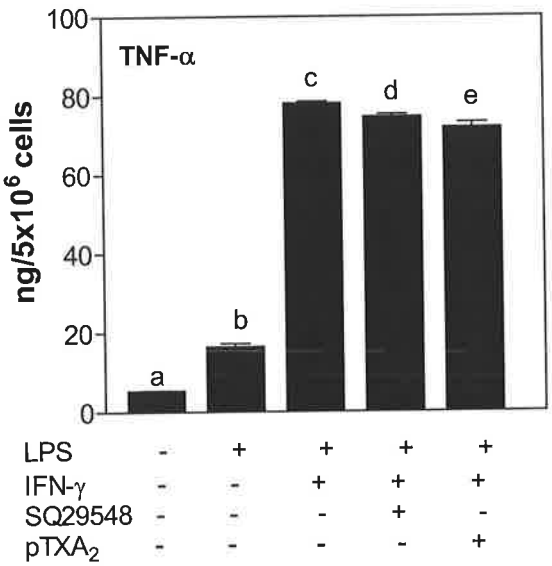


Figure 6.12: Effect of thromboxane receptor blockade on production of TNF-α by monocytes treated with IFN-γ. Where indicated monocytes ($5x10^6$ in 2.5mls) were treated with SQ29548 (10μM) or $pTXA_2$ (10μM) for 30 minutes. IFN-γ (150ng/ml) was then added and after a further 4 hours LPS (200ng/ml) was added. Supernatants were collected 18 hours after the addition of LPS. Bars with different letters are significantly different from each other; $p<0.05$ ANOVA followed by Neuman-Keuls test for multiple comparisons.

6.2.9 Effect of IL-17 on monocyte cytokine production

The addition of rIL-17 to freshly prepared human peripheral blood monocytes resulted in up-regulation of monocyte TNF-α and IL-1β production (Figure 6.13). The up-regulation of monocyte cytokine production was only seen at doses higher than those observed in T cell supernatants (Figure 6.4). Whether T cell derived IL-17, in combination with TNF-α contributed to the up-regulation of monocyte IL-1β and TNF-α production in the transwell system is worthy of examination.

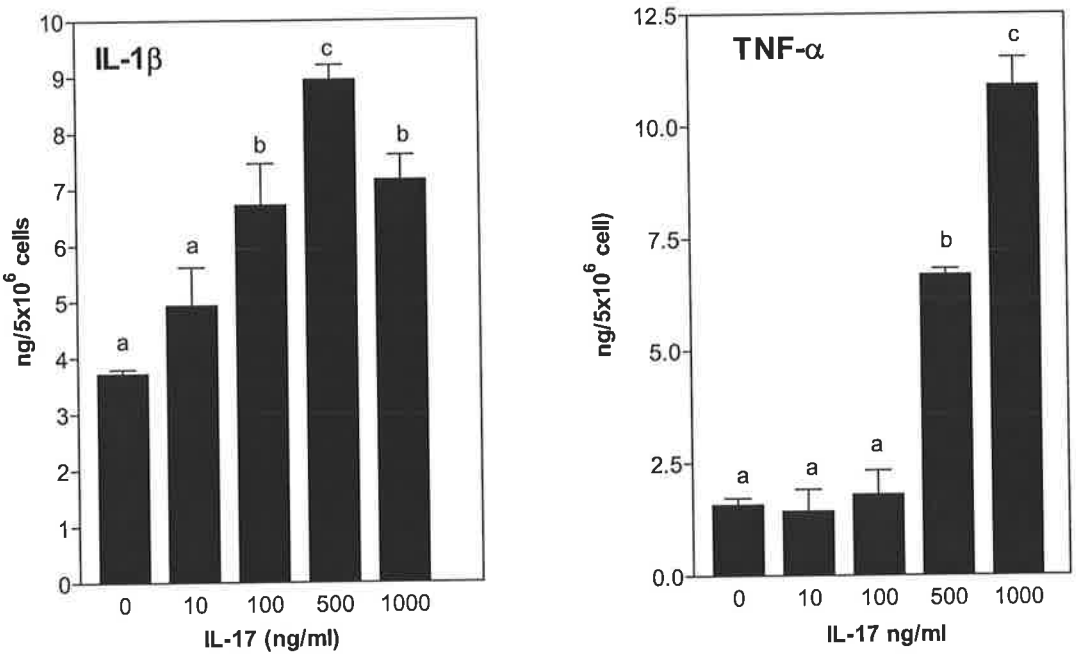


Figure 6.13: Effect of IL-17 on monocyte TNF-α and IL-1β production. Freshly prepared monocytes ($5x10^6$ in 2.5mls) were incubated with IL-17 (0-1000ng/ml). After 18 hours supernatants were collected and TNF-α and IL-1β measured by ELISA. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

6.3 Discussion

As discussed in the preceding chapters, the history of studies on T cells and prostaglandin production has been equivocal with regard to whether T cells express COX and whether they can synthesize eicosanoids. Since the discovery of COX-2 there have been reports that COX-2 can be detected using Western immunoblot in human peripheral blood T cells and the Jurkat T cell line upon stimulation with antibodies directed against CD3 and/or CD28 (Iniguez et al 1999; Pablos et al 1999; Bosticardo et al 2001). While I have detected COX-1 in human peripheral blood T cells, I have not detected COX-2 in T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 using either Western immunoblot or flow cytometry (Chapter 4). The results presented in this Chapter indicate that T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 can up-regulate monocyte COX-2 and that this can be inhibited by treatment of the T cells with CsA prior to stimulation. Furthermore, this paracrine effect of T cells on monocytes is due to soluble mediators because it occurs in the absence of direct cell-cell contact. Therefore, it is worth considering whether the reports of T cell COX-2 are artifacts resulting from detection of monocyte COX-2 up-regulated by T cell specific stimuli.

Bosticado et al report the presence of COX-2 in human peripheral blood T cells isolated by density gradient centrifugation, a technique that is likely to result in substantial monocyte contamination, and they make no comment on T cell purity (Bosticardo et al 2001). While Pablos et al detected COX-2 in OKT3 (i.e. anti-CD3) stimulated T cells using Western immunoblot, COX-2 was not be detected using immunocytochemistry of peripheral blood mononuclear cells or rheumatoid synovial T cells (Pablos et al 1999). The authors suggest this may be due to antibody sensitivity

and the particular activation status of rheumatoid synovial T cells. Iniguez et al argued that the COX-2 detected in their T cell preparations could not be due to contaminating monocytes, because they used a T cell specific stimulus, namely anti-CD3/anti-CD28, and the effect was inhibited by CsA. However, my results indicate that the COX-2 could be due to monocytes, even when T cell specific stimuli are used. Furthermore, Iniguez et al argue that were the detected COX-2 arising from contaminating monocytes, PGE_2 production would also be up-regulated resulting in inhibition of cell proliferation, IFN-γ and IL-2 production, and CD25 expression. Thus inhibition of COX-2 derived PGE_2 with NS398, a COX-2 specific inhibitor, would have produced opposite effects to the observed reduction in cell proliferation, and IFN-γ and IL-2 production (Iniguez et al 1999). However, I have demonstrated that despite up-regulation of monocyte COX-2, no PGE_2 could be detected. The most likely explanation is that the stimulus does not result in release of AA from the cell membrane. Unfortunately the antibody available for $cPLA_2$, lacked sensitivity and therefore I am unable to comment on whether this enzyme was activated. This question warrants further investigation.

The present results provide an alternative explanation for these disparate findings in that COX-2 detected on Western immunoblot could arise from contaminating monocytes in which COX-2 has been up-regulated by T cells stimulated with immobilized anti-CD3 and soluble anti-CD28. Despite T cell purification there are usually some monocytes present within T cell isolates. Monocytes express large amounts of COX-2 when exposed to inflammatory stimuli and a relatively small number of contaminating monocytes could create a COX-2 signal on Western immunoblot of T cell preparations. But when more specifically localizing techniques,

such as immunocytochemistry or flow cytometry were used, no COX-2 was detected in T cells. Overall, I conclude that T cell COX-2 expression is not present and that reports of T cell COX-2 have an alternate explanation.

It has been suggested in animal models of arthritis that chronic high intensity COX expression in the inflamed synovium may be T cell dependent (Sano et al 1992). The present results support a role for T cells in up-regulation of monocyte COX-2 through the production of soluble mediators. T cells produce a number of cytokines that are candidates for the paracrine up-regulation of monocyte COX-2 observed in this study. I have demonstrated that T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 produce IFN-γ, TNF-α, and IL-17. Furthermore CsA can inhibit their production. While IFN-γ is often regarded as a pro-inflammatory cytokine, it has also been reported to have anti-inflammatory properties (see section 1.5.3.2) and its potential influence on monocyte COX-2 expression is therefore of special interest.

It has been reported that IFN-γ alone or in synergy with LPS, up-regulates COX-2 in mouse peritoneal macrophages (Blanco et al 2000). IFN-γ alone has been reported to up-regulate COX-2 expression and PGE_2 production in human epidermal keratinocytes (Matsuura et al 1999). In contrast, there are reports that IFN-γ alone has no effect on COX-2 mRNA in U937 monocytic cells (Arias-Negrete et al 1995) or on COX-2 expression or PGE_2 production in LPS-stimulated human monocytes (Endo et al 1996).

Differing results may depend on the cell type used and experimental conditions with regard to IFN-γ addition. Treatment of cells with IFN-γ, commencing 24 hours prior to

stimulation with LPS, has been shown to result in marked accumulation of COX-2 mRNA in U937 cells (Arias-Negrete et al 1995). In comparison, it has been reported that treatment with IFN-γ prior to stimulation reduces murine peritoneal macrophage PGE_2 production (Boraschi et al 1984). In human monocytes, IFN-γ treatment prior to stimulation with LPS or IL-β reduced PGE_2 and TXB_2 production. The authors suggested the inhibition of eicosanoid production by IFN-γ must be at or before the level of AA release (Browning and Ribolini 1987). Subsequently it has been reported that IFN-γ inhibits phospholipase activity in ConA simulated human monocytes (Wahl et al 1990). However, in U937 macrophages treatment with IFN-γ prior to stimulation with IL-1β, but not LPS, has also been reported to inhibit COX-2 expression and activity (Barrios-Rodiles and Chadee 1998).

My results indicate that IFN-γ is not responsible for up-regulation of human peripheral blood monocyte COX-2 by humoral factors released from T cells stimulated with immobilized anti-CD3 and soluble anti-CD28. In fact, inhibition of T cell derived IFN-γ with a specific IFN-γ neutralizing antibody enhanced the expression of COX-2 in monocytes co-incubated with T cells stimulated with immobilized anti-CD3 and soluble anti-CD28. This augmentation of monocyte COX-2 in the presence of IFN-γ neutralizing antibody was modest. A more obvious suppressive effect of IFN-γ on monocyte COX-2 expression was revealed with incubation of monocytes with IFN-γ before the primary stimulus. These results suggest that IFN-γ is a paracrine negative regulator of monocyte COX-2 expression in T cell-monocyte interactions.

IL-17 is another T cell derived cytokine with effects that can be considered pro-inflammatory. It has been shown to induce PGE_2 production in epithelial cells, endothelial cells, and primary cultured synovial fibroblasts (Fossiez et al 1996). In human macrophages IL-17 induces the release of IL-1β, TNF-α (Jovanovic et al 1998), and matrix metalloproteinases (Shalom-Barak et al 1998). In human chondrocytes IL-17 induces nitric oxide production along with an increase in transcription and de novo translation of the genes encoding COX-2 and IL-1β (Shalom-Barak et al 1998). In adherent human macrophages IL-17 has been reported to increase COX-2 expression and PGE_2 production (Jovanovic et al 2000). My results are in agreement with these findings in that IL-17 resulted in a dose dependent increase in human peripheral blood monocyte COX-2 expression and eicosanoid production. It has previously been suggested that IL-17 has a limited role in T cell driven inflammatory processes (Fossiez et al 1996). My results show that at high concentration IL-17 can induce monocyte COX-2 expression and eicosanoid production. At lower concentrations (≤10μg/ml), which are still above that produced by activated T cells, there is minimal effect on monocyte COX-2 expression. However, IL-17 neutralizing antibody at least partially reduced the up-regulation of monocyte COX-2 in interactions between stimulated T cells and monocytes. These results suggest that T cell derived IL-17 is a paracrine up-regulator of monocyte COX-2 and that it is acting in combination with other soluble mediator(s) produced by activated T cells.

There are a number of other potential mediators released by stimulated T cells that may be involved in the induction of monocyte COX-2 that I have not examined. TNF-α has been reported to both up-regulate monocyte COX-2 expression (Jones et al

1993; Pouliot et al 1997) and to have no effect (Nusing and Ullrich 1992). Whether T cell derived TNF-α is a paracrine regulator of monocyte COX-2 in the transwell system was not investigated. In addition, it may be that IL-17 and TNF-α have a synergistic effect on monocyte COX-2 expression. This remains an area of interest and warrants further investigation. However, although T cells produce TNF-α, the relatively large amounts present in the rheumatoid joint are primarily monocyte/macrophage derived (Chu et al 1991). Thus, paracrine effects of T cell derived TNF-α on monocyte COX-2 expression and activity observed *in vitro* using the transwell system are likely to be negligible in comparison to autocrine and paracrine effects of monocyte derived TNF-α that may occur *in vivo*.

GM-CSF, which can be produced by T cells, has a number of immunomodulatory effects. In human monocytes GM-CSF alone has been reported to have no effect on PGE_2 synthesis (Hart et al 1988). With the recent development of GM-CSF receptor antagonists and a specific GM-CSF neutralizing antibody, further investigation of the role of GM-CSF in T cell-monocyte interactions could be undertaken.

Direct cell-cell contact is an important means of intercellular communication and direct contact between T cells and monocytes has been shown to induce monocyte cytokine production (Isler et al 1993; Li et al 1995; Vey et al 1996; McInnes et al 1997; Parry et al 1997; Sebbag et al 1997; Brennan et al 2002) and MMP expression (Lacraz et al 1994). Furthermore, addition of T cell derived cytokines such as IFN-γ or GM-CSF has been reported to enhance contact dependent T cell induction of monocyte TNF-α (Sebbag et al 1997).

Fixed cell preparations can be used to explore the role of cell surface adhesion molecules in intercellular signaling. While the fixation process is not compatible with cell viability, the adhesion molecules and cell associated cytokines fixed on the surface may remain capable of engaging their cognate receptors on live cells added after removing the fixing agent. The system allows detection of changes in function and synthetic activity of the unfixed target cells, which can be attributed to signaling through surface adhesive interactions. As a model for heterotypic T cell-monocyte adhesive interactions, this model has serious limitations, since the surface adhesive interactions are fortified by and provide a focus for transfer of cytokines and the soluble signaling agents, whose release may be directed specifically to sites of intercellular adhesion (Springer 1994). The failure of fixed T cells to up-regulate monocyte COX-2 in the present studies needs to be interpreted in this context. T cells fixed after stimulation with immobilized anti-CD3 and soluble anti-CD28 have been reported to induced monocyte IL-10 release through cell-cell contact (Parry et al 1997), whereas T cells fixed following stimulation with cytokines (IL-15 or IL-2 alone, or combinations of IL-15 or IL-2 with IL-6 and TNF-α) failed to induce monocyte IL-10 release (Sebbag et al 1997). These authors also found duration of T cell stimulation prior to fixation and addition of soluble cytokines altered monocyte responses to fixed cells. Thus differing conditions of T cell stimulation may result in differences in the array or configuration of surface molecules through which T cells may communicate with monocytes via adhesive interactions. In my studies fixed, activated T cells failed to induce monocytes to express COX-2 or TNF-α (data not shown). This did not appear to be due to a technical failure in adequate fixation since activated T cells fixed by the same method are capable of altering synoviocytes

(Chapter 7). It is possible that fixation of T cells may have altered function of membrane ligands important in the induction of monocyte COX-2.

In addition to the up-regulation of monocyte COX-2 expression by soluble mediators produced by T cells stimulated with immobilized anti-CD3 and soluble anti-CD28, production of both IL-1β and TNF-α was increased. While the addition of IFN-γ alone resulted in inconsistent production of small amounts of TNF-α by fresh human peripheral blood monocytes, treatment with IFN-γ prior to LPS stimulation enhanced monocyte TNF-α production. This effect has previously been reported (Hayes et al 1995) and a variety of mechanisms are thought to be involved. IFN-γ has been shown to reduce IL-10 mRNA and protein expression in LPS-stimulated monocytes, an effect that was associated with an increase in TNF-α production. Furthermore, IL-10 inhibited TNF-α production by LPS-stimulated monocytes, while IL-10 neutralizing antibody increased TNF-α production (Donnelly et al 1995). Treatment with IFN-γ has been reported to increase CD14 (LPS receptor) expression by human peripheral blood neutrophils (Takeshita et al 1998) and U937 cells (Ishizuka et al 1995), which may contribute to increased TNF-α production. Finally treatment of monocytes with IFN-γ prior to LPS stimulation has been reported to increase TNF-α transcription and TNF-α mRNA stability (Hayes et al 1995).

The possible role of eicosanoids in modulating monocyte TNF-α production in the presence of IFN-γ has not been well studied. Hart et al reported that IFN-γ alone had no effect on monocyte TNF-α, IL-β, or PGE_2 production, but enhanced LPS-stimulated monocyte TNF-α and IL-1β production. With the addition of LPS and IFN-

γ simultaneously, PGE_2 production was reduced compared to LPS alone. However, there was no correlation between the reduction in PGE_2 and the increase in TNF-α (Hart et al 1989). The authors concluded that a non-COX product was involved in the increase in TNF-α. However, these investigations did not examine the effects of IFN-γ priming nor did they take into account the role of TXA_2, which has been shown subsequently to induce monocyte TNF-α production (Caughey et al 1997).

The present results suggest that treatment of monocytes with IFN-γ prior to stimulation with LPS, results in reduction of both PGE_2 and TXA_2, with an alteration in the balance between these two eicosanoids in favour of TXA_2. However, this effect appeared to have only a minor role in the enhancement of TNF-α production by monocytes treated with IFN-γ prior to LPS stimulation (Figure 6.12).

IL-17 has been reported to have no direct effect on cytokine secretion by human peripheral blood monocytes, suggesting a limited role of IL-17 in T cell driven inflammatory processes (Fossiez et al 1996). In contrast, I found that monocyte TNF-α and IL-1β synthesis were increased in the presence of IL-17. The discrepancy between these findings may be related to concentration, in that only higher concentrations of IL-17 appeared to induce TNF-α and IL-1β production. Given this, the relevance of IL-17 induced cytokine secretion by monocytes to conditions *in vivo* is uncertain. However, lower doses of IL-17 in combination with other cytokines present within the inflamed rheumatoid joint, such as IL-1β or TNF-α may conceivably enhance monocyte cytokine secretion. This question warrants further investigation.

In summary, soluble mediators produced by activated T cells can influence the expression of monocyte COX-2 and cytokine production. The findings potentially reconcile differing reports regarding expression of COX-2 in T cells. The results suggest that the high levels of IL-17 and relatively low levels of IFN-γ, which have been reported in rheumatoid joints may contribute to T cell driven up-regulation of monocyte COX-2. The effects of IL-17 on monocyte COX-2 expression, in addition its implication in joint damage in animal models of arthritis (Lubberts et al 2001; Bush et al 2002a), add to the validation of IL-17 as a therapeutic target in rheumatoid arthritis and other inflammatory diseases.

Chapter 7

Up-regulation of synoviocyte COX-2 expression and eicosanoid production through interactions with T lymphocytes

"When you are a Bear of Very Little Brain, and you Think of Things, you find sometimes that a Thing which seemed very Thingish inside you is quite different when it gets out into the open and has other people looking at it."

A.A.Milne The House at Pooh Corner

7.1 Introduction

In the preceding chapter I observed that T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 could induce monocyte COX-2 expression and cytokine production. Furthermore IL-17 appeared to be an important positive regulator, while IFN-γ was a negative regulator of the effect on monocyte COX-2 expression.

Within the inflamed joint, T cells lie within close proximity to not only monocytes, but also synoviocytes. Therefore there is potential for interaction between T cells and synoviocytes. Direct cell-cell contact between T cells and synoviocytes has been reported to increase synoviocyte PGE_2 production (Burger et al 1998). Furthermore, IL-17 has been shown to enhance the effects of IL-1β on synovial IL-6 production (Chabaud et al 1998) and TNF-α induced IL-1, IL-6, and IL-8 production (Katz et al 2001). In contrast to the pro-inflammatory effects of IL-17, IFN-γ has been reported to

inhibit TNF-α but not IL-1β induced rheumatoid synoviocyte proliferation and collagenase production (Alvaro-Garcia et al 1990).

These observations suggest that T cells may induce synoviocyte COX-2 expression and that IL-17 and IFN-γ may be important positive and negative regulators of this effect respectively. The aim of these studies was to investigate whether T cells are capable of up-regulating synoviocyte COX-2 expression and eicosanoid production and if so by what mechanisms.

7.2 *Results*

7.2.1 Expression of COX-1 and COX-2 in synoviocytes

While our laboratory has worked extensively with monocytes, there had been little previous work with synoviocytes. Therefore I first needed to characterize COX-1 and COX-2 expression and eicosanoid synthesis by cultured human fibroblast-like synoviocytes.

COX-1 was readily detectable in both unstimulated and stimulated synoviocytes by Western immunoblot. Expression of COX-2 could be induced by stimulation of synoviocytes with IL-1β (2ng/ml) (Figure 7.1). The expression of COX-1 and COX-2 was not affected by growth of synoviocytes in RPMI containing amphotericin (data not shown).

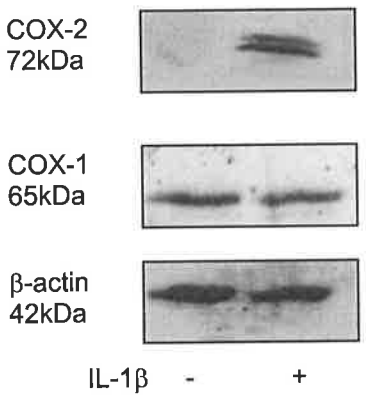


Figure 7.1: Expression of COX-1 and COX-2 in synoviocytes by Western immunoblot. Synoviocytes ($5x10^5$ in 2mls) were incubated for 18 hours in the absence or presence of IL-1β (2ng/ml). Cells were collected and processed for Western immunoblot.

7.2.2 Characterization of eicosanoid production by synoviocytes

The induction of COX-2 by IL-1β was associated with a significant increase in PGE_2 and 6-keto-$PGF_{1\alpha}$ production (Figure 7.2). TXB_2 was not detected (data not shown). To assess the ability of synoviocytes to produce PGE_2 and 6-keto-$PGF_{1\alpha}$ without requirement for endogenous AA, synoviocytes were incubated with or without IL-1β for 18 hours. Cells were then washed with FCS free RPMI and incubated for 30 minutes with or without AA (10μM). Addition of exogenous AA resulted in increased PGE_2 and 6-keto-$PGF_{1\alpha}$ production and the amounts were enhanced by previous treatment of cells with IL-1β (Figure 7.2), correlating with the increase in COX-2 expression (Figure 7.1).

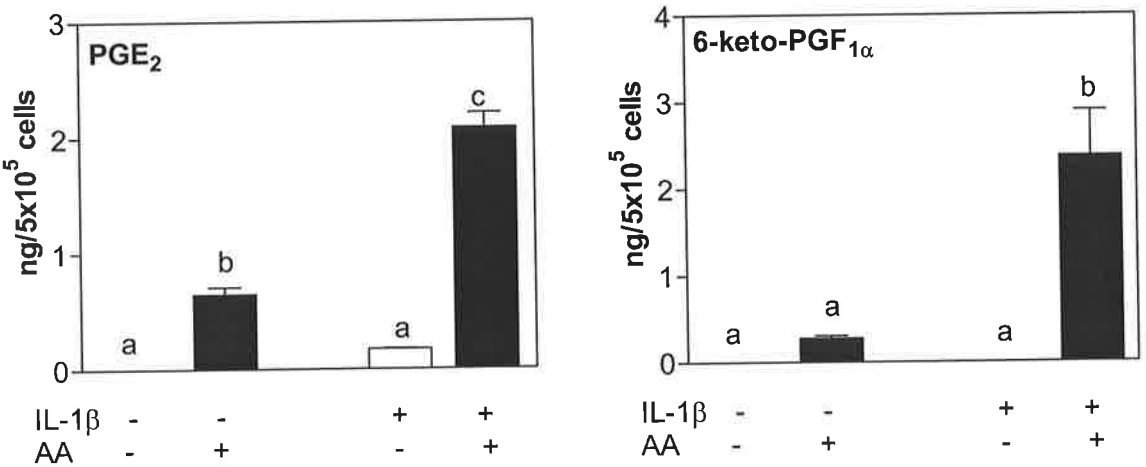


Figure 7.2: Production of PGE_2 and 6-keto-$PGF_{1\alpha}$ by synoviocytes. Cells were incubated in the absence or presence of IL-1β (2ng/ml) for 18 hours. Cells were then washed twice with RPMI (no FCS) and incubated without (hollow bars) or with (solid bars) AA (10μM) for 30 minutes. Supernatants were collected and assayed for eicosanoids. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

COX-1 and COX-2 were inhibited selectively to determine their relative contribution to prostaglandin synthesis. Resting synoviocytes, in which only COX-1 is present were treated with aspirin (55μM) for 30 minutes and washed before induction of COX-2 with IL-1β (2ng/ml). To inhibit COX-2 activity selectively, NS398 (0.5μM) was added. COX-2 inhibition resulted in reduction of PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis, while COX-1 inhibition had no effect on synthesis of these prostaglandins (Figure 7.3).

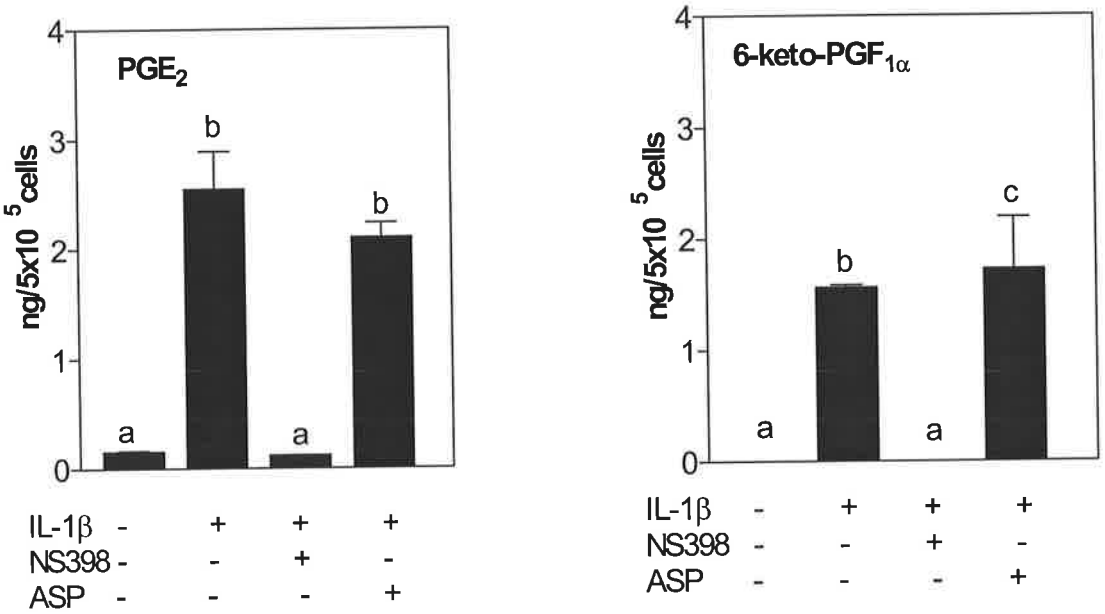


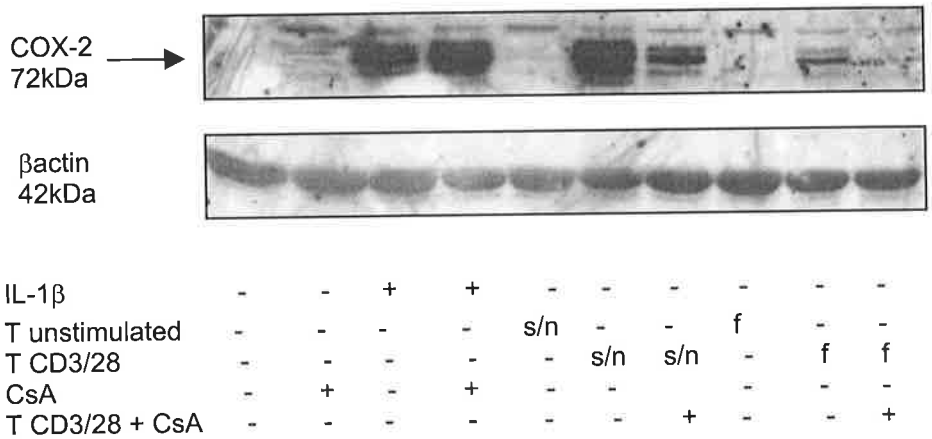
Figure 7.3: Effect of COX-1 and COX-2 inhibition on synoviocyte PGE_2 and 6-keto-$PGF_{1\alpha}$ production. Synoviocytes were incubated in the absence or presence of IL-1β (2ng/ml) for 18 hours following either COX-1 inhibition (transient aspirin pre-treatment; ASP) or COX-2 inhibition (NS398 0.5μM). Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

7.2.3 Effect of T cells on synoviocyte COX-2 expression and eicosanoid production

Having characterized the production of eicosanoids by synoviocytes, I next examined whether T cells were capable of up-regulating synoviocyte COX-2 in a similar fashion to that observed with monocytes (Chapter 6).

T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28. Some T cells were treated with CsA (100ng/ml) commencing 30 minutes, prior to stimulation. After incubation for 18 hours T cells and supernatants were then separated by centrifugation. T cells, fixed with 2% paraformaldehyde, or supernatants were added to synoviocytes. After a further 18 hour incubation supernatants and cells were harvested.

Both supernatants from activated T cells and to a lesser extent, fixed activated T cells up-regulated synoviocyte COX-2 expression and eicosanoid synthesis. The up-regulation of synoviocyte COX-2 expression and eicosanoid synthesis could be inhibited, at least partially, by treatment of T cells with CsA commencing 30 minutes prior to stimulation with immobilized anti-CD3 and soluble anti-CD28 (Figure 7.4). Neither PGE_2 nor 6-keto-$PGF_{1\alpha}$ were detectable in supernatants from T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 (data not shown), confirming that the detected eicosanoids were produced by synoviocytes. Similarly synoviocytes treated with soluble anti-CD28 alone produced no detectable PGE_2 or 6-keto-$PGF_{1\alpha}$ (data not shown).



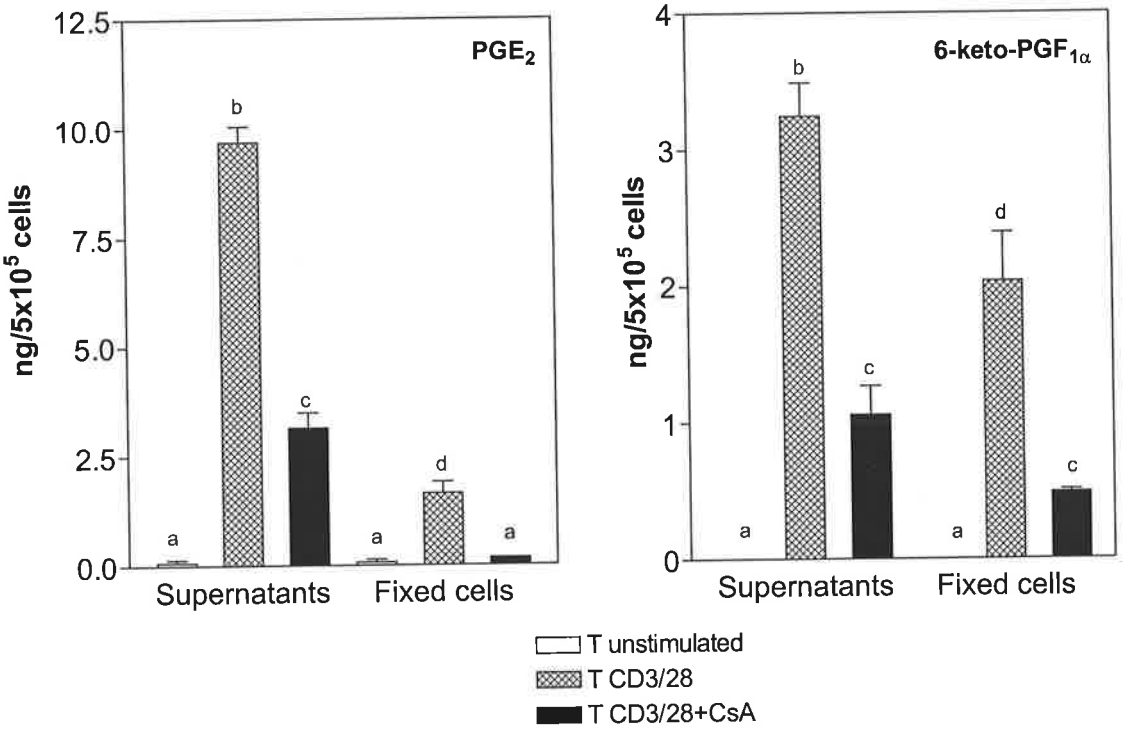


Figure 7.4: Effect of T cells on synoviocyte COX-2 expression and eicosanoid synthesis. Synoviocytes were incubated with either IL-1β (2ng/ml), T cell supernatants (s/n) or fixed T cells (f) from unstimulated T cells (T unstimulated), T cells treated with immobilized anti-CD3 and soluble anti-CD28 for 18 hours (T CD3/28) or T cells treated with CsA (100ng/ml) commencing 30 minutes prior to stimulation with anti-CD3/anti-CD28 (T CD3/28+CsA). Synoviocytes were also treated directly with CsA (CsA). After 18 hours cells and supernatants were collected. Bars with different letters are significantly different from each other; $p<0.05$ ANOVA followed by Neuman-Keuls test for multiple comparisons.

7.2.4 Effect of varying the T cell to synoviocyte ratio on synoviocyte COX-2 expression and eicosanoid production

In the preceding experiments, a ratio of two T cells to one synoviocyte was used. When supernatants from an increasing number of T cells were added to synoviocytes, there was a dose dependent increase in synoviocyte COX-2 expression and eicosanoid production, which was linear within the range 0.5-2 T cells per synoviocyte (Figure 7.5).

7.2.5 Time course for release by T cells of factors that stimulate synoviocytes to express COX-2 and produce eicosanoids

T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 and supernatants collected at 0, 2, 4, 8, and 18 hours. Supernatants were stored at 4^0C until the 18 hour time point. After warming to room temperature, supernatants were added to synoviocytes and incubated for a further 18 hours at 37^0C, 5%CO_2. There was a time-dependent increase in T cell supernatant activity with regard to stimulation of synoviocytes to express COX-2 and produce eicosanoids (Figure 7.6).

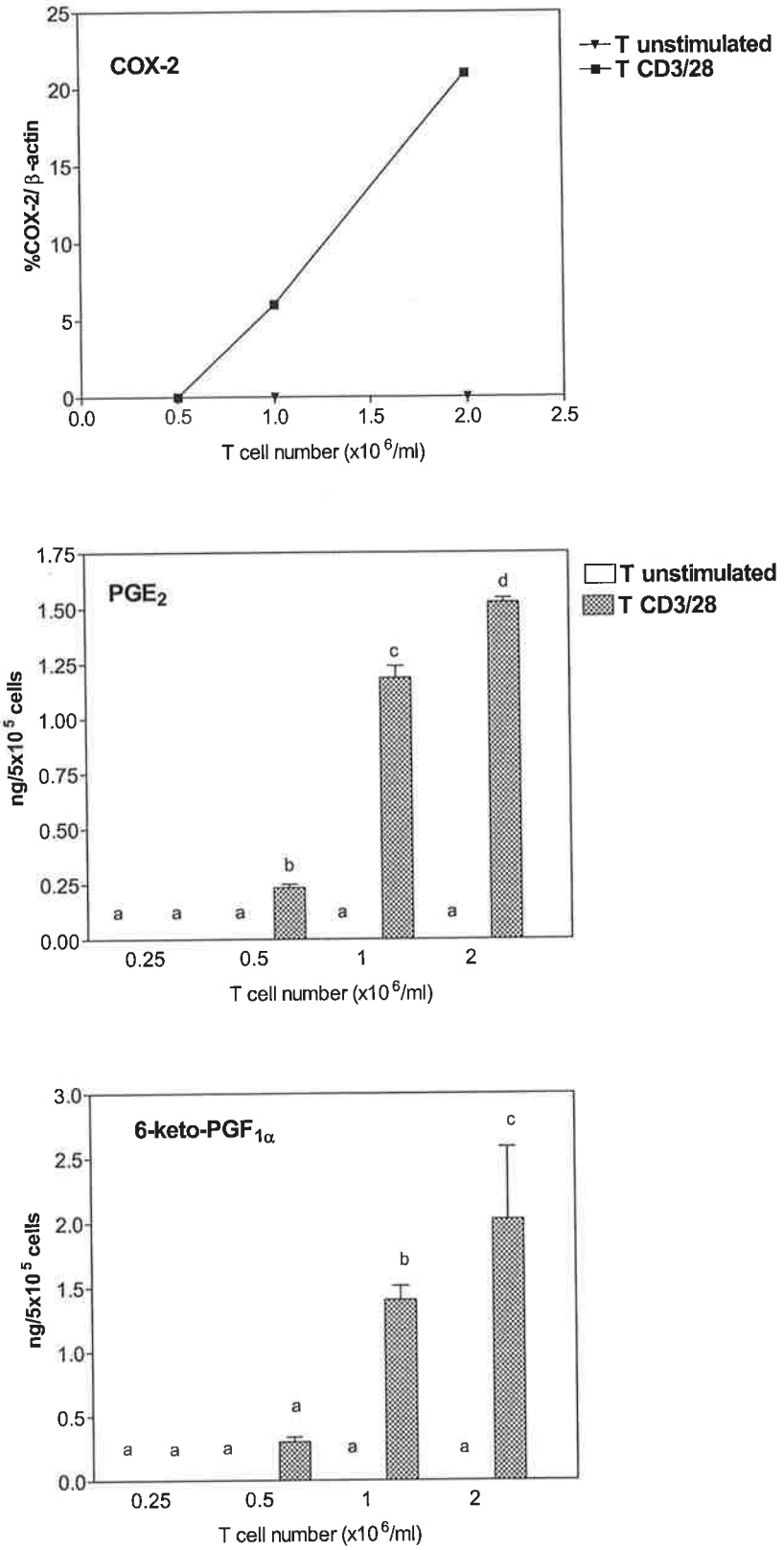


Figure 7.5: Effect on synoviocytes of supernatants obtained from incubating varying numbers of unstimulated T cells (T unstimulated) and T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 (T CD3/28) for 18 hours. COX-2 expression is displayed as a percentage of the intensity of β-actin staining on Western immunoblot. Bars with different letters are significantly different from each other; $p<0.05$ ANOVA followed by Neuman-Keuls test for multiple comparisons.

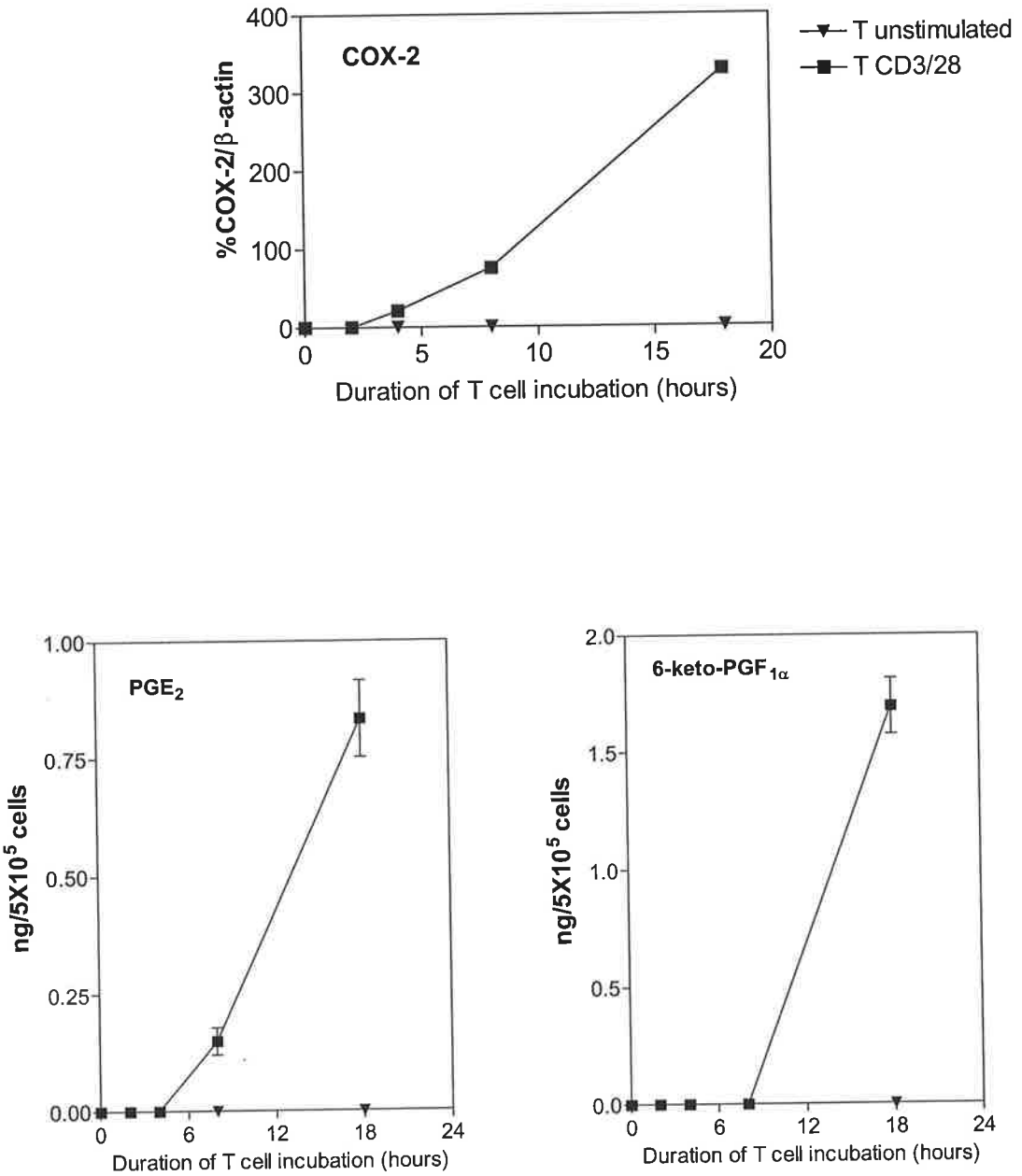


Figure 7.6: Time course for release by T cells of factors that stimulate synoviocytes to express COX-2 and produce eicosanoids. T cells were either unstimulated (T unstimulated) or stimulated with immobilized anti-CD3 and soluble anti-CD28 (T CD3/28) for times shown when supernatants were harvested. Supernatants were added to synoviocytes and incubated for a further 18 hours prior to harvest of cell pellets for Western immunoblot and supernatants for eicosanoid assay. COX-2 expression is shown as the relative densities of COX-2 and β-actin staining on Western immunoblot expressed as a percentage.

7.2.6 Time course of synoviocyte COX-2 expression and eicosanoid production: comparison of IL-1β with T cell supernatants

Synoviocytes were stimulated with either IL-1β (2ng/ml) or supernatants from activated T cells prepared by incubation of T cells in the presence of immobilized anti-CD3 and soluble anti-CD28 for 18 hours. Synoviocytes and supernatants were harvested at 0, 2, 4, 8, 12, and 18 hours. Supernatants were stored at -20^0C until eicosanoid measurement and cell pellets were immediately processed for Western immunoblot. The time course for COX-2 protein expression was similar with the two methods of stimulation. While the release of PGE_2 was slightly delayed in synoviocytes stimulated with T cell supernatants, there was no significant difference at 18 hours (Figure 7.7). Production of 6-keto-$PGF_{1\alpha}$ was less at all time points with synoviocytes stimulated with T cell supernatants compared to IL-1β (Figure 7.7).

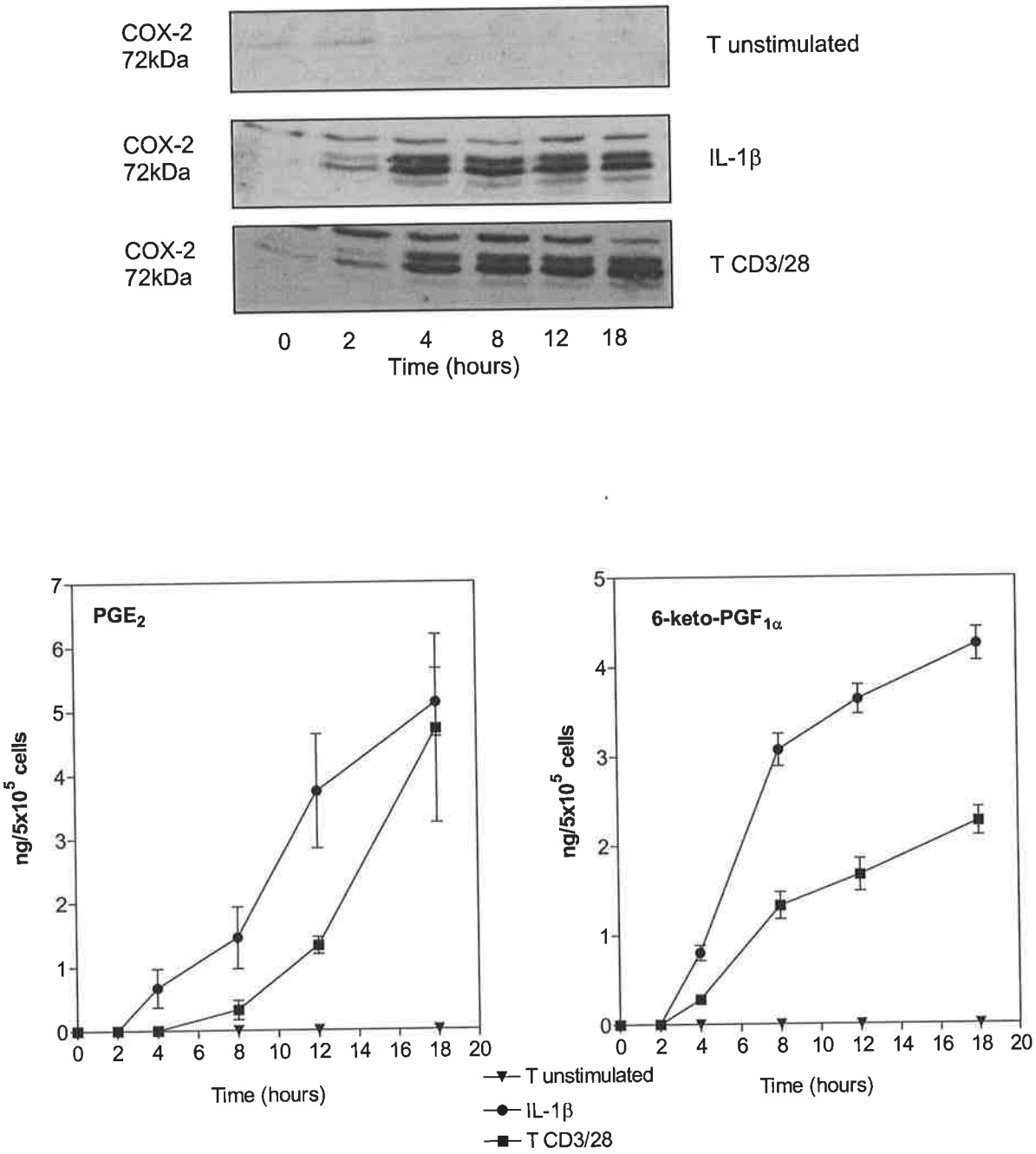


Figure 7.7: Time course of COX-2 expression and eicosanoid production by synoviocytes stimulated with either IL-1β (2ng/ml) or supernatants from T cells that had been cultured in the absence (T unstimulated) or presence of immobilized anti-CD3 and soluble anti-CD28 (T CD3/28) for 18 hours.

7.2.7 Effect of IL-17 on synoviocyte COX-2 expression and eicosanoid production

In the previous chapter, I showed that T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 produced IL-17, IFN-γ, and TNF-α and that production of these lymphokines was inhibited by treatment of T cells with CsA commencing 30 minutes prior to stimulation. IL-1β was not detected in supernatants from activated T cells (data not shown). Both soluble mediators and direct cell-cell interactions appear to contribute to the up-regulation of synoviocyte COX-2 expression and eicosanoid synthesis by activated T cells. However, the effect of supernatants was more potent than that of fixed cells. Therefore, for the purpose of this thesis, I concentrated on the soluble mediators involved, in particular IL-17, TNF-α, and IFN-γ.

It has been reported that IL-17 stimulates synoviocyte PGE_2 production and therefore I examined whether it could be responsible for stimulated T cell induced up-regulation of synoviocyte COX-2.

The addition of rIL-17 to synoviocytes resulted in a dose dependent up-regulation of synoviocyte COX-2 expression and eicosanoid production (Figure 7.8). However, at levels of IL-17 detected in stimulated T cell supernatants (1ng/ml) there was modest effect only.

IL-17 did not induce synoviocyte TNF-α production (data not shown).

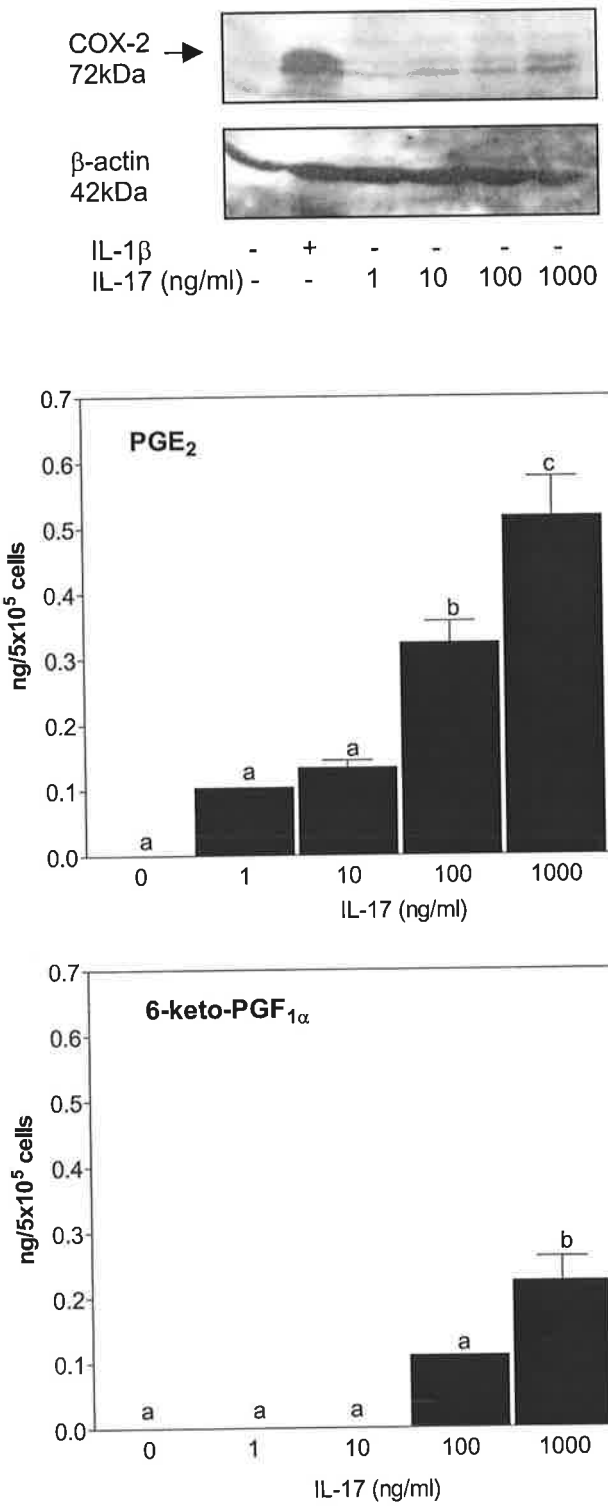


Figure 7.8: Effect of IL-17 on synoviocyte COX-2 expression and eicosanoid production. Synoviocytes ($5x10^5$ in 2mls) were incubated with IL-17 (0-1000ng/ml). After 18 hours cells were pelleted and processed for Western immunoblot and supernatants collected for eicosanoid release. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

IL-17 neutralizing antibody was used to assess the importance of IL-17 in the stimulated T cell supernatants on the up-regulation of synoviocyte COX-2. T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 for 24 hours when supernatants were collected and incubated with or without IL-17 neutralizing antibody (1μg/ml) for 4 hours prior to the addition of supernatants to synoviocytes. The addition of IL-17 neutralizing antibody resulted in partial inhibition of synoviocyte COX-2 expression and a more substantial reduction of eicosanoid production (Figure 7.9).

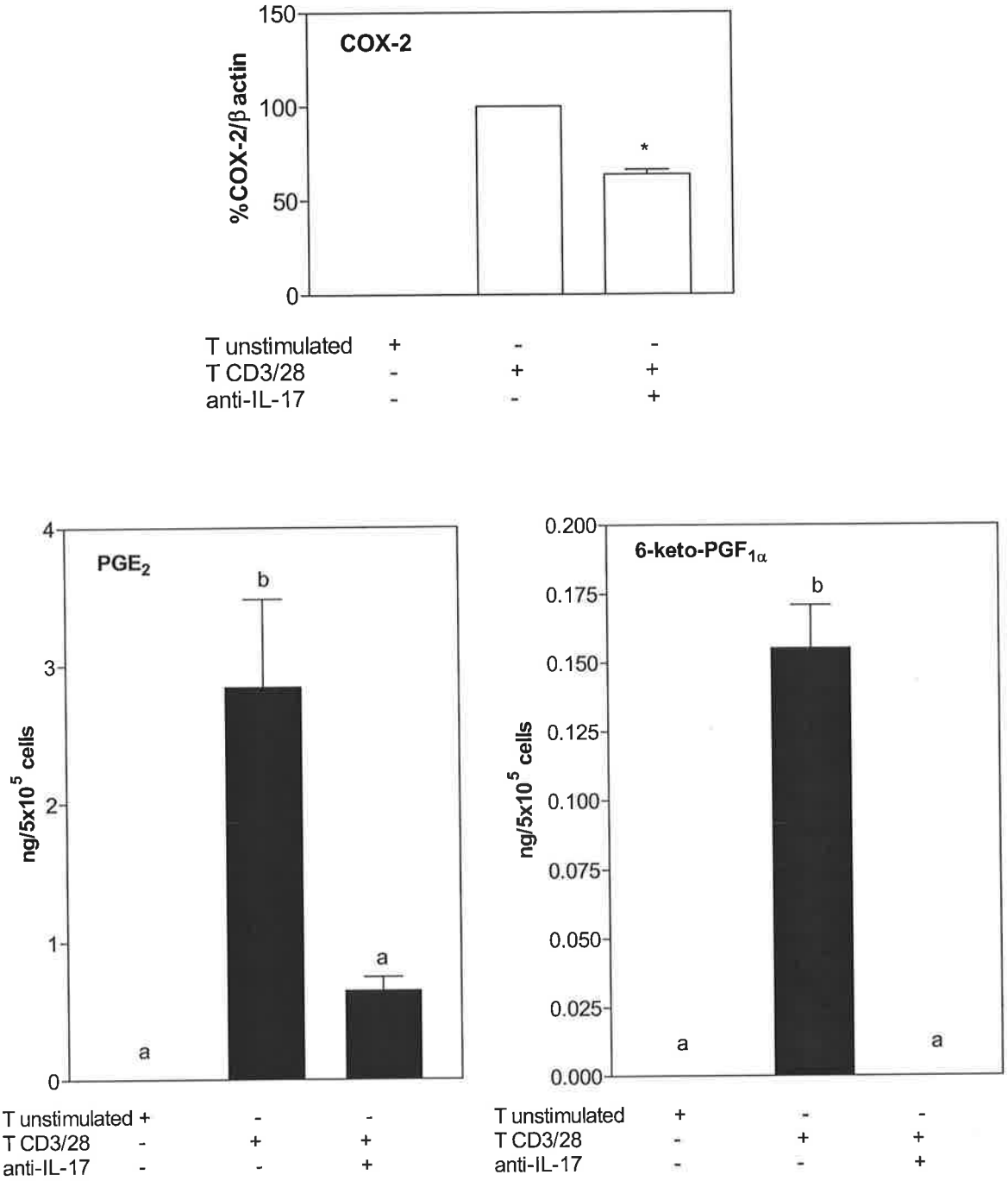


Figure 7.9: Effect of IL-17 neutralizing antibody on synoviocyte COX-2 expression and eicosanoid synthesis during incubation with supernatants from T cells that had been stimulated with immobilized anti-CD3 and soluble anti-CD28 for 24 hours. IL-17 neutralizing antibody (1μg/ml) was incubated with T cell supernatants for four hours. Supernatants were then added to synoviocytes. T unstimulated: supernatants from unstimulated T cells; T CD3/28, supernatants from anti-CD3/anti-CD28 stimulated T cells; anti-IL-17, supernatants from anti-CD3/anti-CD28 stimulated T cells treated with IL-17 neutralizing antibody. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons. (*$p<0.05$ Student t-test).

7.2.8 Effect of T cell derived TNF-α on synoviocyte COX-2 expression and eicosanoid production

TNF-α neutralizing antibody was used to investigate the role of T cell derived TNF-α in the up-regulation of synoviocyte COX-2. Supernatants from T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 for 24 hours were incubated with or without TNF-α neutralizing antibody (1μg/ml) for 4 hours prior to addition of supernatants to synoviocytes. After a further 18 hour incubation cells and supernatants were harvested and processed for Western immunoblot and eicosanoid assay respectively. The presence of TNF-α neutralizing antibody resulted in substantial inhibition of the T cell supernatant stimulating activity for synoviocyte COX-2 expression and eicosanoid production (Figure 7.10). It has previously been shown that TNF-α alone has little effect on synoviocyte COX-2 expression and PGE_2 production (Mino et al 1998), thus the large effects observed with the blockade suggested there may be synergy between TNF-α and another cytokine present within the activated T cell supernatants.

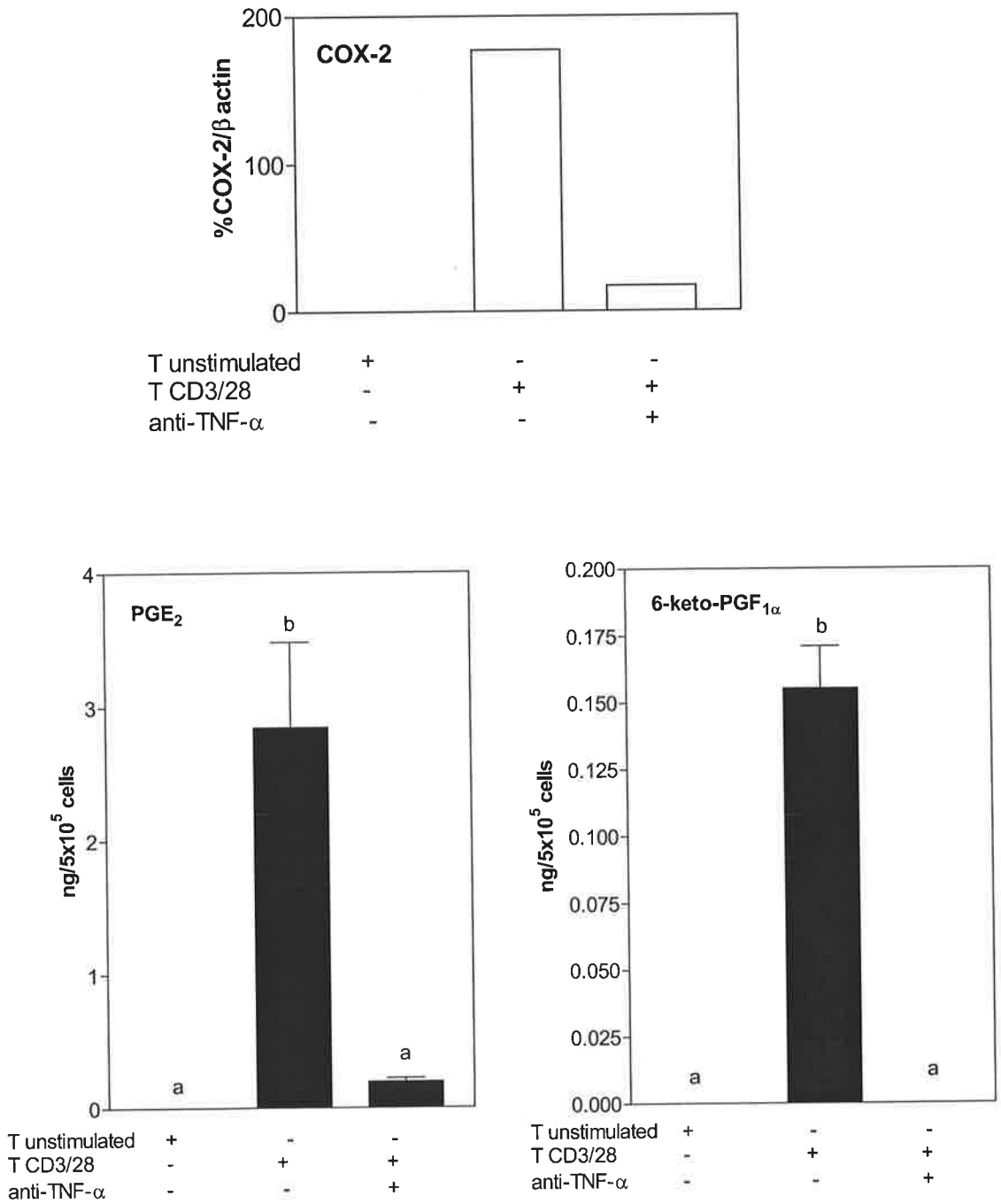


Figure 7.10: Effect of TNF-α neutralizing antibody on COX-2 expression and eicosanoid production by synoviocytes stimulated with T cell supernatants. T cells were stimulated for 24 hours with immobilized anti-CD3 and soluble anti-CD28. After separation from cells, supernatants were incubated with TNF-α neutralizing antibody (1μg/ml) for four hours prior to addition of supernatants to synoviocytes for a further 18 hour incubation. T unstimulated: supernatants from unstimulated T cells; T CD3/28, supernatants from anti-CD3/anti-CD28 stimulated T cells; anti-TNF-α, supernatants from anti-CD3/anti-CD28 stimulated T cells treated with TNF-α neutralizing antibody. Bars with different letters are significantly different from each other; $p<0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

7.2.9 Effect of IL-17 in combination with TNF-α or IL-1β on synoviocyte COX-2 expression and eicosanoid production

To examine whether there was any synergistic effect between TNF-α and IL-17, synoviocytes were incubated with TNF-α (2ng/ml) in the presence or absence of IL-17 (1ng/ml). The addition of TNF-α at 2ng/ml, which is in the range detected in T cell supernatants under these experimental conditions, had no effect on synoviocyte COX-2 expression or PGE_2 production. Similarly the addition of IL-17 at 1ng/ml, which is in the range detected in T cell supernatants under these experimental conditions, had no detectable effect on synoviocyte COX-2 expression or eicosanoid production when used alone. However, the combination of IL-17 and TNF-α resulted in enhanced synoviocyte COX-2 expression and PGE_2 production (Figure 7.11).

To further examine the effects of IL-17, under conditions of co-stimulation likely to apply in vivo, synoviocytes were incubated with IL-1β (2ng/ml) in the presence or absence of IL-17. The presence of IL-17 resulted in enhanced IL-1β stimulated COX-2 expression and PGE_2 production (Figure 7.11).

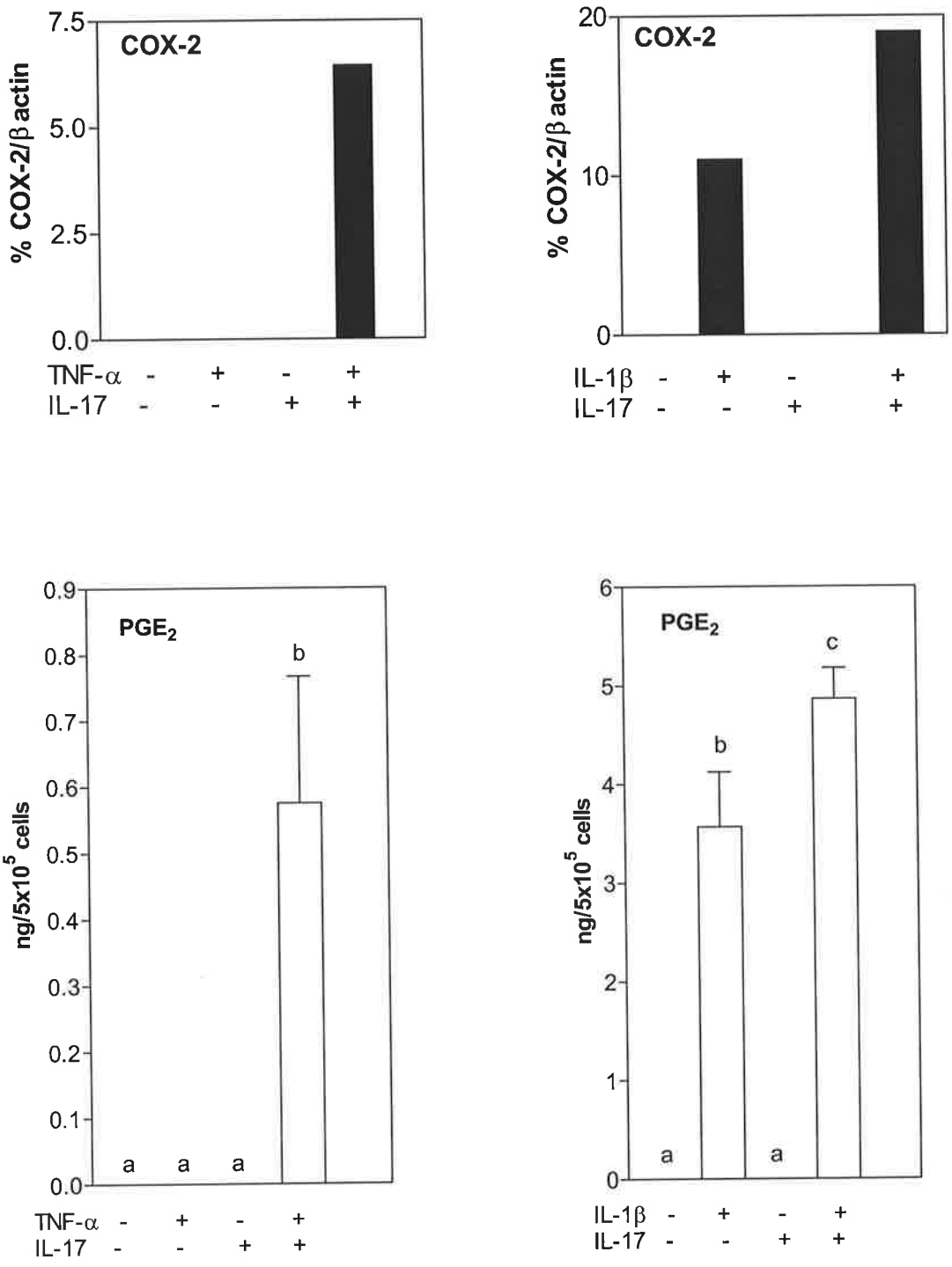


Figure 7.11: Effect of IL-17 on TNF-α and IL-1β stimulated synoviocyte COX-2 expression and PGE_2 production. $5x10^5$ synoviocytes were stimulated with IL-1β (2ng/ml) or TNF-α (2ng/ml) in the absence or presence of IL-17 (1ng/ml). After 18 hours cells were pelleted for Western immunoblot and supernatants processed for eicosanoid assay. Bars with different letters are significantly different (ANOVA followed by Newman-Keuls test for multiple comparisons; $p<0.05$). The data shown are representative of 4 separate experiments.

7.2.10 Effect of IFN-γ on synoviocyte COX-2 expression and eicosanoid production

Addition of IFN-γ alone to synoviocytes did not induce COX-2 expression. Treatment of synoviocytes with IFN-γ (15ng/ml) commencing 4 hours prior to stimulation with IL-1β (2ng/ml) resulted in a non-significant reduction in PGE_2 production by synoviocytes with no apparent change in the level of COX-2 expression (data not shown).

Simultaneous addition of IFN-γ (15ng/ml) and IL-1β (2ng/ml) to synoviocytes resulted in inhibition of synoviocyte PGE_2 and 6-keto-$PGF_{1\alpha}$ production. However, there was no apparent change in the level of COX-2 expression (Figure 7.12).

The reduction in release of both PGE_2 and 6-keto-$PGF_{1\alpha}$ in the presence of IFN-γ suggests that a common step in their synthetic pathway is effected. Two common steps in production of eicosanoids are the release of AA from cell membranes by PLA_2 and conversion of AA to PGH_2 by COX. There was no apparent change in COX-2 expression, therefore release of AA was examined.

[^{3}H]-AA release was increased by the addition of IFN-γ to IL-1β. However, the change was numerically small (Figure 7.12).

7.2.11 Effect of TXA_2 on synoviocyte COX-2 expression

As seen with monocytes, the thromboxane receptor agonist, $cTXA_2$ (0.01-10 μg/ml) did not induce COX-2 expression in synoviocytes (data not shown).

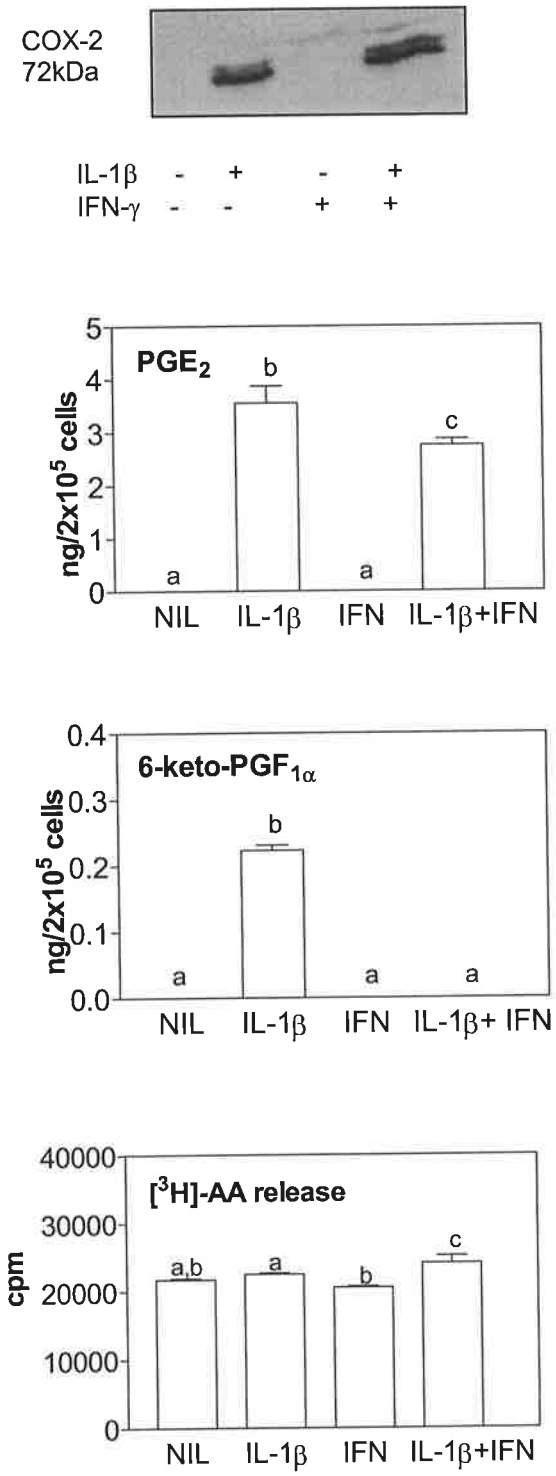


Figure 7.12: Effect of IFN-γ on COX-2 expression, eicosanoid production, and AA release by IL-1β stimulated synoviocytes. Synoviocytes ($2x10^5$ in 1ml) were incubated with [^{3}H]-AA (2μCi/ml) for 24 hours. Cells were then washed and IL-1β (2ng/ml) was added alone or in combination with IFN-γ (15ng/ml). Cells were incubated for a further 18 hours at 37^0C, 5%CO_2. Supernatants were collected and radioactivity determined. Using cells from the same donor, but not pre-treated with [^{3}H]-AA, supernatants and cells were collected for eicosanoid production and COX-2 expression. Bars with different letters are significantly different from each other; $p<0.05$ ANOVA followed by Neuman-Keuls test for multiple comparisons.

7.3 Discussion

The results indicate that both soluble mediators produced by T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 and fixed activated T cells can up-regulate synoviocyte COX-2 expression and eicosanoid production. Furthermore the effect can be at least partially inhibited by treatment of T cells with cyclosporin A.

In rheumatoid joints, T cells and synoviocytes lie in close proximity and therefore heterotypic interactions between these cells can be expected. It has previously been reported that fixed activated T cells can up-regulate synoviocyte PGE_2 production in a direct cell-cell contact dependent manner. The authors go on to suggest that membrane associated TNF-α and IL-1β may be important in this process (Burger et al 1998). Results of the present study are in agreement with these findings in that fixed activated T cells induced synoviocyte COX-2 expression and eicosanoid production. Furthermore, I have shown that soluble mediators may also participate in T cell dependent up-regulation of synoviocyte COX-2 expression and eicosanoid production.

In chapter 6, I demonstrated that T cells, stimulated with immobilized anti-CD3 and soluble anti-CD28, produce IL-17, TNF-α, and IFN-γ. Within our T cell enriched populations, there are a small number of contaminating monocytes (~0.4%) and thus, the TNF-α in the T cell supernatants could be derived from contaminating monocytes. However, IL-1β which is a major monocyte derived cytokine, was not detected in the stimulated T cell preparations, suggesting that the degree of monocyte contamination in the T cell preparations was not likely to be contributing to the TNF-α in the T cell supernatants. Because of their presence within rheumatoid synovial fluid, as well as

their pro-inflammatory actions, IL-17 and TNF-α have interest as T cell derived mediators of rheumatoid joint pathology.

IL-17 is a pro-inflammatory cytokine and many of its actions are similar, although less potent, than those of TNF-α and IL-1β. In the present study, concentrations of exogenous IL-17 found in T cell supernatants yielded no evidence of up-regulation of synoviocyte COX-2 expression or increase in eicosanoid synthesis. On the other hand, antibody neutralization of T cell derived IL-17, at least partially removed the ability of T cell supernatants to up-regulate synoviocyte COX-2 expression and eicosanoid production. Taken together, the results suggest that T cell derived IL-17 may be a paracrine up-regulator of synoviocyte COX-2 expression and eicosanoid synthesis that acts in concert with another agent in the supernatants. For this reason, possible IL-17 interactions with TNF-α were examined.

TNF-α and IL-1β both appear to be important cytokines in RA. In the rheumatoid joint, monocytes/macrophages are the major producers of TNF-α and IL-1β, although as I have shown, activated T cells may also produce TNF-α. The addition of TNF-α neutralizing antibody to supernatants from T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 suppressed the ability of the T cell supernatants to stimulate synoviocyte COX-2 induction and PGE_2 synthesis.

IL-17 has been reported to enhance many of the effects of TNF-α and IL-1β on monocytes and synoviocytes and this had led to suggestions that the main role of IL-17 may be to "fine-tune" the inflammatory process (Katz et al 2001). In skin and synovial fibroblasts IL-17 induced production of IL-1β, IL-6, and IL-8. However, the

observed increases in IL-6 and IL-8 production with IL-17 alone were small when compared to those induced by the either IL-1β or TNF-α. On the other hand, the combination of IL-17 and TNF-α resulted in enhanced expression of IL-1β, IL-6, and IL-8 compared to stimulation with either IL-17 or TNF-α alone (Katz et al 2001). While neither IL-17 nor TNF-α alone had a direct effect, the combination induced synoviocyte production of GM-CSF (Fossiez et al 1996). IL-17 in combination with IL-1β has also been reported to have a synergistic effect on IL-6 production and an additive effect on leukaemia inhibitory factor production by rheumatoid synoviocytes (Chabaud et al 1998). Synoviocyte production of macrophage inflammatory protein (MIP)-3α, a chemokine involved in recruitment of $CD4^+$ T cells, has been reported to be induced by IL-17, IL-1β, or TNF-α alone, with enhanced production with cytokine combinations. In addition, this cytokine stimulated production of MIP-3α was reduced by the presence of IL-4 and IL-13 but not IL-10 (Chabaud et al 2001b). Using explants of human osteoarthritic knee menisci LeGrand et al reported that combinations of IL-17, IL-1β, and TNF-α induced significantly higher levels of nitric oxide release than individual cytokines used alone. IL-1β, but neither TNF-α nor IL-17 alone led to a significant increase in PGE_2 production. However the combination of IL-17 with either IL-1β or TNF-α increased PGE_2 production (LeGrand et al 2001).

In the present study, addition of IL-17 and TNF-α together produced substantial expression of synoviocyte COX-2 and PGE_2 whereas neither cytokine alone at these concentrations produced a substantial effect. Taken together these data suggest that IL-17 and TNF-α act together in T cell supernatants to up-regulate synoviocyte COX-2 expression and PGE_2 production.

While fixed activated T cells also induced synoviocyte COX-2 expression and eicosanoid production the effects were small compared to those of supernatants from stimulated T cells. Burger et al previously reported that plasma membranes from stimulated human peripheral blood T cells, or from a T cell line, induced PGE_2 production by synoviocytes and dermal fibroblasts. The effects were mediated at least in part by membrane associated TNF-α and IL-1β, but not by CD69, CD40 ligand, or CD11b (Burger et al 1998). Heterotypic interactions between activated T cells and synoviocytes appear to be mediated, at least in part, by LFA-1 and ICAM-1 interactions with synoviocyte IL-1β production increasing as a result (Nakatsuka et al 1997).

While I did not observe an effect of unstimulated T cells on synoviocyte COX-2 expression and PGE_2 production, Yamamura et al observed that unfixed resting T cells could activate synoviocytes with induction of IL-6, IL-8 mRNA and protein synthesis, and PGE_2 release (Yamamura et al 2001). Furthermore, the effect of resting T cells on synoviocyte PGE_2 production was enhanced by the addition of exogenous IL-17. While IL-2 and IL-17 could not be detected in their resting T cells, TNF-α was not examined. Therefore it is possible that small amounts of TNF-α were secreted from the T cells, which then synergized with the exogenous IL-17 resulting in enhanced PGE_2 production. Resting T cells have been reported to increase the production of collagen type I but not collagen type III by synoviocytes. Furthermore, fixed activated T cells inhibited synoviocyte production of both collagen type I and III, an effect mediated by membrane associated IFN-γ, TNF-α, and IL-1β (Rezzonico et al 1998). In addition, direct contact between activated T cells and synoviocytes results in an imbalance in the production of MMP-1 and TIMP-1 in favour of MMP-1 (Burger et al

1998). Thus, cell contact dependent interactions between activated T cells and synoviocytes may have effects on cartilage destruction through an increase in matrix catabolism, via increased MMP-1, and reduction in synthesis of extracellular matrix components collagen type I and III. The direct effects of IL-17 on cartilage degradation (see section 1.5.2.2) may further compound these effects.

In keeping with previous reports (Nakajima et al 1990; Temime et al 1991), IFN-γ inhibited IL-1β-stimulated eicosanoid production by synoviocytes. The inhibition of both PGE_2 and 6-keto-$PGF_{1\alpha}$ production suggests that the inhibitory effect of IFN-γ is at a common step in the biosynthetic pathway, most likely COX or PLA_2. In human periodontal ligament fibroblasts, IFN-γ inhibited IL-1β stimulated PGE_2 production with no change in COX-2 expression (Noguchi et al 1999). The present results are in keeping with these findings in that synoviocyte IL-1β induced PGE_2 production was inhibited, while COX-2 expression was unchanged by the presence of IFN-γ.

PLA_2 enzymes mediate the release of AA from cell membranes. In rheumatoid synovial fibroblasts, $cPLA_2$ expression and AA release have been reported to be induced by IL-1β (Hulkower et al 1994). In comparison, I was unable to detect a significant increase in AA release in response to IL-1β, while the combination of IL-1β and IFN-γ led to a slight increase in AA release. These apparent differences may be due to differing methods of detecting AA release. While I used [^{3}H]-AA release by cultured human synoviocytes, Hulkower et al used release of [1-^{14}C]-AA by subcellular fractions (Hulkower et al 1992; Hulkower et al 1994). An alternative explanation is that the terminal eicosanoid synthases, PGE synthase and PGI synthase, are affected by IFN-γ. PGE synthase exists in two forms, cPGES and mPGES (see

section 1.2.2.5). In rheumatoid synovial fibroblasts, IL-1β has been shown to induce expression of mPGES (Stichtenoth et al 2001). Unfortunately I was unable to detect mPGES using Western immunoblot with the available antibody, therefore no comment can be made on the ability of IFN-γ to regulate mPGES expression.

With advances in the understanding of the important role of cytokines in RA, biological agents targeting specific cytokines have become available. Both TNF-α monoclonal antibodies and a soluble TNF-α receptor fusion protein have been shown to reduce disease activity and joint damage in RA (Maini et al 1998; Moreland et al 1999; Weinblatt et al 1999). Recombinant human IL-1β receptor antagonists may also reduce joint erosion in RA (Bresnihan et al 1998; Jiang et al 2000). While these agents have proved efficacy, there is a substantial proportion of cases in which the response is unsatisfactory. This may reflect the importance of other pro-inflammatory cytokines, such as IL-17, which are present within the rheumatoid joint. Furthermore, at least part of their effect may be due to inhibition of the synergistic effects of IL-17 and TNF-α or IL-1β. It may be that combination anti-cytokine therapy may produce a better response than inhibition of a single cytokine. With this in mind it is notable that the combination of IL-17, IL-1β, and TNF-α blockade has greater effect in *in vitro* models of tissue degradation and inflammatory mediator production than inhibition of the individual cytokines alone (Chabaud and Miossec 2001; Chevrel et al 2002).

In summary, soluble mediators produced by activated T cells can influence the expression of synoviocyte COX-2 and eicosanoid production. The results suggest that T cell derived IL-17 in combination with TNF-α (whether T cell or monocyte derived) and IL-1β may contribute to T cell driven up-regulation of synoviocyte COX-2. The

effects of IL-17 on synoviocyte COX-2 expression, as well as the implication that IL-17 is involved in joint damage in animal models of arthritis (Lubberts et al 2001; Bush et al 2002a), provide further evidence for the role of IL-17 as a therapeutic target in rheumatoid arthritis and other inflammatory diseases. The place of IL-17 blockade among multiple anti-cytokine therapies used alone and in combination remains to be elucidated.

Chapter 8

Summary

"The outcome of any serious research can only be to make two questions where one question grew before."

Thorstein Veblen 1908

8.1 Introduction

Lipid mediators (eicosanoids), produced via the cyclooxygenase pathway, as well as peptide mediators (cytokines), mediate the inflammatory process in rheumatoid arthritis. While T cells are abundant within the rheumatoid synovium, there is a paucity of most T cell derived cytokines. This has led to much speculation as to the role of T cells in rheumatoid arthritis. Recently IL-17, a pro-inflammatory T cell cytokine, has been found in relatively large amounts (5-5000pg/ml) in rheumatoid joints. In addition, T cells have been shown to activate monocytes and synoviocytes through direct cell-cell contact. This has given new insights into the role of T cells in RA.

8.2 Salient findings

This thesis has examined the role of T cells in up-regulating monocyte and synoviocyte COX-2 expression and eicosanoid production. As a starting point, it was important to examine T cell COX isotypes and eicosanoid production. Historically, it has been considered that cells do not contain COX and are therefore incapable of

producing eicosanoids. With the recognition that COX exists in two isoforms, COX-1 (constitutively expressed) and COX-2 (inducible by inflammatory stimuli), there has been renewed interest in COX in all cell types. While there have been recent reports that T cells contain both COX-1 and COX-2, the production of eicosanoids via the two COX isotypes has received little attention. In Chapter 4, I confirm the presence of COX-1 in T cells. However, using a variety of techniques, I was unable to identify COX-2 in activated T cells. In addition, I have demonstrated that T cells are capable of producing TXA_2 and small amounts of PGE_2 and PGD_2 via COX-1. In Chapter 5, I examined the effects of TXA_2 on T cell function. I observed that small amounts of T cell derived TXA_2 may have a permissive or enhancing effect on T cell proliferation, while larger amounts of TXA_2 that may be produced by monocytes/macrophages present within an inflammatory environment may act to inhibit T cell proliferation.

In an effort to explain the apparent discrepancy between my findings and those previously reported with respect to the presence of COX-2, I have examined the ability of T cell to up-regulate monocyte COX-2 expression. In Chapter 6, I have demonstrated that activated T cells can up-regulate monocyte COX-2 expression. Furthermore, T cell derived IL-17 was a positive regulator of monocyte COX-2 expression, while IFN-γ was a negative regulator. In Chapter 7, similar effects were observed in the interaction between T cells and synoviocytes in that activated T cells could up-regulate synoviocyte COX-2 expression and IL-17 was a positive regulator. While recombinant IL-17 up-regulated monocyte and synoviocyte COX-2 expression the effect, using doses similar to those found in T cell supernatants, was small when compared to that seen with activated T cell supernatants, suggesting synergy between IL-17 and other cytokines produced by activated T cells. Indeed, synergy between IL-

17 and TNF-α with respect to induction of synoviocyte COX-2 expression was demonstrated. IL-1β, another important monokine found in rheumatoid joints, also displayed synergy with IL-17. In addition, direct cell-cell contact between fixed activated T cells and synoviocytes up-regulated synoviocyte COX-2 expression and eicosanoid production. However, this effect was small compared to that seen with T cell supernatants.

The studies presented in this thesis identify a mechanism through which T cells can contribute to the inflammatory response in rheumatoid arthritis. The results suggest that IL-17, especially in combination with IL-1β and TNF-α, may up-regulate COX-2 expression in both monocytes and synoviocytes.

8.3 Future directions

8.3.1 Role of TXA_2 in T cell function and rheumatoid arthritis

The studies presented in this thesis suggest that TXA_2 may have a role in T cell function. Interestingly at low-moderate concentrations TXA_2 inhibited IFN-γ and yet increased IL-17 production by stimulated T cells. Whether these biphasic effects contribute to the T cell cytokine pattern observed in rheumatoid arthritis is unknown and worthy of further investigation.

In addition thoracic duct lymphocytes from rats with adjuvant-induced arthritis produced larger quantities of TXB_2 and PGE_2 than those from control rats without arthritis. The mechanism for this increase remains to be determined. Further investigation into the expression of COX-2 and the terminal eicosanoid synthases

present in rat thoracic duct lymphocytes is warranted. The transfer of polyarthritis by rat thoracic duct lymphocytes has been well documented. However, whether TXA_2 production by these arthritogenic effector T cells has a role in the transfer of polyarthritis or maintenance of the inflammatory process warrants further investigation. An approach to this question is evaluation of the potential therapeutic effects of thromboxane synthase inhibitors and thromboxane receptor antagonists in experimentally induced polyarthritis.

8.3.2 Role of interleukin-17 in rheumatoid arthritis

To date, IL-17 is the only T cell derived cytokine found in rheumatoid joints in significant amounts. The identification of IL-17, the characterization of its effects, and its isolation in RA joints suggests a pathogenic role for T cells in RA joints.

Whether IL-17 producing T cells present within rheumatoid joints are involved in the initiation of RA remains unknown. However, IL-17 release occurs early in the T cell activation process (Yao et al 1995b) and in rat adjuvant-induced arthritis, IL-17 is increased early in the disease process (Bush et al 2001). Taken together these observations suggest that IL-17 may have a role in the early phase of RA. The ability of arthritogenic rat thoracic duct lymphocytes to produce IL-17 and its effect on the both the induction and effector phases of rat adjuvant-induced arthritis is of interest. Furthermore, if IL-17 is the effector molecule of a new subset of T cells, then finding the stimulus that promotes differentiation of naïve T cells into this subset may provide some insight into the aetiology of RA. In addition, if larger series confirm that IL-17 is present in RA synovial tissue and fluid but not in other forms of inflammatory arthritis as suggested by Honorati and co-workers (Honorati et al 2001), the presence of IL-17

may not only provide clues to aetiology of RA and but also provide a powerful means by which RA can be diagnosed and distinguished from other inflammatory arthropathies.

Whether IL-17 producing T cells are involved in the initiation of RA or not, the presence of IL-17 within rheumatoid joints provides a likely effector mechanism for a direct T cell pro-inflammatory effect. IL-17 has pleiotropic effects on inflammatory mediator production and bone and cartilage degradation in rheumatoid arthritis and thus could be considered a pivotal T cell derived cytokine in RA (Figure 8.1). While many of its effects are small when compared those of IL-1β and TNF-α, it can enhance the effects of both of these cytokines. Therefore, it can be argued that the major role of IL-17 is to modulate the effects of these predominantly macrophage/monocytes derived cytokines. Whether IL-17 has a central role or is merely a modulator of IL-1β and TNF-α activities in rheumatoid joints, does not require resolution for pursuit of IL-17 neutralization as a therapeutic goal. Biological agents targeting both TNF-α and IL-1β are currently in clinical use. While they are certainly effective in many patients, there are a substantial number who have an unsatisfactory response. This may reflect the importance of other pro-inflammatory cytokines, such as IL-17, which are present within the rheumatoid joint. Available data would suggest that inhibition of IL-17 may have benefits in the treatment of inflammatory arthritis.

Further trials into its potential as therapeutic target are thus warranted. Treatment of rat adjuvant-induced arthritis with an IL-17 receptor IgG1 Fc fusion protein resulted in a dose-dependent reduction in paw volume, and reduced radiographic and histology

scores in arthritic rats compared with controls (Bush et al 2002a). Using *ex vivo* RA synovium and bone, the combination of IL-17, IL-1β, and TNF-α blockade has greater effect with respect to inhibition of IL-6 release and collagen degradation, than inhibition of each cytokine individually (Chabaud and Miossec 2001). Incubation of supernatants from stimulated RA peripheral blood mononuclear cells with soluble TNF-α decoy receptors inhibited their ability to induce synoviocyte release of IL-6, MIP-3α, and type 1 collagen. However the combination of soluble receptors directed against IL-1β and IL-17 in addition to TNF-α enhanced the effects (Chevrel et al 2002). Clearly, further animal trials of such combinations are desirable before human studies can begin.

Treatment of RA with combinations of conventional anti-rheumatic agents, such as methotrexate, salazopyrine, and hydroxychloroquine, provides increased benefit over use of single agents (O'Dell et al 1996). The observations with IL-17 suggest that combination biological agent therapies, which target both IL-17 and other cytokines, may have in common with accepted combinations of chemical agent therapy, greatly enhanced efficacy. Whether we are heading toward an era of combination cytokine therapy in the management of rheumatoid arthritis remains to be seen.

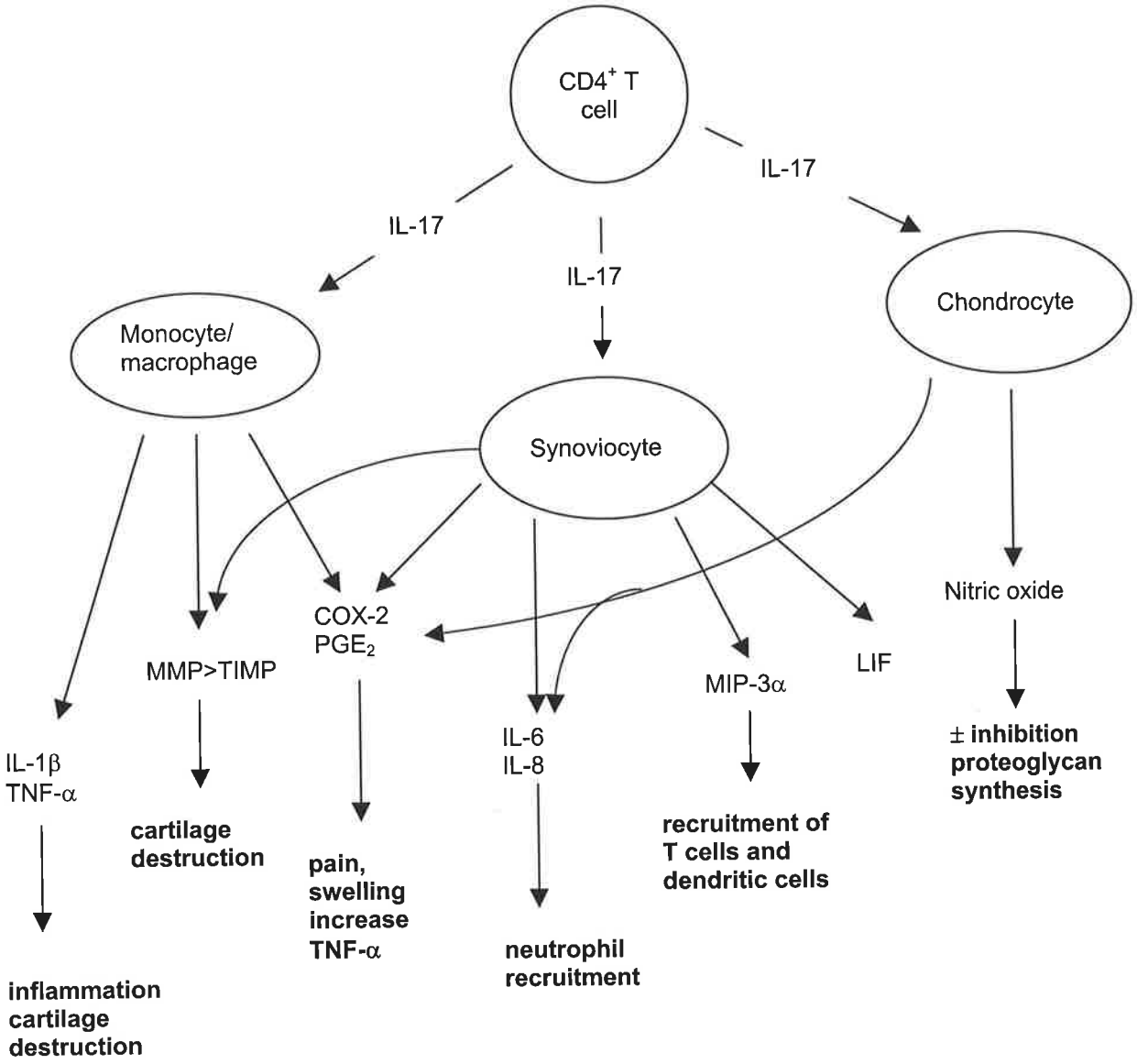


Figure 8.1: Effects of IL-17 in rheumatoid arthritis.

Appendix

Solutions and Reagents

Solutions for flow cytometry

Immunofluorescence (IF) Buffer

1L PBS

20mls FCS

1ml azide 1M

FACS fix

1L PBS

10mls formalin

20gm glucose

0.2gm azide

Dulbecco's PBS (DPBS)

100mls 10X PBS

0.1gm $CaCl_2$ in 100mls milliQ H_2O

0.1gm $MgCl_2$ in 100mls milliQ H_2O

1gm D-glucose

1ml of 1M azide

700mls milliQ H_2O

PBS 10x

8.0gm NaCl

0.2gm KCL

1.15gm Na_2HPO_4

0.2gm KH_2PO_4

Saponin 10%w/v

Add 0.1gm saponin to 10mls milliQ H_2O

Saponin wash buffer

DPBS 1%FCS

saponin 10% weight volume to a final concentration of 0.1% weight volume

Add 5mls of 10%saponin to 495mls DPBS 1%FCS

Solutions for elutriation

Running buffer

1L (1x) Hanks buffered salt solution (HBSS)

2.1gm Tri-sodium citrate

pH 7.3-7.4

10xHBSS

4gm KCl

0.6gm KH_2PO_4

80gm NaCl

3.5gm $NaHCO_3$

0.475gm Na_2HPO_4

Dissolve in 1L milliQ H_2O pH 7.3

Solutions for radioimmunoassay

RIA buffer

1L milliQ H_2O

0.1% gelatin

0.9% NaCl

0.01M Trisbase

pH 7.3

Na_2CO_3 (1mM)

0.106gm Na_2CO_3 1L milliQ H_2O

pH 10

Charcoal dextran

1% Dextran T70

1% Charcoal

[^{3}H]-TXB_2

Supplied as 100μCi/ml in Ethanol (Amersham)

Add 10μL to 10mls PBS

Store at 4^0C

TXB_2 standards

Dissolve 1mg in 1ml Ethanol

Dilute in RIA buffer to 100ng/ml

Store at -20^0C

TXB_2 anti-sera

Dilute 1:4000 with RIA buffer

Store at -20^0C

[^{3}H]-PGE_2

Supplied as 100μCi/ml in Ethanol (Amersham)

Add 15μL to 10mls Na_2CO_3

Store at 4^0C

PGE_2 standards

Dissolve 1mg in 1 ml Ethanol

Dilute in RIA buffer to 100ng/ml

Store at -20^0C

PGE_2 anti-sera

Dilute 1:1500 in RIA buffer

Store at -20^0C

$[^3H]$-6-keto-$PGF_{1\alpha}$

Supplied as 100μCi/ml in Ethanol (Amersham)

Add 25μL to 10mls PBS

Store at 4^0C

6-keto-$PGF_{1\alpha}$ standards

Dissolve 1mg in 1ml Ethanol

Dilute in RIA buffer to 100ng/ml

Store at -20^0C

6-keto-$PGF_{1\alpha}$ anti-sera

Sigma as per manufacturers instructions

Store at -20^0C

Solutions for ELISA

Assay buffer (1%BSA)

50mls PBS

0.5gm BSA

Wash Buffer (0.05% Tween-20)

1L PBS

0.5ml Tween-20

Coating Buffer

1.68gm $NaHCO_3$ in 100mls milliQ H_2O

2.12gm Na_2CO_3 in100mls milliQ H_2O

Add 74mls $NaHCO_3$ to 26mls Na_2CO_3 pH 9.4-9.7

Phosphate buffer

1.0M NaH_2PO_4 and Na_2HPO_4

Citrate buffer (0.2M)

9.6gm citric acid

14.7gm Tri-sodium citrate

500mls milliQ H_2O pH 4.9

Solutions for Western Immunoblot

5x Lamelli

100mls of 3.125M Tris HCl

50mls glycerol

10gm SDS

25mls β-ME

15mls milliQ H_2O

pH 6.8

Lysis Buffer

10mls 10x HBSS

1ml Hepes 1M pH 7.4

2.5mls Triton (20%)

Make to 100mls with milliQ H_2O

PIS

0.17gm PMSF (#P7626 Sigma Chemical Co)

5mg Leupeptin (#L2884 Sigma Chemical Co)

5mg Aprotinin (#A4529 Sigma Chemical Co)

5mls DMSO

Mix well, aliquot and store @ -20^0C

TrisBase 1.5M pH 8.8

90.75gm Trisbase

Dissolve in 400mls milliQ H_2O pH 8.8

Complete to 500mls filter through 0.22μM

TrisBase 0.5M pH 6.8

6.07gm Trisbase

Dissolve in 70mls milliQ H_2O pH 6.8

Complete to 100mls filter through 0.22μM

Ammonium persulphate

Aliquot 0.1gm and add 1ml milliQ H_2O

Acrylamide (30%, 0.5) 500ml

150gm acrylamide

2.5gm bis-acrylamide

Complete to 500mls with milliQ H_2O, filter 0.22μM

Store in brown bottle @ 4^0C

Running buffer (glycine buffer 10x)

60.6gm Trisbase

288.2gm glycine

20gm SDS

Make to 2L with milliQ H_2O

Transfer buffer

15gm TrisBase (final concentration 25mM)

72gm glycine (192mM)

1000mls methanol (20% v/v)

Mix TrisBase and glycine in 2L RO H_2O (pH 8.3), add methanol and complete to 5L with RO H_2O. Store at 4^0C, use three times only.

Tris Buffered Saline (TBS) (10x, 1L)

111gm NaCl

39.4gm Tris HCl

Complete to 1L with milliQ H_2O

TBS-Tween

1L TBS 10x

10L milliQ H_2O

15mls Tween 20

6.5mls NaOH 10M

pH 7.6-8.0

Ponceau Red (0.5%, 500ml)

2.5gm Ponceau S

5mls acetic acid

Make to 500mls with milliQ H_2O and mix well

Stripping buffer for membranes

100mM β-ME

2% SDS

6.25mM Tris HCl pH 6.7

Soak membrane @50^0C for 30 minutes on rocker

Gel preparation

Clean glass plates with 70% ethanol and dry, assemble glass plates with spacers and clamp. Place glass plates into electrophoresis unit (Hoeffer SE400) and secure.

Large resolving gel

8.8mls milliQ H_2O

5mls 1.5M Tris base pH 8.8

6mls acrylamide stock

Degas and then add

200µL 10% SDS

75µL 10% ammonium persulphate

25µL TEMED

Mix gently and pour gel to 4-5cm below top of glass plate. Overlay with isobutanol and allow to set 45-60 minutes. Wash x3 with milliQ H_2O and dry. Insert comb.

Stacking gel

5.7mls milliQ H_2O

2.3mls 0.5M Tris base pH 6.8

1.7mls acrylamide stock

degas and then add

100µL 10% SDS

30µL ammonia persulphate

20µL TEMED

Mix and pour gel. Allow to set 45-60 minutes.

Electrophoresis

Remove comb and wash x3 with milliQ H_2O, dry wells. Assemble glass plates into back of electrophoresis unit. Add 300mls running buffer to lower chamber of unit. Load 10μL of Biorad Broad Range Standard (#161-0318) to 1st lane. Add samples (50μL for monocytes and T cells, 95μL for synoviocytes) to lanes. Top up wells with running buffer. Place upper part of unit onto glass plates and fill with remaining running buffer. Secure lid of unit and run at 16mAmp constant current until samples reach separating gel, then run at 160 volts until tracking dye reaches bottom of gel.

Protein transfer

Cut an appropriate sized piece of PVDF membrane and rinse in methanol. Equilibrate membrane in transfer buffer. Stop electrophoresis and remove gel from between plates. Assemble apparatus for transfer – black cage (-), 2 filter paper pads, gel, PVDF membrane, 1 filter paper pad and grey cage (+). Place in transfer unit (black to black) and transfer at 300mAmps, 4^0C, with constant stirring for at least 16 hours.

Probing membrane

Prepare blocking buffer (2.5gm skim milk powder in 50mls TBS-Tween). Stop transfer and remove PVDF membrane, discard gel. Place membrane in Ponceau red to visualize protein bands. Wash excess dye away with milliQ H_2O. Label membrane, makers, and lanes with red pen and cut off excess membrane. Block membrane in blocking buffer for 1 hour. Rinse briefly with TBS-Tween. Soak in primary antibody for appropriate time.

Wash with TBS-Tween 1 hour. Soak in secondary antibody 1 hour. Wash with TBS-Tween 1 hour.

ECL detection

Combine 6mls of each of the two ECL solutions and soak membrane for 10 minutes. Remove membrane and wrap in cling film before placing inside file cassette. In dark, place film into cassette over membrane and expose for 1-10 minutes.

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Erratum

Reviewer 1

1. The number of experiments has been added to the text legends. IN the case of page 126 activated T cells from 20 different donors were examined for the presence of COX-2 and this has been added to the text.

Figure 4.6 The findings shown are from a single experiment that is representative of three separate experiments.

Figure 4.7 The findings shown are from a single experiment that is representative of two separate experiments.

Figure 4.8 The findings shown are from a single experiment that is representative of three separate experiments.

Figure 4.12 The findings shown are from a single experiment that is representative of two separate experiments.

Figure 5.1 The findings shown are from a single experiment that is representative of two separate experiments.

Figure 5.2 The findings shown are from a single experiment that is representative of two separate experiments.

Figure 5.3 The findings shown are from a single experiment that is representative of three separate experiments.

Figure 5.6 The findings shown are from a single experiment that is representative of three separate experiments.

Figure 5.7 The findings shown are from a single experiment that is representative of two separate experiments.

Figure 5.8 The findings shown are from a single experiment that is representative of two separate experiments.

Figure 5.9 The findings shown are from a single experiment that is representative of three separate experiments.

Figure 5.10 The findings shown are from a single experiment that is representative of three separate experiments.

Figure 5.11 Data from one experiment representative of three is shown.

Figure 5.12 Data from one experiment representative of two is shown

Figure 6.4 The findings shown are from a single experiment that is representative of three separate experiments.

Figure 6.5 The data shown are from one experiment, representative of four separate experiments using monocytes from different donors.

Figure 6.7 The data shown are representative of 4 separate experiments using monocytes from different donors.

Figure 6.9 The data shown are from one experiment, representative of 2 separate experiments using monocytes from different donors.

Figure **6.10** The data shown are from one experiment, representative of 2 separate experiments using monocytes from different donors.

Figure 6.11 The data shown are from one experiment, representative of four separate experiments using monocytes from different donors.

Figure 6.12 The results shown are representative of 2 separate experiments.

Figure 6.13 The results shown are representative of 4 separate experiments.

Figure 7.2 Data shown are representative of 3 separate experiments using synoviocytes from 3 different donors.

Figure 7.3 Data shown are representative of 3 separate experiments using synoviocytes from 3 different donors.

Figure 7.4 Data shown are representative of 4 separate experiments using T cells from 4 different donors and fibroblast like synoviocytes from 4 different donors.

Figure 7.8 Data shown are representative of 4 separate experiments using cells from different donors.

Figure 7.9 Data shown are representative of two separate experiments using T cells from 2 different donors and fibroblast like synoviocytes from 2 different donors.

Figure 7.10 Data shown are representative of two separate experiments using T cells from 2 different donors and fibroblast like synoviocytes from 2 different donors.

Figure 7.12 The results of one experiment, which are representative of three are shown.

2. Page 69 Table 1.7 – the stated IL-10 levels has been corrected to high rather than low.
3. Page 87 - the second to last word "of" in line 6 has been removed.
4. Page 138 – using the available antibody I was unable to detect PGE synthase within human peripheral blood T cells. However, this antibody is poor and in our laboratory has previously been unable to detect PGE synthase in monocytes, which produce large amounts of PGE_2. Thus the inability of the antibody to detect PGE synthase does not necessarily mean PGE synthase is not present in T cells. The text on page 138 has been amended to make this clear.
5. Page 152 – given that indocid inhibits COX-1 and COX-2 it results reduced production of TXB2. As the reviewer states this may be a mechanism by which indocid stops adjuvant arthritis. This is worthy of further investigation.

6. Page 160 Fig 5.5 – I agree that the numbers for T cell proliferation are low however, these numbers are correct.
7. Page 166 line 15 – I agree that $cTXA_2$ is inhibitory even at low doses. This has been corrected in the text.

Reviewer 2

- The statistical tests employed were parametric. The bars on all graphs represent standard error of the mean (SEM) unless otherwise stated.