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**P2X<sub>7</sub>, INFLAMMATION AND  
GASTROINTESTINAL DISEASE**

**By**

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# CONTENTS

Page  
number

ABSTRACT

ACKNOWLEDGEMENTS

PUBLICATIONS

ABBREVIATIONS LIST

LIST OF FIGURES

LIST OF TABLES

## CHAPTER 1. INTRODUCTION

1.1. INFLAMMATORY BOWEL DISEASE	1
1.2. EXISTING AND POTENTIAL NEW TREATMENTS FOR IBD	5
1.2.1. Anti-inflammatory drugs	7
1.2.1.1. <i>Aminosalicylates</i>	
1.2.1.2. <i>Corticosteroids</i>	
1.2.2. Immunosuppressant drugs	7
1.2.2.1. <i>Thiopurines</i>	
1.2.2.2. <i>Methotrexate</i>	
1.2.2.3. <i>Cyclosporin</i>	
1.2.3. Anti-cytokine therapy	8
1.2.3.1. <i>Anti-TNF drugs</i>	
1.2.3.2. <i>Other anti-cytokines</i>	
1.2.4. Regulatory cytokine therapy	10
1.2.5. Other potential therapies	11
1.2.5.1. <i>Adhesion molecules</i>	
1.2.5.2. <i>Galectin-2</i>	
1.2.5.3. <i>GM-CSF</i>	
1.2.5.4. <i>Stem cells</i>	
1.2.5.5. <i>Gastrointestinal parasites</i>	
1.3. INFLAMMATION	12
1.4. THE MUCOSAL IMMUNE SYSTEM	13
1.4.1. Immune responses and IBD	14
1.4.2. Cells of the immune system	17
1.4.2.1. <i>Macrophages</i>	
1.4.2.2. <i>Lymphocytes</i>	
1.4.2.3. <i>Dendritic cells</i>	
1.5. CYTOKINES	24
1.5.1. Proinflammatory cytokines in IBD	24
1.5.2. Immunoregulatory cytokines in IBD	26
1.5.3. Chemokines	31

1.6. THE INTERLEUKIN-1 FAMILY	32
1.6.1. Cleavage of IL-1 $\beta$	35
1.6.2. Secretion of IL-1 $\beta$	38
1.7. PURINE RECEPTORS	41
1.7.1. P2X <sub>7</sub>	49
1.8. AIMS AND OBJECTIVES	56
<b>CHAPTER 2. GENERAL MATERIALS AND METHODS</b>	
2.1. CELL CULTURE AND ISOLATION OF PRIMARY CELLS	58
2.1.1. THP-1 cell culture	58
2.1.2. Isolation of peripheral blood mononuclear cells (PBMCs)	59
2.1.3. Isolation of lamina propria mononuclear cells (LPMCs)	59
2.1.4. Assessment of cell viability	60
2.2. FLOW CYTOMETRY	61
2.2.1. Use of flow cytometry to measure apoptosis and cell death	63
2.3. P2X <sub>7</sub> -STIMULATED INTERLEUKIN (IL)-1 $\beta$ RELEASE	64
2.4. DATA ANALYSIS	65
2.7.1. Analysis of flow cytometry dotplots	65
2.7.2. Analysis of cell cycle histograms	66
2.7.3. Statistical analysis	69
<b>CHAPTER 3. P2X<sub>7</sub> RECEPTOR PORE FORMATION TO DEMONSTRATE THE PRESENCE OF FUNCTIONAL RECEPTORS</b>	
3.1. INTRODUCTION	70
3.2. METHODS	71
3.3. RESULTS AND DISCUSSION	74
3.3.1. EB uptake in THP-1 cells	77
3.3.2. EB uptake in PBMCs	81
3.3.3. EB uptake in LPMCs	83
3.4. SUMMARY OF P2X <sub>7</sub> -STIMULATED EB UPTAKE	85
<b>CHAPTER 4. P2X<sub>7</sub> RECEPTOR STIMULATION: EFFECT ON IL-1<math>\beta</math> RELEASE AND ANNEXIN V BINDING</b>	
4.1. INTRODUCTION	90
4.2. P2X <sub>7</sub> RECEPTOR-STIMULATED IL-1 $\beta$ RELEASE	91
4.2.1. Introduction	91
4.2.2. Method	92
4.2.3. Results and Discussion	94
4.2.3.1. <i>IL-1<math>\beta</math> release from THP-1 cells</i>	
4.2.3.2. <i>IL-1<math>\beta</math> release from PBMCs</i>	
4.2.3.3. <i>IL-1<math>\beta</math> release from LPMCs</i>	



4.2.3.4. <i>Summary of P2X<sub>7</sub>-stimulated IL-1<math>\beta</math> release</i>	
4.3. P2X <sub>7</sub> RECEPTOR-STIMULATED ANNEXIN V BINDING	107
4.3.1. Introduction	107
4.3.1.1. <i>Cell death</i>	
4.3.1.2. <i>Phosphatidylserine (PS) flip</i>	
4.3.2. Method	112
4.3.3. Results and Discussion	1113
4.3.3.1. <i>AV binding in THP-1 cells</i>	
4.3.3.2. <i>AV binding in PBMCs</i>	
4.3.3.3. <i>AV binding in LPMCs</i>	
4.3.3.4. <i>Summary of P2X<sub>7</sub>-stimulated AV binding</i>	
4.4. THE FUNCTIONAL CHARACTERISATION OF THE P2X <sub>7</sub> RECEPTOR	121
<b>CHAPTER 5. P2X<sub>7</sub> RECEPTOR STIMULATION: TIME COURSE OF RESPONSE</b>	
5.1. INTRODUCTION	126
5.2. TIME COURSE OF P2X <sub>7</sub> -STIMULATED AV-BINDING	128
5.2.1. Method	129
5.2.2. Results and Discussion	130
5.2.2.1. <i>Time course of AV binding in THP-1 cells</i>	
5.2.2.2. <i>Time course of AV binding in PBMCs</i>	
5.2.2.3. <i>Time course of AV binding in LPMCs</i>	
5.2.3. Summary of AV binding	142
5.3. USE OF TOTO-3 IODIDE TO INVESTIGATE CELL DEATH IN PBMCs	146
5.3.1. Introduction	146
5.3.2. Method	146
5.3.3. Results and Discussion	149
5.4. LDH RELEASE FROM P2X <sub>7</sub> -STIMULATED CELLS AS A MEASURE OF CELL DEATH	152
5.4.1. Introduction	152
5.4.2. Method	152
5.4.3. Results and Discussion	153
5.5. TIME COURSE OF P2X <sub>7</sub> -STIMULATED IL-1 $\beta$ RELEASE	156
5.5.1. Method	156
5.5.2. Results and Discussion	156
5.5.2.1. <i>Time course of IL-1<math>\beta</math> release from PBMCs</i>	
5.5.2.2. <i>Time course of IL-1<math>\beta</math> release from LPMCs</i>	
5.5.3. Summary of IL-1 $\beta$ release	162
5.6. SUMMARY OF THE EFFECT OF P2X <sub>7</sub> RECEPTOR STIMULATION TIME ON CELL DEATH AND IL-1 $\beta$ RELEASE	163

## **CHAPTER 6. P2X<sub>7</sub> RECEPTOR STIMULATION: REVERSIBILITY OF RESPONSE**

6.1. INTRODUCTION	166'
6.2. METHODS	167
6.2.1. Reversibility of P2X <sub>7</sub> -induced AV binding	167
6.2.2. Release of LDH from cells stimulated for 5 or 30 minutes	168
6.2.3. P2X <sub>7</sub> -stimulated apoptosis determined by cell cycle analysis	168
6.2.4. P2X <sub>7</sub> -stimulated apoptosis determined by Hoechst staining	169
6.2.5. IL-1 $\beta$ release from cells stimulated for 5 or 30 minutes	170
6.3. RESULTS AND DISCUSSION	170
6.3.1. Reversibility of P2X <sub>7</sub> -induced AV binding	170
6.3.1.1. <i>Reversibility of AV binding in THP-1 cells</i>	
6.3.1.2. <i>Reversibility of AV binding in PBMCs</i>	
6.3.1.3. <i>Reversibility of AV binding in LPMCs</i>	
6.3.2. Release of LDH from cells stimulated for 5 or 30 minutes	176
6.3.3. P2X <sub>7</sub> -stimulated apoptosis determined by cell cycle analysis	179
6.3.4. P2X <sub>7</sub> -stimulated apoptosis determined by Hoechst staining	181
6.3.5. IL-1 $\beta$ release from cells stimulated for 5 or 30 minutes	183
6.3.5.1. <i>IL-1<math>\beta</math> release after 5 minutes stimulation</i>	
6.3.5.2. <i>IL-1<math>\beta</math> release after 30 minutes stimulation</i>	
6.4. SUMMARY OF THE REVERSIBILITY OF THE EFFECTS OF P2X <sub>7</sub> STIMULATION	188
6.4.1. P2X <sub>7</sub> -mediated cell death	190

## **CHAPTER 7. EFFECTS OF P2X<sub>7</sub> ANTAGONISTS ON IL-1 $\beta$ RELEASE**

7.1. INTRODUCTION	194
7.1.1. Current P2X <sub>7</sub> antagonists	196
7.1.1.1. <i>oATP</i>	
7.1.1.2. <i>KN62</i>	
7.1.1.3. <i>Other P2X<sub>7</sub> antagonists</i>	
7.1.2. Novel P2X <sub>7</sub> antagonists	202
7.2. EFFECT OF P2X <sub>7</sub> ANTAGONISTS ON SPONTANEOUS IL-1 $\beta$ RELEASE FROM COLONIC MUCOSAL BIOPSIES	204
7.2.1. Introduction	204
7.2.2. Method	205
7.2.3. Results	205
7.2.3.1. <i>IL-1<math>\beta</math> release from biopsies from normal tissue</i>	
7.2.3.2. <i>IL-1<math>\beta</math> release from biopsies from inflamed tissue</i>	
7.2.4. Discussion	207
7.3. EFFECT OF P2X <sub>7</sub> ANTAGONISTS ON P2X <sub>7</sub> -STIMULATED IL-1 $\beta$ RELEASE FROM ISOLATED LPMCs	209

7.3.1. Method	209
7.3.2. Effect of P2X <sub>7</sub> antagonists on IL-1 $\beta$ release from LPMCs isolated from normal tissue	210
7.3.2.1. <i>Results</i>	
7.3.2.2. <i>Discussion</i>	
7.3.3. Effect of P2X <sub>7</sub> antagonists on IL-1 $\beta$ release from LPMCs isolated from inflamed tissue	213
7.3.3.1. <i>Results</i>	
7.3.3.2. <i>Discussion</i>	
7.3.4. Summary and Conclusion	220
<b>CHAPTER 8. DISCUSSION AND FUTURE WORK</b>	
8.1. SUMMARY	222
8.1.1. Characteristics of P2X <sub>7</sub> -stimulated IL-1 $\beta$ release	223
8.1.2. Characteristics of P2X <sub>7</sub> -stimulated PS flip and cell death	226
8.2. CONCLUSIONS AND FUTURE WORK	227
REFERENCES	232

## **ABSTRACT**

The inflammatory bowel diseases, ulcerative colitis and Crohn's disease are characterised by spontaneously relapsing and remitting inflammation, associated with increased mucosal levels of the inflammatory cytokine, interleukin-1 (IL-1) $\beta$ . IL-1 $\beta$  processing and release is mediated by ATP-stimulation of the purine receptor, P2X<sub>7</sub>. P2X<sub>7</sub> is a membrane ion channel highly expressed in immune cells. Signal transduction occurs via rapid cation exchange, plasma membrane depolarisation and increased intracellular calcium. Additionally, prolonged or repeated P2X<sub>7</sub> stimulation leads to formation of a non-selective membrane pore permeable to small molecules, and ultimately to cell death.

The aim of this project was to investigate the properties of the P2X<sub>7</sub> receptor in mononuclear cells, to show that it is associated with IL-1 $\beta$  release in the colon, and that this release can be modified by P2X<sub>7</sub> antagonists. Studies of ethidium bromide uptake, a functional assay, showed that P2X<sub>7</sub> receptors are present on LPMCs and displayed properties similar to those of PBMCs and THP-1 cells. P2X<sub>7</sub> receptor-stimulation released mature IL-1 $\beta$  from LPMCs in a dose-dependent manner that, in IBD patients, matched the severity of their inflammation, and could be markedly reduced by P2X<sub>7</sub> antagonists.

P2X<sub>7</sub> stimulation also results in increased exposure of phosphatidylserine on the outer cell membrane (PS flip), often considered to be a marker of apoptotic cell death. P2X<sub>7</sub>-stimulated PS flip however is reversible and is not associated with cell death following brief stimulation times. Cell death caused by longer stimulation did not have features of apoptosis, was more evident in monocytes than lymphocytes, with LPMCs being less susceptible than PBMCs and THP-1 cells.

These studies have shown that the P2X<sub>7</sub> receptor is intimately involved in the release of IL-1 $\beta$  from human colonic mononuclear cells, that the release is greater in cells from IBD tissue and can be markedly inhibited by P2X<sub>7</sub> antagonists.

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Stevenson, D. J., Lawson, M., Braddock, M., Bowers, K. & Hawkey, C. J. (2002) Purinergic stimulation of the P2X<sub>7</sub> receptor is associated with reversible PS-flip in human PBMCs. *Gut*, **51**, (Suppl III), A293.

Stevenson, D. J., Li, C. K. & Hawkey, C. J. (2003) P2X<sub>7</sub> receptor-mediated release of interleukin-1 $\beta$  (IL-1 $\beta$ ) from human colon and peripheral blood mononuclear cells (PBMCs). *Gut*, **52**, (Suppl VI), A187.

Posters presented at the United European Gastroenterology Week

Stevenson, D., Lawson, M., Braddock, M., Bowers, K. & Hawkey, C. J. (2003) Reversible phosphatidylserine flip and interleukin-1 $\beta$  release in human blood and lamina propria mononuclear cells. *Gastroenterology*, **124**, (4 Suppl 1), A156.

Stevenson, D. J., Bowers, K., Lawson, M. & Hawkey, C. J. (2007) Lamina propria mononuclear cell P2X<sub>7</sub> receptor as an anti-inflammatory target in IBD. *Gastroenterology*, **132**, (4 Suppl 2), A696.

Posters presented at the American Gastroenterological Association

## **ABBREVIATIONS**

<b>APC</b>	<b>Allophycocyanin</b>
<b>APCs</b>	<b>Antigen-presenting cells</b>
<b>5-ASA</b>	<b>5-aminosalicylic acid</b>
<b>ATP</b>	<b>Adenosine tri-phosphate</b>
<b>AV</b>	<b>Annexin V</b>
<b>BzATP</b>	<b>2'-3'-O-(4-benzoylbenzoyl)-adenosine 5'-triphosphate</b>
<b>CD</b>	<b>Crohn's disease</b>
<b>CD3, CD14</b>	<b>Cluster of differentiation markers</b>
<b>Da</b>	<b>Dalton</b>
<b>DCs</b>	<b>Dendritic cells</b>
<b>DTT</b>	<b>Dithiothreitol</b>
<b>EB</b>	<b>Ethidium bromide</b>
<b>EDTA</b>	<b>Ethylenediaminetetraacetic acid</b>
<b>FCS</b>	<b>Foetal calf serum</b>
<b>FITC</b>	<b>Fluorescein isothiocyanate</b>
<b>FS</b>	<b>Forward scatter</b>
<b>GALT</b>	<b>Gut-associated lymphoid tissue</b>
<b>HEK293 cells</b>	<b>Human embryonic kidney cells commonly used in P2X<sub>7</sub> transfection</b>
<b>HBSS</b>	<b>Hanks balanced salt solution</b>
<b>IBD</b>	<b>Inflammatory bowel disease</b>
<b>IBS</b>	<b>Irritable bowel syndrome</b>
<b>IFN-<math>\gamma</math></b>	<b>Interferon-<math>\gamma</math></b>
<b>KN62</b>	<b>1-[N,O-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine</b>
<b>IL-(1)</b>	<b>Interleukin-(1)</b>
<b>IL-1Ra</b>	<b>IL-1 receptor antagonist</b>
<b>LDH</b>	<b>Lactate dehydrogenase</b>
<b>LPMCs</b>	<b>Lamina propria mononuclear cells</b>
<b>LPS</b>	<b>Lipopolysaccharide</b>
<b>MAPK</b>	<b>Mitogen activated protein kinase</b>

NF- $\kappa$ B	Nuclear transcription factor
NK cells	Natural killer cells
NOD2	One of a family of intracellular pattern recognition receptors that is genetically linked to CD
oATP	periodate-oxidised ATP
PAMP	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PI	Propidium iodide
PMA	Phorbol-12-myristate-13-acetate potent tumour promoter, activates protein kinase C
P5P	Pyridoxal-5-phosphate
PPADS	Pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid
PRR	Pattern recognition receptor
PS	Phosphatidylserine
SS	Side scatter
T cells	T lymphocytes: Th - helper T cells; Tr - regulatory T cells
THP-1	Mononuclear cell line expressing P2X <sub>7</sub>
TLR4	Toll-like receptor 4
TNF	Tumour necrosis factor
UC	Ulcerative colitis
YO-PRO, TOTO-3	Fluorescent cyanine dyes



## **List of Figures**

	<b><u>Page no.</u></b>
Fig 1.1. Anatomic distribution of ulcerative colitis and Crohn's disease	2
Fig 1.2. The appearance of normal, CD and UC mucosa: diagrammatic, histological and endoscopic views	3
Fig 1.3. Antigen uptake in the intestine	15
Fig 1.4. Systemic effects of IL-1 $\beta$	27
Fig 1.5. Regulatory cytokines involved in the development of Th lymphocyte populations and the inflammatory response	29
Fig 1.6. The role of the inflammasome in activation of caspase-1 and release of mature IL-1 $\beta$	37
Fig 1.7. Proposed pathways for P2X <sub>7</sub> -stimulated IL-1 $\beta$ cleavage and release	40
Fig 1.8. Schematic representation of the P2Y and P2X receptors	44
Fig 1.9. ATP release and its effects on purine receptors	48
Fig 1.10. Proposed relationship between P2X <sub>7</sub> stimulation, caspase-1 maturation and IL-1 $\beta$ release	51
Fig 1.11. Structure of the principal P2X <sub>7</sub> agonists	53
Fig 1.12. Schematic representation of the signalling complex for the rat P2X <sub>7</sub> receptor expressed in HEK293 cells	55
Fig 2.1. Simplified schematic of the Altra flow cytometer	62
Fig 2.2. Flow cytometric analysis of PBMCs and LPMCs	67
Fig 2.3. Analysis of THP-1 cell cycle data using WinMDI and Cylchred	68
Fig 3.1. Forward Scatter (FS)/Side Scatter (SS) dotplots showing cell shrinkage on stimulation	75
Fig 3.2. An example of EB dotplots for LPMCs	76
Fig 3.3. EB uptake in mononuclear cells stimulated with BzATP or ATP	79
Fig 3.4. The percentage of the total cell population contained within the 'live-gates'	80
Fig 3.5. Subjects showing a lack of response for P2X <sub>7</sub> -stimulated EB uptake in mononuclear cells stimulated with BzATP or ATP	86
Fig 4.1. The principal pathways of LPS signal transduction	93
Fig 4.2. The effect of FCS on the release of IL-1 $\beta$ from THP-1 cells stimulated with BzATP	96
Fig 4.3. The release of IL-1 $\beta$ from PBMCs stimulated with BzATP or ATP	98
Fig 4.4. The release of IL-1 $\beta$ from LPMCs stimulated with BzATP or ATP	100
Fig 4.5. The release of IL-1 $\beta$ from LPMCs isolated from inflamed tissue	102
Fig 4.6. The release of pro-IL-1 $\beta$ from LPMCs isolated from inflamed tissue	104
Fig 4.7. The release of IL-18 from LPMCs isolated from severely inflamed tissue	105
Fig 4.8. The two major apoptotic pathways in mammalian cells	110
Fig 4.9. The generation of PS asymmetry in the cell membrane	111
Fig 4.10. Flow cytometry dotplots for AV/PI binding in lymphocyte- and monocyte-gated PBMCs	114

Fig 4.11. Annexin V binding in mononuclear cells stimulated with BzATP or ATP	116
Fig 4.12. Comparison of BzATP-stimulation of AV binding in PBMCs stimulated for 5 or 20 minutes	118
Fig 4.13. A pictorial comparison of EC <sub>50</sub> values for EB uptake, AV binding and IL-1 $\beta$ release in mononuclear cells stimulated with BzATP or ATP	122
Fig 4.14. Effect of cell number on IL-1 $\beta$ release from LPMCs isolated from normal tissue	125
Fig 5.1. Time course of AV/PI binding in THP-1 cells	131
Fig 5.2. The effect of stimulation time on the unstained population of THP-1 cells	132
Fig 5.3. Time course of AV/PI binding in lymphocyte-gated PBMCs	134
Fig 5.4. Time course of AV/PI binding in monocyte-gated PBMCs	136
Fig 5.5. The effect of stimulation time on the unstained populations of lymphocyte- and monocyte-gated PBMCs	138
Fig 5.6. Time course of AV/PI binding in lymphocyte-gated LPMCs	140
Fig 5.7. Time course of AV/PI binding in monocyte-gated LPMCs	141
Fig 5.8. Changes in the number of cells contained within the gates for the lymphocyte and monocyte populations of PBMCs and LPMCs	143
Fig 5.9. Effect of BzATP stimulation on CD14 staining of PBMC monocytes	147
Fig 5.10. Emission spectra and dotplots of TOTO-3 and PI or EB fluorescence	148
Fig 5.11. Measurement of cell death in BzATP-stimulated PBMCs using EB, PI and TOTO-3	150
Fig 5.12. The release of LDH enzyme from mononuclear cells stimulated with BzATP or ATP	154
Fig 5.13. Time course of IL-1 $\beta$ release from PBMCs	157
Fig 5.14. Time course of IL-1 $\beta$ release from LPMCs	159
Fig 5.15. Comparison of the effects of K <sub>2</sub> ATP or Na <sub>2</sub> ATP as a P2X <sub>7</sub> agonist in LPMCs	161
Fig 6.1. Reversibility of AV binding in THP-1 cells	172
Fig 6.2. Reversibility of AV binding in PBMCs	173
Fig 6.3. Reversibility of AV binding in LPMCs	175
Fig 6.4. Release of LDH from mononuclear cells stimulated with 500 $\mu$ M BzATP for 5 or 30 minutes	177
Fig 6.5. Induction of apoptosis in BzATP-stimulated mononuclear cells, determined by cell cycle analysis	180
Fig 6.6. Induction of apoptosis in BzATP-stimulated mononuclear cells, determined by Hoechst staining	182
Fig 6.7. Release of IL-1 $\beta$ from PBMCs stimulated with BzATP for 5 or 30 minutes	184
Fig 6.8. Release of IL-1 $\beta$ from LPMCs stimulated with BzATP for 5 or 30 minutes	185
Fig 6.9. P2X <sub>7</sub> -stimulated mechanisms of cell death	193
Fig 7.1. P2 receptor antagonists	197

Fig 7.2. P2X <sub>7</sub> receptor antagonists	198
Fig 7.3. Novel P2X <sub>7</sub> receptor antagonists	203
Fig 7.4. Effect of P2X <sub>7</sub> antagonists on IL-1 $\beta$ release from colonic mucosal biopsies maintained in organ culture	206
Fig 7.5. Effect of P2X <sub>7</sub> antagonists on IL-1 $\beta$ release from LPMCs isolated from normal tissue	211
Fig 7.6. Effect of P2X <sub>7</sub> antagonists, oATP and AZ10603690, on IL-1 $\beta$ release from LPMCs isolated from inflamed tissue	215
Fig 7.7. Effect of oATP on IL-1 $\beta$ release from PBMCs stimulated with 200 $\mu$ M BzATP	216
Fig 7.8. Effect of AZ10573295 on IL-1 $\beta$ release from LPMCs isolated from inflamed tissue	218

**List of Tables****Page no.**

Table 1.1.	Current treatments for UC and CD	6
Table 1.2.	Characteristics of macrophage subpopulations	18
Table 1.3.	Characteristics of lymphocyte cell populations	21
Table 1.4.	Characteristics and development of dendritic cell populations	23
Table 1.5.	Regulatory cytokines associated with the mucosal immune response	30
Table 1.6.	Chemokines shown to be increased or induced in tissue from IBD patients	33
Table 1.7.	Distribution and properties of P1 receptors	43
Table 1.8.	Agonist profiles and tissue distribution of human P2Y receptors	45
Table 1.9.	Agonist profiles and tissue distribution of P2X receptors	47
Table 1.10.	Effects of P2X <sub>7</sub> receptor stimulation on immune cells	50
Table 3.1.	Examples of published data for P2X <sub>7</sub> -stimulated pore formation measured by ethidium bromide uptake	71
Table 3.2.	Summary of results for P2X <sub>7</sub> -stimulated ethidium bromide uptake	78
Table 4.1.	Summary of results for P2X <sub>7</sub> -stimulated IL-1 $\beta$ release	95
Table 4.2.	Summary of results for P2X <sub>7</sub> -stimulated total AV-positive binding	115
Table 5.1.	Examples of published data for ATP-stimulated cell death	127
Table 7.1.	Examples of P2X <sub>7</sub> receptor-mediated effects that have been inhibited by oATP and KN62	199
Table 7.2.	Effect of P2X <sub>7</sub> antagonists on IL-1 $\beta$ release from LPMCs isolated from inflamed tissue	214

# CHAPTER 1. INTRODUCTION

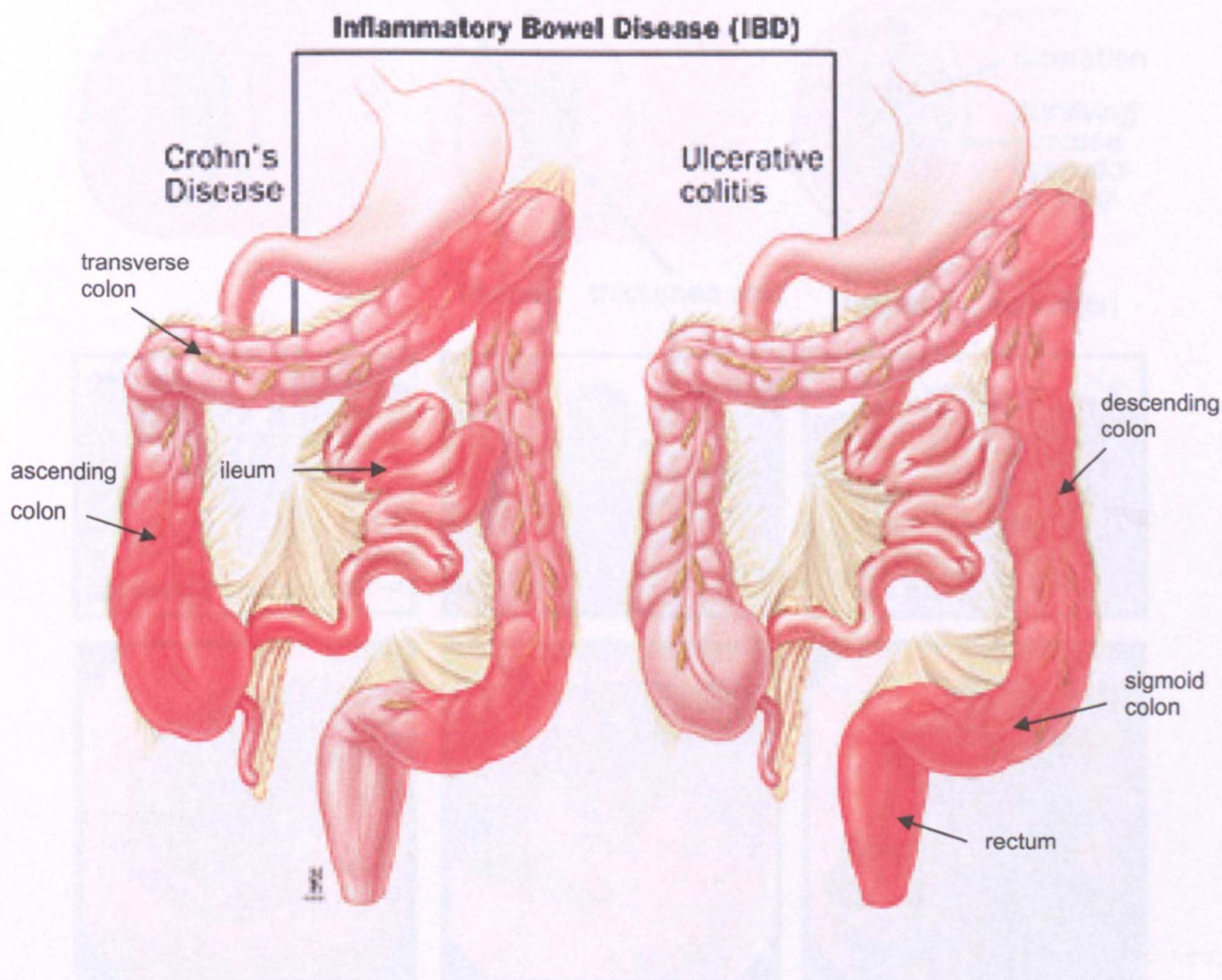
## 1.1. INFLAMMATORY BOWEL DISEASE

Inflammatory Bowel Disease (IBD) is a heterogeneous group of diseases with some common causative factors but different clinical manifestations in terms of disease behaviour, location and response to treatment (Cummings, JRF & Jewell, DP, 2005). The two principal disorders are ulcerative colitis (UC) and Crohn's disease (CD), both characterised by spontaneously relapsing and remitting inflammation of the intestinal tract.

The predominant symptom in UC is bloody diarrhoea, but patients may also suffer from abdominal or rectal pain, fever and weight loss (Harris, M *et al.*, 2006). UC is exclusively confined to the colon and rectum (Fig 1.1.), but the extent of the disease can involve the rectum only (proctitis), the left side of the colon (left-sided or distal colitis), or the entire colon (pancolitis). UC primarily affects the superficial mucosa; exhibiting ulceration, oedema and haemorrhage of varying severity. Some of the characteristics of UC mucosa are shown in Fig 1.2.

Crohn's disease is more variable and may occur anywhere in the gastrointestinal tract, but is most common in the ileum and colon (Fig 1.1.). Unlike UC, ulceration is patchy but involves chronic inflammation of all layers of the intestinal wall, often resulting in the formation of fistulas between loops of the bowel or to the skin (Fig 1.2.). Common symptoms are abdominal pain, diarrhoea, weight loss, fever, anaemia and perianal disease (Bayless, T *et al.*, 2006). A feature of both diseases is the infiltration of inflammatory cells into the gut wall. Acute ulcerative colitis is particularly marked by high numbers of neutrophils that often migrate into the crypt lumen to form micro-abscesses (McKaig, B & Mahida, YR, 2000).

The geographical incidence of IBD varies considerably (Lakatos, PL, 2006). In general, the highest incidence rates (frequency of new cases) and prevalence for both CD and UC are found in Northern and Western Europe and North America (Loftus, EVJ, 2004). In these countries, the incidence rates range from 1.5-20.3 cases per 100,000 person-years for UC and 0.7-15.6 cases per 100,000 person-years for CD (Loftus, EVJ, 2004). Prevalence



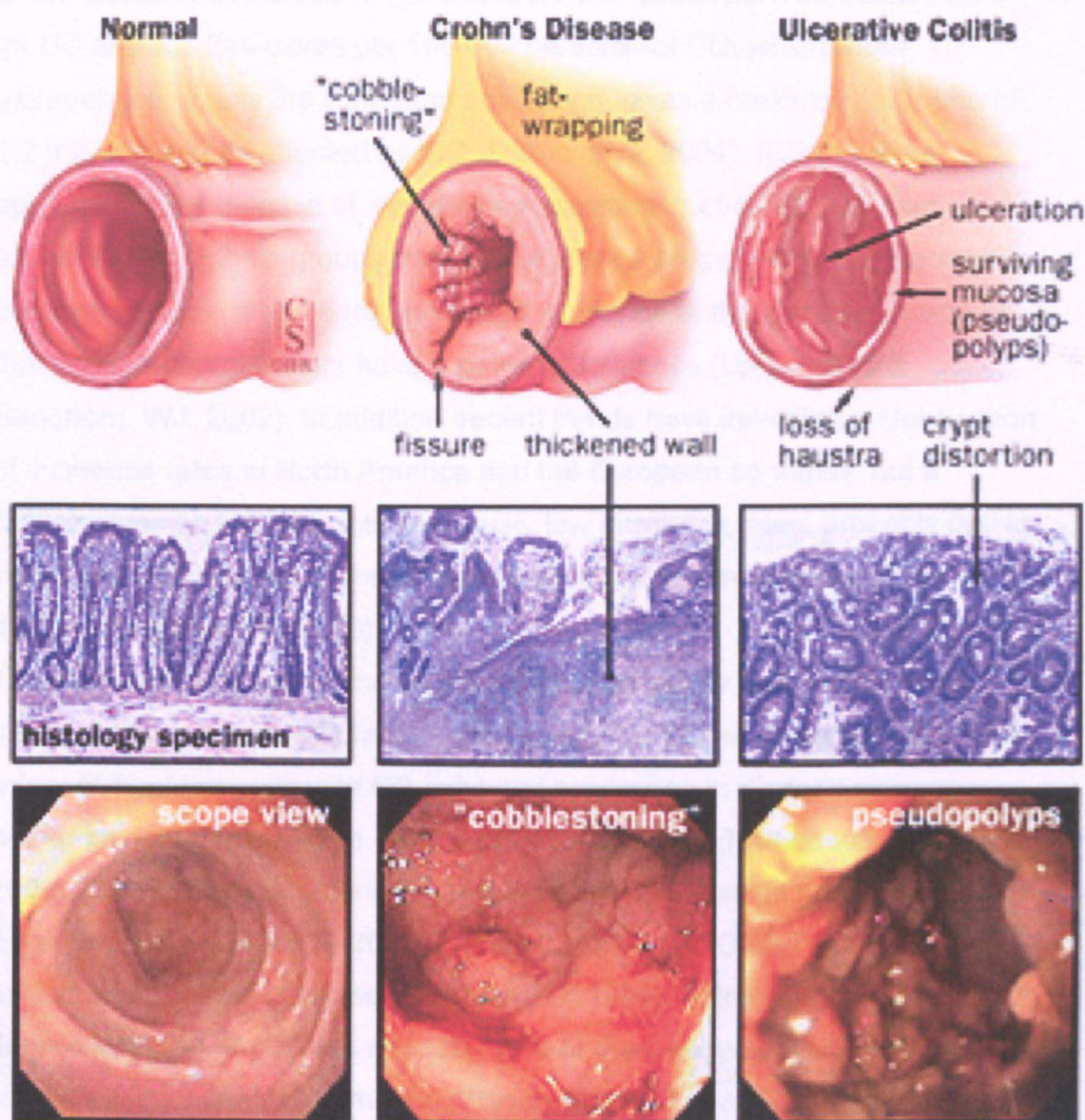
**Fig 1.1. Anatomic distribution of ulcerative colitis and Crohn's disease**

Ulcerative colitis is exclusively confined to the colon and rectum; the diagram shows left-sided colitis involving the rectum, sigmoid colon and descending colon.

Crohn's disease may occur anywhere in the gastrointestinal tract (indicated on the diagram by red patches), but it is most common in the ileum and colon.

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**Fig 1.2. The appearance of normal, CD and UC mucosa: diagrammatic, histological and endoscopic views**

UC exhibits ulceration, oedema and haemorrhage of varying severity, primarily of the superficial mucosa. Severe inflammation can lead to the formation of pseudopolyps, areas of granular tissue that on healing become re-epithelialised and protrude into the lumen.

With CD ulceration is patchy but involves chronic inflammation of all layers of the intestinal wall. The mucosa takes on a "cobblestone" appearance as a result of the deep ulceration and swelling of the surrounding tissue.

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of the diseases in Europe range from 21.4-243 cases per 100,000 persons for UC and 8.3-214 cases per 100,000 persons for CD, which when extrapolated across the European population, gives a maximum estimate of 2.2 million persons affected by IBD (Loftus, EVJ, 2004). IBD therefore appears to be a disease of westernised societies, particularly in urban environments. Ethnic groups from low incidence areas who have migrated to other geographic areas show increased incidence of the disease, indicating that environmental factors have a powerful influence (Loftus, EVJ & Sandborn, WJ, 2002). In addition, recent trends have indicated a stabilisation of incidence rates in North America and the European countries, but a progressive rise in areas with previously low incidence rates, probably due to increased urbanisation of these areas and increased awareness of the disease (Lakatos, PL, 2006).

The causes of IBD are unknown but they are generally believed to result from predisposing genetic factors, particularly for CD. In a study on identical twins, 50% of the pairs with CD exhibited similarities in disease diagnosis and behaviour compared to 18% of the pairs with UC (Halfvarson, J *et al.*, 2003). One of the characteristics of IBD is altered intestinal permeability, and it has been shown that 10-20% of healthy relatives of Crohn's patients also exhibit an abnormal increase (Hollander, D, 1999). In another study on first-degree relatives of patients with CD, 49% of them showed signs of subclinical intestinal inflammation (Thjodleifsson, B *et al.*, 2003).

Over the last few years, genome linkage studies of families affected with IBD identified nine susceptibility loci on different chromosomes (Ahmad, T *et al.*, 2004). Since then, more recent studies have identified further susceptibility genes including the interleukin 23 receptor gene (Tremelling, M *et al.*, 2007) and four novel loci (Parkes, M *et al.*, 2007). The most significant genetic association for CD has been the discovery of genetic polymorphisms of the NOD2/CARD15 gene. NOD proteins are a subset of pattern-recognition receptors which help to regulate the host response to pathogens, and a mutation in the gene has been shown to confer susceptibility to CD (Cho, JH, 2004). NOD2 is expressed mainly by macrophages and dendritic cells and mediates intracellular recognition of MDP (muramyl dipeptide), a building block for bacterial cell walls (Maeda, S *et al.*, 2005). There are three common



genetic variants of NOD2 associated with CD; the relative risk of developing CD from carrying one variant is between 1.5 and 3, increasing to 20-40 in people carrying two variants. NOD2 variants are also associated with disease development, carriers demonstrating younger age at onset, presence of ileal involvement and a tendency to develop strictures (Ahmad, T *et al.*, 2004).

Development of IBD is thought to result from complex interactions between environmental and genetic factors that stimulate an abnormal activation of the mucosal immune system. In healthy people the intestine becomes inflamed in response to a potential pathogen, but then returns to a normal state once the pathogen has been eradicated. However, in individuals with IBD the inflammation is not down-regulated and the mucosal immune system remains chronically activated, leading to chronic inflammation of the intestine (Hanauer, SB, 2006). Other theories about the pathogenesis of IBD have suggested that it may be caused by a dysfunctional immune response to commensal bacteria, or a defective mucosal barrier allowing unrestrained uptake of antigens and proinflammatory molecules (Hendrickson, BA *et al.*, 2002; Sartor, RB, 1997b)

## **1.2. EXISTING AND POTENTIAL NEW TREATMENTS FOR IBD**

Successful treatment of IBD involves the control of active inflammation to obtain clinical remission and then maintaining it for as long as possible. Long-term management is governed by the type and severity of disease (Table 1.1.), and will include drugs to treat symptoms, such as antidiarrhoeal agents, as well as anti-inflammatory agents (Podolsky, DK, 2002). Treatment is generally stepped, adding more potent agents if less active drugs fail to give the required response. Because the inflammation in IBD is thought to be caused by an abnormal immune response, the mainstay of treatment in the past has been anti-inflammatory and immunosuppressant drugs. More recently, a number of drugs have been produced with inflammatory mediators as specific targets.

	Ulcerative colitis	Crohn's Disease
Mild disease	Aminosalicylates	Aminosalicylates Metronidazole Budesonide or Ciprofloxacin
Moderate disease	Aminosalicylates Corticosteroids	Corticosteroids Azathioprine or Mercaptopurine
Severe disease	Corticosteroids Cyclosporine	Corticosteroids Methotrexate Infliximab
Refractory disease	Corticosteroids plus Azathioprine or Mercaptopurine	Infliximab
Remission	Aminosalicylates Azathioprine or Mercaptopurine	Azathioprine or Mercaptopurine, Mesalamine, Metronidazole

**Table 1.1. Current treatments for UC and CD**

Long-term management of IBD requires the use of several different treatments and is governed by the type and severity of disease.

Adapted from Podolsky, DK, (2002)

## **1.2.1. Anti-inflammatory drugs**

### **1.2.1.1. Aminosalicylates**

Aminosalicylates such as sulphasalazine and mesalazine have long been used in UC for maintenance of remission, and are suitable for treatment of mild or moderately active UC and CD. The active moiety is 5-aminosalicylic acid (5-ASA) which is administered in different formulations that govern its site of release. This may depend on time, pH, or bacterial enzymes in the large bowel (Carter, MJ *et al.*, 2004). 5-ASA acts by inhibiting the production of prostaglandins, leukotrienes and inflammatory cytokines, decreasing the activation of the nuclear transcription factor NF- $\kappa$ B, and scavenging reactive oxygen species. 5-ASA has been shown to exert its effects through increased PPAR- $\gamma$  (peroxisome proliferators-activated receptor- $\gamma$ ) expression (Rousseaux, C *et al.*, 2005). PPAR- $\gamma$  is highly expressed in colonic epithelial cells and is a key receptor in the regulation of bacterial-induced inflammation. It acts by reducing NF- $\kappa$ B activity and its expression is highly impaired in patients with UC (Dubuquoy, L *et al.*, 2003).

### **1.2.1.2. Corticosteroids**

In more severe cases of IBD when 5-ASA-based compounds are inadequate, corticosteroids such as prednisolone, hydrocortisone and budesonide are used. Corticosteroids are only used to control acute inflammatory activity and are of no benefit to patients in remission (Malchow, H *et al.*, 1984). They are usually administered for as short a period as possible, due to their serious side effects. Budesonide is a newer corticosteroid which is rapidly metabolised by the liver, reducing its potential for causing systemic side effects (Kane, SV *et al.*, 2002). Corticosteroids act by inhibiting several inflammatory pathways; they suppress interleukin transcription and arachidonic acid metabolism, and stimulate apoptosis of lymphocytes in the lamina propria (Carter, MJ *et al.*, 2004).

## **1.2.2. Immunosuppressant drugs**

Immunosuppressant drugs are used for maintenance, particularly to reduce the need for corticosteroid treatment, but may put the patient at risk of infection as well as having side effects of their own.

### **1.2.2.1. Thiopurines**

Azathioprine and its active metabolite mercaptopurine are effective for both active disease and maintenance of remission in CD and UC (Sandborn, WJ *et al.*, 2000; Pearson, DC *et al.*, 2000). They act by inducing T cell apoptosis by modulating cell signalling (Tiede, I *et al.*, 2003), and have a relatively slow onset of action, requiring 4 to 12 weeks to achieve a therapeutic effect.

Thiopurines need to be monitored carefully since they cause a dose-related suppression of bone marrow leading to leucopenia (Podolsky, DK, 2002).

### **1.2.2.2. Methotrexate**

Methotrexate is used in CD patients who are resistant or intolerant to thiopurines. Its anti-inflammatory effects are thought to be mediated by adenosine, whose release is promoted by methotrexate through inhibition of adenosine deaminase (Cronstein, BN, 1997). Its side effects are initially gastrointestinal (nausea, vomiting, diarrhoea and stomatitis) but it may also lead to hepatotoxicity and pneumonitis (Fraser, AG, 2003).

### **1.2.2.3. Cyclosporin**

Cyclosporin is effective in the management of severe UC in patients who would otherwise undergo colectomy. It is a highly specific inhibitor of T cell activation and prevents clonal expansion of T cell subsets. It acts by inhibiting calcineurin (a protein phosphatase that stimulates growth and differentiation of the T cell response) and blocks signalling pathways triggered by antigen recognition (Matsuda, S & Koyasu, S, 2000). Major complications with the drug are reported in 0-17% of patients and these include renal impairment, infections and neurotoxicity (Durai, D & Hawthorne, AB, 2005).

## **1.2.3 Anti-cytokine therapy**

Cytokines are small soluble proteins released by cells that control other cell behaviour, interaction and communication. They play a central role in the immune system and are involved in a variety of immunological, inflammatory and infectious diseases (see section 1.5). A greater understanding of the inflammatory mechanisms underlying IBD has allowed the development of new treatments, many of which are aimed at inflammatory cytokines. The most prominent one is infliximab, an anti-Tumour Necrosis Factor (TNF)

monoclonal antibody.

### **1.2.3.1. Anti-TNF drugs**

Infliximab was originally designed to bind soluble TNF in the circulation or interstitium, but it is now known that it acts by binding membrane-bound TNF in immune cells and inducing apoptosis. This induction of apoptosis has been demonstrated both in T cells (ten Hove, T *et al.*, 2002) and in monocytes (Lugering, A *et al.*, 2001). It therefore eliminates not only the cytokine but also the TNF-positive cell population. Controlled trials with infliximab have demonstrated efficacy in both active and fistulating CD (Targan, SR *et al.*, 1997; Hanauer, SB *et al.*, 2002; Present, DH *et al.*, 1999). The use of infliximab in UC is not so established, although trials have shown that it induces a clinical response in patients with active colitis (Rutgeerts, P *et al.*, 2005) and reduces colectomy rate in those with severe colitis (Jannerot, G *et al.*, 2005).

Because infliximab acts in an immunosuppressive way, treatment is associated with side effects including severe infections such as tuberculosis and other opportunistic infections. In other patients autoimmune diseases such as lupus erythematoses or multiple sclerosis have been seen (Stange, EF, 2006). One of the problems of biological therapies such as infliximab is the formation of antibodies to the drug itself (immunogenicity). This can affect both the efficacy of the drug as well as its safety, and patients may suffer serum sickness-like reactions, or occasionally anaphylactic reactions (Vermeire, S, 2006). Infliximab is a mouse/human chimeric monoclonal antibody which is approximately 75% human and 25% murine (Sandborn, WJ, 2003). To try and overcome the problem of immunogenicity, newer antibodies have been developed which are more humanised. Certolizumab is a polyethylene glycol conjugate of the antigen-binding fragment of a humanised anti-TNF- $\alpha$  monoclonal antibody; adalimumab is a monoclonal antibody that is 100% human. Both of these antibodies have undergone clinical trials and have been shown to be effective in Crohn's disease and well tolerated (Schreiber, S *et al.*, 2005; Hanauer, SB *et al.*, 2006).

Etanercept is a genetically engineered fusion protein consisting of the human TNF receptor fused with the Fc domain of human IgG1. It is a completely

human molecule and binds soluble TNF- $\alpha$  but is not effective in the treatment of Crohn's disease (Sandborn, WJ *et al.*, 2001). This confirms the importance of the binding of these antibodies to membrane-bound TNF- $\alpha$  for their efficacy.

### **1.2.3.2. Other anti-cytokines**

Work on experimental colitis in mice has shown increased apoptosis in T cells when treated with anti-interleukin (IL)-6 (Atreya, R *et al.*, 2000) or anti-IL-12 (Fuss, IJ *et al.*, 1999) antibodies. More recently, an anti-IL-12p40 antibody has been shown to reduce secretion of IL-12p70 and IL-23 in cells from CD patients, and also reduce IL-23-induced production of IL-17 and IL-6 (Fuss, IJ *et al.*, 2006). IL-12 and IL-23 both contain an identical p40 chain bound to a p35 or a p19 chain respectively, and it is now thought that IL-23 may be a more important cytokine in the development of IBD than IL-12, and that IL-12 antibodies may actually be working against IL-23 (Yen, D *et al.*, 2006; Fuss, IJ *et al.*, 2006).

### **1.2.4. Regulatory cytokine therapy**

An alternative to blocking inflammatory cytokines is to administer regulatory cytokines, and this has been tried for both IL-10 and IL-11. Administration of recombinant human IL-11 to patients with Crohn's disease produced remission in approximately 36% of the patients compared with 16% of the placebo-treated controls (Sands, BE *et al.*, 2002). Trials with IL-10 showed clinical improvement but not remission in patients with chronic active CD (Schreiber, S *et al.*, 2000), whereas 23% of patients with moderate CD achieved improvement and remission (Fedorak, RN *et al.*, 2000). These effects were found to be dose-dependent, higher doses of IL-10 being less effective, and it has since been shown that high doses of IL-10 upregulate the production of IFN- $\gamma$ , a pro-inflammatory cytokine (Tilg, H *et al.*, 2002). Another drawback with these two cytokines is that they have to be administered intravenously and the cytokine may be cleared from the bloodstream before reaching its target.

## **1.2.5. Other potential therapies**

### **1.2.5.1. Adhesion molecules**

Adhesion molecules regulate trafficking of leucocytes between the blood and the tissues. In IBD, adhesion molecules are upregulated and increased recruitment of leucocytes into the inflamed tissue amplifies the immune response. Natalizumab is a humanised monoclonal antibody against  $\alpha 4$  integrin that produced small improvements in response and remission rates when administered to Crohn's disease patients (Sandborn, WJ *et al.*, 2005). Unfortunately a few patients developed progressive multifocal leukoencephalopathy, a fatal viral disease affecting people with severe immune deficiency, and the drug was withdrawn from the market for a re-evaluation of its safety (Keeley, KA *et al.*, 2005).

### **1.2.5.2. Galectin-2**

Galectin-2 is a lectin expressed in intestinal epithelial cells that binds to T cells and initiates apoptosis. Studies in experimental models of murine colitis showed that galectin-2 induced apoptosis of activated but not resting T cells. It also caused downregulation of proinflammatory, but upregulation of anti-inflammatory cytokine expression in T cells (Sturm, A *et al.*, 2006).

### **1.2.5.3. GM-CSF**

In some patients with Crohn's disease a defect in neutrophil function is thought to be a causative factor (Sands, BE, 2006) and this has led to GM-CSF (granulocyte-macrophage-colony-stimulating factor) being tested as a potential treatment. Trials carried out so far suggest that GM-CSF can decrease disease severity and improve quality of life for patients (Dieckgraefe, BK & Korzenik, JR, 2002; Korzenik, JR *et al.*, 2005).

### **1.2.5.4. Stem cells**

Hematopoietic stem cell transplantation (HSCT) has been used for several years in the treatment of severe autoimmune diseases and a few reports have suggested that it may be of significant benefit in IBD (Tyndall, A & Hawkey, CJ, 2006). In a phase I study in 12 patients with refractory CD, eleven achieved sustained clinical remission and only one developed a recurrence of active CD after 15 months (Oyama, Y *et al.*, 2005).

### **1.2.5.5. Gastrointestinal parasites**

The hygiene hypothesis proposes that raising children in extremely hygienic environments with less exposure to parasitic infections may negatively affect the development of the immune system and predispose them to immunologic diseases such as IBD (Moreels, TG & Pelckmans, PA, 2005).

Gastrointestinal infections with helminths induce a Th2 immune response and also activate regulatory T cells which contribute to immune suppression. Since CD has been shown to be a Th1-mediated disease, it has been proposed that infection with helminths may modulate the inflammatory response. Trials in patients infected with helminth ova showed that 72% of CD patients achieved remission, and in UC patients 43% responded to treatment (Summers, RW *et al.*, 2005; Summers, RW *et al.*, 2004).

Many of the newer treatments for IBD are based on inhibition of inflammatory mediators or promotion of suppressors of inflammation. Development of these treatments has been possible through a greater understanding of the inflammatory process.

## **1.3. INFLAMMATION**

Inflammation is a complex reaction of the body in response to damage to its cells and microcirculation. The basic symptoms have been known since ancient Greek and Roman times and were described as redness (*rubor*), swelling (*tumor*), heat (*calor*), pain (*dolor*) and loss of function (*function laesa*) (Sedgwick, AD & Willoughby, DA, 1985). Any harmful stimulus can provoke an inflammatory reaction but there are three main groups:

- i. physical, which may be mechanical, irradiation or extremes of temperature
- ii. chemical, contact with any irritating substance
- iii. infection, by any living organism.

The process of inflammation has a very close relationship with the immune system, the cells of which are widely distributed throughout the body. The progressive development of the inflammatory response is controlled by inflammatory mediators; soluble diffusible molecules which act both locally at the site of tissue damage or infection, and at more distant sites. Bacterial products and toxins act as exogenous mediators, the most important of these



being endotoxin (lipopolysaccharide, LPS) of Gram-negative bacteria.

Endogenous mediators are produced within the immune system itself and may be derived from normal plasma proteins, e.g. complement or clotting proteins, or may be released at the site of injury from various cell types, e.g. cytokines, chemokines.

The immediate inflammatory response is vasodilation, leading to increased blood supply, and increased capillary permeability allowing the entry of fluids and plasma proteins into the tissue. This causes the characteristic heat, redness and swelling. One of the first vasoactive substances to be released in acute inflammation is histamine and this plays an important role in the earliest stages of inflammation (Spector, WG & Willoughby, DA, 1963).

Vascular permeability is maintained by the sequential release of other mediators such as bradykinin, 5-hydroxytryptamine and prostaglandins. Over the next few hours following the acute response, leucocytes start to migrate from small blood vessels and accumulate in the surrounding tissues. This occurs through the action of cytokines and complement fragments, which affect the adhesive properties of the endothelium, and also by chemokines which attract the cells to the site. Initially the main cell types are neutrophils, followed by monocytes which rapidly differentiate into macrophages, and it is these cells and their local actions which cause the pain of inflammation.

Lymphocytes will also be involved, activated by antigen which drains from the site of infection via the afferent lymphatics (Janeway, CA *et al.*, 2001).

In order to reduce the possibility of damage to surrounding tissues, the body must have mechanisms to stop the inflammatory reaction once the damaging agent is removed. If the immune response is not controlled, the tissue may be damaged by the cells and the mediators involved in the immune response, and the inflammatory reaction may become harmful rather than useful. In many diseases such as IBD a large part of the tissue damage is caused by the inflammatory response itself (Playfair, JHL & Chain, BM, 2001).

#### **1.4. THE MUCOSAL IMMUNE SYSTEM**

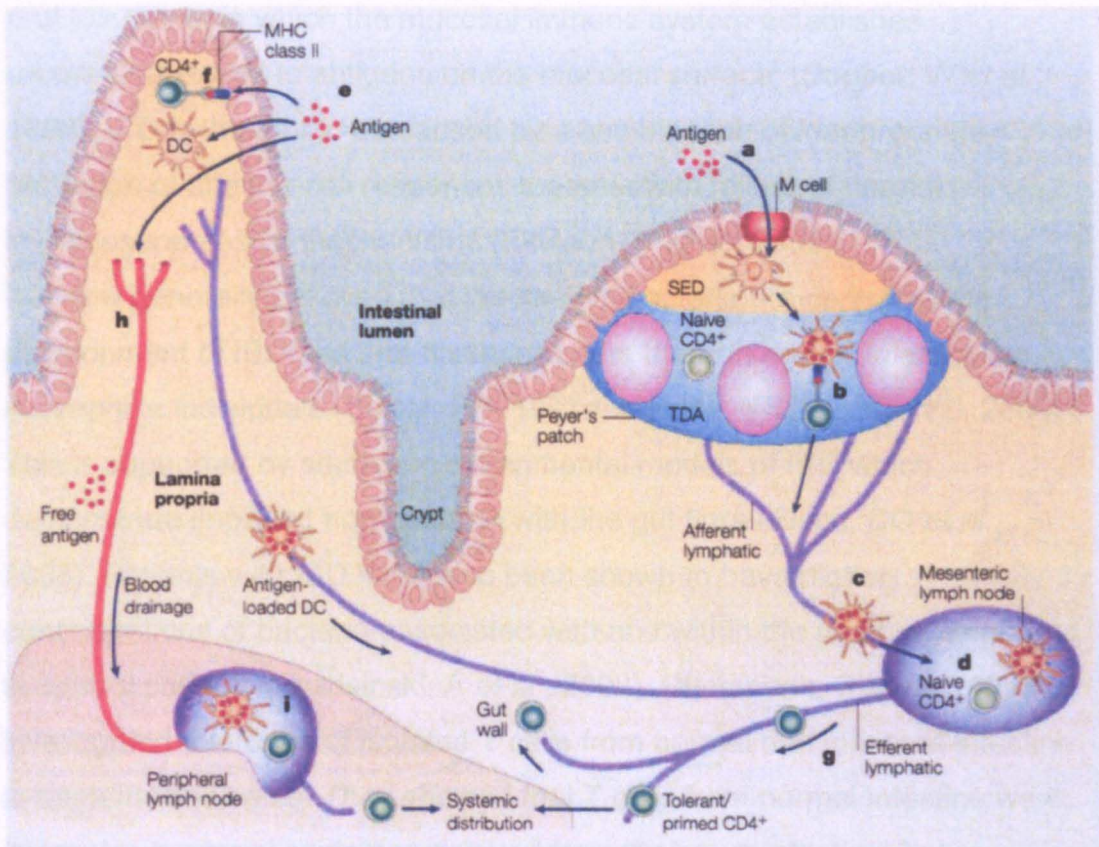
The intestinal mucosa is divided into distinct compartments of different cell types: the surface epithelium, the lamina propria and the muscularis

mucosae. The mucosal immune system consists of gut-associated lymphoid tissue (GALT), and immune cells distributed diffusely throughout the lamina propria and epithelium. The GALT are organised structures which act as primary lymphoid organs aiding the development of T lymphocytes (cryptopatches), or secondary lymphoid organs involved in the induction of the mucosal immune response (Peyer's patches, isolated lymphoid follicles) (Newberry, RD & Lorenz, RG, 2005). The mucosal surface of the gut is continually exposed to potentially harmful antigens from the environment, food and bacteria. The mucosal immune system therefore has to control the balance between responsiveness and non-responsiveness, and it is the antigen-presenting cells (APCs) and the cytokines they secrete, that play a major role in controlling the mucosal immune response (Mahida, YR, 1997; McKaig, B *et al.*, 2000).

APCs are so called because they can take up antigens which cross the epithelial barrier, process them and present the antigen on their cell surface bound to major histocompatibility (MHC) class II molecules (transmembrane proteins expressed at the cell surface). Only macrophages, dendritic cells and B lymphocytes constitutively express MHC class II molecules as well as important costimulatory molecules, and are called professional APCs (Brandtzaeg, P, 2001). All are present in the GALT and scattered throughout the lamina propria (Fig 1.3.). Antigens can cross the epithelium through direct transport by M cells (specialised epithelial cells which are scattered amongst the epithelium covering the lymphoid tissue), or as a result of breaks in the integrity of the epithelial barrier. It has also been shown that dendritic cells can migrate into the epithelium and extend cellular processes into the lumen to sample the contents (Rescigno, M *et al.*, 2001). The presentation of antigens by APCs leads to activation of T lymphocytes which migrate from the Peyer's patches via the circulation to the lamina propria. In a healthy individual, these cells would naturally die by apoptosis, but if the same antigen was present in the lamina propria then the lymphocyte response would be amplified leading to inflammation (Brandtzaeg, P, 2001).

#### **1.4.1. Immune responses and IBD**

As described above, the human intestinal mucosa has to maintain a delicate



**Fig 1.3. Antigen uptake in the intestine**

- Antigen enters the tissue through the M cells in the follicle-associated epithelium and is taken up by local dendritic cells (DC).
- The antigen is presented directly to T cells ( $CD4^+$ ) in the Peyer's patch or
- the antigen-loaded dendritic cell enters the draining lymph and presents antigen to T cells in the mesenteric lymph node (d).
- A similar process may occur when antigen enters the tissue through the epithelium.
- It is also possible that epithelial cells expressing MHC class II may act as local APCs.
- The activated T cells leave the mesenteric lymph node and home back to the intestinal mucosa via the blood stream.
- Antigen can also gain direct access to the bloodstream from the gut and interact with T cells in peripheral lymphoid tissues (i).

SED: subepithelial dome

TDA: thymus-dependent area

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balance between the ability to react to potential pathogens, but not to common gut constituents. This is normally achieved by a process known as oral tolerance, in which the mucosal immune system establishes unresponsiveness to antigens on the mucosal surface, (Strober, W *et al.*, 1998). IBD is thought to be caused by a combination of inappropriate activation of effector-cell responses together with failure of normal immunosuppressive mechanisms (Eksteen, B *et al.*, 2005).

It is now generally believed that the luminal bacteria are central to the development of IBD and that disease occurs through loss of tolerance in susceptible individuals (Sartor, RB, 1997a; Mahida, YR & Rolfe, VE, 2004). This is supported by studies in experimental models of IBD which demonstrate impaired homeostasis with the gut flora (Elson, CO *et al.*, 2005). Patients with IBD have also been shown to have higher concentrations of bacteria associated with and within the mucosa compared to control patients (Swidsinski, A *et al.*, 2002). Duchmann, R *et al.*, (1995) investigated tolerance of isolated T cells from normal and inflamed intestine to bacterial sonicates. They showed that T cells from normal intestine were tolerant to bacterial sonicates cultured from their own intestine, but proliferated in the presence of sonicates from other individuals. In contrast, T cells isolated from IBD intestine strongly proliferated in response to bacteria from their own mucosa. More recently, it has been shown that the bacterial flora composition of CD patients, but not UC patients, is significantly altered from that of healthy controls (Gophna, U *et al.*, 2006). However, the imbalance was found in both inflamed and non-inflamed tissues of the CD patients so is unlikely to be the sole cause of the inflammation.

Development of disease is thought to occur through defects in one or more of the mechanisms involved in host tolerance for the intestinal flora. The first of these is the innate immune response involving phagocytic cells and the epithelial barrier itself. Zareie, M *et al.*, (2001) showed that isolated lamina propria cells from Crohn's patients spontaneously secreted the inflammatory cytokine TNF- $\alpha$ , and reduced the epithelial resistance of a co-cultured monolayer of T84 cells. The second mechanism is regulation and suppression mediated by regulatory T cells. Animal studies have shown that

transfer of naive T cells into immune-deficient mice produces colitis which can be prevented by co-transfer, or cured by subsequent transfer of regulatory T cells (Coombes, JL *et al.*, 2005).

The result of an abnormal innate response or defective regulatory mechanism is an excessive T cell response which, if left unchecked, leads to acute and chronic inflammation in the intestine.

## **1.4.2. Cells of the Immune System**

### **1.4.2.1. Macrophages**

Macrophages are part of the first line of host defence against gut microorganisms and other antigenic stimuli. They protect the mucosa against harmful pathogens, phagocytose dead cells and foreign debris, and regulate the inflammatory response to bacteria and antigens which have crossed the epithelial layer. They are derived from stem cells in the bone marrow that differentiate into monocytes, and are subsequently released into the blood. After circulating for 2-3 days, monocytes migrate into the tissues where they mature into macrophages and remain as resident cells for weeks to months before undergoing apoptosis (Abbas, AK *et al.*, 1997). In recent years it has become clear that there are different populations of macrophages with distinct biological functions. The characteristics of these subpopulations are shown in Table 1.2.

In the gut, macrophages are located in the lamina propria, mainly concentrated in a band beneath the luminal epithelium (Pavli, P *et al.*, 1996). In normal mucosa, resident tissue macrophages display a very different phenotype to the monocytes from which they are derived (Smith, PD *et al.*, 2005). They retain the ability to phagocytose and kill microorganisms, but do not produce pro-inflammatory cytokines in response to phagocytosis. They also lack surface CD14, the receptor for bacterial LPS, thus limiting LPS-induced cytokine production (Smith, PD *et al.*, 2001). Normal intestinal macrophages also lack APC function, partly due to absence of the co-stimulatory molecules necessary for T cell activation (Rugtveit, J *et al.*, 1997). Hence, despite residing in a tissue in close contact with large numbers of potentially immunostimulatory bacteria, normal gut tissue macrophages provide an efficient scavenging host defence function without

	Classically activated	Alternately activated	Type II activated
Activating signals	IFN- $\gamma$ followed by binding to TLR (microbe or LPS)	IL-4 or IL-13	Binding to Fc $\gamma$ R followed by binding to TLR
Secretory products	IL-1, TNF, IL-6, IL-12 chemokines arachidonic acid & prostaglandins	IL-10, IL-1ra	IL-10, IL-1, TNF, IL-6
Characteristics	enhanced antigen-presentation (MHC class II upregulated)  phagocytic  enhanced ability to kill and degrade intracellular microbes by toxic oxygen and NO production  inflammatory prolonged cytokine production leads to tissue damage	MHC class II upregulated but inefficient antigen-presentation  mannose receptor upregulated leading to endocytosis and phagocytosis  no NO production  produce arginase, promoting cell growth and repair  anti-inflammatory regulatory and recovery function	MHC class II upregulated  phagocytic  produce toxic oxygen and NO  anti-inflammatory IL-10 inhibits actions of classically activated type

**Table 1.2. Characteristics of macrophage subpopulations**

The three subpopulations of macrophages are known as classically activated, alternately activated and type II activated. Classically activated macrophages induce T cells to produce IFN- $\gamma$  and hence promote a Th1 response. Type II activated macrophages are named for their ability to preferentially induce Th2 immune responses since they induce T cells to produce high levels of IL-4.

TLR = Toll-like receptor

Fc $\gamma$ R is the receptor for the constant region of Immunoglobulin G. Antibodies that bind to the surface of invading pathogens (opsonisation) also bind to the Fc receptors and initiate phagocytosis.

Mosser, DM, (2003); Gordon, S, (2003); Ma, J *et al.*, (2003)



promoting mucosal inflammation.

In active IBD there is an increase in the mucosal macrophage population thought to be derived from circulating monocytes (Rugtveit, J *et al.*, 1994; Allison, MC *et al.*, 1988). The recruitment of these monocytes is through receptor-binding of chemotactic ligands such as chemokines, secreted by cells in the inflamed tissue (Smith, PD *et al.*, 2005). The macrophage population from inflamed tissue has been shown to exhibit greater heterogeneity than that of normal mucosa (Mahida, YR *et al.*, 1989a; Allison, MC & Poulter, LW, 1991). Rogler, G *et al.*, (1997) investigated the phenotype of macrophages isolated from IBD tissue compared to those of normal tissue and found increased expression of CD14, CD16 (a receptor for the Fc portion of IgG), CD11b and c (adhesion molecules), and HLA-DR (MHC class II histocompatibility antigen). In another study, Mahida, YR *et al.*, (1989a) demonstrated that macrophages from normal tissue were located just below the epithelium, had high lysosomal enzyme content and were likely to be 'scavenger' cells. In inflamed UC and CD tissue however, the cells became distributed throughout the lamina propria. They also showed the presence of two macrophage populations not present in normal tissue. The first were CD16<sup>+</sup> macrophages whose function are unknown, although CD16<sup>+</sup>-monocytes have been shown to differentiate into lymph-homing dendritic cells (Randolph, GJ *et al.*, 2002). The second population were epithelioid cells which are modified macrophages and are the main component of granulomas, often found in CD but not in UC. Epithelioid cells have a reduced phagocytic but greater secretory capacity and granulomas are thought to be crucial antigen-presenting sites (Matsumoto, T *et al.*, 2001). An increase in epithelioid cells, clustered at sites of tissue damage has also been demonstrated by Allison, MC *et al.*, (1991).

#### **1.4.2.2. Lymphocytes**

Lymphocytes are cells that specifically recognise and respond to an individual antigen. Naive lymphocytes (i.e. ones which have not yet been exposed to their particular antigen) are released into the blood stream and migrate from there into one of the organised lymphoid tissues, awaiting contact with their respective antigens. If this fails to occur, they pass back into the blood stream via the efferent lymphatics and are transported on to

another lymphatic organ. Once the naive lymphocyte binds to its specific antigen, it starts to proliferate and differentiate within the lymphoid tissue. The activated cells produced enter the blood circulation, but they now preferentially leave the blood in the same type of tissue as that in which they were activated, a process known as homing. There they are able to respond to any antigen still present (Salmi, M & Jalkanen, S, 2005).

Lymphocytes are divided into two major subpopulations; B lymphocytes (B cells), which are produced and mature in the bone marrow, and T lymphocytes (T cells), which are produced in the bone marrow but mature in the thymus (Playfair, JHL *et al.*, 2001; Abbas, AK *et al.*, 1997). T cells have been further subdivided into populations that have distinct functions; helper cells (Th), cytotoxic cells (Tc), regulatory cells (Tr), and intraepithelial cells (IELs). The characteristics of the different lymphocyte populations are shown in Table 1.3.

In the gut, naive lymphocytes are mostly located in Peyer's patches where they become exposed to antigens transported through the epithelium and presented by dendritic cells (Fig 1.3.). After returning to the circulation, they preferentially home to the lamina propria to execute a response (Salmi, M *et al.*, 2005). T cells constitute approximately one-third of the cells in the intestinal lamina propria and are capable of producing high levels of cytokines, predominantly IFN- $\gamma$  (Monteleone, I *et al.*, 2002). This may be because T cells derived from Peyer's patches are likely to be activated by bacterial antigens which stimulate dendritic cells to produce IL-12 and promote a Th1-type response (Nagata, S *et al.*, 2000). Persistent stimulation and inflammation is avoided by the presence of regulatory cells secreting TGF- $\beta$  and IL-10 (Groux, H *et al.*, 1997; Annacker, O & Powrie, F, 2002). Also, Boirivant, M *et al.*, (1996) showed that, compared with peripheral blood T cells, lamina propria T cells exhibited increased susceptibility to apoptosis which was enhanced on stimulation. The cells are therefore removed before they can mediate a harmful response.

Crohn's disease is characterised by excessive numbers of activated T cells in the mucosa, and studies have demonstrated a resistance to apoptosis in mucosal T cells leading to prolonged survival, accumulation of cells and



B lymphocytes (B cells)		Antigen receptors are membrane-bound antibodies. Differentiate into plasma cells which secrete antibody. (Playfair, JHL <i>et al.</i> , 2001)
T lymphocytes (T cells)	Helper T cells (Th)	Act on other immune cells to promote the immune response. Characterisation based on the cytokines they release: Th1 cells - proinflammatory. Promote differentiation and proliferation of T cells and activate other cells such as macrophages. Enhance activity of cytotoxic T cells. Th2 cells - promote 'allergic' response. Stimulate B cells to produce antibody. Promote eosinophil recruitment. (Neurath, MF <i>et al.</i> , 2002) Th17 cells - recently discovered proinflammatory subset so-named because they secrete IL-17. Produced in response to IL-23 and TGF- $\beta$ (Harrington, LE <i>et al.</i> , 2006).
	Cytotoxic T cells	Detect and destroy virus-infected cells by lysis or induction of apoptosis. (Playfair, JHL <i>et al.</i> , 2001)
	Regulatory T cells (Tr)	Inhibit proliferation of other cells and suppress immune responses. 'Natural' Tr cells are thought to have a role in preventing autoimmune disease (Nagler-Anderson, C <i>et al.</i> , 2004). Tr1 cells require IL-10 for their formation and once mature secrete large amounts of it (Groux, H & Powrie, F, 1999). Th3 cells secrete TGF- $\beta$ and are thought to be involved in maintenance of tolerance to dietary antigen (Weiner, HL, 2001). CD8+ Tr cells are thought to be involved in oral tolerance (Mowat, AM, 2003).
	Intraepithelial lymphocytes (IELs)	Dispersed as single cells within the mucosal epithelium. Effector memory cells with regulatory features, normally quiescent but can respond rapidly to harmful antigens. Protect against epithelial pathogens and promote healing after injury. (Cheroutre, H, 2005)
Natural killer cells (NK cells)		Detect cells expressing low levels of MHC class I molecules, such as virally infected or cancerous cells, and kill them Secrete IFN- $\gamma$ and TNF- $\alpha$ . (Moretta, L <i>et al.</i> , 2002)

**Table 1.3. Characteristics of lymphocyte cell populations**

hence perpetuation of inflammation (Ina, K *et al.*, 1999; Boirivant, M *et al.*, 1999).

#### **1.4.2.3. Dendritic cells**

Dendritic cells (DCs) are APCs present in small numbers in most tissues. Their role is to acquire antigen and transport it to the lymphoid tissue where they activate naive T cells; one DC can affect between 300-1000 T cells (Stagg, AJ *et al.*, 2003). DC precursors are derived in the bone marrow and migrate to the tissues via the peripheral blood. These immature cells are highly efficient at antigen uptake by endocytosis, but express low levels of MHC class II on their cell surface. However in response to damage or inflammatory signals such as microbial products and cytokines, they undergo maturation and migrate to the lymph nodes. During maturation, their expression of MHC class II is upregulated, turning them into potent stimulators of T cells (Stagg, AJ *et al.*, 2003; Banchereau, J & Steinman, RM, 1998). DCs express many costimulatory molecules which interact with receptors on T cells to enhance adhesion and signalling (e.g. B7.1, B7.2, CD40), and these are also upregulated on maturation. As with the other immune cells, there are also subsets of dendritic cells and most of the information about their development has come from isolation and culture of their precursor cells. Their characteristics are shown in Table 1.4.

In the human colon, dendritic cells have been shown to form a network throughout the lamina propria and beneath the basement membrane of the crypts (Pavli, P *et al.*, 1996). The precise role of these cells is still not completely understood, and studies have been complicated by the different subpopulations which exist, but they appear to be critical for regulation of immunity in the gut. For instance, it is now known that as well as promoting responses to foreign antigens, DCs are also involved in tolerance to self-antigens (Mowat, AM, 2003). DCs express a series of surface receptors which recognise common structural elements of microbes. They appear to be able to differentiate between different microorganisms and respond in different ways (Stagg, AJ *et al.*, 2003). Resident tissue DCs are not normally activated by harmless antigens and have regulatory functions, maintaining homeostasis and suppressing responses to commensal bacteria. However during inflammation DCs are recruited from the blood which express different

	DC1	DC2
Precursor	pDC1 Monocytes (derived from myeloid progenitor cells)	pDC2 Plasmacytoid cells (derived from lymphoid progenitor cells)
Immature cell	imDC1 Produced by culture in the presence of GM-CSF and IL-4, or after bacterial phagocytosis.	imDC2 Produced by culture in the presence of IL-3 or following an innate immune response to viral stimulation.
Mature cell	mDC1 Matured by stimulation with pro-inflammatory cytokines or microbial products such as LPS. Secrete IL-12, inducing strong Th1 and cytotoxic lymphocyte responses.	mDC2 Cells matured with IL-3 promote Th2 responses and production of CD8 <sup>+</sup> Tr cells.  Cells matured by viral stimulation secrete IFN- $\alpha$ and $\beta$ , and promote Th cells to produce IFN- $\gamma$ and IL-10.

**Table 1.4. Characteristics and development of dendritic cell populations**

The table shows the stages of development of the two subsets of dendritic cells and the conditions required to produce them.

Liu, Y-J, (2001; Shortman, K & Liu, Y-J, (2002)

pattern-recognition receptors (PRRs) capable of recognising pathogen-associated molecular patterns (PAMPs) of invading bacteria (Granucci, F & Ricciardi-Castagnoli, P, 2003). Hart, AL *et al.*, (2005) studied DC populations in lamina propria mononuclear cells (LPMCs) isolated from IBD patients and healthy controls. They found that in IBD, DCs expressed higher levels of CD40 (a marker of maturation/activation), expression of PRRs was upregulated, and that DCs from Crohn's disease (but not UC) produced pro-inflammatory cytokines.

The ability of immune cells to affect each other's functions provides an important amplification mechanism for specific immunity, but when uncontrolled can also lead to damaging inflammation (Abbas, AK *et al.*, 1997). The way in which the cells influence each other is principally by the release of cytokines.

## **1.5. CYTOKINES**

Cytokines are small soluble proteins which act through specific receptors to influence the activation, differentiation or growth of other cells. Nearly all nucleated cells are capable of synthesising them, and of responding to them. They have autocrine, paracrine and endocrine activities that may be exerted directly or indirectly by stimulating the release of other effector molecules. Cytokines are primarily involved in host responses to disease and infection and have been grouped according to their biological function rather than their structure (Dinarello, CA, 2000).

### **1.5.1. Proinflammatory cytokines in IBD**

These cytokines promote inflammation by upregulating the enzymes associated with the production of inflammatory mediators such as prostaglandins, leukotrienes, nitric oxide and platelet-activating factor. Three inflammatory cytokines have been shown to be produced spontaneously from IBD mucosa at levels significantly higher than that of normal mucosa (Ligumsky, M *et al.*, 1990; Reimund, JM *et al.*, 1996). The cytokines originate mainly from LPMCs (Youngman, KR *et al.*, 1993; Reinecker, H-C *et al.*, 1993), and their production appears to correlate with the degree of

inflammation (Ishiguro, Y, 1999).

**Interleukin (IL)-1 $\beta$**  is a key multifunctional cytokine which is produced by, and acts on nearly all tissues and organs of the body (Dinarello, CA, 1996). It is a potent mediator and activates many immune and inflammatory cells including T cells and NK cells (Rogler, G & Andus, T, 1998a). It also induces expression of endothelial adhesion molecules, essential for adhesion of leukocytes to the endothelial surface prior to migration into the tissues (Dinarello, CA, 2000). IL-1 $\beta$  is a principal cytokine in IBD and has been shown to be present at higher levels in biopsies from patients with active disease compared to healthy controls (Ligumsky, M *et al.*, 1990). A similar increase has been demonstrated in IL-1 $\beta$  secretion from isolated mononuclear cells from active IBD tissue (Mahida, YR *et al.*, 1989b). This study also showed that depletion of macrophages from the cell population appreciably reduced the amount of IL-1 $\beta$  produced, demonstrating that these cells are a major source of this cytokine. IL-1 $\beta$  release is a principal topic of this project and is discussed in more detail in section 1.6.

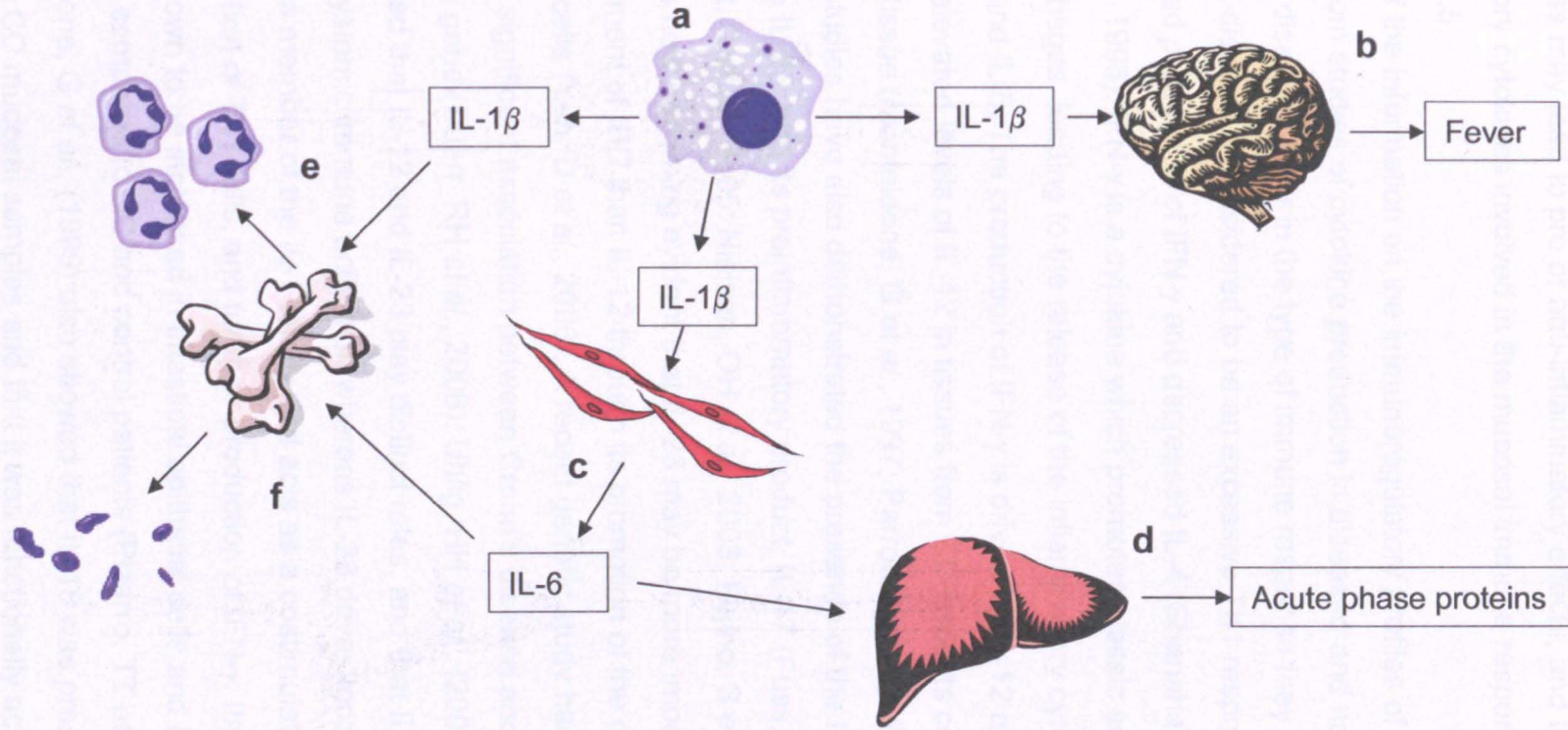
**IL-6** is secreted by a number of cell types including monocytes, activated T cells, B cells, fibroblasts, endothelium and mesangial cells (Radford-Smith, G & Jewell, DP, 1994). It is not normally produced unless cells are appropriately stimulated; such stimuli are bacterial lipopolysaccharide, viral infection, and other proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ . IL-6 promotes the differentiation of B cells into antibody-producing plasma cells, and promotes IL-2 production in activated T cells (Ito, H, 2003). Both tissue and serum levels of IL-6 are significantly higher in active IBD compared with controls (Mitsuyama, K *et al.*, 1991), even patients with only moderately active CD show elevated serum IL-6 (Gross, V *et al.*, 1992). Increased production by macrophages and T cells of IL-6 and its soluble receptor (sIL-6R), leads to increased formation of the complex IL-6-sIL-6R which binds to T cell membranes and promotes induction of anti-apoptotic genes. This leads to resistance of LPMCs to apoptosis thereby increasing the T cell population and augmenting the inflammation (Atreya, R & Neurath, MF, 2005).

**TNF- $\alpha$**  is a highly inflammatory cytokine secreted mainly by activated macrophages and monocytes. It has many actions but those relevant to IBD are its ability to recruit circulating inflammatory cells, induction of oedema, activation of granulocytes and T cells, and granuloma formation. It shares many of its activities with IL-1 $\beta$  except that TNF induces apoptotic cell death, a property which gave it its name (van Deventer, SJH, 1997). Spontaneous secretion of TNF- $\alpha$  from isolated LPMCs from IBD tissue is much lower than that of IL-1 $\beta$  and IL-6 (Reinecker, H-C *et al.*, 1993), and a study of TNF- $\alpha$  mRNA levels found no difference between IBD and control groups (Stevens, C *et al.*, 1992). Cappello, M *et al.*, (1992) also showed that TNF mRNA was located in cells in deeper lamina propria whereas IL-1 mRNA was in subepithelial macrophages. It would thus appear that TNF- $\alpha$  plays a less prominent role in IBD than IL-1 $\beta$  or IL-6.

As well as activating inflammatory cells within the intestinal mucosa, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  are responsible for a characteristic accompanying systemic response (Fig 1.4). Effects include fever, anorexia, increased circulating white blood cells (leukocytosis), increased blood platelets (thrombocytosis), induction of the hepatic acute phase response (production of plasma proteins whose function is to restore homeostasis), and stimulation of the hypothalamic/pituitary/adrenal axis (part of the neuroendocrine system that controls reaction to stress) (Sartor, RB, 1994). IL-1 $\beta$  appears to be a primary stimulator of diarrhoea, a major symptom of intestinal inflammation. It induces the release of prostaglandins from subepithelial mesenchymal cells which promotes anion secretion from epithelial cells. It also increases gut motility by the release of corticotrophin-releasing hormone, and prostaglandins which stimulate small intestinal contractions. IL-1 $\beta$  and TNF- $\alpha$  also stimulate proliferation of intestinal smooth muscle cells and fibroblasts, contributing to fibrosis, an important complication of CD (Sartor, RB, 1994).

### **1.5.2. Immunoregulatory cytokines in IBD**

Some cytokines play a more regulatory role by controlling the development of immune cell populations, or by inhibiting or stimulating the release of other



**Fig 1.4. Systemic effects of IL-1 $\beta$**

- IL-1 $\beta$  is secreted by many cell types including macrophages.
- IL-1 $\beta$  enters the circulation and activates IL-1 receptors in the hypothalamic vascular network resulting in the synthesis of COX-2. COX-2 causes elevated brain PGE<sub>2</sub> levels which activates the thermoregulatory centre and leads to fever.
- IL-1 $\beta$  activates IL-1 receptors on the endothelium resulting in rashes and production of IL-6.
- Circulating IL-6 stimulates production of acute phase proteins from liver hepatocytes.
- IL-1 $\beta$  acts on the bone marrow to increase mobilisation of granulocyte progenitors and mature neutrophils.
- IL-1-induced IL-6 increases platelet production resulting in thrombocytosis.

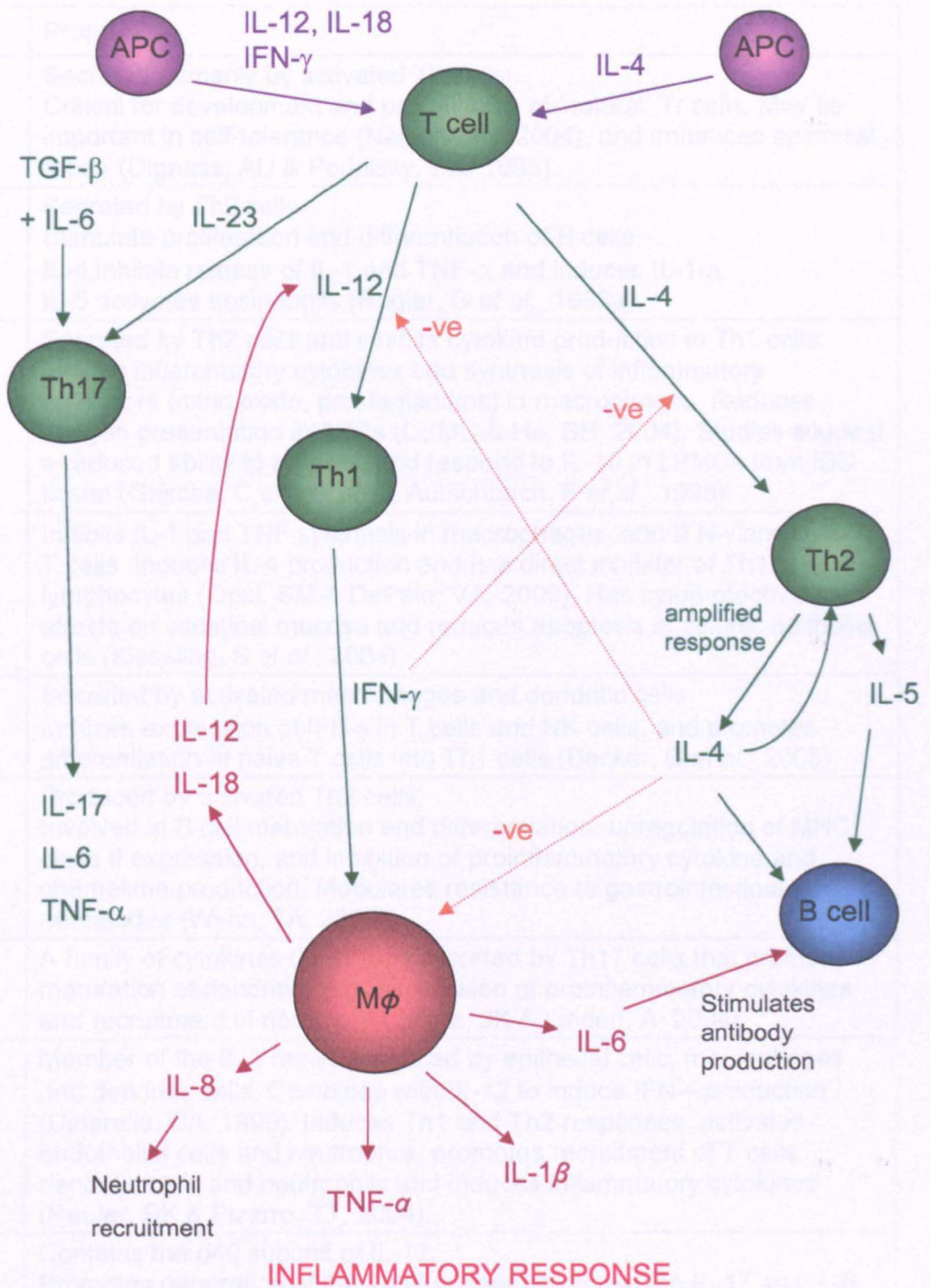
Dinarello, CA, (2005)

cytokines. A simplified diagram of the role of regulatory cytokines in the development of Th lymphocyte populations and their control of pro-inflammatory cytokine release is shown in Fig 1.5. The actions of regulatory cytokines may lead to pro or anti-inflammatory effects, and the principal regulatory cytokines involved in the mucosal immune response are shown in Table 1.5.

Much of the information on the immunoregulatory profiles of UC and CD has come from studies of cytokine production in diseased and normal tissue, and the two diseases differ in the type of immune response they express.

Crohn's disease is considered to be an excessive Th1 response, leading to increased production of IFN- $\gamma$  and decreased IL-4 (Shanahan, F, 2002; Fuss, IJ *et al.*, 1996). IFN- $\gamma$  is a cytokine which promotes classic activation of macrophages, leading to the release of the inflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$  and IL-6. The production of IFN- $\gamma$  is driven by IL-12 and studies have shown elevated levels of IL-12 in tissues from CD patients compared to normal tissue (Monteleone, G *et al.*, 1997; Parronchi, P *et al.*, 1997). More recent studies have also demonstrated the presence of the IL-12-related cytokine IL-23, and its proinflammatory product, IL-17 (Fuss, IJ *et al.*, 2006; Schmidt, C *et al.*, 2005; Nielsen, OH *et al.*, 2003; Fujino, S *et al.*, 2003). In fact, it is now becoming evident that IL-23 may be more important for the development of IBD than IL-12 through its promotion of the development of Th17 T cells (Yen, D *et al.*, 2006). A recent genetic study has demonstrated a highly significant association between Crohn's disease and the IL23 receptor gene (Duerr, RH *et al.*, 2006). Uhlig, HH *et al.*, (2006) have suggested that IL-12 and IL-23 play distinct roles, and that IL-12 has a key role in systemic immune activation whereas IL-23 drives local inflammation. IL-18 is a member of the IL-1 family and acts as a costimulatory factor for the proliferation of Th1 cells, and for their production of IFN- $\gamma$ . Its expression has been shown to be increased in intestinal epithelial cells and LPMCs from CD patients compared to UC and control patients (Pizarro, TT *et al.*, 1999). Monteleone, G *et al.*, (1999) also showed that IL-18 was present at higher levels in CD mucosal samples and that it was functionally active. In cultures of LPMCs in which they downregulated the IL-18, they demonstrated a





**Fig 1.5. Regulatory cytokines involved in the development of Th lymphocyte populations and the inflammatory response**

Activation of APCs by different stimuli (see tables 1.2 and 1.4) determines the cytokines they release. These cytokines in turn stimulate naïve T cells to differentiate into Th1, Th2 or Th17 populations.

APC: Antigen presenting cell

Th: T helper lymphocyte populations

M $\phi$ : macrophage

Cytokine	Properties
IL-2	Secreted primarily by activated Th cells. Critical for development and proliferation of 'natural' Tr cells. May be important in self-tolerance (Nelson, BH, 2004), and enhances epithelial repair (Dignass, AU & Podolsky, DK, 1996).
IL-4 & IL-5	Secreted by Th2 cells. Stimulate proliferation and differentiation of B cells. IL-4 inhibits release of IL-1 and TNF- $\alpha$ and induces IL-1ra. IL-5 activates eosinophils (Rogler, G <i>et al.</i> , 1998a).
IL-10	Secreted by Th2 cells and inhibits cytokine production in Th1 cells. Inhibits inflammatory cytokines and synthesis of inflammatory mediators (nitric oxide, prostaglandins) in macrophages. Reduces antigen presentation in APCs (Li, MC & He, SH, 2004). Studies suggest a reduced ability to produce and respond to IL-10 in LPMCs from IBD tissue (Gasche, C <i>et al.</i> , 2000; Autschbach, F <i>et al.</i> , 1998).
IL-11	Inhibits IL-1 and TNF synthesis in macrophages, and IFN- $\gamma$ and IL-2 in T cells. Induces IL-4 production and is a direct inhibitor of Th1 lymphocytes (Opal, SM & DePalo, VA, 2000). Has cytoprotective effects on intestinal mucosa and reduces apoptosis in colonic epithelial cells (Kiessling, S <i>et al.</i> , 2004)
IL-12	Secreted by activated macrophages and dendritic cells. Induces expression of IFN- $\gamma$ in T cells and NK cells, and promotes differentiation of naive T cells into Th1 cells (Becker, C <i>et al.</i> , 2005).
IL-13	Produced by activated Th2 cells. Involved in B cell maturation and differentiation, upregulation of MHC class II expression, and inhibition of proinflammatory cytokine and chemokine production. Modulates resistance to gastrointestinal nematodes (Wynn, TA, 2003).
IL-17	A family of cytokines (IL-17A-F) secreted by Th17 cells that promote maturation of dendritic cells, expression of proinflammatory cytokines and recruitment of neutrophils (Kolls, JK & Linden, A, 2004).
IL-18	Member of the IL-1 family produced by epithelial cells, macrophages and dendritic cells. Combines with IL-12 to induce IFN- $\gamma$ production (Dinarello, CA, 1999). Induces Th1 and Th2 responses, activates endothelial cells and neutrophils, promotes recruitment of T cells, dendritic cells and neutrophils and induces inflammatory cytokines (Reuter, BK & Pizarro, TT, 2004).
IL-23	Contains the p40 subunit of IL-12. Promotes generation of the Th17 subset which release IL-17 and IL-6 (Yen, D <i>et al.</i> , 2006).
IL-27	Contains a protein related to the p40 subunit of IL-12. Thought to limit the intensity and duration of T cell activation (Becker, C <i>et al.</i> , 2005).
IFN- $\gamma$ (Interferon- $\gamma$ )	Produced by Th1 and NK cells. Potent activator of macrophages and promotes T and B cell differentiation. Increases MHC class I & II expression and activates neutrophils, NK cells and vascular endothelial cells (Rogler, G <i>et al.</i> , 1998a).

**Table 1.5. Regulatory cytokines associated with the mucosal immune response**

decrease in IFN- $\gamma$  expression.

In ulcerative colitis the picture is less clear but it has been considered to have some features of a Th2-mediated disease (Bouma, G & Strober, W, 2003; Farrell, RJ & Peppercorn, MA, 2002). UC is associated with the production of autoantibodies, which have been used to differentiate between disease subgroups (Saxon, A *et al.*, 1990; Das, KM *et al.*, 1993; Duerr, RH & Neigut, DA, 1995). As Th2 cells are generally more involved in the activation of B cells and induction of a humoral immune response than Th1 cells, the presence of autoantibodies is more likely to indicate a Th2-mediated response. The characteristic cytokine of the Th2 response is IL-4 and although there is no evidence that T cells from UC secrete elevated amounts of IL-4, increased expression of IL-4 mRNA has been demonstrated (Inoue, S *et al.*, 1999). Secretion of IL-5, another Th2 cytokine, has been shown to be elevated in UC but not in CD (Fuss, IJ *et al.*, 1996; Fuss, IJ *et al.*, 2004). It has been suggested that IL-13 is an important effector cytokine in UC in that it has been shown to impair epithelial barrier function by affecting tight junctions, inducing apoptosis and inhibiting repair (Heller, F *et al.*, 2005). Others have shown elevated levels of IL-13 produced by NK T cells from UC patients (Fuss, IJ *et al.*, 2004), but contradictory studies have shown decreased levels of IL-13 and suggest disease may be as a result of impaired regulatory properties (Vainer, B *et al.*, 2000; Kadivar, K *et al.*, 2004).

### 1.5.3. Chemokines

Chemokines are a family of cytokines whose main function is to control the movement and activation of leucocytes. They are small proteins (7-15kDa) with 20-70% homology in their amino acid sequences, and have been subdivided into families (named CXC, CC, C and CX<sub>3</sub>C) based on the arrangement of their N-terminal cysteine residues (Zlotnik, A & Yoshie, O, 2000). Functionally chemokines are broadly divided into 'inflammatory' or 'homeostatic', although some homeostatic chemokines have been shown to be upregulated during inflammation. The inflammatory chemokines are inducible, and are synthesised and secreted by infiltrating leucocytes and

tissue cells on stimulation. The main stimuli for chemokine production are proinflammatory cytokines, bacterial products such as LPS, and viral infection. Homeostatic chemokines are constitutively expressed in lymphoid tissue, and are involved in basal lymphocyte trafficking between lymphoid organs and the tissues (Papadakis, KA, 2004).

Chemokines are important in the trafficking of dendritic cells. Immature DCs express several chemokine receptors which respond to chemokines present in the tissues and help to keep them located there. On maturation however, these receptors are downregulated and the cells upregulate expression of a receptor which binds a chemokine expressed in T cell-rich areas. This results in the movement of DCs, activation of T cells and induction of the adaptive immune response (Sozzani, S *et al.*, 1998; Dieu, MC *et al.*, 1998).

Similarly chemokines play a role in the differentiation of T cells into Th1 or Th2 cells. They can activate T cells directly, or indirectly by affecting the dendritic cells and the cytokines they produce. Th1 and Th2 cells have been shown to express different chemokine receptors (Luther, SA & Cyster, JG, 2001).

Chemokines also play a vital role in leucocyte adhesion and extravasation during inflammation. The chemokines secreted in any particular tissue will control the type of inflammatory cells which infiltrate that tissue. Ulcerative colitis and Crohn's disease are characterised by chronic inflammation with additional acute inflammatory flare-ups. In the chronic phase, the gut mucosa is infiltrated with macrophages and lymphocytes, whereas during the acute phase there are additional increased levels of neutrophils, particularly in ulcerative colitis (Luster, AD, 1998). Chemokines which have been shown to be increased or induced in patients with IBD are shown in Table 1.6.

## **1.6. THE INTERLEUKIN (IL)-1 FAMILY**

IL-1 is a highly inflammatory cytokine, often acting in conjunction with other cytokines or cell mediators. The three classical members of the IL-1 family are IL-1 $\alpha$  (IL-1F1), IL-1 $\beta$  (IL-1F2), and IL-1 receptor antagonist (IL-1Ra, IL-1F3) (Dinarello, CA, 1996).

The two agonists, IL-1 $\alpha$  and IL-1 $\beta$ , are synthesised as 31kDa precursors (pro-IL-1) that are processed to 17kDa mature forms by specific intracellular

Chemokine	Site of production	Action
CCL2 (MCP-1)	Macrophages, smooth muscle, endothelial cells	Recruits monocytes, dendritic cells and activated T cells (Grimm, MC <i>et al.</i> , 1996).
CCL3 (MIP-1 $\alpha$ )	Epithelial cells, endothelial cells, mucosal inflammatory cells	Recruit activated T cells. (Banks, C <i>et al.</i> , 2003; Mazzucchelli, L <i>et al.</i> , 1996)
CCL4 (MIP-1 $\beta$ )		
CCL5 (RANTES)	IELs, subepithelial lamina propria	
CCL7 (MCP-3)	Predominantly epithelial cells	Recruits monocytes, dendritic cells and activated T cells (Wedemeyer, J <i>et al.</i> , 1999).
CCL11 (Eotaxin)	Epithelial and endothelial cells, peripheral blood eosinophils	Recruits eosinophils and basophils (Garcia-Zepeda, EA <i>et al.</i> , 1996).
CCL20 (MIP-3 $\alpha$ )	Epithelial cells, particularly follicle-associated epithelium of Peyer's patches	Recruits dendritic cells, B cells and memory T cells (Puleston, J <i>et al.</i> , 2005)
CXCL1-3 (GRO- $\alpha,\beta,\gamma$ )	Epithelial cells, macrophages, myofibroblasts	GRO- $\alpha$ has synergistic effects on IL-8-induced neutrophil activation (Imada, A <i>et al.</i> , 2001). Increased expression of CXCL1-3 and their receptors in IBD (Puleston, J <i>et al.</i> , 2005).
CXCL5 (ENA-78)	Epithelial cells of inflamed mucosa	Recruits and activates neutrophils (Z'Graggen, K <i>et al.</i> , 1997).
CXCL8 (IL-8)	Constitutively expressed in the gut by macrophages, epithelial cells, fibroblasts	Recruits neutrophils. Thought to be important in formation of crypt abscesses. Increased expression of IL-8 and its receptor in active UC (Mahida, YR <i>et al.</i> , 1992; Williams, EJ <i>et al.</i> , 2000). Expression correlates with disease severity (Daig, R <i>et al.</i> , 1996).
CXCL10 (IP-10)	Expressed in lamina propria cells of normal colon	Recruits activated T cells. Has a role in basal trafficking of lymphocytes in the gut but is upregulated in inflammation (Ugucconi, M <i>et al.</i> , 1999).

**Table 1.6. Chemokines shown to be increased or induced in tissue from IBD patients**

MCP: Monocyte Chemoattractant Protein; MIP: Macrophage Inflammatory Protein; RANTES: Regulated on Activation, Normal T-cell Expressed and Secreted; GRO: Growth-Related gene product; ENA: Epithelial Neutrophil-Activating peptide; IL: Interleukin; IP: Interferon-inducible Protein; IEL: Intraepithelial lymphocyte

proteases. IL-1 $\beta$  is biologically active only in its mature form, which is exported in large quantities on stimulation. Both forms of IL-1 $\alpha$  are active, but it is produced in much lower amounts than IL-1 $\beta$ , and is not actively secreted (Hazuda, DJ *et al.*, 1988).

IL-18 (IL-1F4) and IL-33 (IL-1F11) are regulatory cytokines; IL-18 stimulates activation of Th1 cell differentiation and IFN- $\gamma$  secretion (Table 1.5) and the endothelium-derived IL-33 induces expression of Th2-associated cytokines (Carriere, V *et al.*, 2007). More recently, new members of the IL-1 family have been identified, numbered IL-1F5 - IL-1F10, whose functions have yet to be fully elucidated (Barksby, HE *et al.*, 2007).

The IL-1 receptor binds both IL-1 $\alpha$  and IL-1 $\beta$  and is also composed of a family of three proteins, the type I receptor (IL-1RI), the type II receptor (IL-1RII) and the receptor accessory protein (IL-1RAcP). Of the two IL-1 receptors, just IL-1RI is signal transducing, and this only occurs after binding of the accessory protein. The IL-1RII receptor has been described as a 'decoy' receptor since it has no functional activity on binding IL-1, and by preventing IL-1 from binding to the functional IL-1RI receptor, it helps to regulate its activity. It has a high affinity for IL-1 $\beta$  but much lower for IL-1 $\alpha$  and IL-1Ra, which is consistent with a regulatory role.

The receptor antagonist (IL-1Ra) is also a natural regulator of IL-1 since it binds to both the IL-1 receptors, but inhibits binding of the accessory protein, thus blocking signalling (Sims, JE, 2002). IL-1Ra exists as three structural isoforms. The secreted form (sIL-1Ra) is a 17kDa protein primarily produced by mononuclear cells, and the 18kDa intracellular form (icIL-1RaI) is found in keratinocytes and other epithelial cells, monocytes, tissue macrophages, fibroblasts and endothelial cells (Arend, WP, 2002). A second 16kDa intracellular form (icIL-1RaII) has also been detected, thought to be derived by alternative translation initiation from the mRNA of sIL-1Ra (Malyak, M *et al.*, 1998). icIL-1RaI has been shown to have no effect intracellularly on IL-1 mediated signalling, but inhibits IL-1 responses only when released from the cell (Evans, I *et al.*, 2006).

In the intestinal mucosa, levels of total IL-1Ra protein have been found to be greater in the epithelial cells than the LPMCs in both normal and IBD

subjects (Cominelli, F & Pizarro, TT, 1996). sIL-1Ra was detectable in LPMCs from both normal and inflamed tissue, but only in epithelial cells from inflamed tissue. In contrast, icIL-1Ra was virtually undetectable in both LPMCs and epithelial cells from normal tissue but induced during active inflammation in both cell types (Cominelli, F *et al.*, 1996). The authors suggested that production of IL-1Ra from epithelial cells in particular, may be a mechanism of controlling the harmful effects of IL-1 produced during an inflammatory response. A mucosal imbalance between IL-1 and IL-1Ra has been shown to be present in patients with IBD. The ratio IL-1Ra/IL-1 decreased markedly in both CD and UC patients when compared to control subjects, and it was also found to correlate with severity of disease (Casini-Raggi, V *et al.*, 1995; Dionne, S *et al.*, 1998). Furthermore, the same studies found that the ratio did not decrease in inflammatory control patients, suggesting that the imbalance may be of pathogenic importance in IBD. An association has also been demonstrated between UC and allele 2 of the IL-1Ra gene (Mansfield, JC *et al.*, 1994). The association appeared to be greatest in patients with total colitis and was not seen in CD. Other groups have shown similar associations, one suggesting that the presence of allele 2 is a genetic marker for severity of disease (Bioque, G *et al.*, 1996), and another that the predisposition to UC caused by the allele may have an ethnic association (Tountas, NA *et al.*, 1999). A study in India (Mittal, RD *et al.*, 2005) found an association between allele 2 and patients with CD but not with UC, the opposite result to studies from the West, which they also proposed was due to ethnic differences and genetic heterogeneity.

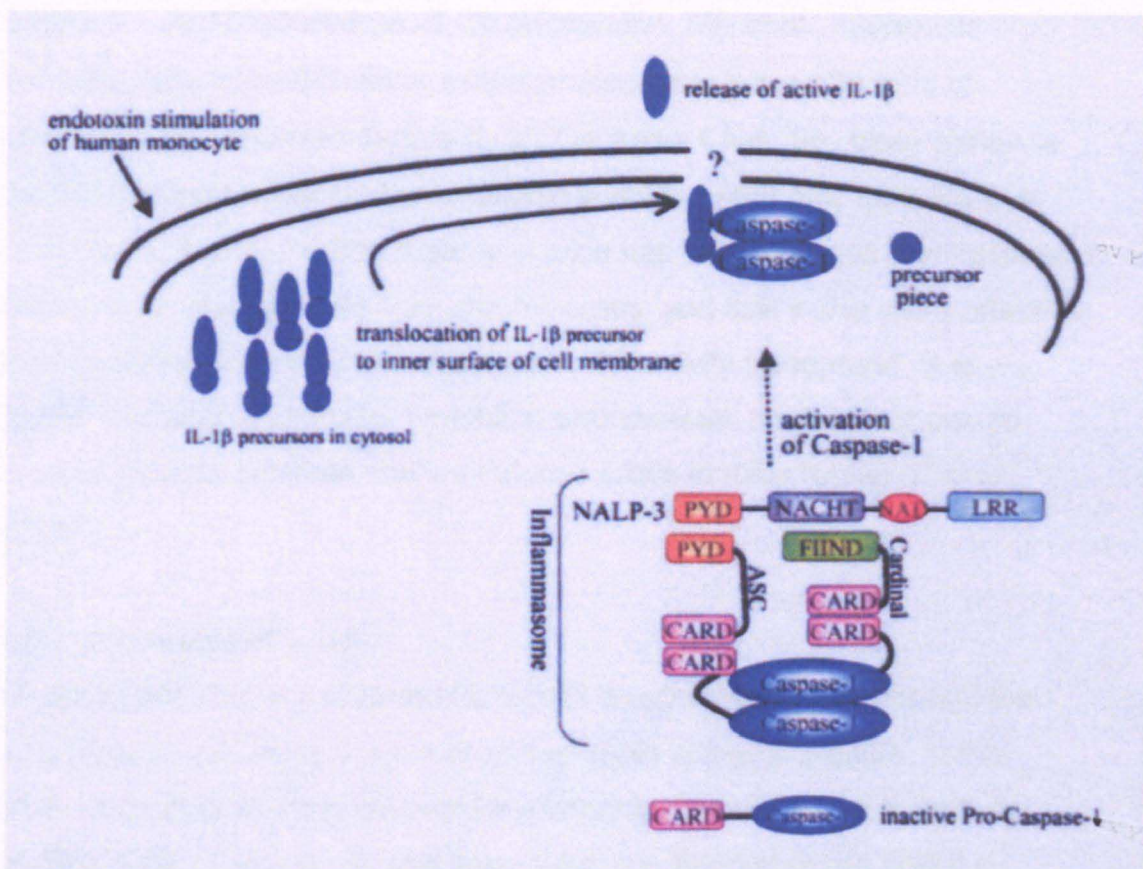
### **1.6.1. Cleavage of IL-1 $\beta$**

Biological activity of IL-1 $\beta$  requires cleavage of the inactive pro-IL-1 $\beta$ . IL-1 $\beta$  is cleaved to its mature form by a specific protease enzyme, IL-1 $\beta$  converting enzyme (ICE). ICE is a member of a family of intracellular cysteine proteases known as caspases (cysteine proteases cutting after aspartic acid, (Alnemri, ES *et al.*, 1996)). ICE (caspase-1) cleaves pro-IL-1 $\beta$  after the aspartic acid residue at position 116, to produce the biologically active 17kDa form (Wilson, KP *et al.*, 1994).

Caspase-1 is constitutively expressed in many cell types and like IL-1 $\beta$ , is also produced as an inactive precursor (Siegmund, B, 2002). The active enzyme is a heterodimer composed of 10kDa and 20kDa proteins. It is generated by cleavage of the 45kDa inactive pro-enzyme at four aspartic acid residues, resulting in removal of a 13kDa amino-terminal domain, and a 2kDa protein separating the 10- and 20kDa subunits (Ayala, JM *et al.*, 1994). Maturation of caspase-1 occurs through a caspase-activating complex called the inflammasome (Martinon, F *et al.*, 2002). The inflammasome is a scaffold of interacting proteins which bind pro-caspase enzymes through caspase-recruitment domains (Fig 1.6.). The NALP3 inflammasome binds only pro-caspase-1 and is restricted to immune cells; once bound, activation of pro-caspase-1 occurs through autocatalysis (Martinon, F & Tschopp, J, 2004). Since excessive IL-1 $\beta$  production is harmful, it is not surprising that as well as controlling the activity of IL-1 $\beta$ , cells also have regulatory mechanisms for controlling caspase-1 activity. The LRR region of the NALP3 protein is thought to bind to the NACHT region exerting an autoinhibitory effect, and the cell also contains several proteins (COP, ICEBERG, DASC) that interfere with pro-caspase-1 binding to the inflammasome (Martinon, F *et al.*, 2004). A single amino acid mutation in the NACHT region of the NALP3 protein has been shown to be involved in hereditary fever syndromes and chronic inflammatory diseases (Agostini, L *et al.*, 2004). Macrophages from such patients secrete IL-1 $\beta$  even in the absence of a stimulus and it is thought that the mutation prevents the autoinhibitory effect of LRR binding to the NACHT domain (Agostini, L *et al.*, 2004; Dinarello, CA, 2004). Another protein, proteinase inhibitor 9, has been shown to inhibit caspase-1 by blocking its active site (Annand, RR *et al.*, 1999), and nitric oxide is also a potent inhibitor of caspase-1, preventing the release of IL-1 $\beta$  and IL-18 from macrophages (Kim, YM *et al.*, 1998).

In the human intestinal mucosa, lamina propria macrophages have been shown to be the predominant caspase-1-expressing cell type (McAlindon, ME *et al.*, 1999). This is consistent with the finding that macrophages are also responsible for the release of mature IL-1 $\beta$  in inflamed mucosa (Mahida,





**Fig 1.6. The role of the inflammasome in activation of caspase-1 and release of mature IL-1 $\beta$**

Monocytes stimulated with substances such as bacterial endotoxin (LPS) accumulate pro-IL-1 $\beta$  in the cytosol. Pro-caspase-1 is constitutively expressed in these cells and is activated by the inflammasome.

The caspase recruitment domain (CARD) on pro-caspase-1 interacts with CARD of an adapter protein (ASC). The pyrin domain (PYD) of ASC binds to the PYD of NALP-3. NALP-3 is composed of a NACHT domain, a NALP-associated domain (NAD) and a region of leucine-rich repeats (LRR). The CARD of caspase-1 also interacts with CARD of a protein called Cardinal, which binds to NACHT in NALP-3 via its N-terminal domain (FIIND). Formation of the inflammasome results in activation of caspase-1 by autocatalysis which then associates with pro-IL-1 $\beta$  at the inner surface of the cell membrane. Cleavage of pro-IL-1 $\beta$  occurs and active IL-1 $\beta$  is secreted from the cell.

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YR *et al.*, 1989b). Studies on LPMCs isolated from normal and IBD mucosa showed that cells from normal tissue only expressed the inactive p45 form of caspase-1 and produced pro-IL-1 $\beta$  (McAlindon, ME *et al.*, 1998b). In contrast, cells from IBD tissue also expressed the active p20 form of caspase-1 and secreted mature IL-1 $\beta$ . Caspase-1 has also been shown to be strongly expressed in normal colonic epithelium but only in its inactive form (Jarry, A *et al.*, 1999). A study in mice has demonstrated that caspase-1 deficiency protected them from chronic colitis, and that it was more effective than treatment with IL-1Ra or blocking IL-18 activity (Siegmund, B *et al.*, 2001). Similarly, a caspase-1 inhibitor, pralnacasan, has been shown to prevent dextran sulphate sodium-induced colitis in mice (Loher, F *et al.*, 2004).

### **1.6.2. Secretion of IL-1 $\beta$**

Most proteins that are secreted from cells do so via transport through the endoplasmic reticulum; a process that requires a 'signal peptide' at the amino-terminus. IL-1 $\beta$  is unusual for a secreted protein in that it does not contain a signal sequence, and accumulates in the cytoplasm until it is processed and released. The mechanisms governing the maturation and release of active IL-1 $\beta$  are not well understood, but it is known that two separate stimuli are required.

In the human colon there is a complex population of microorganisms, largely composed of Gram-negative bacteria (MacDonald, TT, 1995). LPS is the main component of the outer membrane of Gram-negative bacteria, and is a potent stimulator of inflammation. LPS stimulation of monocytes and macrophages induces many genes which express inflammatory mediators such as cytokines and chemokines, including the production of large amounts of pro-IL-1 $\beta$  (Guha, M & Mackman, N, 2001). This pro-cytokine, however, is not released in its mature form unless the cells receive a second stimulus that will promote maturation of caspase-1.

Agents which have been shown to induce processing and release of IL-1 $\beta$  include antimicrobial peptides (Perregaux, DG *et al.*, 2002), nigericin (Cheneval, D *et al.*, 1998) and adenosine 5'-triphosphate (ATP) (Perregaux,

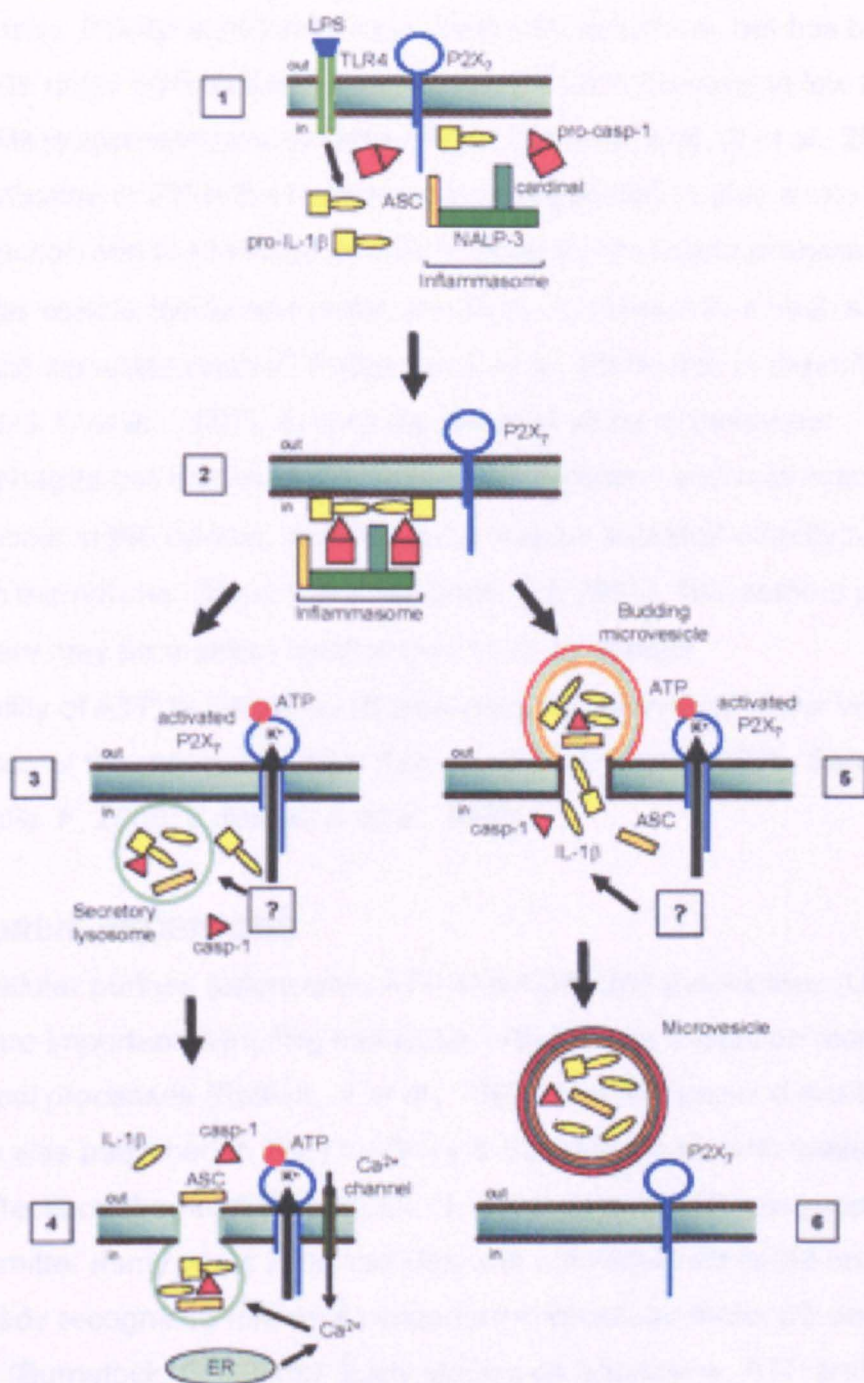
DG & Gabel, CA, 1998b; Perregaux, DG *et al.*, 2000). All of these agents are able to promote major changes to the intracellular ionic environment, in particular efflux of potassium ions ( $K^+$ ) from the cell which has been shown to be necessary for caspase-1 maturation (Cheneval, D *et al.*, 1998; Perregaux, DG & Gabel, CA, 1994).

Some studies suggest that caspase-1 may also play a part in the secretion of IL-1 $\beta$  from the cell. In LPS-stimulated monocytes, Singer, II *et al.*, (1995) used immuno-electron microscopy to demonstrate localisation of caspase-1 on the external cell membrane in conjunction with IL-1 $\beta$ . Others have shown an association between extracellular release of mature 20kDa caspase-1 subunits and IL-1 $\beta$  processing in stimulated monocytes (Laliberte, RE *et al.*, 1999).

Two groups have proposed vesicular mechanisms for IL-1 $\beta$  secretion (Fig 1.7.). Andrei, C *et al.*, (2004) demonstrated localisation of pro-IL-1 $\beta$  and pro-caspase-1 in secretory lysosomes from ATP-stimulated human monocytes. They suggested that ATP-induced  $K^+$  efflux is crucial for the exocytosis of these lysosomes and the secretion of mature IL-1 $\beta$ . The process also required activation of three phospholipase enzymes: phosphatidylcholine-specific phospholipase C and calcium-independent and -dependent phospholipase A<sub>2</sub>. Calcium-independent phospholipase A<sub>2</sub> has been shown to be involved in processing of pro-IL-1 $\beta$  to the mature form (Walev, I *et al.*, 2000), whereas the other two enzymes are required for secretion (Andrei, C *et al.*, 2004). A more recent study has shown that inhibitors of histone deacetylases prevented exocytosis of IL-1 $\beta$ -containing secretory lysosomes (Carta, S *et al.*, 2006). The inhibition appeared to be due to disruption of a microtubule network required for activation of calcium-dependent phospholipase A<sub>2</sub>.

MacKenzie, A *et al.*, (2001) using a monocytic cell line (THP-1 cells) stimulated with ATP, found that the monocytes shed microvesicles from their plasma membrane within 2-5 seconds of stimulation. Two minutes after stimulation the vesicles were shown to contain mature IL-1 $\beta$  which later appeared in the vesicle-free supernatant. Formation of the microvesicles was accompanied by phosphatidylserine (PS) flip and loss of membrane





**Fig 1.7. Proposed pathways for P2X<sub>7</sub>-stimulated IL-1 $\beta$  cleavage and release**

1. Activation of TLRs by LPS causes accumulation of pro-IL-1 $\beta$  and formation of the inflammasome.
2. Pro-IL-1 $\beta$  and the inflammasome localise below the inner leaflet of the plasma membrane.
3. Activation of P2X<sub>7</sub> triggers K<sup>+</sup> efflux leading to the formation of secretory lysosomes containing some components of the inflammasome. During this process, procaspase-1 is cleaved and pro-IL-1 $\beta$  is converted to mature IL-1 $\beta$ .
4. The lysosome content is secreted in a process requiring P2X<sub>7</sub>-dependent K<sup>+</sup> efflux, Ca<sup>2+</sup> increase, activation of PC-specific phospholipase C and phospholipase A2.

Alternatively:

5. Activation of P2X<sub>7</sub> and K<sup>+</sup> efflux triggers budding of small membrane blebs (microvesicles) that trap some of the inflammasome components. During this process, procaspase-1 is cleaved and pro-IL-1 $\beta$  is converted to mature IL-1 $\beta$ .
6. Eventually the membrane blebs pinch off and diffuse into the pericellular space.

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asymmetry. PS-flip is normally associated with apoptosis, but has been shown to occur constitutively on a subset of T cells expressing low levels of CD45RB (transmembrane tyrosine phosphatase) (Elliott, JI *et al.*, 2005).

Redistribution of PS in the membrane was suggested to play a role in signal transduction and to modulate activity of several membrane proteins.

A similar vesicle-mediated mechanism for IL-1 $\beta$  release has been shown in microglia stimulated with ATP (Bianco, F *et al.*, 2005) and in dendritic cells (Pizzirani, C *et al.*, 2007). In contrast, a recent study in peritoneal macrophages has shown that activation of caspase-1 and maturation of pro-IL-1 $\beta$  occur in the cytosol, and that IL-1 $\beta$  may be secreted directly across the plasma membrane (Brough, D & Rothwell, NJ, 2007). The authors proposed that there may be multiple mechanisms of IL-1 $\beta$  release.

The ability of ATP to initiate IL-1 $\beta$  processing is believed to occur via activation of the purine receptor, P2X<sub>7</sub> (Ferrari, D *et al.*, 1997b; Sanz, JM & Di Virgilio, F, 2000; Colomar, A *et al.*, 2003).

## 1.7. PURINE RECEPTORS

Extracellular purines (adenosine, ATP and ADP) and pyrimidines (UTP and UDP) are important signalling molecules with diverse effects on many biological processes (Ralevic, V *et al.*, 1998). The first paper describing their actions was published in 1929 by Drury & Szent-Gyorgyi, who investigated their effects on the heart (Burnstock, G, 2004). When ATP was proposed as a transmitter many years later, the idea was not widely accepted because of its already recognised role as an important intracellular molecule and energy source (Burnstock, G, 2006b). Early studies on adenosine, ATP and UTP focussed on their cardiovascular effects, but later research also investigated effects of purines on platelet aggregation (Born, GV, 1962) and on mast cells (Cockcroft, S & Gomperts, BD, 1980). Since then, many diverse responses to extracellular purines and pyrimidines have been reported in a wide range of biological systems, including smooth muscle contraction, neurotransmission, exocrine and endocrine secretion, the immune response, inflammation, platelet aggregation, pain, and modulation of cardiac function (Ralevic, V *et al.*, 1998).

Purinergic receptors were first formally recognised in 1978 by Burnstock, who proposed their division into two main families; P1 receptors that respond to adenosine and P2 receptors that recognise ATP, ADP, UTP and UDP (Ralevic, V *et al.*, 1998). P1 receptors are all G-protein coupled and have been divided into four subtypes, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>; their distribution and properties are shown in Table 1.7.

P2 receptors have been divided into two sub-families dependent on whether they are G-protein coupled (P2Y), or membrane ion channels (P2X) (Abbracchio, MP & Burnstock, G, 1994). Further sub-divisions have been made based on molecular structure of cloned receptors, and currently six mammalian P2Y receptors (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub> and P2Y<sub>12</sub>) and seven P2X receptor subunits (P2X<sub>1-7</sub>) are recognised (Fig 1.8).

P2Y receptors respond to nucleotides either by activating phospholipase C and releasing intracellular calcium, or by affecting adenylyl cyclase and altering cAMP levels (Burnstock, G, 2006b). Many cells express multiple P2Y receptor subtypes; some are activated principally by nucleotide diphosphates, others by triphosphates. Likewise, some P2Y receptors are activated by both purine and pyrimidine nucleotides, whereas others are activated by purine nucleotides alone (Burnstock, G, 2006b). P2Y receptors are widely distributed (Table 1.8) and have many biological effects including vasodilation, hormone secretion, platelet aggregation and regulation of cell function.

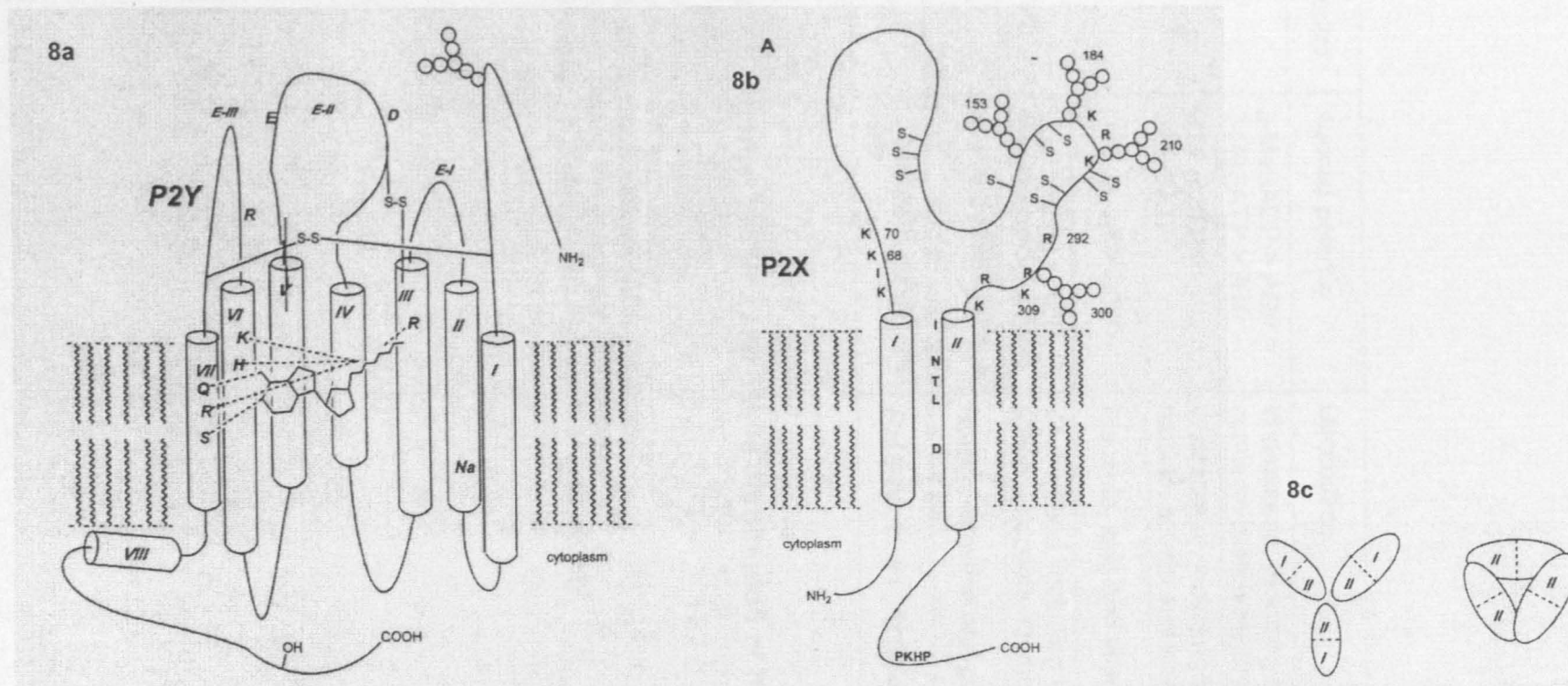
P2X receptors are abundantly distributed and functional responses have been seen in neurons, glia, epithelia, endothelia, and bone, muscle and haematopoietic tissues (North, RA, 2002). They are involved in smooth muscle contractility, neuroendocrine secretion and modulation of synaptic transmission. They are also thought to be involved in pain perception and are key factors in sensing tissue damage and inflammatory stimuli (North, RA, 2002). P2X receptors are composed of subunits which have two hydrophobic transmembrane domains with short intracellular amino- and carboxyl-terminals and a large extracellular region containing ten cysteine residues (Fig 1.8.b) (Burnstock, G, 1997). The functional receptors are thought to be oligomeric structures; studies of P2X<sub>1</sub>, P2X<sub>2</sub> and P2X<sub>3</sub> have shown them to be composed of three subunits (Fig 1.8.c) (Nicke, A *et al.*, 1998; Mio, K *et al.*,

	Distribution	Effects
A <sub>1</sub>	Widely distributed but particularly ubiquitous within the CNS.	Reduces neurotransmitter release and neuronal firing. In the heart it reduces the rate, muscle conductivity, and force of contraction. Reduction of lipolysis in adipose tissue, and reduction of urine formation.
A <sub>2A</sub>	CNS, vascular smooth muscle, endothelium, immune tissues, platelets.	Acts on endothelial and smooth muscle cells to produce vasodilation. Interacts with dopamine receptor signalling in the CNS.
A <sub>2B</sub>	Present in practically every cell but in small numbers and need relatively high concentrations of adenosine to evoke a response. Present at higher levels in parts of the intestine and bladder.	May be involved in regulation of vascular tone and mast cell function.
A <sub>3</sub>	Widely distributed but expression levels are generally low.	Anti-inflammatory response in neutrophils, eosinophils and macrophages by direct effect on cell degranulation or the production of anti-inflammatory cytokines. In basophils, degranulation and mediator release lead to bronchospasm and asthma. In cardiomyocytes, neuronal cells and bone marrow cells activation induces cytoprotective effects in vitro. In vivo, agonists act as cardio- and neuroprotective agents and attenuate ischemic damage (Fishman, P & Bar-Yehuda, S, 2003).

**Table 1.7. Distribution and properties of P1 receptors**

Ralevic, V *et al.*, (1998); Klotz, K-N, (2000)





**Fig 1.8. Schematic representation of the P2Y and P2X receptors**

- P2Y receptors have 7 transmembrane domains, an internal C-terminal chain and an external N-terminal chain. The diagram shows the human P2Y<sub>1</sub> receptor which binds ATP via transmembrane domains 3, 6 and 7 and extracellular loops 2 and 3.
- P2X receptors have 2 transmembrane domains and both terminal chains are intracellular. In the extracellular domain there are 10 highly conserved cysteine residues (-S) thought to form disulphide bridges. Conserved positively charged residues (K and R) at positions 68, 70, 292 and 309 are important for ATP binding in the P2X<sub>1</sub> receptor.
- Possible arrangements of P2X subunits. P2X receptors are thought to be arranged in trimers, intersubunit recognition is dependent on residues in the second transmembrane domain.

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Receptor	Agonist potency	Distribution
P2Y <sub>1</sub>	2-MeSADP > ADP > 2-MeSATP > ATP	Widespread, including heart, vascular, connective, immune and neural tissues.
P2Y <sub>2</sub>	UTP = ATP >> 2-MeSATP	Widespread, including lung, heart, skeletal muscle, spleen and kidney.
P2Y <sub>4</sub>	UTP >> ATP,UDP	Placenta, lung and vascular smooth muscle.
P2Y <sub>6</sub>	UDP > UTP > ADP > 2-MeSADP >> ATP	Widespread, including lung, heart, aorta, spleen, placenta, thymus, intestine and brain.
P2Y <sub>11</sub>	ATP > 2-MeSATP >>> ADP	Spleen, intestine and granulocytes.
P2Y <sub>12</sub>	2-MeSADP > ADP >> ATP	Platelets, brain (Nicholas, RA, 2001).

**Table 1.8. Agonist profiles and tissue distribution of human P2Y receptors**

ADP; Adenosine 5'-diphosphate

ATP; Adenosine 5'-triphosphate

2-MeSADP (ATP); 2-methylthioADP (ATP)

UTP (UDP); Uridine 5'-triphosphate (diphosphate)

von Kugelgen, I & Wetter, A, (2000); Ralevic, V *et al.*, (1998)

2005), and the second trans-membrane domain appears to be critical for subunit interactions (Torres, GE *et al.*, 1999b).

The native receptor assembly is unknown for most cell types, but studies with cloned P2X receptors have shown that all of the receptors are able to produce homo-oligomers except P2X<sub>6</sub>. Most of the P2X subunits associate with each other to form stable hetero-oligomers, particularly P2X<sub>5</sub> which associates with all of the other receptor subunits, but P2X<sub>7</sub> forms only homo-oligomeric units (Torres, GE *et al.*, 1999a). The seven cloned receptors show distinct pharmacological profiles (Table 1.9), but for all of them their naturally occurring agonist is ATP. Signal transduction occurs via fast influx of sodium (Na<sup>+</sup>) and calcium (Ca<sup>2+</sup>) ions and K<sup>+</sup> efflux, leading to depolarisation of the plasma membrane and an increase in intracellular Ca<sup>2+</sup> concentration (Di Virgilio, F *et al.*, 2001).

The role of P2 receptors in the immune system has been widely investigated over the last ten years. Nucleotides are now considered to be inflammatory mediators, released as danger signals to the immune system (Gallucci, S & Matzinger, P, 2001). They are concentrated to micromolar or even millimolar levels within the cytoplasm of every living cell, but the extracellular concentration is very low (Di Virgilio, F *et al.*, 2003). Stressed or damaged cells can locally release nucleotides which may be detected by APCs, activating them and initiating an immune response (Gallucci, S *et al.*, 2001). Once released, the nucleotides are degraded by ecto-nucleotidase enzymes expressed on the plasma membranes of most cells (Zimmermann, H, 2000). This results in the formation of adenosine, which has been shown to have an anti-inflammatory function via the A<sub>2A</sub> P1 receptor (Ohta, A & Sitkovsky, M, 2001). Hence there is an inbuilt mechanism for controlling any inflammatory response produced (Fig 1.9.).

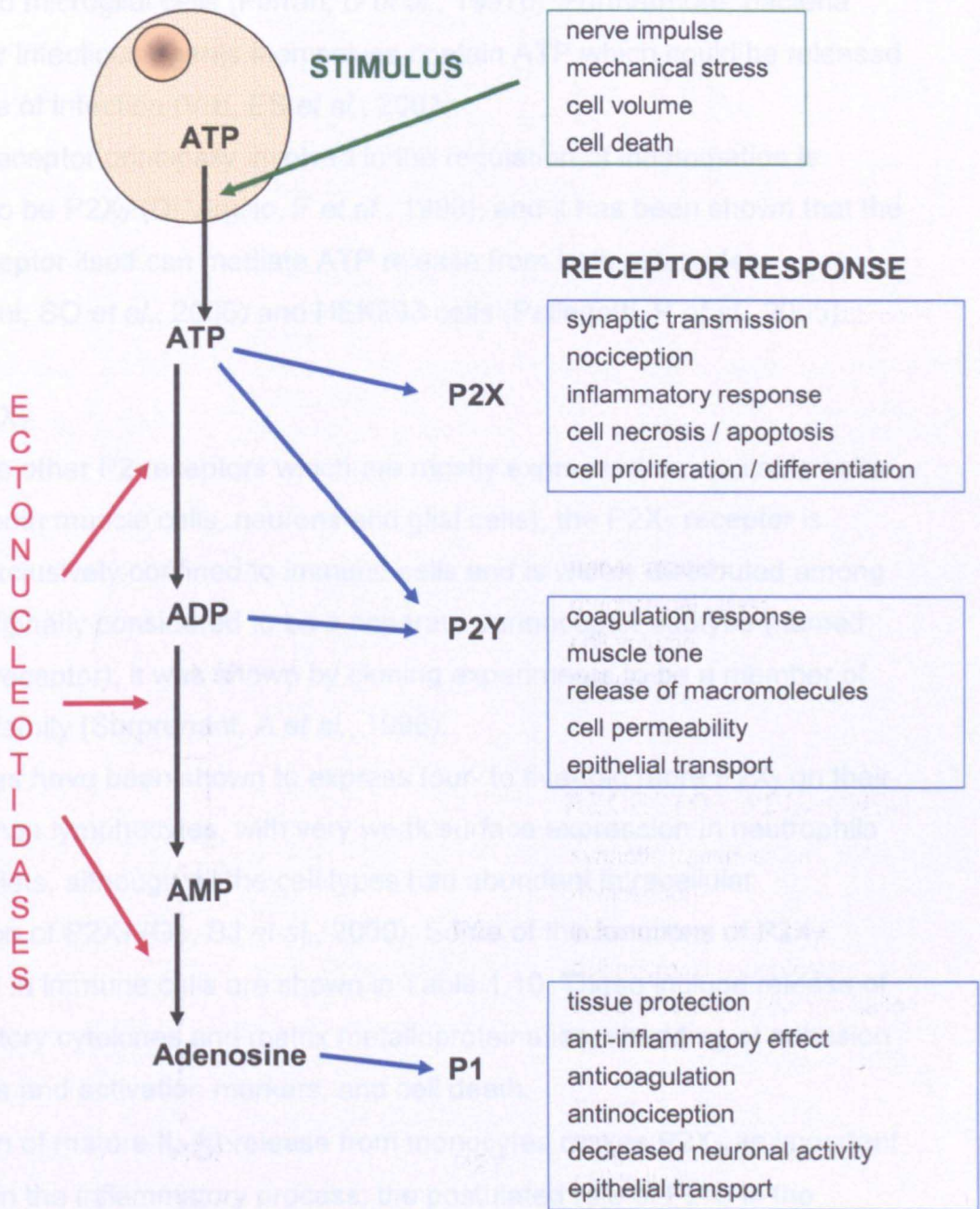
Apart from cellular damage, ATP can be released by other mechanisms. Tissue damage is generally associated with vascular injury which leads to platelet adherence and degranulation, releasing high levels of ATP (Hechler, B *et al.*, 2005). Endothelial cells of the vessel walls have also been shown to release ATP both constitutively, and when stimulated with Ca<sup>2+</sup>-mobilizing agents or by mechanical stress (Schwiebert, LM *et al.*, 2002). Macrophages release ATP when infected with *Mycobacterium tuberculosis* (Sikora, A *et al.*,

	Agonist activity of cloned receptors	Distribution of native receptors
hP2X <sub>1</sub> rP2X <sub>1</sub>	ATP > $\alpha,\beta$ -meATP 2-MeSATP > ATP > $\alpha,\beta$ -meATP	CNS, principal P2X receptor expressed in smooth muscle.
rP2X <sub>2</sub>	2-MeSATP > ATP, $\alpha,\beta$ -meATP inactive	The only P2X receptor found in the adrenal medulla. Widespread in brain and spinal cord, also found in bladder, intestine, vas deferens.
hP2X <sub>3</sub> rP2X <sub>3</sub>	2-MeSATP > ATP > $\alpha,\beta$ -meATP 2-MeSATP > ATP > $\alpha,\beta$ -meATP	Expressed only by a subset of sensory neurons
hP2X <sub>4</sub> rP2X <sub>4</sub>	ATP > 2-MeSATP > $\alpha,\beta$ -meATP ATP > 2-MeSATP > $\alpha,\beta$ -meATP	The only P2X receptor expressed by acinar cells of salivary glands. Expressed in brain, spinal cord, sensory ganglia, lung, bronchial epithelium, thymus, bladder, adrenal gland, testis, vas deferens.
rP2X <sub>5</sub>	ATP > 2-MeSATP > ADP	Neural tissue but not expressed in the brain.
rP2X <sub>6</sub>	ATP > 2-MeSATP > ADP	CNS, uterus, ovary, bronchial epithelia.
hP2X <sub>7</sub> rP2X <sub>7</sub>	BzATP > ATP BzATP > ATP > 2-MeSATP > ADP	Principally cells of haematopoietic origin

**Table 1.9. Agonist profiles and tissue distribution of P2X receptors**

Seven P2X receptor proteins (principally human and rat) have been cloned. The ion channels formed from homomeric association of the subunits when expressed in cells have been functionally characterised and show distinct agonist profiles. P2X<sub>7</sub> is the only P2X receptor for which BzATP is a more potent agonist than ATP (Ralevic, V & Burnstock, G, 1998).

ADP; Adenosine 5'-diphosphate  
 ATP; Adenosine 5'-triphosphate  
 2-MeSATP; 2-methylthioATP  
 $\alpha,\beta$ -meATP;  $\alpha,\beta$ -methyleneATP  
 BzATP; 2'(3')-O-(4-benzoylbenzoyl)-ATP



**Fig 1.9. ATP release and its effects on purine receptors**

The inflammatory response is initiated in immune cells by the release of ATP from stressed or damaged cells and stimulation of the P2X<sub>7</sub> receptor. The response is controlled by breakdown of ATP to adenosine by ectonucleotidases leading to stimulation of anti-inflammatory P1 receptors. Novak, I, (2003)

1999), and cytotoxic T cells release ATP as part of their killing process (Filippini, A *et al.*, 1990; Blanchard, DK *et al.*, 1995). Activation with LPS has also been shown to release ATP from both macrophages (Sperlagh, B *et al.*, 1998) and microglial cells (Ferrari, D *et al.*, 1997c). Furthermore, bacteria and other infectious agents themselves contain ATP which could be released at the site of infection (Vizi, ES *et al.*, 2001).

The P2 receptor principally involved in the regulation of inflammation is thought to be P2X<sub>7</sub> (Di Virgilio, F *et al.*, 1998), and it has been shown that the P2X<sub>7</sub> receptor itself can mediate ATP release from both astrocytes (Suadicani, SO *et al.*, 2006) and HEK293 cells (Pellegatti, P *et al.*, 2005).

### 1.7.1. P2X<sub>7</sub>

Unlike the other P2 receptors which are mostly expressed on excitable cells (e.g. smooth muscle cells, neurons and glial cells), the P2X<sub>7</sub> receptor is almost exclusively confined to immune cells and is widely distributed among them. Originally considered to be a separate purinoceptor subtype (named the P2Z receptor), it was shown by cloning experiments to be a member of the P2X family (Surprenant, A *et al.*, 1996).

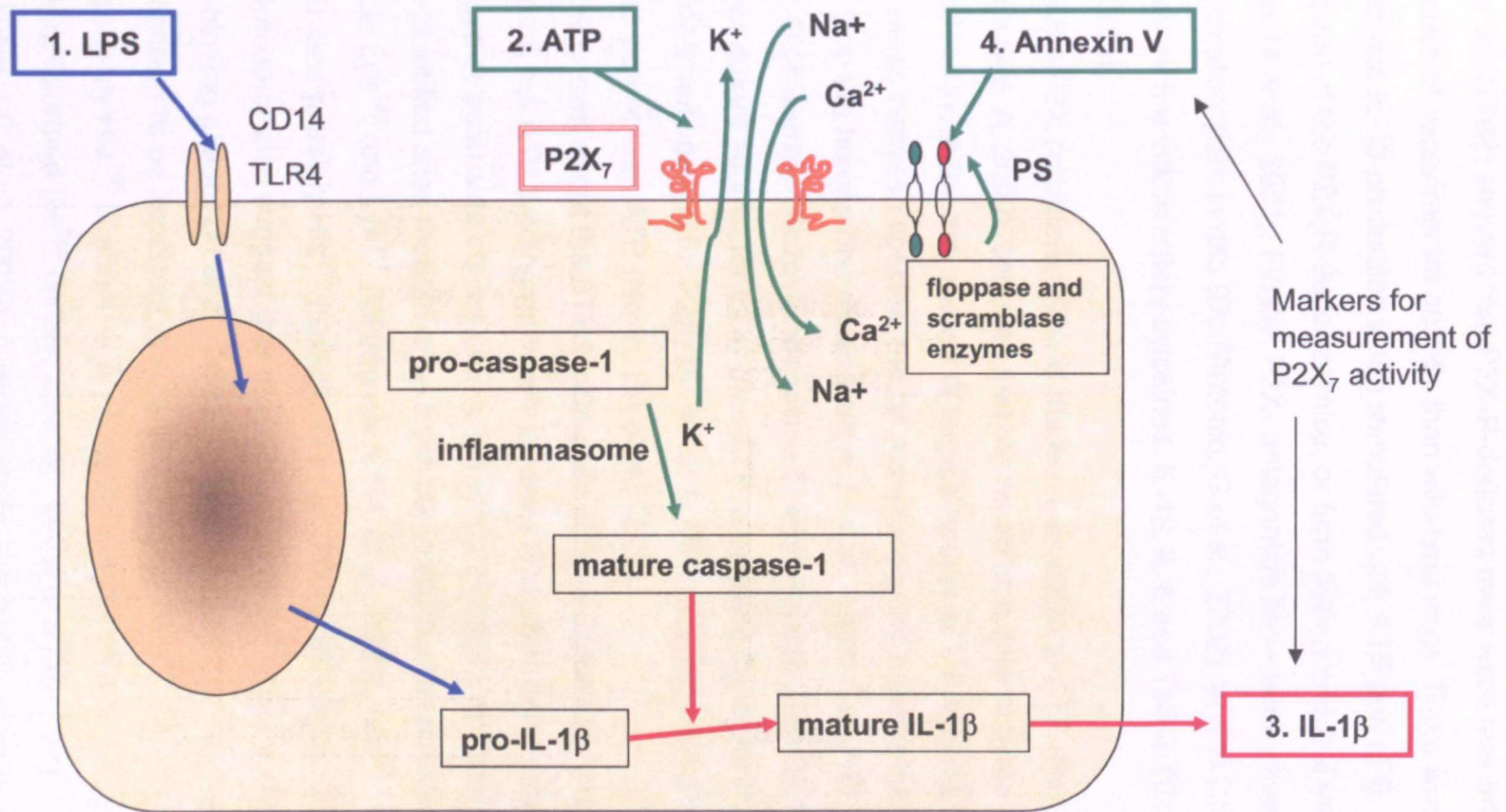
Monocytes have been shown to express four- to five-fold more P2X<sub>7</sub> on their surface than lymphocytes, with very weak surface expression in neutrophils and platelets, although all the cell types had abundant intracellular expression of P2X<sub>7</sub> (Gu, BJ *et al.*, 2000). Some of the functions of P2X<sub>7</sub> receptors in immune cells are shown in Table 1.10. These include release of inflammatory cytokines and matrix metalloproteinases, shedding of adhesion molecules and activation markers, and cell death.

Promotion of mature IL-1 $\beta$  release from monocytes makes P2X<sub>7</sub> an important receptor in the inflammatory process; the postulated role of P2X<sub>7</sub> in the release of IL-1 $\beta$  is shown in Fig 1.10. The fact that P2X<sub>7</sub> is an important inflammatory receptor has been demonstrated by several studies. Firstly, incubation of THP-1 monocyte cells with the inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ , increased amounts of P2X<sub>7</sub> mRNA (Humphreys, BD & Dubyak, GR, 1998a). The two cytokines were found to have a greater effect when added together, and IFN- $\gamma$  has also been shown to have a similar synergistic

Cell type	Agonist	Action	Reference
Human PBMC monocytes	ATP, BzATP	Rapid release of MMP-9, a matrix metalloproteinase which assists passage of cells through the basement membrane into areas of inflammation.	Gu, BJ & Wiley, JS, (2006)
Human monocyte-derived macrophages		Formation of multinucleated giant cells by concanavalin A $\pm$ IFN- $\gamma$ inhibited by P2X <sub>7</sub> antagonist oATP.	Falzoni, S <i>et al.</i> , (1995)
Human monocyte-derived macrophages	ATP, BzATP	Killing of intracellular bacteria.	Lammas, DA <i>et al.</i> , (1997)
J774 mouse macrophages		P2X <sub>7</sub> hyper-expressing clones more susceptible to spontaneous cell death.	Chiozzi, P <i>et al.</i> , (1996);
Mouse peritoneal macrophages	ATP	Induces DNA fragmentation and apoptotic cell death.	Hogquist, KA <i>et al.</i> , (1991a)
Human monocyte-derived macrophages	ATP	Release of mature IL-1 $\beta$	Ferrari, D <i>et al.</i> , (1997b);
Mouse peritoneal macrophages	ATP		Perregaux, DG <i>et al.</i> , (1994); Perregaux, DG <i>et al.</i> , (2001)
LG14 B-lymphoblastoid cells		Promotes proliferation of cells grown in the absence of exogenous growth factors. Growth inhibited by oATP and apyrase.	Baricordi, OR <i>et al.</i> , (1999)
Human PBMC lymphocytes	ATP, BzATP	Induces shedding of L-selectin (CD62L, an adhesion molecule important in transendothelial migration) and CD23 (a B cell activation marker).	Gu, B <i>et al.</i> , (1998);
Mouse splenic lymphocytes	ATP	Induces shedding of L-selectin and CD21 (involved in activation of B cells).	Sengstake, S <i>et al.</i> , (2006);
Mouse splenic lymphocytes	ATP	Induces shedding of CD27 (a co-stimulatory receptor involved in T cell expansion and generation of T and B cell memory).	Moon, H <i>et al.</i> , (2006)
Mouse splenic lymphocytes	ATP	Induces cell death.	Aswad, F <i>et al.</i> , (2005);
Mouse T lymphocytes	BzATP, ATP	Regulation of differentiation and cell death.	Chused, TM <i>et al.</i> , (1996)
Human dendritic cells	ATP	Release of mature IL-1 $\beta$ and TNF- $\alpha$	Ferrari, D <i>et al.</i> , (2000)
D2SC/1 mouse dendritic cells	ATP	Induces apoptosis	Coutinho-Silva, R <i>et al.</i> , (1999)
Human dendritic cells	ATP	Induces rapid shedding of CD23	Sluyter, R & Wiley, JS, (2002)

**Table 1.10. Effects of P2X<sub>7</sub> receptor stimulation on immune cells**





**Fig 1.10. Proposed relationship between P2X<sub>7</sub> stimulation, caspase-1 maturation and IL-1β release**

1. Bacterial endotoxin promotes the formation of pro-IL-1β within the cell.
2. Activation of P2X<sub>7</sub> by ATP promotes K<sup>+</sup> efflux leading to inflammasome formation and maturation of caspase-1.
3. Mature caspase-1 cleaves pro-IL-1β leading to release of mature IL-1β.
4. At the same time, influx of Ca<sup>2+</sup> leads to PS flip (detected by AV binding), a possible mechanism for IL-1β release or a marker of cell apoptosis.

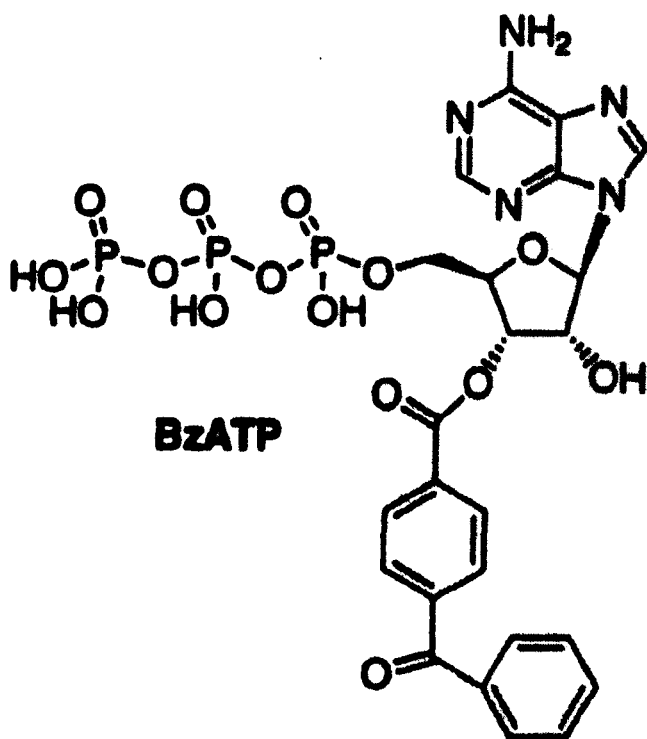
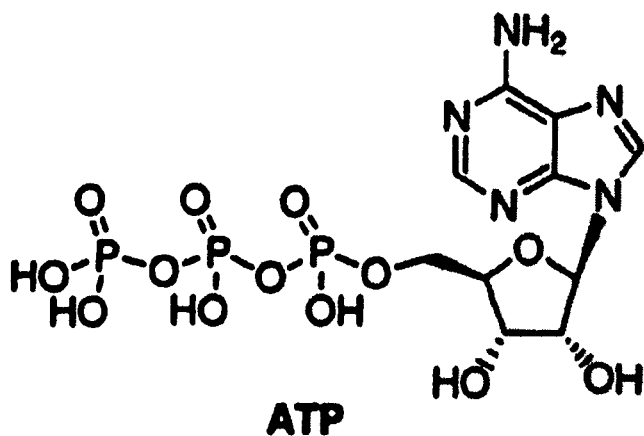
effect with LPS (Humphreys, BD & Dubyak, GR, 1996). Secondly, Labasi, JM *et al.*, (2002) showed that P2X<sub>7</sub>R-deficient mice were less affected by induction of experimental arthritis than wild-type mice. There was also no detectable IL-1 $\beta$  production when stimulated with ATP and LPS, either from the blood of the P2X<sub>7</sub>R-deficient mice, or from their peritoneal macrophages (Solle, M *et al.*, 2001). Finally, P2X<sub>7</sub> antagonists have been shown to inhibit inflammatory pain in rats (Dell'Antonio, G *et al.*, 2002), and to inhibit the release of the inflammatory cytokines, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (Gourine, AV *et al.*, 2005).

Of all the P2X receptors, P2X<sub>7</sub> is the least sensitive to ATP (North, RA & Surprenant, A, 2000), ensuring that the receptor is only activated in extreme conditions involving the release of large amounts of the agonist. Its sensitivity also varies between species, the rat receptor having a greater affinity for ATP than the human one (Rassendren, F *et al.*, 1997). The ATP analogue 2'(3')-O-(4-benzoylbenzoyl)-adenosine 5'-triphosphate (BzATP, Fig 1.11.) is a more potent agonist for P2X<sub>7</sub> than ATP, a defining factor for P2X<sub>7</sub> activity. BzATP is not specific for P2X<sub>7</sub> but at other P2X receptors it is equipotent with or less potent than ATP (North, RA *et al.*, 2000).

Little is known about the ATP binding site in the extracellular loop of the receptor, but ATP binding generally involves hydrogen bond formation with charged or polar side chains (Jiang, LH *et al.*, 2000b). Point mutation studies have identified sites thought to be involved; in the human receptor these include Lys<sup>193</sup> and Lys<sup>311</sup> (Worthington, RA *et al.*, 2002), Arg<sup>307</sup> (Gu, BJ *et al.*, 2004), and possibly His<sup>155</sup> (Cabrini, G *et al.*, 2005). Studies on the rat P2X<sub>7</sub>receptor also suggest that residues close to Ile<sup>67</sup> may be important in ATP-binding (Jiang, LH *et al.*, 2000b). Other sites on the P2X<sub>7</sub> receptor have been shown to be important in subunit assembly (Glu<sup>496</sup> (Gu, BJ *et al.*, 2001) and possibly His<sup>155</sup> (Cabrini, G *et al.*, 2005)), or in trafficking of the receptor to the cell surface (Ile<sup>568</sup> (Wiley, JS *et al.*, 2003) and Arg<sup>578</sup> and Lys<sup>579</sup> (Denlinger, LC *et al.*, 2003)). A recent study has identified the extracellular residue Thr<sup>283</sup> as being critical for mediation of P2X<sub>7</sub> receptor ion channel activity (Young, MT *et al.*, 2006).

In addition to ion channel activity, prolonged or repeated stimulation of the





**Fig 1.11. Structure of the principal P2X<sub>7</sub> agonists**

a. ATP: adenosine 5'-triphosphate, the natural agonist for the P2X<sub>7</sub> receptor.

b. BzATP: 2'(3')-O-(4-benzoylbenzoyl)-adenosine 5'-triphosphate.

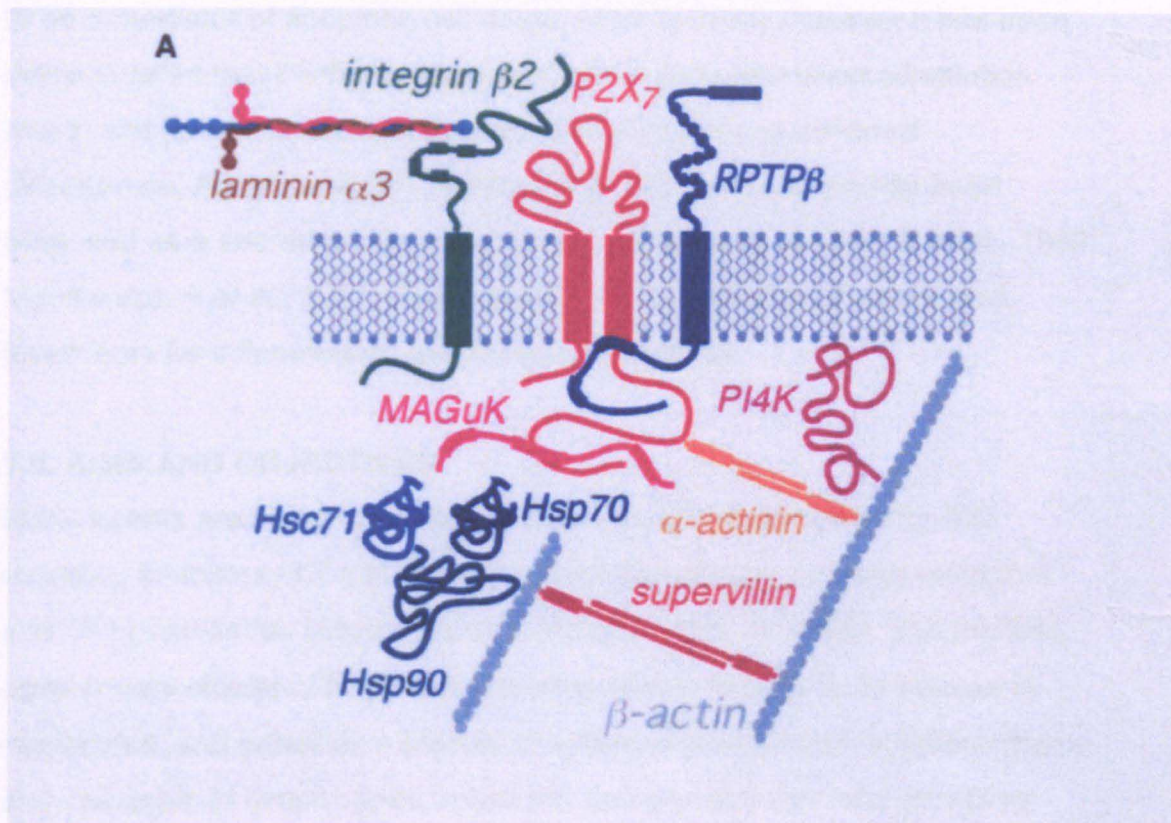
P2X<sub>7</sub> is characterised by the fact that it is the only P2X receptor at which BzATP is more potent than ATP.

P2X<sub>7</sub> receptor leads to the formation of a non-selective membrane pore that is permeable to small molecules. In lymphocytes the membrane pore is much smaller than that of other cells, only allowing permeation of molecules of approximately 300Da in size compared to approximately 900Da in other cells (Wiley, JS *et al.*, 1993). P2X<sub>7</sub> is structurally different from other P2X receptors in that it has a much longer carboxyl terminal, and this appears to be essential for pore formation (Surprenant, A *et al.*, 1996; Virginio, C *et al.*, 1999a). A study of the rat P2X<sub>7</sub> receptor demonstrated that 95% of the C-terminal chain was required for pore formation, but only a small portion for ion channel activity (Smart, ML *et al.*, 2003). Similarly Cheewatrakoolpong, B *et al.*, (2005) identified a splice variant of human P2X<sub>7</sub> lacking the C-terminus which demonstrated normal ion channel activity but was unable to form a pore.

Although it appears to have no role in the ion channel activity of P2X<sub>7</sub>, the C-terminal chain appears to be important in receptor function containing the sites for receptor trafficking as well as pore formation. Denlinger, LC *et al.*, (2001) also showed that it contains a region (amino acids 573-590) sharing strong amino acid homology with the LPS binding site of LPS-binding protein. They suggested that internalised LPS may play a regulatory role in receptor trafficking, and this is supported by the fact that the point mutations involved in trafficking lie within the LPS-binding region.

Kim, M *et al.*, (2001) have identified a rat P2X<sub>7</sub> signalling complex involving 11 proteins connected to the receptor via the C-terminal chain (Fig 1.12.). Some of the proteins are thought to initiate cytoskeletal rearrangements following receptor activation and others exert feedback control of the ion channel function. The residue at position 451 has been identified as a potential site of interaction with one or more of the proteins forming the signalling complex (Young, MT *et al.*, 2006).

Activation of P2X<sub>7</sub> by ATP is associated with increased exposure of the membrane phospholipid, phosphatidylserine (PS), on the outer surface of the cell membrane (Harada, H *et al.*, 2000; MacKenzie, A *et al.*, 2001). PS is normally located predominantly on the inner surface of the cell membrane, but during early apoptosis it translocates from the inner to the outer layer of the plasma membrane (PS-flip) where it is thought to trigger recognition and



**Fig 1.12. Schematic representation of the signalling complex for the rat P2X<sub>7</sub> receptor expressed in HEK293 cells**

11 proteins have been identified which interact with the rat P2X<sub>7</sub> receptor. These are the extracellular matrix protein laminin α3, membrane-spanning proteins integrin β2 and receptor protein tyrosine phosphatase β (RPTPβ), and 8 intracellular proteins, namely α-actinin 4, β-actin, supervillin, three heat shock proteins (Hsp90, Hsc71, Hsp70), phosphatidylinositol 4-kinase 230 (PI4K), and membrane-associated guanylate kinase P55 (MAGuK).

Reprinted by permission from Macmillan Publishers Ltd: EMBO Journal, Kim, M *et al.*, Proteomic and functional evidence for a P2X<sub>7</sub> receptor signalling complex, 6347-58. Copyright (2001).

phagocytosis by macrophages (Fadok, VA *et al.*, 1992). Naito, M *et al.*, (1997) suggested that this PS externalisation was a downstream event of caspase activation since it could be blocked by caspase inhibitors. For many years, therefore, ATP-stimulation of the P2X<sub>7</sub> receptor has been considered to be a mediator of apoptotic cell death. More recently however it has been demonstrated that PS-flip is reversible, particularly with short stimulation times, and its role as an apoptotic receptor has been questioned (MacKenzie, AB *et al.*, 2005). Alternatively, P2X<sub>7</sub> stimulation has been proposed as a secretory mechanism for IL-1 $\beta$  release (Andrei, C *et al.*, 1999; MacKenzie, A *et al.*, 2001) thus making P2X<sub>7</sub> a potential target for new treatments for inflammatory conditions such as IBD.

### **1.8. AIMS AND OBJECTIVES**

Many agents are being investigated as prospective treatments for IBD including inhibitors of T cell activation, proinflammatory cytokine receptors and Th1 polarisation (Ardizzone, S & Bianchi Porro, G, 2005). The multiple downstream effects of P2X<sub>7</sub> receptor stimulation, namely IL-1 $\beta$  release in monocytes, and adhesion molecule shedding and regulation of differentiation and cell death in lymphocytes, make this receptor another very attractive anti-inflammatory target.

Very little work has been carried out so far on the function of the P2X<sub>7</sub> receptor in the human colon. Colonic mucosal macrophages and T cells have been shown to express functional P2X<sub>7</sub> receptors associated with both apoptosis and the release of IL-1 $\beta$  (Li, CKF *et al.*, 2001). This project aims to investigate the characteristics of the P2X<sub>7</sub> receptor in human colonic LPMCs isolated from both normal and inflamed tissue. The pharmacological properties of the receptor will be investigated in terms of its agonist and antagonist profiles, in particular looking at the effects of receptor stimulation on IL-1 $\beta$  secretion and cell death. P2X<sub>7</sub>-stimulated PS flip will be detected using Annexin V binding, with propidium iodide to identify dead cells. This will be backed up by other methods for measuring cell apoptosis and death to ascertain whether P2X<sub>7</sub> mediated PS flip is associated with apoptotic cell death in LPMCs.

Comparisons of LPMC receptor activity will be made with a known P2X<sub>7</sub>-expressing monocyte cell line (THP-1 cells) and also with peripheral blood mononuclear cells (PBMCs). Since inflamed tissue contains a higher proportion of newly recruited cells from the circulation, it is to be expected that LPMCs from inflamed tissue will show characteristics closer to those of PBMCs than will LPMCs from normal tissue. The ability of novel P2X<sub>7</sub> antagonists to inhibit IL-1 $\beta$  release will also be investigated and their potential as new treatments for IBD considered.

## CHAPTER 2. GENERAL MATERIALS AND METHODS

### 2.1. CELL CULTURE AND ISOLATION OF PRIMARY CELLS

THP-1 cells are a monocyte cell line derived from the peripheral blood of a one-year-old male with acute monocytic leukaemia (Tsuchiya, S *et al.*, 1980). They express a well-characterised P2X<sub>7</sub> receptor which responds to ATP (Spranzi, E *et al.*, 1993) and therefore make a good model system for comparing receptor responses with those of PBMCs and LPMCs. They have been used to study many aspects of P2X<sub>7</sub> function including IL-1 $\beta$  release (Verhoef, PA *et al.*, 2005; Grahames, CB *et al.*, 1999), pore formation (Donnelly-Roberts, D *et al.*, 2004) and other functional studies (Wilson, HL *et al.*, 2002; Humphreys, BD *et al.*, 1998a; Buell, G *et al.*, 1998).

LPMCs were isolated from colonic tissue obtained from freshly resected operation specimens; normal tissue was acquired from cancer patients (>5cm from the tumour), and inflamed tissue from patients with Crohn's disease or ulcerative colitis. In most cases of inflamed tissue, there was also an accompanying specimen of uninflamed tissue. All patients gave written informed consent and approval was obtained from the Nottingham Local Research Ethics Committee. PBMCs were isolated from fresh venous blood taken from healthy volunteers and prevented from coagulating with 3.2% buffered sodium citrate.

#### 2.1.1. THP-1 cell culture

A sample culture donated by AstraZeneca R&D Charnwood (Loughborough, UK), was maintained in suspension in RPMI 1640 containing 25mM HEPES (Sigma-Aldrich, Poole, Dorset), supplemented with 10% v/v foetal calf serum (FCS), 2mM L-glutamine, and 50 $\mu$ g/ml gentamicin (all supplied by Invitrogen Co., Paisley) (hereafter called RPMI medium). Experimental culture flasks were seeded at  $1 \times 10^5$  cells/ml and used when the cell number reached approximately  $1 \times 10^6$  cells/ml (3-5 days). Cultures were propagated by dilution (1:10) in fresh medium.

### **2.1.2. Isolation of peripheral blood mononuclear cells (PBMCs)**

PBMCs were isolated using Histopaque-1077 (Sigma-Aldrich), a solution of polysucrose and sodium diatrizoate adjusted to a density of  $1.077 \pm 0.001\text{g/ml}$ . During centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and rapidly sediment, whereas lymphocytes and monocytes remain at the plasma-Histopaque interface (Sigma Diagnostics Inc., 1999).

Anticoagulated whole blood was carefully pipetted onto Histopaque-1077 and centrifuged at  $400 \times g$  for 30min at room temperature. The opaque interface containing the mononuclear cells was carefully transferred into clean tubes and washed with RPMI 1640 containing 25mM HEPES but with no added supplements. Cells were centrifuged at  $250 \times g$  for 10min, washed again and resuspended in RPMI 1640. PBMCs were used within one hour after preparation and were kept at room temperature prior to use. The cell population (estimated from flow cytometry scatter plots) was composed of approximately 75-80% lymphocytes, 5-10% monocytes and cell debris.

### **2.1.3. Isolation of lamina propria mononuclear cells (LPMCs)**

LPMCs were isolated from human colonic mucosa following the method of Mahida, YR *et al.*, (1997). The tissue sample was collected in cold RPMI 1640, and transported to a Class II safety cabinet for processing. The mucosa was washed several times in Hanks Balanced Salt Solution without calcium and magnesium ions ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS, Invitrogen), with gentle scraping to remove mucus, blood and digested material. The mucosa was dissected from the underlying muscularis mucosae in small strips and washed approximately six times by gentle shaking in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS. The mucosal strips were incubated in a shaking water bath at  $37^\circ\text{C}$  for 15min in 50ml of 1mM dithiothreitol (DTT, Sigma-Aldrich) in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS to remove surface mucus, followed by five washes in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS.

To remove epithelial cells, the strips were transferred into 50ml of pre-warmed 1mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS, and incubated at  $37^\circ\text{C}$  for 30min in a shaking water bath. The strips were washed five times as before, and then the EDTA

incubation and wash repeated a further two times, with a final wash in RPMI 1640. The strips were cut into approximately 1cm<sup>2</sup> pieces, placed in cell culture dishes containing RPMI medium and incubated overnight at 37°C in 5% CO<sub>2</sub>. After incubation the dishes were checked carefully to ensure that LPMCs had migrated out of the tissue, and for any bacterial contamination. The tissue was transferred into approximately 30ml RPMI 1640 and shaken gently to dislodge any cells still attached to it. The LPMCs were removed from the culture dish by repeated gentle pipetting, and the cell suspension transferred to 50ml conical tubes. The tissue washings and the cell suspension were pooled, centrifuged at 350 x g for 5min and resuspended in RPMI 1640. The LPMCs were used within one hour after preparation and were kept at room temperature prior to use.

Cell populations from normal tissue (estimated from flow cytometry scatter plots) were approximately 65-75% lymphocytes, 5-10% monocytes/macrophages and 15% neutrophils. In tissue from inflamed patients, the neutrophil population rose to approximately 25%. These numbers are consistent with phenotypic characterisation studies carried out on migrating cell populations from both normal and IBD tissue using combinations of monoclonal antibodies specific for different cell types. In normal tissue, the predominant cell population was found to be T cells (approximately 68%), and of the remaining cells, approximately 3% were B cells, 10% macrophages and 8% eosinophils (Mahida, YR *et al.*, 1997). In IBD tissue, the cell populations were found to be similar, except that the proportion of T cells was reduced (approximately 45%), and there was a neutrophil population of approximately 23% (McAlindon, ME *et al.*, 1998a).

#### **2.1.4. Assessment of cell viability**

Prior to experimentation, all of the cell suspensions were checked for viability by mixing an aliquot with an equal volume of Trypan blue (Sigma-Aldrich) and counting in a haemocytometer.

Viability was calculated as follows:

$$\text{Viability} = \frac{\text{number of live cells}}{\text{total number of cells}} \times 100 \%$$



The results are shown below:

cell type	% viability (mean $\pm$ se)	n
THP-1s	97.1 $\pm$ 0.6	4
PBMCs	95.4 $\pm$ 0.9	3
LPMCs	95.7 $\pm$ 1.8	3

Viability for LPMCs was determined with cells from normal tissue. The data shows that the cells were viable prior to the start of the experiments and were not damaged by the isolation procedures.

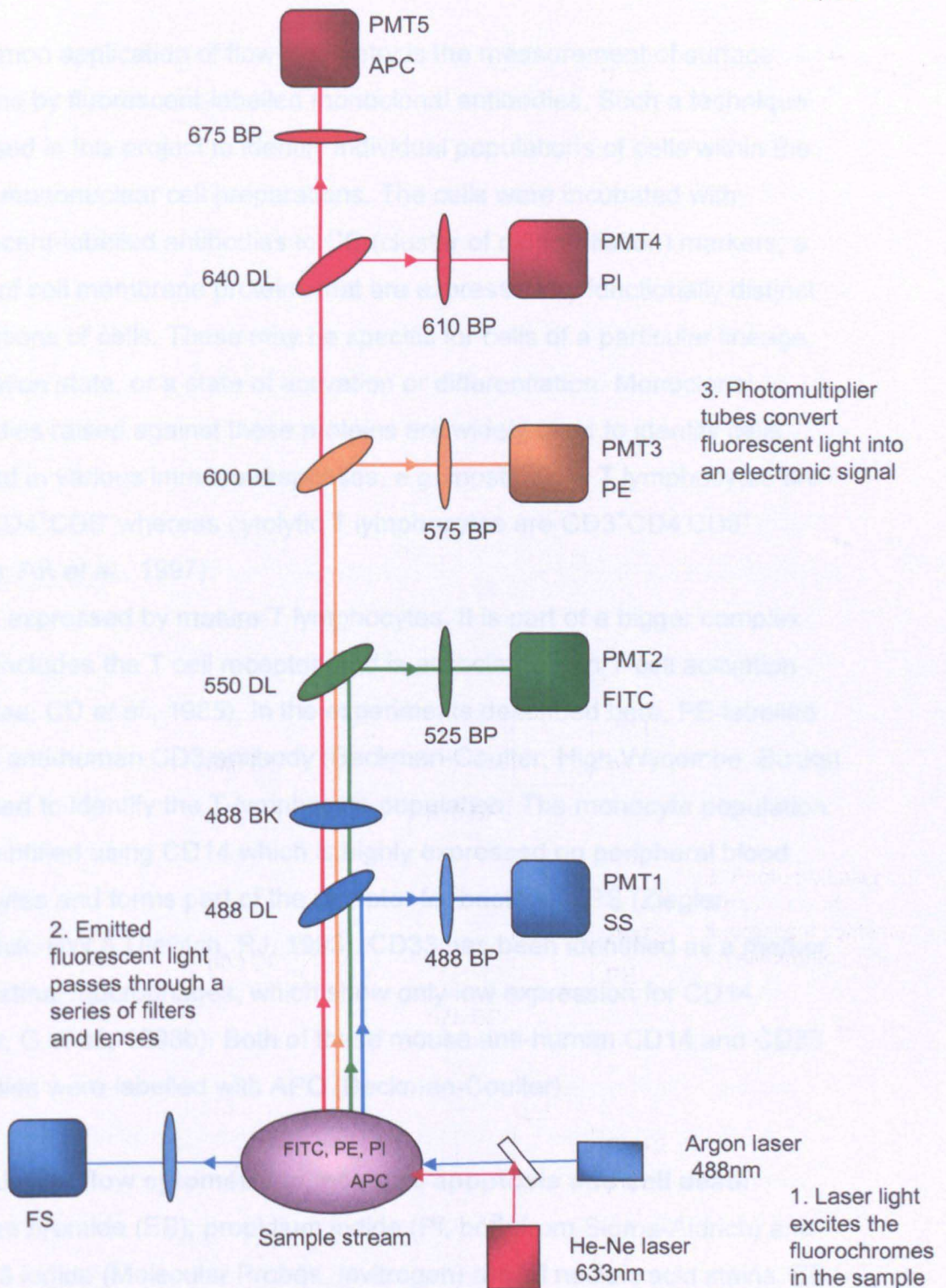
## 2.2. FLOW CYTOMETRY

Flow cytometry is a method for analysis of single cells using laser light and fluorescent labels. The labelled cells travel through the cytometer in suspension within a sheath fluid, which allows them to be presented one at a time to the excitation lasers (Fig 2.1.). The light that is scattered or emitted by the cells is then detected by a series of mirrors, beam splitters and filters, allowing specific bands of fluorescence to be measured (Ormerod, MG, 1999).

The combination of fluorescent labels with monoclonal antibody technology has made flow cytometry a powerful tool in immunobiology (Nihei, OK *et al.*, 2000b). In studies of the P2X<sub>7</sub> receptor, flow cytometry has been widely used to study cell parameters such as cell viability, morphology, intracellular calcium and apoptosis (Nihei, OK *et al.*, 2000b).

Fluorescence can be used to examine any cell component or function that can be detected with a fluorescent compound. The following fluorochromes were used in this project:

	Excitation wavelength (nm)	Emission wavelength (nm)
Fluorescein isothiocyanate (FITC)	490	525
Propidium iodide (PI)	536	620
Ethidium bromide (EB)	518	605
Phycoerythrin (PE)	565	575
Allophycocyanin (APC)	650	660
TOTO-3 iodide	642	660



**Fig 2.1. Simplified schematic of the Altra flow cytometer**

- BK:** Laser-blocking filter, transmits the fluorescence wavelengths but not the laser wavelength.
- BP:** Bandpass filters, transmit light within a specified range.
- DL:** Dichroic lenses, act as long pass filters and reflect the blocked light to the detectors.
- PMT:** Photomultiplier tube, light sensitive sensors which convert light energy into electronic signals proportional to the original fluorescence intensity. These are translated by a computer into data (Ormerod 1999).
- FS:** Forward Scatter, laser light passing through the sample around the cells, a measure of cell size.
- SS:** Side Scatter, laser light scattered by the cells in the sample, a measure of cell granularity.

A common application of flow cytometry is the measurement of surface antigens by fluorescent-labelled monoclonal antibodies. Such a technique was used in this project to identify individual populations of cells within the mixed mononuclear cell preparations. The cells were incubated with fluorescent-labelled antibodies to CD (cluster of differentiation) markers; a group of cell membrane proteins that are expressed by functionally distinct populations of cells. These may be specific for cells of a particular lineage, maturation state, or a state of activation or differentiation. Monoclonal antibodies raised against these proteins are widely used to identify cells involved in various immune responses, e.g. most helper T lymphocytes are CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> whereas cytolytic T lymphocytes are CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> (Abbas, AK *et al.*, 1997).

CD3 is expressed by mature T lymphocytes. It is part of a bigger complex which includes the T cell receptor, and is associated with T cell activation (Tsoukas, CD *et al.*, 1985). In the experiments described here, PE-labelled mouse anti-human CD3 antibody (Beckman-Coulter, High Wycombe, Bucks) was used to identify the T-lymphocyte population. The monocyte population was identified using CD14 which is highly expressed on peripheral blood monocytes and forms part of the receptor for bacterial LPS (Ziegler-Heitbrock, HW & Ulevitch, RJ, 1993). CD33 has been identified as a marker for intestinal macrophages, which show only low expression for CD14 (Rogler, G *et al.*, 1998b). Both of these mouse anti-human CD14 and CD33 antibodies were labelled with APC (Beckman-Coulter).

### **2.2.1. Use of flow cytometry to measure apoptosis and cell death**

Ethidium bromide (EB), propidium iodide (PI, both from Sigma-Aldrich) and TOTO-3 iodide (Molecular Probes, Invitrogen) are all nucleic acid stains. EB and PI are structurally similar dyes that have the ability to intercalate double-stranded DNA or RNA leading to a 20- to 30-fold increase in fluorescence (Haugland, RP, 2002). TOTO-3 is a cyanine dimer dye that exhibits strong fluorescence on binding to DNA (Molecular Probes, 2000). All three dyes are generally excluded from viable cells and have been widely used for the measurement of cell death. EB however is also small enough to pass through the P2X<sub>7</sub>-induced pore of living cells, and this property has been

utilised to demonstrate the presence of functional P2X<sub>7</sub> receptors on the cell surface (detailed in chapter 3).

Annexin V (AV) is a calcium-dependent phospholipid-binding protein with a high affinity for cell membrane phosphatidylserine (PS). This property has been widely used for the detection of early apoptosis in a flow-cytometric method using FITC-labelled AV (Vermes, I *et al.*, 1995; van Engeland, M *et al.*, 1998; Tait, JF *et al.*, 1999). The combination of AV-FITC with PI is commonly used to distinguish between early apoptotic cells, which have an intact cell membrane and will therefore only stain with AV-FITC, and necrotic cells that will show positive staining for both dyes.

Annexin V-FITC was used here to investigate P2X<sub>7</sub>-induced PS-flip together with PI to measure cell death, in particular looking at concentration-response curves (chapter 4), the effect of stimulation time (chapter 5) and the reversibility of AV binding (chapter 6). Experimental details for the individual incubations are detailed in the respective chapters.

Samples were measured in a Beckman-Coulter Altra flow cytometer with a blue laser (488nm) to excite the FITC, PI, EB and PE, and a red laser (633nm) to excite APC and TOTO-3.

### **2.3. P2X<sub>7</sub>-STIMULATED INTERLEUKIN (IL)-1 $\beta$ RELEASE**

For each experiment carried out to measure AV binding, parallel sets of samples were set up to measure IL-1 $\beta$  release. For the IL-1 $\beta$  experiments, cells were resuspended in RPMI 1640 containing 0.1% v/v FCS. Serum contains LPS-binding protein which is essential for the binding of LPS to CD14 (Muta, T & Takeshige, K, 2001). However serum also contains many other proteins including soluble CD14, and was therefore added to the medium at a low concentration to minimise any effect on the results. Cells were pre-incubated with LPS for three hours to induce formation of pro-IL-1 $\beta$  prior to P2X<sub>7</sub> receptor stimulation to release mature IL-1 $\beta$ . For some of the experiments intracellular IL-1 $\beta$  was also measured by lysing the cells in 0.1% saponin (chapters 6 and 7).

IL-1 $\beta$  was assayed by sandwich ELISA, according to the manufacturer's instructions, using a DuoSet<sup>®</sup> development kit for human IL-1 $\beta$  (R&D

Systems, Abingdon, Oxon). Absorbance was measured at 450/540nm using a Multiskan Ascent<sup>®</sup> microplate photometer (Thermo Electron Corporation, Basingstoke, Hampshire) with Ascent software v2.4.

Some of the samples were also assayed for pro-IL-1 $\beta$  (Quantikine<sup>®</sup> human pro-IL-1 $\beta$  immunoassay kit, R&D Systems) and IL-18 (Module set, Bender MedSystems, Vienna, Austria).

The Quantikine<sup>®</sup> pro-IL-1 $\beta$  kit is stated to be specific for pro-IL-1 $\beta$  with no significant cross-reactivity with IL-1 $\beta$  or IL-1 $\alpha$ . For the IL-1 $\beta$  DuoSet<sup>®</sup> kit, the manufacturer states no cross-reactivity with IL-1 $\alpha$  and 3.8% cross-reactivity with rat IL-1 $\beta$ . No data was supplied for cross-reactivity with human pro-IL-1 $\beta$ , so this was tested by assaying the standards from the pro-IL-1 $\beta$  kit. The results are shown below:

#### pg pro-IL-1 $\beta$ /ml

added	measured	% of added
1500	206.73	13.8
750	102.95	13.7
375	53.23	14.2
187.5	27.76	14.8
93.8	14.1	15.0
46.9	5.28	11.3
23.4	0	

The IL-1 $\beta$  kit measured 13.8% (mean) of the pro-IL-1 $\beta$  added; hence it is possible that a small proportion of the IL-1 $\beta$  measured in the assays was due to pro-IL-1 $\beta$ .

## 2.4. DATA ANALYSIS

### 2.4.1. Analysis of flow cytometry dotplots

Flow cytometry data was analysed using WinMDI v2.8 (Windows Multiple Document Interface for flow cytometry (2000), Scripps Research Institute, La Jolla, USA). Cells passing through the flow cytometer are recorded individually as events and can be displayed as dotplots, where each dot represents an individual cell, or histograms, which show the frequency

distribution of the intensity of the fluorescent signal. For the THP-1 cells 10,000 events were collected per sample; for the PBMCs and LPMCs 50,000 events were collected. For the mixed cell populations, individual lymphocyte and monocyte populations were identified either by plotting side scatter (SS) versus CD-marker (Fig 2.2.a & b, R1 and R2), or by gating the populations on the forward scatter (FS) versus SS plots (Fig 2.2.c & d). These gates were then applied to the ethidium or the AV/PI plots. Using gates from the FS/SS plot will not include dead cells in the selected populations, since being smaller; they will have a smaller forward scatter. For the time course experiments, where cell death was of particular interest, AV/PI plots were gated using the regions from the SS v CD-marker plots which will include both live and dead cells.

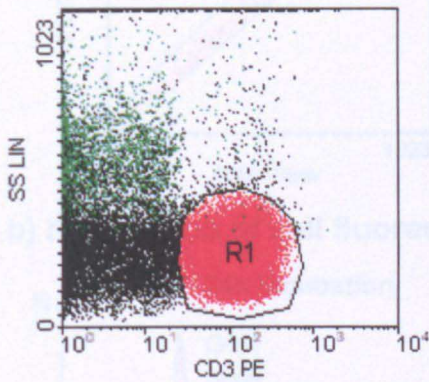
#### **2.4.2. Analysis of cell cycle histograms**

Cell cycle analysis measures the DNA content of a cell by measuring the fluorescence of PI bound to DNA in the nucleus and can be used as a method to identify apoptotic cells. The cell cycle consists of four distinct phases. Quiescent (G0) and G1 cells have one copy of DNA and therefore will all have the same fluorescence intensity. S phase cells are duplicating their DNA and thus exhibit increasing levels of fluorescence. G2 and M phase cells are preparing for or undergoing mitosis so have two copies of DNA and hence twice the fluorescence intensity. However, if two G1 cells pass through the laser beam together they will appear to have the same fluorescence as a single G2 cell and this has to be corrected for (Anon, 1999).

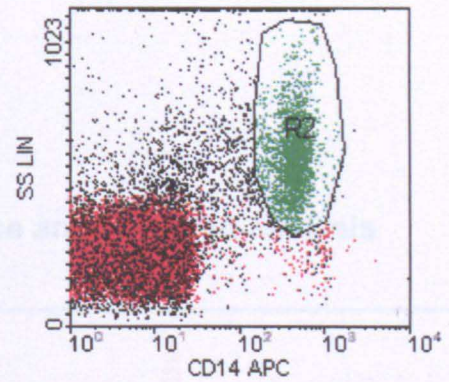
As a cell passes through the laser beam, its fluorescent signal increases to a maximum and then decreases, and the area under the curve gives the total fluorescence of the particle. Two G1 cells passing through the laser beam together will exhibit the same maximum fluorescence as one G2 cell but the area under the curve will be larger. This property can be used to differentiate between these two events by plotting the maximum fluorescence (peak height) against the peak area (Fig 2.3.a). Aggregates of cells have a larger peak area relative to peak height and can be gated out.



a) CD3-PE stained PBMCs

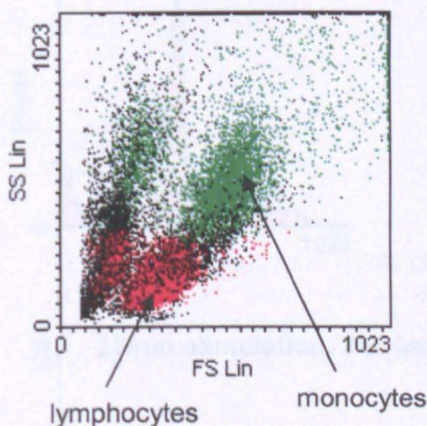


b) CD14-APC stained PBMCs

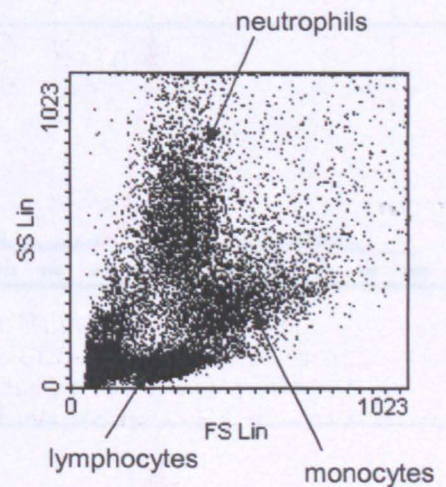


**a & b.** SS/CD-marker dotplots of PBMCs. R1 indicates a region of lymphocytes showing strong staining with CD3-PE, but low side scatter. R2 indicates a region of monocytes, which stain strongly with CD14-APC, and show high side scatter due to their granular nature.

c) PBMCs



d) LPMCs



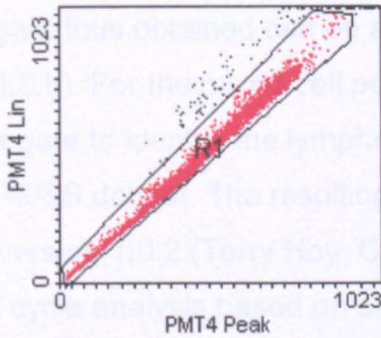
**c.** FS/SS dotplot of PBMCs showing lymphocyte and monocyte cell populations identified by the CD markers. PBMC monocytes are larger and more granular than lymphocytes, reflected by their higher FS and SS. The two distinct populations of each cell type indicate live and dead/dying cells. Apoptotic and dead cells are smaller due to cell shrinkage and breakdown and hence have a smaller FS.

**d.** LPMCs have a similar FS/SS pattern to PBMCs but also contain neutrophils.

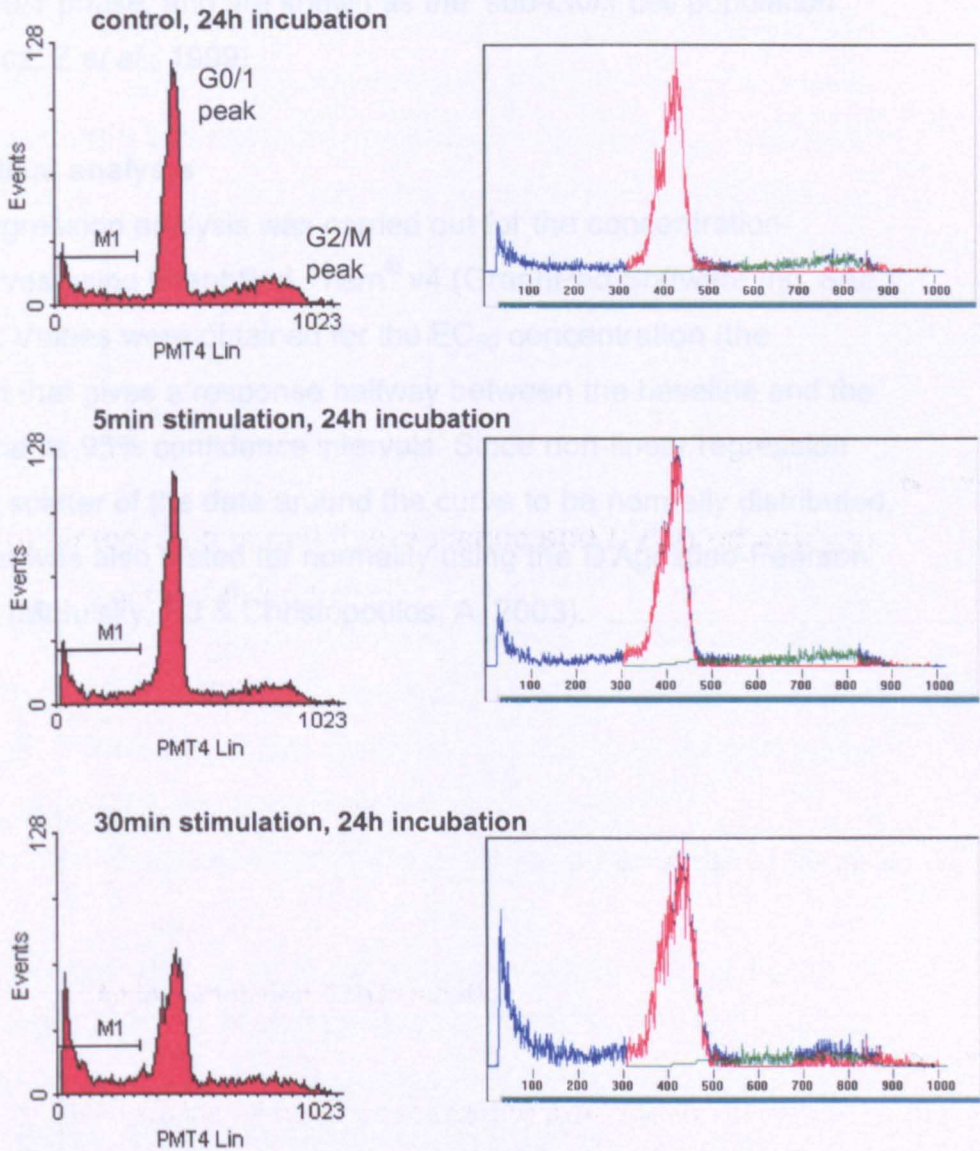
**Fig 2.2.** Flow cytometric analysis of PBMCs and LPMCs



## a) single cell gate



## b) histograms of cell fluorescence and Cylchred analysis



**Fig 2.3. Analysis of THP-1 cell cycle data using WinMDI and Cylchred**

a) A dotplot of peak height v peak area allows a gate to be drawn around the population of single cells. Cell aggregates have a greater peak area relative to peak height and can be excluded from the gate.

b) Histograms of the fluorescence of the gated single cells show the phases of the cell cycle. The M1 region marks the sub-G0/1 population which contains the apoptotic cells. Cylchred analysis of the histograms identifies the phases of the cell cycle.



The single-cell gate thus obtained can be applied to the fluorescence histogram (Fig 2.3.b). For the mixed cell populations the single-cell gate was combined with a gate to identify the lymphocyte and monocyte populations taken from the FS/SS dotplot. The resulting gated histograms were analysed using Cylchred version 1.0.2 (Terry Hoy, Cardiff University) (Fig 2.3.b); software for cell cycle analysis based on algorithms by Watson, JV *et al.*, (1987) and Ormerod, MG *et al.*, (1987). Apoptotic cells have less DNA than cells in the G0/1 phase, and are known as the 'sub-G0/1 cell population' (Darzynkiewicz, Z *et al.*, 1999).

### **2.4.3. Statistical analysis**

Non-linear regression analysis was carried out for the concentration-response curves using GraphPad Prism<sup>®</sup> v4 (GraphPad Software Inc, San Diego, USA). Values were obtained for the EC<sub>50</sub> concentration (the concentration that gives a response halfway between the baseline and the maximum) and its 95% confidence intervals. Since non-linear regression assumes the scatter of the data around the curve to be normally distributed, residuals data was also tested for normality using the D'Agostino-Pearson omnibus test (Motulsky, HJ & Christopoulos, A, 2003).

## CHAPTER 3. P2X<sub>7</sub>-STIMULATED PORE FORMATION TO DEMONSTRATE THE PRESENCE OF FUNCTIONAL RECEPTORS

### 3.1. INTRODUCTION

As described in section 1.7.1, P2X<sub>7</sub> has the ability to form a non-selective membrane pore that is permeable to small molecules. This property has been utilised as a method to demonstrate the presence of functional P2X<sub>7</sub> receptors on the cell surface by measuring the uptake of small fluorescent dyes such as ethidium bromide (EB) into the cells. Ethidium bromide is often used to detect dead cells by flow cytometry. It normally enters the cell through breaks in the membrane, but is also small enough to pass through the P2X<sub>7</sub>-induced pore of living cells. Once inside the cell it intercalates double-stranded DNA or RNA with a 20- to 30-fold increase in its fluorescence (Haugland, RP, 2002).

Theaker, J *et al.*, (2000) characterised EB uptake in BzATP-stimulated THP-1 cells. They found that for each concentration of BzATP used, EB uptake increased in a linear fashion with time, reaching a maximum at approximately 30 minutes. EC<sub>50</sub> values were similar during both the initial linear response phase and also at 90 minutes when the reaction had reached a plateau. They proposed that the size of the agonist-concentration dependent plateau could be ascribed to the number of cells responding to BzATP, demonstrating that EB uptake is a suitable measure of P2X<sub>7</sub> activity and pore formation.

Table 3.1. shows the wide range of cell types in which EB uptake has been used to demonstrate P2X<sub>7</sub>-induced pore formation. It is important to show that dye uptake is via the P2X<sub>7</sub>-activated pore and not simply by a process such as endocytosis, and to this end several of these studies have incorporated a P2X<sub>7</sub> antagonist and demonstrated reduced uptake.

The mechanism of P2X<sub>7</sub>-stimulated pore formation is unknown. One study in THP-1 cells suggested that it does not involve insertion or initial movement of receptors in the membrane but that the receptors cluster together after prolonged activation for 40 minutes (Connon, CJ *et al.*, 2003). In contrast,

Reference	cell type	agonist	antagonist
Falzoni, S <i>et al.</i> , (1995)	human peripheral blood monocytes and macrophages (derived in culture with IFN- $\gamma$ )	ATP	
Wiley, JS <i>et al.</i> , (1993)	human peripheral blood lymphocytes	ATP	suramin
Gargett, CE <i>et al.</i> , (1997a)	human peripheral blood lymphocytes	BzATP, ATP	KN-62
Gu, BJ <i>et al.</i> , (2000)	human PBMCs	ATP	
Sluyter, R <i>et al.</i> , (2004)	human PBMCs	ATP	
Gartland, A <i>et al.</i> , (2001)	human osteoblasts	ATP	
Georgiou, JG <i>et al.</i> , (2005)	human Langerhans cells	BzATP, ATP	
Humphreys, BD <i>et al.</i> , (1996)	THP-1 cells	ATP	
Nihei, OK <i>et al.</i> , (2000a)	murine dendritic cells	ATP	oATP
Chen, YW <i>et al.</i> , (2005)	rat peritoneal cells	BzATP, ATP	
Chaib, N <i>et al.</i> , (2000)	rat submandibular acinar cells	BzATP, ATP	
Tatham, PER & Lindau, M, (1990)	rat peritoneal mast cells	ATP	
Courageot, MP <i>et al.</i> , (2004)	BALB/c mice thymocytes	ATP	
Takenouchi, T <i>et al.</i> , (2005)	mouse microglial cells	BzATP, ATP	
Chessell, IP <i>et al.</i> , (1997)	NTW8 mouse microglial cells	ATP	PPADS
Bisaggio, RD <i>et al.</i> , (2001)	2BH4 murine thymic epithelial cells	ATP	
Lundy, PM <i>et al.</i> , (2004)	CHO cells (K1 strain)	BzATP	oATP

**Table 3.1. Examples of published data for P2X<sub>7</sub>-stimulated pore formation measured by ethidium bromide uptake**

an earlier study concluded that P2X<sub>7</sub> pore formation did not involve receptor density changes or clustering (Smart, ML *et al.*, 2002).

The C-terminus of the P2X<sub>7</sub> receptor has been shown to be critical for pore formation (Surprenant, A *et al.*, 1996; Smart, ML *et al.*, 2003). North, RA, (2002) suggested that the pore is not formed by a gradual increase in permeability of the P2X<sub>7</sub> receptor itself, but that the C-terminus initiates a signal transduction that couples P2X<sub>7</sub> to a separate but closely associated pore-forming protein within the membrane. Several studies support this theory.

Firstly, calmidazolium (an ion channel inhibitor) blocked BzATP-stimulated ion currents but had no effect on YO-PRO (a cyanine dye of similar size to EB) uptake in HEK293 cells expressing the rat P2X<sub>7</sub> receptor (Virginio, C *et al.*, 1997). Secondly, maitotoxin, a potent marine toxin, has been shown to activate an ion channel with distinct properties to that of P2X<sub>7</sub>, but also induces formation of a pore with characteristics indistinguishable from those of the P2X<sub>7</sub>-induced pore (Schilling, WP *et al.*, 1999; Lundy, PM *et al.*, 2004). The third piece of evidence comes from studies of *Xenopus* oocyte cells expressing the P2X<sub>7</sub> receptor. Cells transfected with the rat P2X<sub>7</sub> receptor demonstrated no uptake of YO-PRO iodide following BzATP stimulation for up to 20 minutes, but similar experiments performed with HEK293 cells did show increased fluorescence on stimulation with BzATP (Petrou, S *et al.*, 1997). Similarly, Klapperstuck, M *et al.*, (2000) compared human P2X<sub>7</sub> expressed in *Xenopus* oocytes with the native receptor of human B lymphocytes and found that the transfected receptor was unable to induce pore formation. Finally YO-PRO uptake has been shown to vary considerably among different transfections of HEK293 cells while ion currents are comparable (North, RA, 2002).

P2X<sub>7</sub>-stimulated pore formation in mouse peritoneal macrophages and a mouse thymic epithelial cell line (2BH4), has been shown to require a sustained increase in intracellular Ca<sup>2+</sup> and activation of MAP (mitogen-activated protein) kinases (Faria, RX *et al.*, 2005). Other studies have also shown involvement of MAP kinase-dependent pathways in P2X<sub>7</sub> signal transduction (Donnelly-Roberts, D *et al.*, 2004; Amstrup, J & Novak, I, 2003; Humphreys, BD *et al.*, 2000). In addition, Amstrup, J *et al.*, (2003) showed

that the N-terminus of the receptor was important for activation of the kinases while the C-terminus was important for  $\text{Ca}^{2+}$  entry.

It therefore appears that pore formation by the P2X<sub>7</sub> receptor is dependent on  $\text{Ca}^{2+}$  entry into the cell promoted by the C-terminus, and N-terminal activation of a signalling pathway involving MAP kinases. Provided the host cell contains the necessary components for pore formation, activation of the signalling pathway couples P2X<sub>7</sub> to a pore-forming protein within the membrane. Recent studies have suggested that pannexin-1 may be that pore-forming protein (Locovei, S *et al.*, 2007; Pelegrin, P & Surprenant, A, 2006). Pannexin-1 is a member of a new group of proteins whose function is unknown but which may be responsible for ATP release from erythrocytes (Locovei, S *et al.*, 2006). It has been shown to co-immunoprecipitate with P2X<sub>7</sub> protein from human and mouse macrophages, and exhibit hemichannel function (non-selective ion permeability to molecules <1kDa) when overexpressed (Pelegrin, P *et al.*, 2006). The same authors reported that the hemichannel activity of pannexin-1 was non-functional in the absence of P2X<sub>7</sub> stimulation. In a more recent study, pannexin-1 has been shown to be required for caspase-1 activation (Pelegrin, P & Surprenant, A, 2007), suggested to be a result of its ability to transport bacterial components into the cytoplasm which prompt formation of the inflammasome (Kanneganti, TD *et al.*, 2007). It would appear therefore, that pannexin-1 is intimately associated with the activity of the P2X<sub>7</sub> receptor.

Pore formation is not unique to the P2X<sub>7</sub> receptor and the P2X<sub>2</sub>, P2X<sub>4</sub> and the heteromeric P2X<sub>2</sub>/P2X<sub>3</sub> receptors have all been shown to form similar pores (Virginio, C *et al.*, 1999b; Khakh, BS *et al.*, 1999). However, P2X<sub>7</sub> is the only P2X receptor at which BzATP is a more potent agonist than ATP, and the combination of this property together with pore formation are defining factors for P2X<sub>7</sub> activity.

It has been shown previously that macrophages and T cells isolated from human colonic mucosa exhibit P2X<sub>7</sub> receptor activity (Li, CKF *et al.*, 2001). The characteristics of EB uptake in LPMCs were studied to demonstrate the presence of functional P2X<sub>7</sub> receptors in both the lymphocyte- and

monocyte-gated cells, and were compared to those of PBMCs and THP-1 monocyte cells.

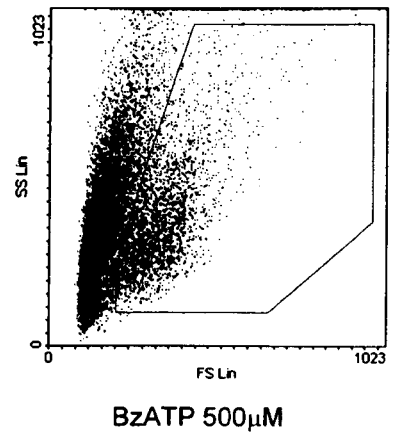
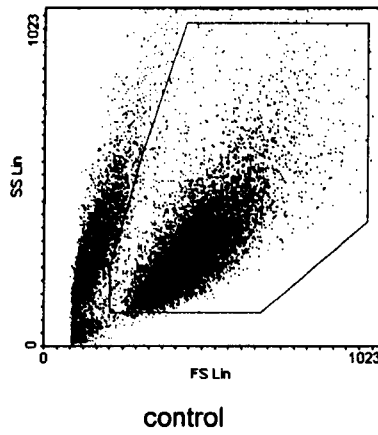
### 3.2. METHODS

Concentration-response curves for EB uptake were carried out for each cell type to confirm the presence of P2X<sub>7</sub> receptors; LPMCs were isolated from normal tissue. Cells were resuspended in RPMI 1640 at a concentration of  $2 \times 10^6$  cells/ml. The cells were incubated for 20min at 37°C with ATP or BzATP (Sigma-Aldrich) over a total concentration range of 5µM to 10mM. The agonists were incubated in the presence of 2µM ethidium bromide (Sigma-Aldrich) in a total incubation volume of 100µl; unstimulated cells were also incubated as a control. The reaction was stopped by adding 400µl of phosphate-buffered saline (PBS, Sigma-Aldrich), since P2X<sub>7</sub> activity has been shown to be inhibited by extracellular Na<sup>+</sup> ions (Wiley, JS *et al.*, 1993; Li, Q *et al.*, 2005), and the cells were placed on ice. The cells were centrifuged at 350 x g for 5min and resuspended in 40µl of PBS. CD markers were added to the mixed cell populations and incubated for 30min on ice in the dark. Cold PBS (250µl) was added and the cells were analysed by flow cytometry.

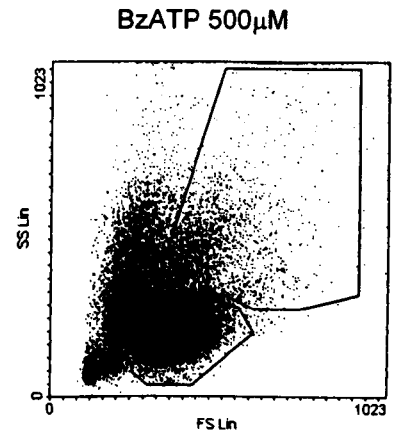
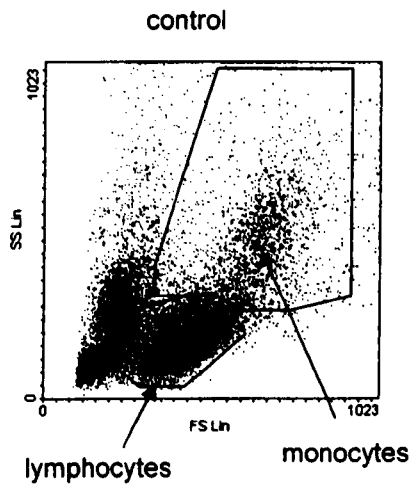
### 3.3. RESULTS AND DISCUSSION

The lymphocyte and monocyte populations of the PBMCs and LPMCs were identified using CD markers and gated on the forward scatter (FS)/side scatter (SS) dotplots. Since gates drawn this way exclude dead cells, the THP-1 cells were also gated for 'live cells' to enable a truer comparison with the results for the PBMCs and LPMCs. Because all of the cell types shrink on stimulation with BzATP or ATP (Fig 3.1.), the gates had to be drawn large enough to allow for this. For each cell population, ethidium fluorescence versus SS was plotted, and a quadrant applied to identify the percentage of the gated cell population binding positively for EB (Fig 3.2.). For all the flow cytometry experiments, because the cell populations were identified by gating post-experiment rather than specific characterisation or isolation, the cell types have been described as 'lymphocyte-gated cells' or 'monocyte-

## a) THP-1 cells



## b) PBMCs



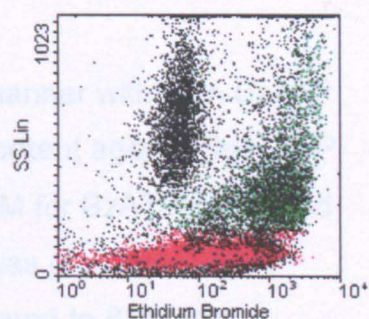
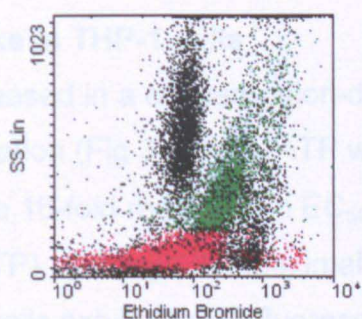
**Fig 3.1. Forward Scatter (FS)/Side Scatter (SS) dotplots showing cell shrinkage on stimulation**

Cells stimulated with BzATP or ATP shrink on stimulation shown by a decrease in FS. Gates drawn around cell populations therefore have to be large enough to include such movement. As can be seen in fig b, with the mixed cell populations this can be particularly difficult as the cell populations tend to converge.

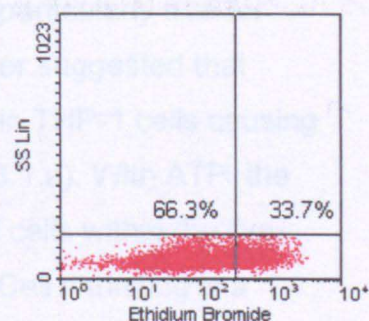
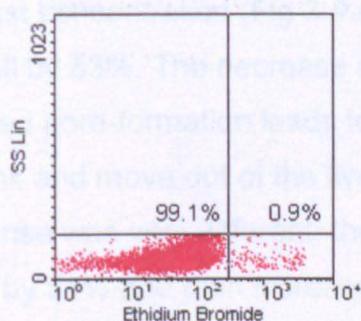
## i. Unstimulated controls

## ii. BzATP-stimulated cells

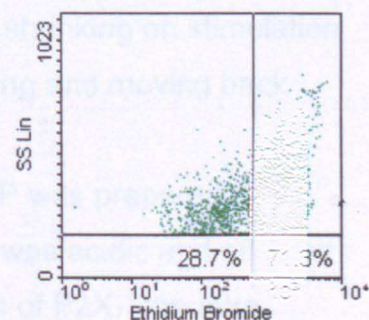
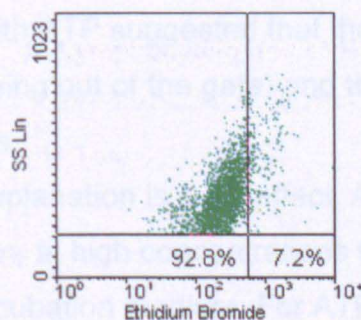
## a) Total LPMCs



## b) Lymphocyte-gated LPMCs



## c) Monocyte-gated LPMCs

**Fig 3.2. An example of EB dotplots for LPMCs**

Figures show EB fluorescence plotted against side scatter. Unstimulated cells are shown on the left and cells stimulated with 1mM BzATP on the right. The increase in EB staining on stimulation of both lymphocyte- and monocyte-gated cells can clearly be seen. The large unstained population (fig a) are neutrophils, which do not respond to P2X<sub>7</sub> stimulation.



gated cells' respectively. In the case of LPMCs, the term 'monocyte-gated cells' also refers to mature tissue macrophages. The data for EB uptake is summarised in Table 3.2.

### 3.3.1. EB uptake in THP-1 cells

EB uptake increased in a concentration-dependent manner with both BzATP and ATP stimulation (Fig 3.3.a). BzATP was a more potent agonist than ATP as shown by the 16-fold reduction in EC<sub>50</sub> value (24µM for BzATP compared to 0.4mM for ATP), although the maximal response was greater with ATP (96% of gated cells exhibiting EB fluorescence compared to 83% with BzATP).

The number of cells contained within the live-gate decreased markedly with increased agonist concentration (Fig 3.4.a); this was particularly true for BzATP which fell by 83%. The decrease in cell number suggested that BzATP-stimulated pore-formation leads to cell death in THP-1 cells causing the cells to shrink and move out of the live-gate (Fig 3.1.a). With ATP, the pattern of response was very different; the number of cells within the live-gate fell initially by 54% and then increased by 43%. Cell shrinking is a characteristic of apoptosis and cell death, but has also been shown to be associated with P2X<sub>7</sub>-stimulated vesicle-shedding from THP-1 cells and hence loss of cell membrane (MacKenzie, A *et al.*, 2001). The change in cell number seen with ATP suggested that the cells were shrinking on stimulation (and hence moving out of the gate) and then recovering and moving back into the live-gate.

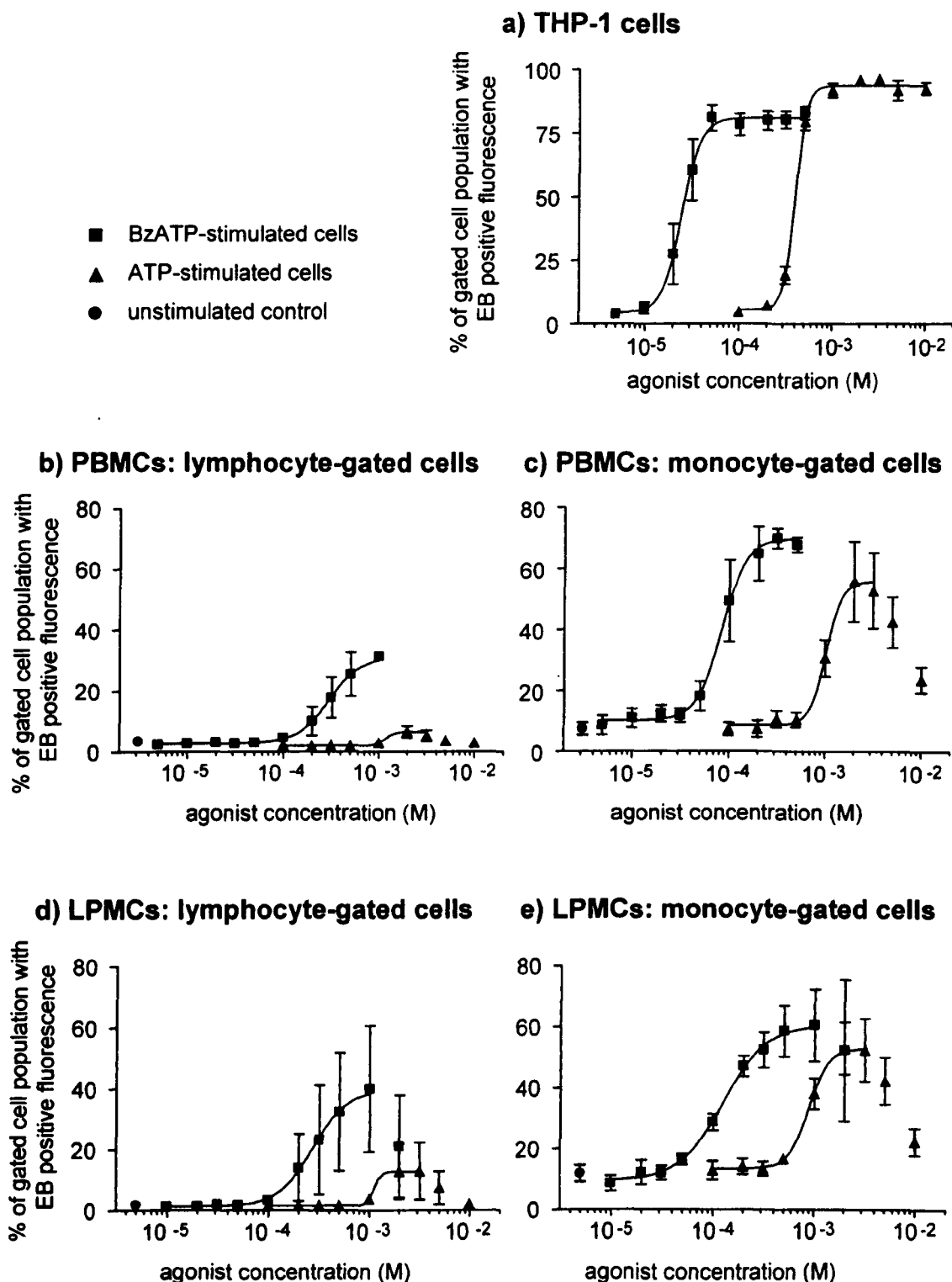
One possible explanation is a pH effect. Although ATP was prepared in a buffered medium, at high concentrations the solution was acidic and affected the pH of the incubation medium. For ATP stimulation of P2X<sub>7</sub>, the tetra-anionic (ATP<sup>4-</sup>) form has been shown to be the active moiety (Steinberg, TH & Silverstein, SC, 1987b). Virginio, C *et al.*, (1997) measured the concentration of ATP<sup>4-</sup> under different conditions of ion concentration and pH, and showed that reducing the pH from 7.3 to 5.3 resulted in a 50% reduction in ATP<sup>4-</sup> concentration. Also Steinberg, TH *et al.*, (1987a) found that lucifer yellow uptake into J774 macrophage cells was barely detectable

	EC <sub>50</sub> value with 95% confidence intervals		Maximum and minimum responses (% of cell population) (mean ± sem)		Concentration at which maximum response occurred (mean ± sem)		Hillslope (mean ± sem)	
	BzATP	ATP	BzATP	ATP	BzATP	ATP	BzATP	ATP
THP-1 cells	24µM 21 - 29µM	0.40mM 0.38 - 0.42mM	83 ± 2.6 3.7 ± 1.2	96 ± 1.0 4.7 ± 0.9	163 ± 113µM	2.7 ± 0.2mM	4.3 ± 1.2	7.3 ± 0.7
PBMCs: monocytes	84µM 67 - 106µM	1.02mM 0.8 - 1.3mM	70 ± 3.2 8.6 ± 3.1	56 ± 13 7.2 ± 2.1	267 ± 33µM	2.0 ± 0.0mM	3.4 ± 1.1	5.3 ± 6.1
PBMCs: lymphocytes	301µM 231 - 392µM	1.18mM	31 2.3 ± 0.8	6.5 ± 2.0 2.1 ± 0.5	667 ± 167µM	2.0 ± 0.0mM	2.6 ± 0.9	8.5
Normal LPMCs: monocytes	127µM 88 - 182µM	0.89mM 0.66 - 1.2mM	60 ± 12 8.5 ± 2.6	53 ± 8.5 13 ± 3.1	1.2 ± 0.4mM	2.7 ± 0.3mM	2.1 ± 0.6	4.5 ± 3.0
Normal LPMCs: lymphocytes	277µM 140 - 545µM	1.12mM	40 ± 21 1.3 ± 0.8	13 ± 9.4 1.5 ± 0.5	1.2 ± 0.4mM	2.3 ± 0.3mM	2.5 ± 2.0	12.5

**Table 3.2. Summary of results for P2X<sub>7</sub>-stimulated ethidium bromide uptake**

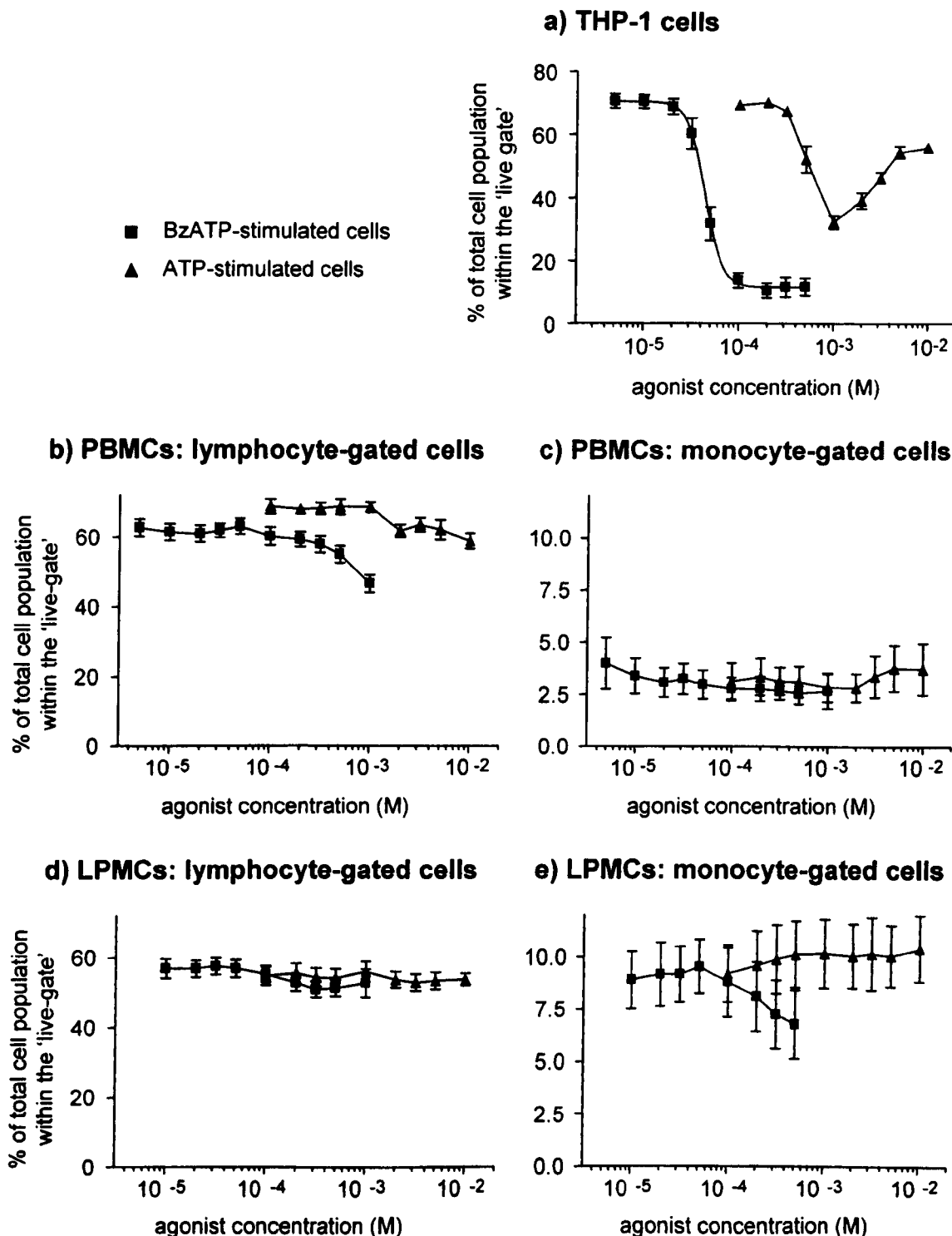
The table shows the EC<sub>50</sub> values taken from the concentration response curves and the Hillslope calculated from the curve. The mean maximum and minimum responses for each cell type with each agonist are shown, together with the mean of the agonist concentrations at which each subject achieved the maximum response.

BzATP was a more potent agonist than ATP, as shown by the EC<sub>50</sub> values. Lymphocyte cells were less reactive than monocyte cells demonstrating higher EC<sub>50</sub> values and lower maximum responses. The monocyte cells demonstrated an order of reactivity of THP-1 > PBMCs > LPMCs.



**Fig 3.3 EB uptake in mononuclear cells stimulated with BzATP or ATP.**

EB uptake was measured by flow cytometry after 20min stimulation with either BzATP or ATP in RPMI 1640. The number of cells with positive EB fluorescence was expressed as a percent of the gated cell population. Figures show the mean  $\pm$  sem of 3 or 4 (THP-1) separate experiments.



**Fig 3.4. The percentage of the total cell population contained within the 'live-gates'**

The lymphocyte and monocyte populations were gated from the FS/SS dotplots and the number of cells contained within each live gate were expressed as a percent of the total cell population. For the PBMCs and LPMCs the figures show little effect of ATP stimulation on cell number, but BzATP stimulation caused a decrease in the number of cells gated for all of the cell types. Results with THP-1 cells were markedly different showing a large reduction in gated cell number with BzATP stimulation, and a reduction followed by a recovery after ATP stimulation.

below pH 6.5. Although the incubation medium was not reduced to pH values as low as these, it is possible that at higher ATP concentrations, less ATP<sup>4-</sup> was available and stimulation of the receptor was reduced. Reduced stimulation would mean less vesicle shedding and allow the membrane to recover and replace itself. However, reduced receptor stimulation should also mean reduced EB uptake, but this was not the case at high ATP concentrations.

The ATP concentration at which the cell numbers began to rise again corresponded to the concentration at which EB uptake reached a maximum in the live-gated cells, suggesting that the pore is fully open. ATP has a molecular weight small enough to pass through the P2X<sub>7</sub> pore of monocytes and once inside the cell it could stimulate cell metabolism and regeneration of membrane (Di Virgilio, F, 2000). Transfection of P2X<sub>7</sub> into HEK cells has been shown to increase cellular ATP levels and promote growth, but only in cells with full pore-forming function (Adinolfi, E *et al.*, 2005). The pannexin-1 protein thought to be involved in pore formation has already been shown to be involved in ATP transport across red cell membranes (Locovei, S *et al.*, 2006) and could potentially play a similar role in THP-1 cells. If this was the case, a similar recovery with BzATP might also be expected to occur. However, BzATP is supplied as a triethylammonium salt with a molecular weight >1000, and would therefore be too large to pass through the membrane pore.

### **3.3.2. EB uptake in PBMCs**

EB uptake in both lymphocyte- and monocyte-gated cell populations of PBMCs increased in a concentration-dependent manner in response to BzATP and ATP stimulation (Fig 3.3.b & c). For both cell types, ATP acted as a partial agonist, particularly with the lymphocyte-gated cells where the response to ATP was very low. BzATP was a more potent agonist than ATP, characteristic of P2X<sub>7</sub> stimulation. In monocyte-gated cells BzATP potency was approximately 10-fold that of ATP, with EC<sub>50</sub> values of 84µM and 1.02mM respectively. In lymphocyte-gated cells BzATP potency was approximately 4-fold (EC<sub>50</sub> values of 301µM for BzATP and 1.18mM for

ATP); although because of the lack of response to ATP the regression analysis could only give an estimate of the EC<sub>50</sub> value.

The response to stimulation of the lymphocyte-gated cell population was less than the monocyte-gated cells; only 31% responded to BzATP compared to 70% of the monocytes. This was even more marked for ATP stimulation with only 6.5% responding compared to 56% of monocytes. Gu, BJ *et al.*, (2000) showed that the uptake of ethidium correlates closely with the surface expression of P2X<sub>7</sub> receptors and that monocytes express 4-5 times more than B- or T-lymphocytes. They induced uptake of ethidium in human PBMCs with 1mM ATP, and found that it was 5-fold greater for monocytes than for lymphocytes. This supports the data presented here given that the monocyte-gated cells were 2-8 times more responsive than lymphocyte-gated cells.

EB uptake in response to ATP stimulation was decreased at higher concentrations of the agonist. The decreased response was not seen with BzATP stimulation of PBMCs but this is probably because the maximum concentration was insufficient to demonstrate the effect. PBMCs required higher concentrations of BzATP to stimulate EB uptake than the THP-1 cells, and as a result the top plateau on the concentration curves was not well defined.

Reduced responses with ATP can often be due to the presence of nucleotidase enzymes. Many cells express apyrase enzymes which break down ATP to ADP, AMP and adenosine, as soon as it is released (Zimmermann, H, 2000). CD39 (ecto-apyrase) has been shown to hydrolyse ATP and modulate its effects in B cells (Nie, K *et al.*, 2005), endothelial cells (Imai, M *et al.*, 2000), and dendritic cells (Berchtold, S *et al.*, 1999). Ecto-nucleotidase enzymes in hippocampal slices have also been shown to break down BzATP to Bz-adenosine (Kukley, M *et al.*, 2004).

It is also possible that the reduced EB uptake with ATP was due to the effect of decreased pH as described in section 3.2.1. If high concentrations of ATP lead to reduced pH and hence less available ATP<sup>4-</sup>, it is possible that stimulation of the P2X<sub>7</sub> receptor could be reduced resulting in decreased EB uptake.

Another possible explanation might be the inclusion of dead cells within the 'live-cell gate'. As described in section 3.3, the live cell gates have to be drawn large enough to allow for cell shrinkage on stimulation. In the case of a single cell line such as THP-1 cells it is relatively easy to achieve this and still keep distinct cell populations. In the dotplots for the mixed cell types however, the lymphocyte and monocyte populations are quite close together (see Fig 3.1.b) making it more difficult to differentiate between them. It is likely therefore that there is some overlap and inclusion of dead cells within the live gate which could account for some apparent reduction in EB uptake. The number of cells within the live-gates was plotted for both lymphocyte- and monocyte-gated cells to see if there was a similar pattern to that of THP-1 cells (Fig 3.4.b & c). BzATP stimulation produced a decrease in the number of gated cells for both populations, with a slightly greater effect on monocyte-gated cells (33% decrease) than lymphocyte-gated cells (26% decrease), but much less than that seen with THP-1 cells. ATP stimulation also produced a decrease in lymphocyte-gated cell number (14%) but less than that with BzATP. Like THP-1 cells, PBMC monocytes stimulated with ATP showed a slight fall in gated-cell number followed by an increase, but not as distinctive. The results therefore suggest that PBMCs were less susceptible to cell death at higher concentrations of agonist than THP-1 cells.

### 3.3.3. EB uptake in LPMCs

P2X<sub>7</sub>-stimulated EB uptake increased in a concentration-dependent manner for both lymphocyte and monocyte-gated cell populations (Fig 3.3.d & e), but as seen with PBMCs, ATP was only a partial agonist. BzATP was approximately seven times more potent than ATP in monocyte-gated cells (EC<sub>50</sub> values of 127 $\mu$ M and 0.89mM for BzATP and ATP respectively), and four times in the lymphocyte-gated population (EC<sub>50</sub> of 277 $\mu$ M for BzATP and 1.12mM for ATP). For LPMCs, the maximal BzATP concentration used was increased to 2mM and at this concentration there was a reduced response as seen with PBMCs and ATP. The response to ATP stimulation also produced bell-shaped curves similar to those of PBMCs. The number of

cells within the live-gates was plotted for both lymphocyte- and monocyte-gated cells and is shown in Fig 3.4.d & e. Only BzATP stimulation produced a decrease at higher concentrations of agonist (10% for lymphocyte-gated cells and 26% for monocyte-gated cells), and this was less than that seen with PBMCs. This suggests that LPMCs are less susceptible to P2X<sub>7</sub>-induced cell death than PBMCs.

The response of LPMCs to P2X<sub>7</sub>-stimulated EB uptake was therefore very similar to that seen with PBMCs, particularly for the lymphocyte-gated cells. LPMC monocytes required higher concentrations of agonist to produce a response than the PBMC monocytes, particularly with BzATP stimulation, and the EC<sub>50</sub> value was also greater. This could reflect downregulation of the receptor in colonic tissue macrophages in accordance with their non-inflammatory characteristics. It is also possible however that it may be an artefact of the cell gating procedure. With LPMCs gating is more difficult due to the presence of a large neutrophil population. At high concentrations of agonist when the cell populations are closer to each other, it is possible that neutrophils, which do not respond to P2X<sub>7</sub>, may be included in the monocyte gate. This would reduce the number of responsive cells within the gate and therefore produce an apparently reduced response compared to that of PBMCs.

EB uptake in LPMCs was very variable between subjects, particularly for the lymphocyte-gated population as shown by the wide error bars on the graph (Fig 3.3.d). This was mainly due to one subject whose responses were much lower than the other two. Each individual's maximal responses for each cell type with each agonist are shown below:

subject	lymphocyte-gated cells		monocyte-gated cells	
	BzATP	ATP	BzATP	ATP
1	78%	32%	69%	62%
2	4%	1.6%	45%	37%
3	38%	4.5%	75%	63%

It is clear that the responses of the lymphocyte-gated cells are more diverse than the monocyte-gated cells. This may be due to differences in pore size of

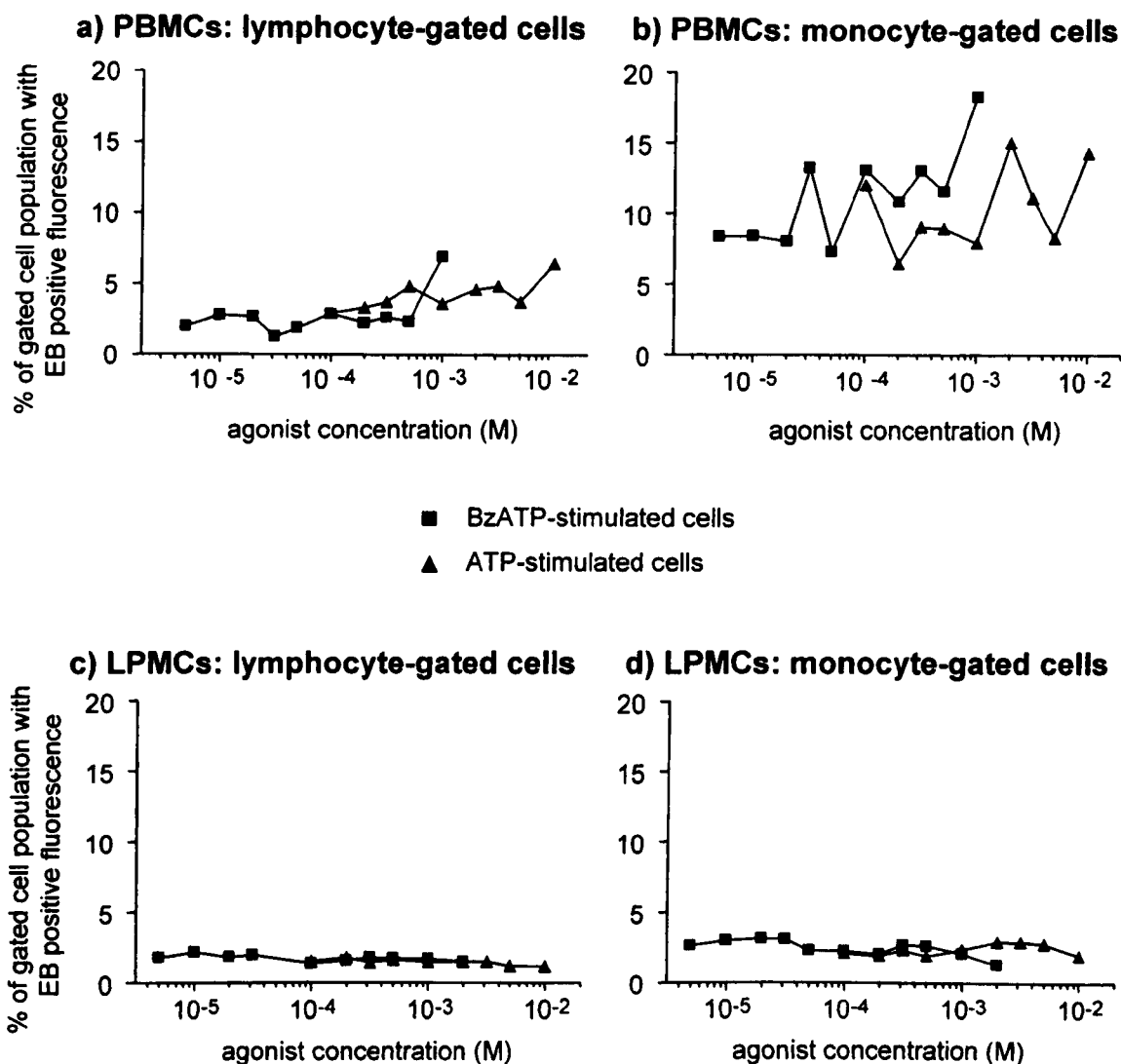


the two cell types. The lymphocyte pore is thought to be approximately 300-400Da in size (Wiley, JS *et al.*, 1993) whereas the monocyte pore is approximately 800-900Da (Steinberg, TH *et al.*, 1987a). Increase in pore size occurs gradually with increasing ATP concentration (Tatham, PER *et al.*, 1990), but because the ethidium ion (MW 314) is at the limit of the lymphocyte pore size it will require maximal stimulation to allow entry of the ion into the cell. Different subjects do not all respond to agonist stimulation in the same way and a concentration that will produce a maximal effect in one subject may be sub-maximal in another. As a result, when the mean data is plotted large error bars are produced, as seen in Fig 3.3.d.

Interestingly, for both the PBMCs and LPMCs, one of the subjects failed to respond to stimulation with BzATP or ATP and showed very little or no EB uptake (Fig 3.5.). Studies have shown a genetic component in P2X<sub>7</sub> function and several structural polymorphisms have been discovered. In around 20% of the population a Glu<sup>496</sup> to Ala polymorphism has been found, located in the carboxyl terminus of the receptor, which leads to loss of function in homozygous individuals and approximately 50% reduction in heterozygous individuals (Gu, BJ *et al.*, 2001). A second polymorphism, Ile<sup>568</sup> to Asn, is located within the trafficking motif of the carboxyl tail of the receptor and prevents normal surface expression (Wiley, JS *et al.*, 2003), and a third has been found (Arg<sup>307</sup> to Gln) which is thought to be located in the area essential for ATP binding (Gu, BJ *et al.*, 2004). As well as being responsible for a lack of response, the existence of polymorphisms could also explain the LPMC data for subject 2; if a heterozygous individual displays a reduced response this could account for the lower observed values.

### **3.4. SUMMARY OF P2X<sub>7</sub>-STIMULATED EB UPTAKE**

The results showed that all of the cells demonstrated a concentration-dependent increase in EB uptake on stimulation with P2X<sub>7</sub> agonists, and for all of them BzATP was a more potent agonist than ATP. This is consistent with the presence of functional P2X<sub>7</sub> receptors and shows that colonic mucosal mononuclear cells, as well as PBMCs and THP-1 cells, are capable of P2X<sub>7</sub>-stimulated pore formation.



**Fig 3.5. Subjects showing a lack of response for P2X<sub>7</sub>-stimulated EB uptake in mononuclear cells stimulated with BzATP or ATP**

In both PBMC and LPMC cell types, one subject failed to respond to stimulation and showed very little or no EB uptake. Even in the absence of a response, PBMC monocytes (fig b) demonstrated a higher level of basal activity than the other cell types confirming their more active nature.

The EC<sub>50</sub> values for each agonist were similar for the PBMCs and LPMCs, being approximately 1mM for ATP and 100-300μM for BzATP. Comparable responses to ATP stimulation have been demonstrated in studies on EB uptake in mouse dendritic cells (Nihei, OK *et al.*, 2000a), mouse microglial cells (Takenouchi, T *et al.*, 2005), and HEK293 cells transfected with recombinant P2X<sub>7</sub> (Ferrari, D *et al.*, 2004). Similarly, Tsukimoto, M *et al.*, (2005) demonstrated a concentration dependent increase in EB uptake in DT40 cells (a chicken B-cell line) transfected with P2X<sub>7</sub>, when they were stimulated with 10-500μM BzATP.

Studies of human PBMC lymphocytes have shown increased EB uptake stimulated by both BzATP and ATP, in which BzATP was five times more potent than ATP, and ATP was only a partial agonist, the maximal response for ATP being only 70% of that for BzATP (Gargett, CE *et al.*, 1997a). These results are consistent with the lymphocyte data presented here.

In contrast, Falzoni, S *et al.*, (1995) studied human PBMC monocytes and mature macrophages (derived from monocytes by culture for five days with IFN-γ) and found that ethidium fluorescence increased in macrophages stimulated with ATP, but that monocytes did not respond. This differs from the results presented here in which the LPMC tissue macrophages demonstrated less stimulated EB uptake than PBMC monocytes, suggesting downregulation of the receptor during maturation of the cells in the tissue. The difference may be due to the mode of maturation. IFN-γ is a cytokine that promotes classic activation of macrophages and the production of an inflammatory response (Rogler, G *et al.*, 1998a). In the normal colon however, resident tissue macrophages do not have pro-inflammatory properties (Smith, PD *et al.*, 2005) and it is therefore likely that a receptor such as P2X<sub>7</sub>, which is involved in the release of the inflammatory cytokine IL-1β, would have low levels of expression.

For all the cell types studied BzATP was a more potent agonist than ATP, but was relatively more so in monocyte-gated cells (7-20 times) as opposed to lymphocyte-gated cells (4 times). Monocytes also showed greater maximum responses to the two agonists than lymphocytes, particularly with ATP where the maximum response with monocytes was 4-8 times greater

than that with lymphocytes. It is clear that lymphocytes do not respond as readily to P2X<sub>7</sub>-stimulation, and this is supported by a study on mouse thymocytes stimulated with ATP in which EB did not permeate the cells at all (Pizzo, P *et al.*, 1991).

The THP-1 monocyte cell line appeared to be more responsive to P2X<sub>7</sub>-stimulation than the monocyte cells of PBMCs and LPMCs. The EC<sub>50</sub> values for both agonists with THP-1 cells were lower than those with the isolated primary cells; 24µM for BzATP and 0.4mM for ATP compared with approximately 100µM and 1.0mM for BzATP and ATP respectively. The maximum response with each agonist was also higher in THP-1 cells. One reason for this could be cell size. Monocytes are bigger cells than lymphocytes (approximately 4.6µm compared to 3.3µm) (Yang, J *et al.*, 1999) and THP-1 cells are bigger than monocytes (Holmes, D & Morgan, H, 2002). If P2X<sub>7</sub> expression is related to cell size, this might explain the magnitude of response of the three cell types. Another possibility is that THP-1 cells are a leukaemic cell line and may demonstrate an uncontrolled response to receptor stimulation. Similarly, if pore formation depends on the presence of other membrane proteins in the cell, different levels of activity could reflect the degree of expression of the associated pore-forming protein in the different cell types.

In their study of PBMC lymphocytes, Gargett, CE *et al.*, (1997a) plotted Hill analyses of BzATP- and ATP-stimulated uptake of EB. Hill analysis is a way of quantifying the steepness of the concentration-response curve and provides information about the nature of ligand-receptor interactions (Colquhoun, D, 1998). A value for the Hill coefficient greater than one normally indicates positive cooperativity, i.e. binding of one ligand facilitates binding of subsequent ligands at other sites on the receptor. For ligand-gated ion channels such as the P2X receptors which are composed of subunits, the Hill coefficient is almost always greater than one. This is because each of the subunits carries an agonist binding site and usually more than one of them need to be occupied for the receptor to be activated (Gibb, AJ, 2003). Gargett, CE *et al.*, (1997a) calculated Hill coefficients of 3.1 and 2.1 for BzATP and ATP respectively for P2X<sub>7</sub>-stimulated PBMC lymphocytes. This

is similar to the value obtained here for lymphocyte-gated PBMCs stimulated with BzATP ( $2.6 \pm 0.9$ ); due to the poor response with ATP an accurate value for the Hill coefficient could not be calculated. In fact for all of the cell types, concentration-response curves for EB uptake gave values for the Hill coefficient greater than two. This suggests that binding of more than one  $\text{ATP}^{4-}$  molecule is required for receptor activation (Tatham, PER *et al.*, 1990).

In summary, the data presented here has shown that LPMCs isolated from colonic mucosa express functional  $\text{P2X}_7$  receptors with properties similar to those of PBMCs and THP-1 cells. Lymphocyte cells appeared to be less responsive than monocyte cells and monocyte cells appeared to express an order of reactivity, with THP-1 cells being the most reactive and LPMCs the least. THP-1 cells had the lowest  $\text{EC}_{50}$  value ( $24\mu\text{M}$ ), required the lowest BzATP concentration to achieve a maximum response ( $163\mu\text{M}$ ) but reached the highest percent response (83%). In contrast, LPMC monocytes required millimolar concentrations of BzATP to achieve a maximum 60% response. This may reflect both the cell size and the expression levels of the receptor and the pore-forming protein in the different cells.

## CHAPTER 4. P2X<sub>7</sub> RECEPTOR STIMULATION: EFFECT ON IL-1 $\beta$ RELEASE AND ANNEXIN V BINDING

### 4.1. INTRODUCTION

Stimulation of the P2X<sub>7</sub> receptor results in the release of IL-1 $\beta$  and induction of PS-flip, a marker of apoptotic cell death (Ferrari, D *et al.*, 1997b; Perregaux, DG *et al.*, 1994; Chiozzi, P *et al.*, 1996; Hogquist, KA *et al.*, 1991a), both of which are important targets for anti-inflammatory drugs. IL-1 $\beta$  is a potent inflammatory cytokine shown to be present at higher levels in IBD tissue (Ligumsky, M *et al.*, 1990), and both aminosalicylates and corticosteroids exert their anti-inflammatory actions by inhibiting cytokine production (Carter, MJ *et al.*, 2004). They act by preventing activation of nuclear transcription factor, NF- $\kappa$ B, a key regulator of the expression of many genes involved in immune and inflammatory responses in the gut, including that of IL-1 $\beta$  (Dijkstra, G *et al.*, 2002).

Several of the standard treatments for IBD act by inducing apoptosis. Lamina propria T cells in the gut exhibit a susceptibility to apoptosis that is decreased in cells from inflamed tissue (Boirivant, M *et al.*, 1996; Boirivant, M *et al.*, 1999). Corticosteroids (Carter, MJ *et al.*, 2004) and thiopurines (Tiede, I *et al.*, 2003) both induce T cell apoptosis, and corticosteroids have also been shown to induce monocyte apoptosis (Schmidt, M *et al.*, 1999). The anti-TNF drug infliximab also induces both T cell and monocyte apoptosis. It acts by upregulating transcription of Bax and Bak, two proapoptotic members of the Bcl-2 family (see section 4.3.1.1.) (Lugering, A *et al.*, 2001; ten Hove, T *et al.*, 2002).

Since the P2X<sub>7</sub> receptor appears to have a role both in cytokine release and cell death, affecting the activity of the receptor may provide a way of controlling these processes, and hence potential treatment for inflammatory diseases. Therefore the characteristics of P2X<sub>7</sub>-stimulated IL-1 $\beta$  release and AV-binding (as a measure of PS-flip) were investigated.

## 4.2. P2X<sub>7</sub> RECEPTOR-STIMULATED IL-1 $\beta$ RELEASE

### 4.2.1. Introduction

Cells do not normally contain large amounts of inflammatory cytokines waiting to be released (Dinarello, CA, 2000). An initial stimulus is required to 'prime' the cells and in the gut this is generally bacterial LPS. The intestinal mucosa forms the primary barrier against the many bacteria present within the gut lumen, and it is important that the body recognises any penetration of this barrier by bacteria or their products. The cells of the innate immune system are able to recognise bacterial wall components such as LPS and induce cytokine secretion leading to an inflammatory response (Beutler, B *et al.*, 2003). LPS stimulation of monocytes and macrophages induces many genes which express inflammatory mediators such as cytokines and chemokines, including the production of large amounts of pro-IL-1 $\beta$  (Guha, M *et al.*, 2001).

LPS is a glycolipid which forms a major structural component of the outer wall of Gram-negative bacteria. It consists of a lipid component (lipid A) and a hydrophilic heteropolysaccharide. Lipid A anchors the molecule within the cell wall and is responsible for the biological toxicity of LPS (Fenton, MJ & Golenbock, DT, 1998). When bacteria multiply or when they die and break up, LPS is released and acts as an extremely potent toxin. Macrophages are of primary importance in LPS recognition and can be activated by concentrations as low as 1pg/ml (Fenton, MJ *et al.*, 1998).

After its release, LPS forms a complex with a plasma protein, LBP (LPS-binding protein), which enhances the binding of LPS to CD14 (Guha, M *et al.*, 2001). CD14 is a glycoprotein expressed on the surface of monocytes, macrophages and weakly on neutrophils. It is anchored to the plasma membrane by a glycosylphosphatidylinositol linkage, but also exists in a soluble form (Guha, M *et al.*, 2001). Its role is to facilitate contact between LPS and its signalling molecule, TLR4 (Toll-like receptor 4) (Jiang, Q *et al.*, 2000). The TLRs are a group of receptors named after their similarity to the *Drosophila* protein Toll, and act as primary sensors of microbial infection. They have a cytoplasmic domain homologous to the IL-1 receptor and signal



through activation of the pro-inflammatory transcription factor, NF- $\kappa$ B (Beutler, B *et al.*, 2003) (Fig 4.1.). LPS has been shown to upregulate CD14 and TLR4, and trigger a physical association between them (Jiang, Q *et al.*, 2000). The LPS signalling complex also includes a small molecule (MD-2), which is physically associated with TLR4 on the cell surface and is essential for the correct intracellular distribution of TLR4 as well as its recognition of LPS (Nagai, Y *et al.*, 2002).

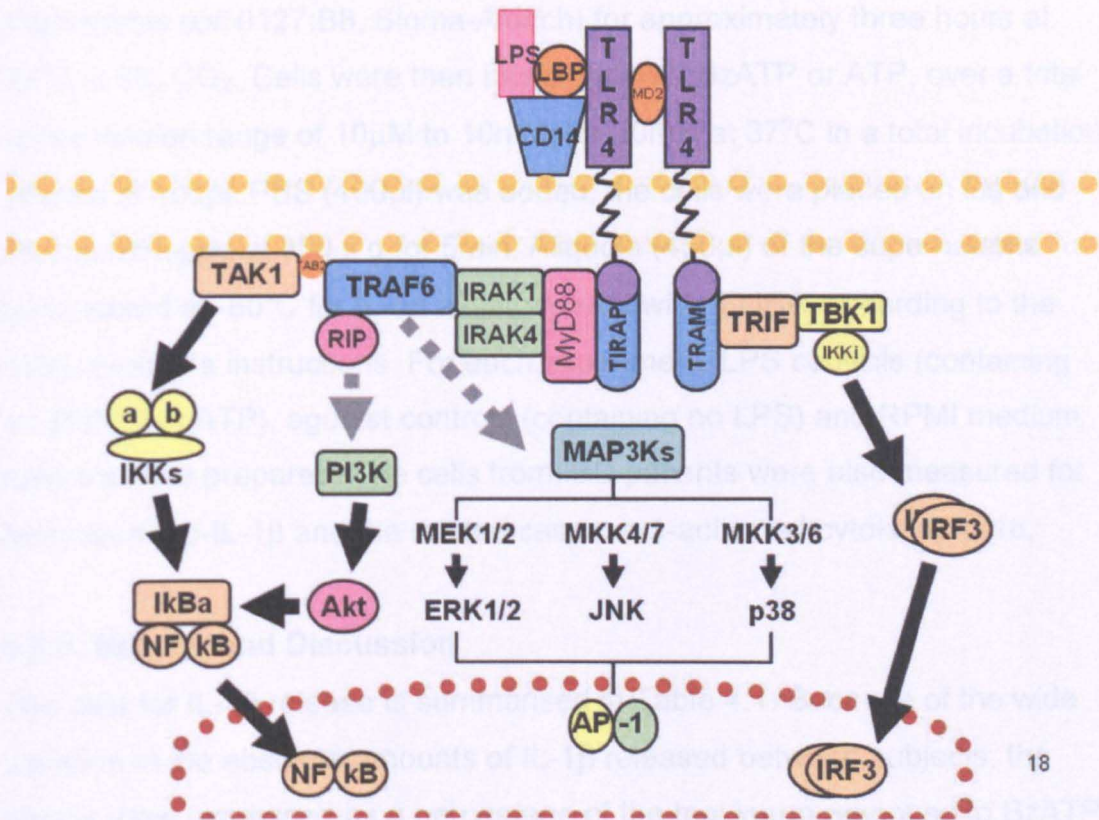
TLR signalling results in recruitment of leukocytes, bacterial phagocytosis and induction of inflammatory cytokines and chemokines (Dobrovolskaia, MA & Vogel, SN, 2002). TLR4 expression has been shown to be increased in inflamed intestinal mucosa and localised to macrophages (Hausmann, M *et al.*, 2002). Others have shown strong upregulation of TLR4 in intestinal epithelial cells from UC and CD tissue compared to normal mucosa (Cario, E & Podolsky, DK, 2000). More recently associations have been made between polymorphisms of the TLR4 gene that lead to impaired LPS signalling, and occurrence of CD and UC (Franchimont, D *et al.*, 2004; Torok, HP *et al.*, 2004; Oostenbrug, LE *et al.*, 2005).

In the experiments described here, cells were incubated with LPS for three hours to induce formation of pro-IL-1 $\beta$ , followed by stimulation of the P2X<sub>7</sub> receptor to release mature IL-1 $\beta$ . LPS also has the ability to release mature IL-1 $\beta$ , and it has been suggested that it does this by inducing ATP release and hence P2X<sub>7</sub> stimulation (Ferrari, D *et al.*, 1997c). An incubation period of three hours with LPS was sufficient to induce formation of intracellular pro-IL-1 $\beta$  without releasing substantial amounts of IL-1 $\beta$  into the supernatant.

#### **4.2.2. Method**

For the LPMCs, IL-1 $\beta$  release was studied in cells isolated from normal colonic tissue collected from colon cancer patients (tissue >5cm from the tumour site), and also from tissue collected from IBD patients. Wherever possible, samples of tissue from both inflamed and uninflamed sections of the colon were obtained from each IBD patient. Cells isolated from inflamed tissue were not pre-incubated with LPS.

Cells were resuspended in RPMI 1640 containing 0.1% v/v FCS at a



18

**Fig 4.1. The principal pathways of LPS signal transduction**

LPS binds to TLR4 via CD14 and LBP. TLR-4 signalling occurs via two pathways; the 'MyD88-dependent' and the 'MyD88-independent' pathways. Both result in activation of NF-κB and the MAP kinase cascade. Translocation of NF-κB to the nucleus leads to production of numerous cytokines, including pro-IL-1β. Beutler, B *et al.*, (2003)

concentration of  $1 \times 10^6$  cells/ml, and incubated with LPS ( $1\mu\text{g/ml}$ , *Escherichia coli* 0127:B8, Sigma-Aldrich) for approximately three hours at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Cells were then incubated with BzATP or ATP, over a total concentration range of  $10\mu\text{M}$  to  $10\text{mM}$ , for 20min at  $37^\circ\text{C}$  in a total incubation volume of  $100\mu\text{l}$ . PBS ( $400\mu\text{l}$ ) was added, the cells were placed on ice and then centrifuged at  $350 \times g$  for 5min. Aliquots ( $450\mu\text{l}$ ) of the supernatants were stored at  $-80^\circ\text{C}$  for IL- $1\beta$  assay by sandwich ELISA, according to the manufacturer's instructions. For each experiment, LPS controls (containing no BzATP or ATP), agonist controls (containing no LPS) and RPMI medium controls were prepared. The cells from IBD patients were also measured for release of pro-IL- $1\beta$  and the related caspase-1-activated cytokine, IL-18.

### 4.2.3. Results and Discussion

The data for IL- $1\beta$  release is summarised in Table 4.1. Because of the wide variation in the absolute amounts of IL- $1\beta$  released between subjects, the results were expressed as a percentage of the maximum response to BzATP for each individual.

#### 4.2.3.1. IL- $1\beta$ release from THP-1 cells

THP-1 cells did not release any IL- $1\beta$  when incubated with BzATP or ATP. This is because they are an immature monocyte cell line expressing little or no CD14 and are therefore unable to respond to LPS priming (Abrink, M *et al.*, 1994).

A single experiment was carried out to test the cells for IL- $1\beta$  release with BzATP stimulation (Fig 4.2.). The THP-1 cells released only  $4\text{pg IL-}1\beta/10^6$  cells whereas comparative experiments with PBMCs and LPMCs released  $1870 \pm 420$  and  $750 \pm 320$   $\text{pg IL-}1\beta/10^6$  cells respectively. Increasing the FCS content of the medium from 0.1% to 10% increased the amount of IL- $1\beta$  released to  $42$   $\text{pg IL-}1\beta/10^6$  cells, but this was still low compared to the PBMCs and LPMCs.

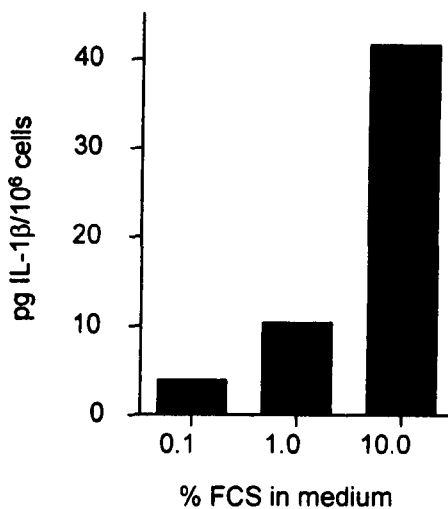
THP-1 cells can be induced to differentiate into mature cells with macrophage-like function by incubation with various compounds (Tsuchiya, S *et al.*, 1982): one commonly used is a phorbol diester, phorbol 12-

	EC <sub>50</sub> value with 95% confidence intervals		Maximum and minimum responses (% of BzATP max) (mean ± sem)		Concentration at which maximum response occurred (mean ± sem)		Maximum pg IL-1β released per 10 <sup>6</sup> cells (mean ± sem)		Hillslope (mean ± sem)	
	BzATP	ATP	BzATP	ATP	BzATP	ATP	BzATP	ATP	BzATP	ATP
PBMCs	39μM 31 - 50μM	0.8mM 0.5 - 1.2mM	93 ± 7 2 ± 0.5	67 ± 10 2 ± 0.6	112 ± 31μM	2.3 ± 0.3mM	2509 ± 777	1767 ± 508	3.8 ± 1.5	5.8 ± 4.1
Normal LPMCs	112μM 81 - 153μM	1.1mM 0.02 - 53mM	92 ± 4 14 ± 6	53 ± 10 16 ± 7	300 ± 71μM	1.7 ± 0.3mM	748 ± 322	379 ± 240	2.9 ± 1.1	12.6 ± 151
Inflamed LPMCs	80μM 62 - 103μM	1.4mM 0.7 - 2.9mM	94 ± 3 17 ± 6	110 ± 41 16 ± 6	217 ± 40μM	4.3 ± 1.2mM	2311 ± 1254	1787 ± 914	3.5 ± 1.2	2.9 ± 2.2
Uninflamed LPMCs	88μM 60 - 128μM	1.1mM 0.7 - 1.7mM	88 ± 7 10 ± 0.6	106 ± 14 8 ± 2	325 ± 63μM	2.3 ± 0.3mM	600 ± 180	934 ± 278	2.9 ± 1.2	7.3 ± 13.2

**Table 4.1. Summary of results for P2X<sub>7</sub>-stimulated IL-1β release**

The table shows the EC<sub>50</sub> values taken from the concentration response curves and the Hillslope calculated from the curve. The mean maximum and minimum responses for each cell type with each agonist are shown, together with the mean of the agonist concentrations at which each subject achieved the maximum response. The absolute amounts of IL-1β released (pg/10<sup>6</sup> cells) corresponding to the maximum response are also shown. Normal LPMCs were isolated from tissue from cancer patients. Inflamed and uninflamed LPMCs were isolated from tissue from IBD patients, from actively inflamed and uninflamed sections respectively.

BzATP was 10-20 times more potent than ATP, as shown by the EC<sub>50</sub> values. PBMCs released much greater amounts of IL-1β than LPMCs from normal or uninflamed tissue, and at lower concentrations of agonist. Release of IL-1β from LPMCs from inflamed tissue was similar to that of PBMCs.



**Fig 4.2. The effect of FCS on the release of IL-1 $\beta$  from THP-1 cells stimulated with BzATP**

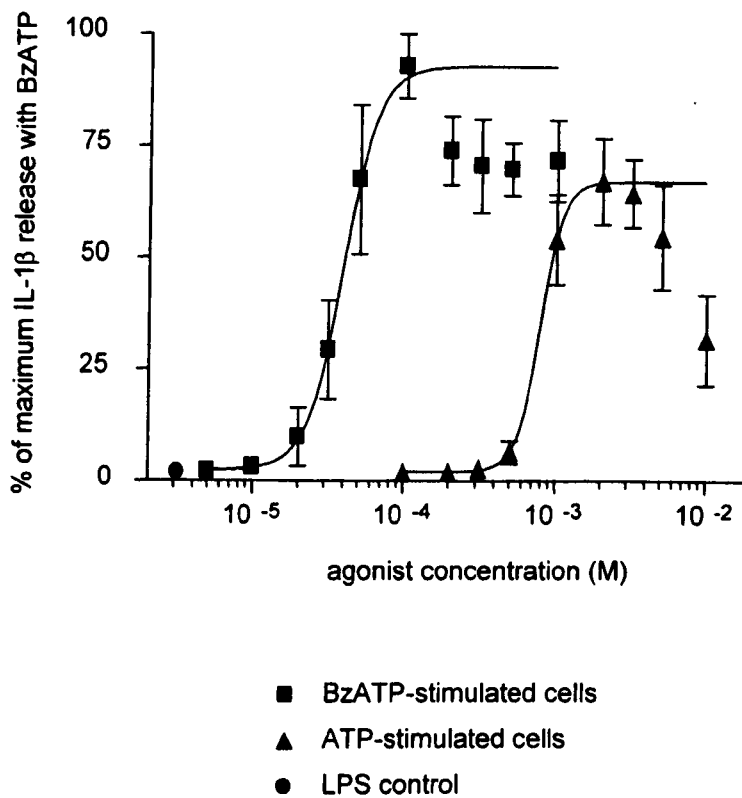
THP-1 cells were resuspended at  $2 \times 10^6$  cells/ml in RPMI containing 0.1, 1 or 10% FCS and incubated with LPS ( $1 \mu\text{g/ml}$ ) for 3h. Cells were stimulated for 20min with BzATP ( $200 \mu\text{M}$ ) and the amount of IL-1 $\beta$  released into the supernatant was measured by ELISA. Results are from a single experiment and are expressed as pg IL-1 $\beta$  /  $10^6$  cells.

myristate-13-acetate (TPA/PMA) (MacKenzie, A *et al.*, 2001; Cochran, FR & Finch-Arietta, MB, 1989). Functional studies of IL-1 $\beta$  release from THP-1 cells generally incorporate an activation step involving incubation with substances such as PMA or with naturally-occurring compounds such as IFN- $\gamma$ . IL-1 $\beta$  release from matured THP-1 cells has been demonstrated many times in the literature (MacKenzie, A *et al.*, 2001; Gudipaty, L *et al.*, 2003; Donnelly-Roberts, D *et al.*, 2004; Verhoef, PA *et al.*, 2005) and therefore experiments on IL-1 $\beta$  release in this project were confined to PBMCs and LPMCs.

#### **4.2.3.2. IL-1 $\beta$ release from PBMCs**

PBMCs generally released large amounts of IL-1 $\beta$ ; up to 4800pg/10<sup>6</sup> cells in one subject, and this release occurred in a concentration-dependent manner for both agonists (Fig 4.3.). BzATP was approximately twenty times more potent than ATP with an EC<sub>50</sub> value of 39 $\mu$ M compared with 0.8mM for ATP. With both agonists the release occurred over a narrow concentration range and the values for the Hill slope were high (5.8 for ATP and 3.8 for BzATP), suggesting multiple agonist binding sites as seen with EB uptake. High values for the Hill slope also arise when the response measured is indirect, i.e. when a sequence of cellular events link receptor activation to the observed response. This is because the Hill equation makes the assumption that response is linearly related to receptor occupancy, whereas with an indirect response this is usually not the case (Jenkinson, DH, 2003). Release of mature IL-1 $\beta$  requires a complex sequence of events involving maturation of caspase-1 via formation of the inflammasome followed by maturation and secretion of IL-1 $\beta$  itself. Combined with the subunit structure of the P2X<sub>7</sub> ion channel which also produces a Hill coefficient >1, it is not surprising that the values obtained here for the Hill slope were so high.

ATP acted as a partial agonist, although both agonists demonstrated reduced release of IL-1 $\beta$  at concentrations greater than the maximum. Other studies have found a similar reduction in IL-1 $\beta$  release at high ATP concentrations (Verhoef, PA *et al.*, 2003; Ferrari, D *et al.*, 1997b). One possible reason could be that high agonist concentrations cause necrotic cell



**Fig 4.3. The release of IL-1 $\beta$  from PBMCs stimulated with BzATP or ATP**

LPS-primed cells in RPMI 1640 + 0.1% FCS were stimulated for 20min with BzATP or ATP. IL-1 $\beta$  released into the supernatant was measured by ELISA and expressed as a percent of the maximum amount released by BzATP. Results show the mean  $\pm$  sem of four separate experiments.

death and release of unprocessed pro-IL-1 $\beta$  instead of mature IL-1 $\beta$ .

Verhoef, PA *et al.*, (2005) proposed that the reduction in IL-1 $\beta$  release is due to chloride ions present in the medium which enter the cell and attenuate the ATP response. Replacement of chloride ions with a non-permeant anion such as gluconate produced a 10-fold increase in IL-1 $\beta$  release. They suggested that chloride ions may alter the conformation of the receptor in such a way as to limit ATP binding, or may regulate inflammasome assembly and hence inhibit caspase-1 processing of pro-IL-1 $\beta$ .

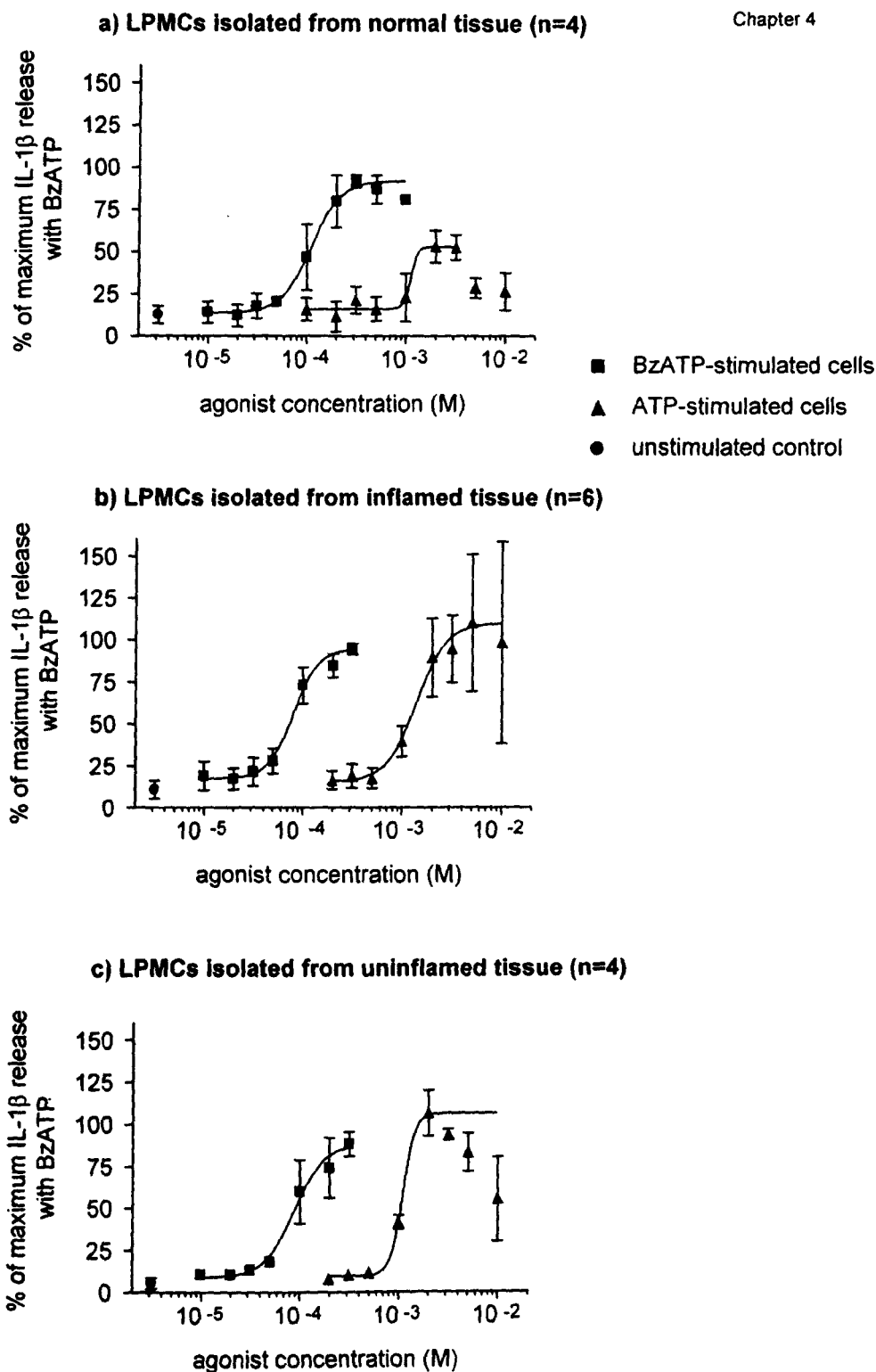
#### **4.2.3.3. IL-1 $\beta$ release from LPMCs**

##### ***i. mature IL-1 $\beta$ release***

IL-1 $\beta$  release from normal tissue (cancer patients) and from inflamed and uninflamed tissue (IBD patients) is shown in Fig 4.4. The release of IL-1 $\beta$  was concentration-dependent in cells from all tissue types, and occurred over a narrow concentration range. This was particularly marked with ATP stimulation of normal and uninflamed cells where there was only one concentration point on the slope resulting in inconclusive curve fit and very high values for the Hill slope (12.6 for normal and 7.3 for uninflamed LPMCs). BzATP was 10-15 times more potent than ATP, with EC<sub>50</sub> values of 80-112 $\mu$ M compared with 1.1-1.4mM for ATP. As seen with PBMCs, IL-1 $\beta$  release decreased at higher concentrations of agonist. The decreased response was not seen with BzATP stimulation of LPMCs from IBD patients, probably because the concentration range was not high enough.

In cells from normal tissue, ATP acted as a partial agonist, but in cells from inflamed tissue this was not the case. The efficacy of a partial agonist depends on the ratio between receptor occupancy and the ability of the bound receptor to elicit a response. These factors may vary in different tissues and efficacy is therefore tissue dependent (Jenkinson, DH, 2003). Since P2X<sub>7</sub> has inflammatory properties, it may be that ATP binding to the receptor in normal tissue is controlled such that the response is reduced and only partial. Perhaps in IBD tissue this control is absent, leaving ATP to elicit a full receptor response and produce a greater inflammatory effect.





**Fig 4.4. The release of IL-1 $\beta$  from LPMCs stimulated with BzATP or ATP**

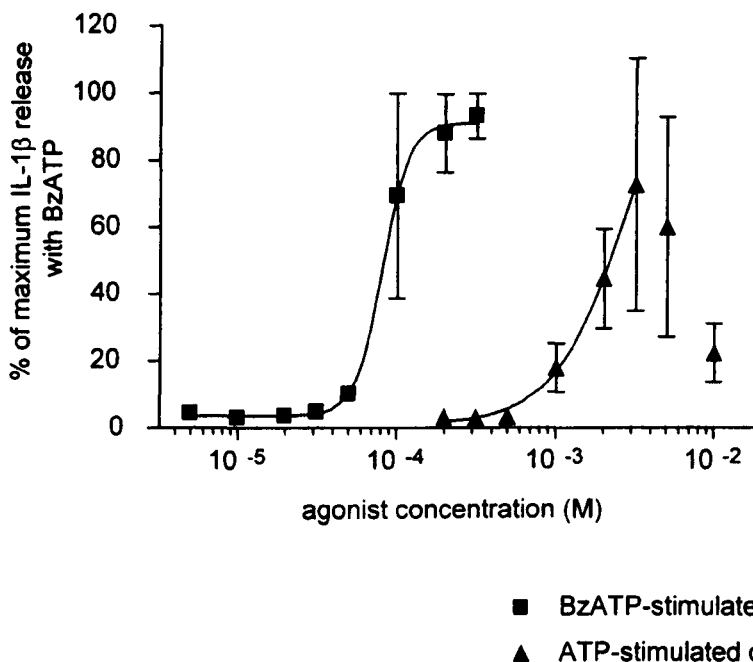
LPS-primed cells in RPMI 1640 + 0.1% FCS were stimulated for 20min with BzATP or ATP (cells and control from inflamed tissue were not treated with LPS). IL-1 $\beta$  released into the supernatant was measured by ELISA and expressed as a percent of the maximum amount released by BzATP. Results show the mean  $\pm$  sem of separate experiments.

Normal LPMCs were isolated from tissue from cancer patients. Inflamed and uninflamed LPMCs were isolated from tissue from IBD patients, from actively inflamed and uninflamed sections respectively.

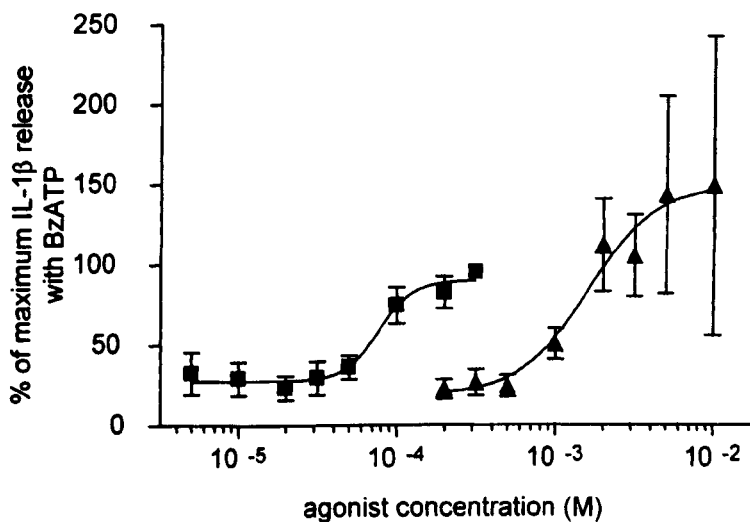
LPMCs from inflamed tissue (Fig 4.4.b) showed a wide variation in ATP-stimulated IL-1 $\beta$  release, particularly at high concentrations. This is because the amounts released by the six subjects with ATP differed extensively with respect to their responses to BzATP-stimulation. This can be seen even more clearly when, based on the histopathology reports, the data for the inflamed tissue was divided into those subjects who exhibited severe inflammation and those who were only mildly inflamed (Fig 4.5.). Of the two subjects with severe inflammation (Fig 4.5.a), ATP behaved in one as a partial agonist reaching only 35% of the BzATP response, whereas in the other it acted as a full agonist reaching 110% of the BzATP response. Four subjects had moderate inflammation and ATP acted as a partial agonist in only one (Fig 4.5.b). For the other three subjects ATP acted as a full agonist, and in one subject ATP released three times the amount of IL-1 $\beta$  as BzATP. This shows that the ability of ATP to act as a full agonist does not depend on the severity of the inflammation, but may be a feature of the disease itself and reflect variations in the receptor responses from different subjects.

LPMCs from normal tissue secreted much lower levels of IL-1 $\beta$  than PBMCs ( $748 \pm 322$  versus  $2509 \pm 777$  pg/ $10^6$  cells), confirming the non-inflammatory character of normal colonic tissue macrophages. In inflamed tissue however, where there is usually an influx of cells from the peripheral blood, the amount secreted was much higher ( $2311 \pm 1254$  pg/ $10^6$  cells) and similar to that seen in PBMCs ( $2509 \pm 777$  pg/ $10^6$  cells). The maximum amounts of IL-1 $\beta$  released from each patient matched the severity of their inflammation. Patients with severe active UC (n=2) released a mean of  $6492 \pm 919$ pg/ $10^6$  cells with BzATP and  $4383 \pm 1792$ pg with ATP, whereas those with moderate UC (n=3) released only  $363 \pm 220$ pg and  $576 \pm 310$ pg/ $10^6$  cells with BzATP and ATP respectively. The single CD patient, who had only slight inflammation, released  $591$ pg/ $10^6$  cells with BzATP and  $498$ pg/ $10^6$  cells with ATP. These values confirm the importance of IL-1 $\beta$  as an inflammatory cytokine in IBD and its usefulness as a therapeutic target, particularly in cases of severe inflammation.

**a) LPMCs isolated from severely inflamed tissue (n=2)**



**b) LPMCs isolated from moderately inflamed tissue (n=4)**



**Fig 4.5. The release of IL-1 $\beta$  from LPMCs isolated from inflamed tissue**

Graphs show the data from Fig 4.4.b separated into results for severely inflamed tissue and moderately inflamed tissue based on the histopathology reports. The wide variation in the responses to ATP stimulation can clearly be seen. For the severely inflamed tissue where there were only 2 subjects, the error bars show the responses of each individual.

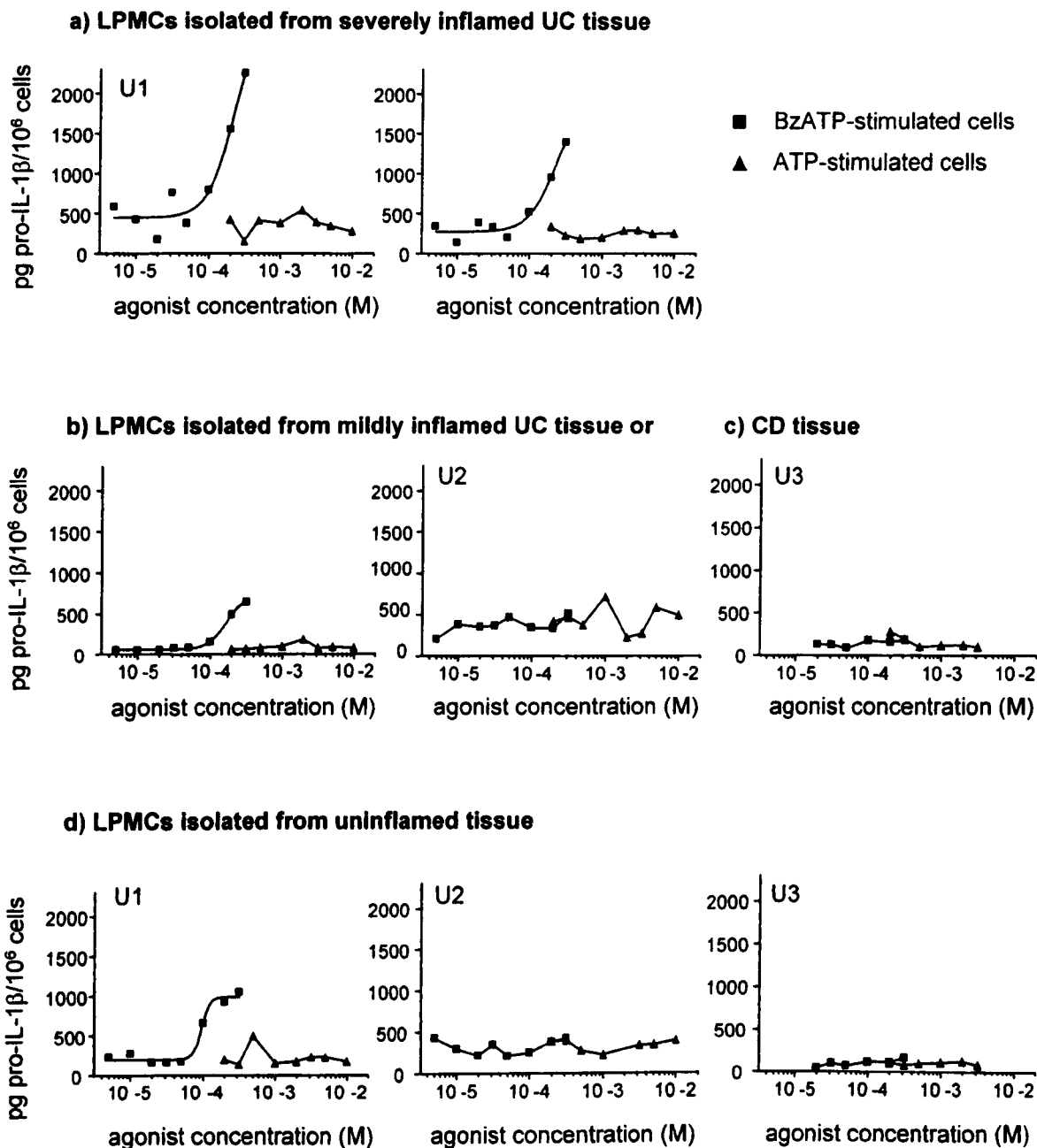
### ***ii. pro-IL-1 $\beta$ release***

The results for pro-IL-1 $\beta$  release from individual subjects are shown in Fig 4.6. For the two patients with severely inflamed UC (Fig 4.6.a), BzATP-stimulated LPMCs released pro-IL-1 $\beta$  in a concentration-dependent manner with the amount released reaching a maximum of 1400-2250pg/10<sup>6</sup> cells. This was equal to approximately 30% of the maximum amount of IL-1 $\beta$  released. The release of pro-IL-1 $\beta$  did not occur until BzATP concentration was greater than 100 $\mu$ M, a concentration at which release of mature IL-1 $\beta$  in these patients was at or near maximum (Fig 4.5.a). This shows that in severely inflamed tissue pro-IL-1 $\beta$  starts to be released together with mature IL-1 $\beta$  at BzATP concentrations near maximal for IL-1 $\beta$  release, although not to the same extent. One possible explanation for the release of pro-IL-1 $\beta$  could be a toxic effect of high concentrations of BzATP leading to necrotic cell death and release of cell contents. 100 $\mu$ M BzATP was the concentration at which the monocyte-gated cell population started to decrease in number (Fig 3.4.e) suggesting that may be the concentration at which cells start to die.

For the patients with moderate inflammation (Fig 4.6.b & c) the responses to BzATP-stimulation differed considerably. One patient demonstrated a concentration-dependent response of a similar pattern to that seen with severely inflamed tissue, but in this patient the release of mature IL-1 $\beta$  was very low (232 pg/10<sup>6</sup> cells maximum). It may be that in this patient the cells died on stimulation and released pro-IL-1 $\beta$  into the supernatant. The other two patients showed no increase in pro-IL-1 $\beta$  release when stimulated with BzATP, but did release greater amounts of mature IL-1 $\beta$  (793 and 590 pg/10<sup>6</sup> cells maximum). ATP did not markedly increase pro-IL-1 $\beta$  release in any of the patients. This may be because the concentration range was not high enough. If pro-IL-1 $\beta$  is not released until IL-1 $\beta$  release is near-maximal, then ATP concentrations greater than 10mM would be necessary.

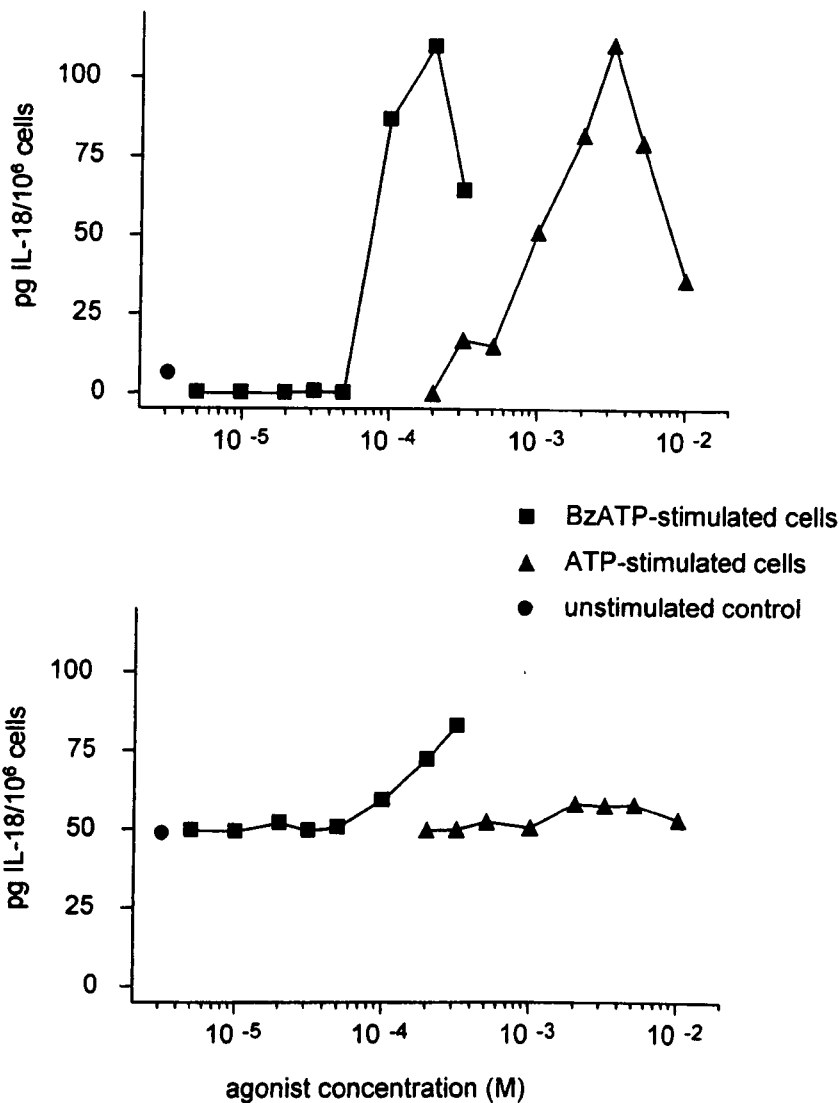
### ***iii. IL-18 release***

Very little IL-18 was released into the cell supernatant and only samples from the severely inflamed UC patients produced measurable amounts. The results are shown in Fig 4.7. and show very different profiles for each patient.



**Fig 4.6. The release of pro-IL-1 $\beta$  from LPMCs isolated from inflamed tissue**

Supernatants collected for measurement of IL-1 $\beta$  release from IBD patients (fig 4.4.b & c) were also assayed for pro-IL-1 $\beta$ . Figures show the results for individual patients. The numbers U1, U2 and U3 indicate where a sample of uninfamed tissue was obtained from the same patient as the inflamed tissue. The amount of pro-IL-1 $\beta$  released was clearly related to the degree of inflammation.



**Fig 4.7. The release of IL-18 from LPMCs isolated from severely inflamed tissue**

Supernatants collected for measurement of IL-1 $\beta$  release from the two patients with severely inflamed tissue were assayed for IL-18. The results are expressed as pg IL-18/10<sup>6</sup> cells and each figure shows the results from one patient. Very little IL-18 was released from either patient although the pattern of release was very different.

One patient demonstrated a clear concentration-dependent release of IL-18 on stimulation with both agonists, whereas the other had a higher baseline secretion but little increase on stimulation. These results suggest that intestinal inflammatory cells secrete very little IL-18. This is supported by a study of IL-18 mRNA expression in IBD tissue which was up-regulated, particularly in CD, but the expression was much greater in intestinal epithelial cells than in LPMCs (Pizarro, TT *et al.*, 1999).

#### **4.2.3.4. Summary of P2X<sub>7</sub>-stimulated IL-1 $\beta$ release**

Both PBMCs and LPMCs displayed a concentration-dependent increase in IL-1 $\beta$  release and for both cell types BzATP was a more potent agonist than ATP. This is consistent with a P2X<sub>7</sub>-mediated response. The principal IL-1 $\beta$ -secreting cells are the monocytes and macrophages, and several comparable studies have been performed using these cell types. For example, studies on ATP stimulation of PBMC monocytes (Elssner, A *et al.*, 2004), PBMC differentiated macrophages (Ferrari, D *et al.*, 1997b) or macrophage cell lines (Verhoef, PA *et al.*, 2003) have all shown a concentration-dependent increase in IL-1 $\beta$  release over the range 0.05-5mM ATP. Similarly, a study using matured THP-1 cells demonstrated BzATP-stimulated IL-1 $\beta$  release with an EC<sub>50</sub> value of approximately 130 $\mu$ M, a value consistent with the ones reported here for PBMCs and LPMCs (Buell, G *et al.*, (1998).

The results clearly showed that LPMCs isolated from normal colonic tissue did not readily produce IL-1 $\beta$  in response to P2X<sub>7</sub> stimulation. Secretion of IL-1 $\beta$  increased dramatically however in cells from inflamed tissue to levels similar to those secreted by PBMCs, confirming that inflamed tissue contains a greater proportion of cells derived from the circulation. This was demonstrated not only by the amount of IL-1 $\beta$  released, which was 3-4½ times that of normal LPMCs, but also by the EC<sub>50</sub> value which was reduced by up to two-thirds in cells from inflamed tissue (80 $\mu$ M compared to 112 $\mu$ M in normal LPMCs) and was closer to the value obtained for PBMCs (39 $\mu$ M). The variable nature of cytokine secretion between patients as seen in these results also confirms the nature of IBD, in that characteristics of the disease

vary widely between subjects, with many factors involved in the nature of its presentation. There is a need therefore for treatments that can target the inflammatory processes in different ways since one that is effective in one patient may not be in another. Inhibition of P2X<sub>7</sub>-stimulated IL-1 $\beta$  release offers another mechanism for such treatment.

### 4.3. P2X<sub>7</sub> RECEPTOR-STIMULATED ANNEXIN V BINDING

#### 4.3.1. Introduction

Some studies have associated P2X<sub>7</sub>-stimulated IL-1 $\beta$  release with cell death (Hogquist, KA *et al.*, 1991a; Brough, D *et al.*, 2007). The receptor's ability to promote membrane PS flip has suggested that it mediates death by apoptosis, but the demonstrated reversibility of PS flip has made this less certain (MacKenzie, AB *et al.*, 2005).

##### 4.3.1.1. Cell death

Cell death can occur via one of two fundamentally different processes; namely apoptosis or necrosis.

**i. Necrosis** is a degenerative process usually triggered by extreme trauma or injury to the cell. Integrity is lost in both internal organelle and plasma membranes, resulting in the release of cell contents into the surrounding environment. This may lead to injury to neighbouring cells, and the infiltration of cytokine-producing immune cells into the area, generating an inflammatory response (Allen, RT *et al.*, 1997). The term necrosis generally refers to the changes that have occurred in cells after they have died regardless of how death has occurred. Two principal mechanisms of necrotic death are oncosis and pyroptosis.

Oncosis is the opposite of apoptosis in that death occurs by cellular and organelle swelling, blebbing and increased membrane permeability. The process ultimately leads to depletion of cellular energy stores, either as a result of interference with ATP generation or uncontrolled energy consumption (Majno, G & Joris, I, 1995).

Pyroptosis is a relatively new term that was originally devised for cell death induced by *Salmonella* and *Shigella* infection of host macrophages



(Cookson, BT & Brennan, MA (2001)). It is a pro-inflammatory pathway mediated by the activation of caspase-1 and leads to membrane breakdown and processing of IL-1 $\beta$  and IL-18.

ii. **Apoptosis** (programmed cell death) is a regulated event involving a series of structural and biochemical changes within the cell (Saraste, A & Pulkki, K, 2000). Nuclear chromatin condenses and becomes confined to the margin of the nucleus, followed by progressive condensing and fragmentation of the nucleus itself. Cell cytoplasm also condenses leading to cell shrinkage, and the cell detaches from surrounding tissues. The plasma membrane becomes convoluted and forms extensions which seal and separate from the cell. This process is called budding, and the 'apoptotic bodies' thus formed are rapidly phagocytosed by macrophages (Saraste, A *et al.*, 2000; Allen, RT *et al.*, 1997). Because the break-up of the cell occurs with no release of cellular contents, apoptosis is not generally associated with inflammation (Savill, J *et al.*, 2002).

All of these structural changes occur in the cell as a result of the activity of caspase enzymes (Thornberry, NA & Lazebnik, Y, 1998). The first member of the family to be identified was caspase-1, which is not involved in apoptosis but is responsible for the cleavage of IL-1 $\beta$  to its mature form (section 1.6.1.). Many other members of the family however, are involved both in the breaking up of the cell (known as effectors, caspase-3, -6 & -7), or in the instigation of this disassembly (known as initiators, caspase-2, -8, -9 & -10) (Thornberry, NA *et al.*, 1998).

Caspases are constitutively expressed as inactive proenzymes and require cleavage to form the active enzyme (Zimmermann, KC & Green, DR, 2001). Activation of the initiator caspases in response to pro-apoptotic signals leads to the proteolytic cleavage and generation of mature effector caspases (Zimmermann, KC *et al.*, 2001). Once activated, effector caspases cleave proteins which support the nuclear membrane, initiate DNA fragmentation, and cleave proteins involved in the cytoskeleton and in the attachment of cells to their neighbours, all facilitating cell disassembly (Thornberry, NA *et al.*, 1998).

Signalling pathways leading to caspase activation are characterised as extrinsic or intrinsic (Fig 4.8.). The extrinsic pathway involves the binding of extracellular ligands to cell surface receptors (e.g. TNF, Fas, TRAIL) resulting in the recruitment of cytosolic adaptor proteins (e.g. FADD), activation of initiator caspases and subsequent activation of effector caspases (Schafer, ZT & Kornbluth, S, 2006; Menaker, RJ & Jones, NL, 2003).

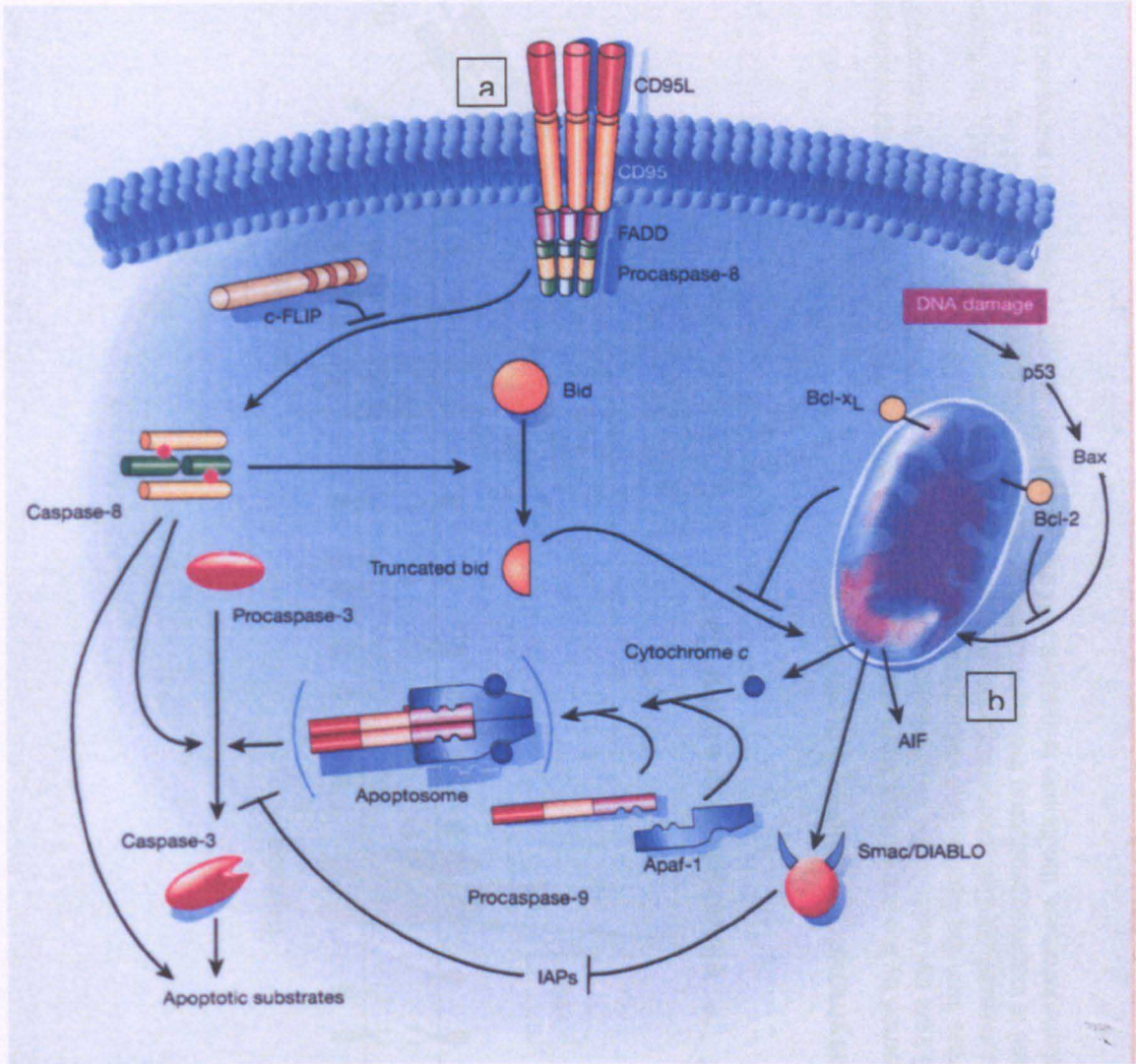
The intrinsic pathway acts via the mitochondria and can be initiated by factors such as cytotoxic agents and DNA damage. The process results in mitochondrial outer membrane permeabilisation and cytochrome c release, and is regulated by proteins of the Bcl-2 family (Schafer, ZT *et al.*, 2006). The Bcl-2 proteins can be subdivided into pro- and anti-apoptotic members depending on whether they promote (e.g. Bax, Bak) or impede (e.g. Bcl-2, Bcl-X<sub>L</sub>) cytochrome c release (Opferman, JT & Korsmeyer, SJ, 2003). Once cytochrome c is released it forms part of a complex called the 'apoptosome', together with a protein called Apaf-1 (apoptosis protease-activating factor-1) and initiator procaspase-9. The apoptosome then activates effector caspases leading to the breakdown of the cell and formation of apoptotic bodies (Schafer, ZT *et al.*, 2006).

#### **4.3.1.2. Phosphatidylserine (PS) flip**

The phagocytosis of apoptotic bodies occurs as a result of a change in the phospholipid bilayer of the plasma membrane early in apoptosis.

Phosphatidylserine (PS) is normally located predominantly on the inner surface of the cell membrane, but during early apoptosis, it translocates from the inner to the outer layer of the plasma membrane (PS-flip) where it triggers recognition by macrophages (Fadok, VA *et al.*, 1992).

The distribution of PS in the cell membrane is controlled by the activities of three enzymes; a PS-specific aminophospholipid translocase (flipase), a lipid scramblase and a non-specific floppase (Fig 4.9.) (Diaz, C *et al.*, 1996). The activity of the enzymes is governed by the intracellular Ca<sup>2+</sup> concentration (Martinez, MC *et al.*, 1999). At physiological Ca<sup>2+</sup> concentrations (<100nM), the flipase and floppase are active but the scramblase is inactive, producing the asymmetric distribution of PS on the inner layer of the membrane. At elevated Ca<sup>2+</sup> concentrations however, the flipase is inactive and the



**Fig 4.8. The two major apoptotic pathways in mammalian cells**

a. The extrinsic pathway involves formation of a death-inducing signalling complex initiated by binding of ligand to a member of the death-receptor family (e.g. CD95, TNF receptor I). The complex binds procaspase-8 via an adaptor molecule FADD (Fas-associated death domain protein), resulting in caspase-8 activation.

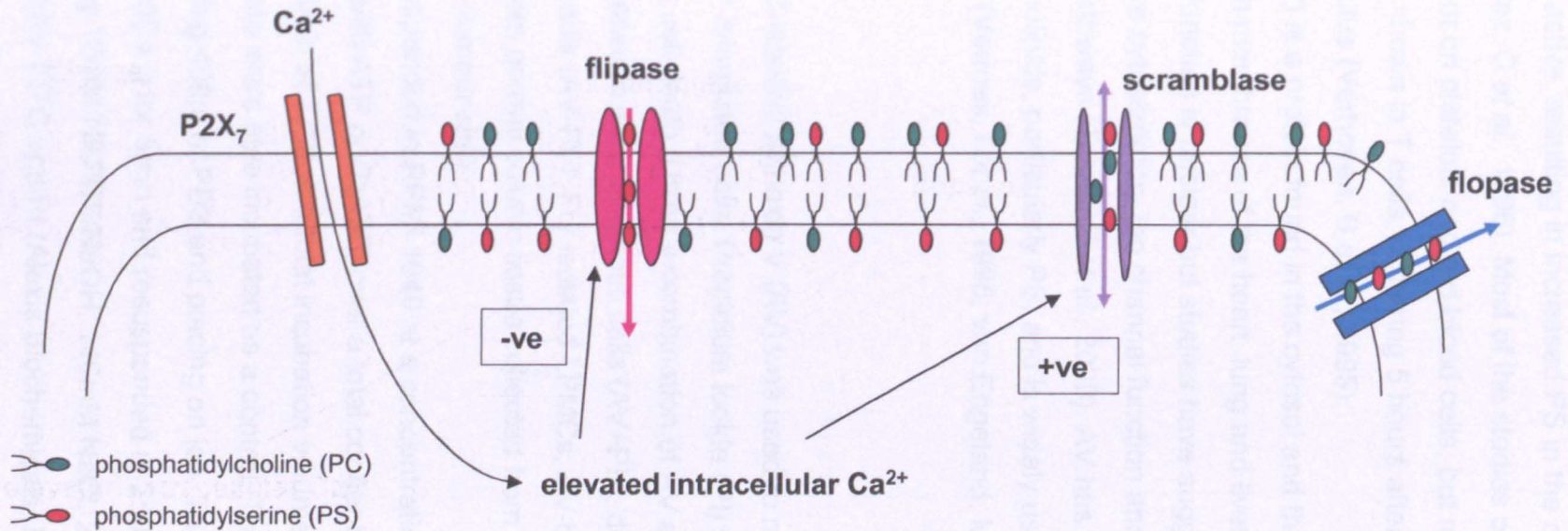
b. The intrinsic pathway, or mitochondrial pathway, involves activation of proteins of the Bcl-2 family. The balance between the activities of pro- and anti-apoptotic members of this family regulates release of cytochrome c from the mitochondria. Cytochrome C combines with Apaf-1 (apoptosis protease-activating factor-1) and procaspase-9 to form the apoptosome, resulting in activation of caspase-9.

Integration of the two pathways is through Bid, a pro-apoptotic member of the Bcl-2 family. Active caspase-8 cleaves Bid which then translocates to the mitochondria and promotes release of cytochrome c.

The active caspase-8 and -9 promote cleavage of effector caspases such as procaspase-3 resulting in the breakdown of the cell.

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**Fig 4.9. The generation of PS asymmetry in the cell membrane**

PS asymmetry in the membrane is governed by the activities of three enzymes. The flipase is an ATP-dependent aminophospholipid translocase which rapidly transports PS from the outer to the inner membrane. The floppase is also ATP-dependent and transports PS to the outer membrane but at a slower rate than the flipase. The scramblase transports PS in both directions. Activity of the enzymes is dependent on intracellular  $\text{Ca}^{2+}$  concentrations. At physiological  $\text{Ca}^{2+}$  concentrations (<100nM), the flipase and floppase are active but the scramblase is inactive, producing the asymmetric distribution of PS on the inner layer of the membrane. At elevated  $\text{Ca}^{2+}$  concentrations however, the flipase is inactive and the scramblase is active, resulting in increased PS in the outer layer of the membrane.

Diaz, C & Schroit, AJ, (1996)

scramblase is active, resulting in increased PS in the outer layer of the membrane (Diaz, C *et al.*, 1996). Most of the studies on these enzymes have been carried out on platelets and red blood cells, but reduced flipase activity has also been shown in T cells, occurring 5 hours after application of an apoptotic stimulus (Verhoven, B *et al.*, 1995).

Annexin V (AV) is a protein found in the cytosol and the nucleus and also associated with membranes of the heart, lung and liver (Tzima, E & Walker, JH, 2000). Its function is unclear but studies have suggested roles in regulation of the cytoskeleton, ion channel function and involvement in signal transduction pathways (Tzima, E *et al.*, 2000). AV has a strong affinity for anionic phospholipids, particularly PS, and is widely used in assays to detect apoptotic cells (Vermes, I *et al.*, 1995; van Engeland, M *et al.*, 1998; Tait, JF *et al.*, 1999).

#### **4.3.2. Method**

Binding of FITC-labelled Annexin V (AV) was used to measure PS-flip in BzATP- or ATP-stimulated cells. Propidium iodide (PI) was also incorporated as a marker for cell death. Using a combination of AV and PI it was possible to distinguish between early apoptotic cells (AV+PI-), dead cells (AV+PI+) and unstained cells (AV-PI-). For isolated LPMCs, AV-binding was studied in cells isolated from normal colonic tissue collected from colon cancer patients (>5cm from the tumour site).

Cells were resuspended in RPMI 1640 at a concentration of  $2 \times 10^6$  cells/ml and incubated with ATP or BzATP, over a total concentration range of  $10\mu\text{M}$  to  $10\text{mM}$ , for 20min at  $37^\circ\text{C}$  in a total incubation volume of  $100\mu\text{l}$ .

Unstimulated cells were also incubated as a control. The reaction was stopped by adding  $400\mu\text{l}$  of PBS and placing on ice. The cells were centrifuged at  $350 \times g$  for 5min and resuspended in  $250\mu\text{l}$  of cold binding buffer containing  $10\text{mM}$  HEPES/NaOH,  $140\text{mM}$  NaCl,  $2.5\text{mM}$   $\text{CaCl}_2$ , pH 7.4 with  $5\mu\text{l}$  each of AV-FITC and PI (Alexis Biochemicals, Bingham, Notts). The cells were kept on ice and analysed by flow cytometry.

### 4.3.3. Results and Discussion

Cell populations were gated on the FS/SS dotplots as shown in Fig 3.1. For each gated cell population, AV versus PI binding was plotted, and a quadrant applied to identify the percentage of the gated population binding positively for each stain (Fig 4.10.). Values for AV+PI- (upper left quadrant, apoptotic cells) and AV+PI+ (upper right quadrant, dead cells) were added together to give total AV-positive binding. Different quadrants were used for each cell population as monocytes bind approximately twice the number of annexin V molecules per cell as lymphocytes (Tait, JF *et al.*, 1999). The data for AV binding is summarised in Table 4.2.

#### 4.3.3.1. AV binding in THP-1 cells

Total AV-positive binding increased in a concentration-dependent manner with both BzATP and ATP stimulation (Fig 4.11.a). BzATP was a more potent agonist than ATP as shown by the ten-fold reduction in EC<sub>50</sub> value (100µM for BzATP compared with 1.3mM with ATP), but the maximal response was similar for both agonists. The response to ATP occurred over a narrower concentration range than that to BzATP and this was reflected in the values for the Hill slope (4.2 for ATP and 2.3 for BzATP).

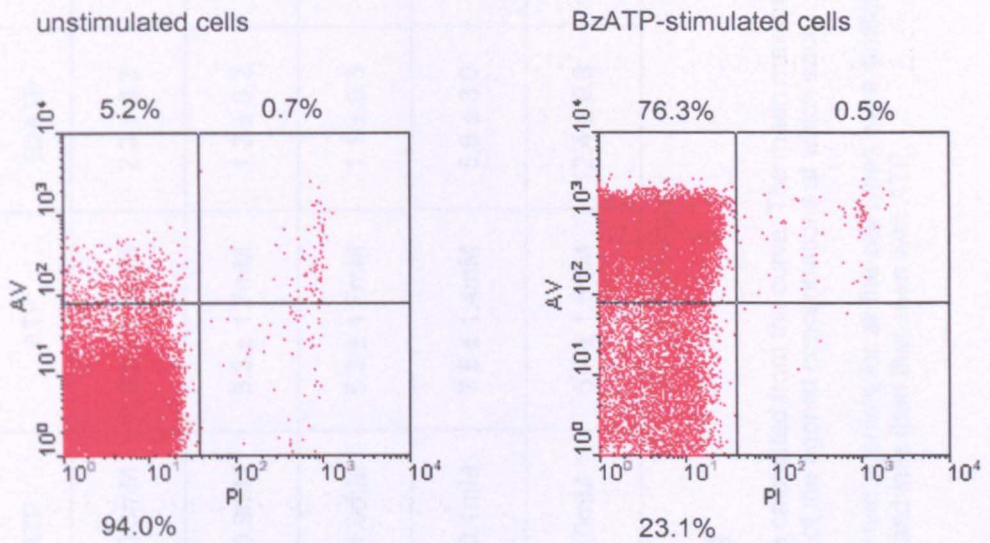
#### 4.3.3.2. AV binding in PBMCs

Total AV-positive binding in both lymphocyte and monocyte-gated cell populations increased in a concentration-dependent manner (Fig 4.11.b & c). BzATP was approximately eight times more potent than ATP in monocyte-gated cells with EC<sub>50</sub> values of 210µM and 1.6mM respectively. In lymphocyte-gated cells this was reduced to approximately four times (EC<sub>50</sub> values of 246µM for BzATP and 1.1mM for ATP).

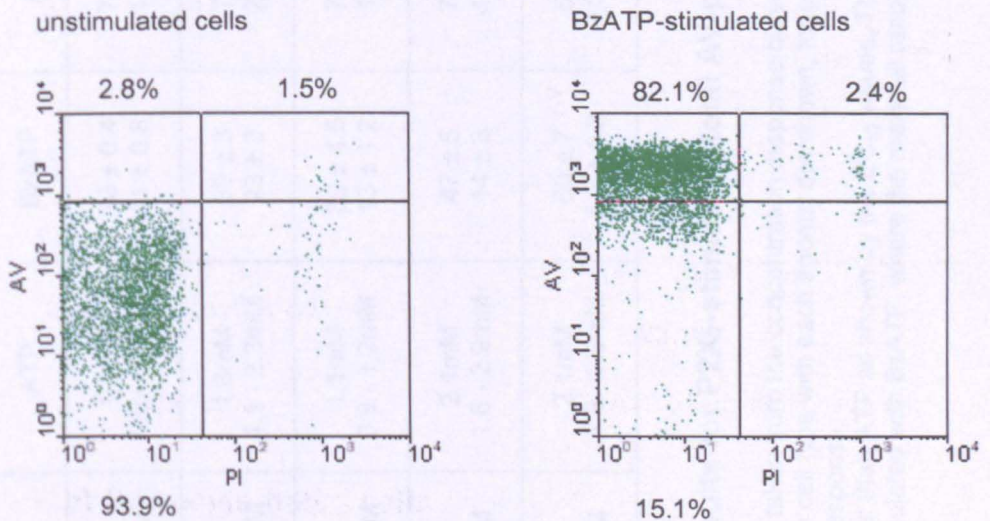
ATP acted as a partial agonist in both cell types, producing only 80% and 88% of the BzATP response in monocytes and lymphocytes respectively. Its agonist activity was greater than that seen with IL-1β release however, where the ATP response was only 72% of the BzATP response. Both cell types responded to ATP over a narrower concentration range than BzATP, and this was reflected in values for the Hill slope which were 4.3 and 4.4 for



### a) Lymphocyte-gated cells



### b) Monocyte-gated cells



**Fig 4.10. Flow cytometry dotplots for AV/PI binding in lymphocyte- and monocyte-gated PBMCs**

Each gated cell population was analysed for AV/PI binding. Unstimulated and maximally-stimulated cells were used to set the position of the quadrants. Cells above the horizontal line are AV-positive (apoptotic cells) and those to the right of the vertical line are PI-positive (dead cells). The figures show the percentage of the gated cell population in each quadrant.

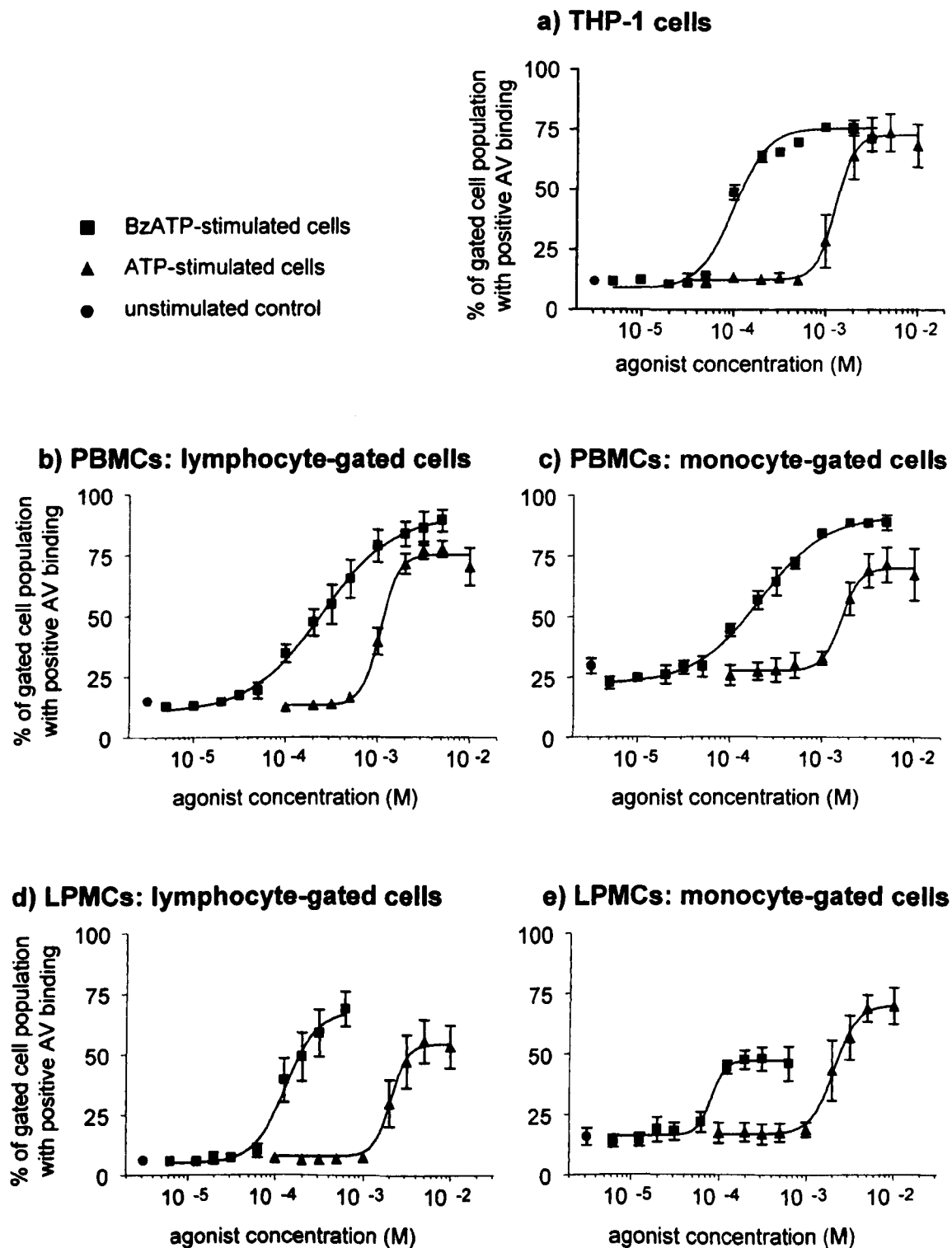
	EC <sub>50</sub> value with 95% confidence intervals		Maximum and minimum responses (% AV+ binding) (mean ± sem)		Concentration at which maximum response occurred (mean ± sem)		Hillslope (mean ± sem)	
	BzATP	ATP	BzATP	ATP	BzATP	ATP	BzATP	ATP
THP-1 cells	100µM 87 - 114µM	1.3mM 1.0 - 1.6mM	76 ± 0.4 11 ± 0.8	74 ± 8 12 ± 3	1.5 ± 0.3mM	2.5 ± 0.3mM	2.3 ± 0.3	4.2 ± 1.3
PBMCs: monocytes	210µM 163 - 272µM	1.6mM 1.1 - 2.3mM	89 ± 3 23 ± 3	72 ± 7 26 ± 4	2.5 ± 0.9mM	5.3 ± 1.7mM	1.2 ± 0.2	4.3 ± 2.3
PBMCs: lymphocytes	246µM 168 - 359µM	1.1mM 0.9 - 1.2mM	89 ± 4.5 13 ± 1.2	78 ± 3 12 ± 1	3.2 ± 1.0mM	5.3 ± 1.7mM	1.1 ± 0.3	4.4 ± 1.7
Normal LPMCs: monocytes	83µM 58 - 119µM	2.1mM 1.6 - 2.9mM	47 ± 5 14 ± 3	70 ± 8 17 ± 4	0.3 ± 0.1mM	7.5 ± 1.4mM	5.6 ± 3.0	3.3 ± 1.6
Normal LPMCs: lymphocytes	131µM 94 - 184µM	2.1mM 1.6 - 2.6mM	69 ± 7 5.7 ± 1.2	55 ± 9 6.3 ± 1.4	0.6 ± 0mM	5.7 ± 1.5mM	2.4 ± 0.9	4.4 ± 2.7

**Table 4.2. Summary of results for P2X<sub>7</sub>-stimulated total AV-positive binding**

The table shows the EC<sub>50</sub> values taken from the concentration response curves and the Hillslope calculated from the curve. The mean maximum and minimum responses for each cell type with each agonist are shown, together with the mean of the agonist concentrations at which each subject achieved the maximum response.

BzATP was a more potent agonist than ATP as shown by the EC<sub>50</sub> values. The responses to the two agonists for all the cell types were similar except for LPMC monocytes stimulated with BzATP, where the maximal response was very low and less than that seen with ATP.





**Fig 4.11. Annexin V binding in mononuclear cells stimulated with BzATP or ATP.**

Total AV-positive binding was measured by flow cytometry after 20min stimulation with either BzATP or ATP in RPMI 1640. Cells staining positive were expressed as a percent of the gated cell population; results show the mean  $\pm$  sem of four separate experiments.

ATP but only 1.2 and 1.1 for BzATP for monocyte- and lymphocyte-gated cells respectively.

Interestingly, the concentration-response curves with BzATP appeared to be less steep than all of the other curves produced so far. The values for the Hill slope have been greater than two for every other measured parameter, but were only 1.2 and 1.1 for BzATP-stimulated AV binding in PBMCs. Also, most of the  $EC_{50}$  values for BzATP stimulation have been in the region of  $100\mu\text{M}$ , whereas the values for AV binding in PBMCs were greater than  $200\mu\text{M}$ .

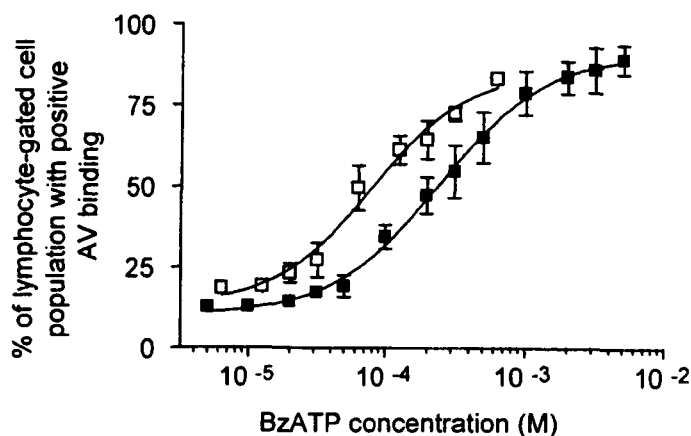
In experiments performed at the beginning of the project, concentration-response curves were carried out using PBMCs stimulated with BzATP for five minutes. These results were compared with the ones for 20min stimulation and are shown in Fig 4.12. AV binding curves for both lymphocyte- and monocyte-gated cells showed a leftward shift with 5min stimulation suggesting that BzATP was a more potent agonist with a shorter stimulation time. This was reflected in the  $EC_{50}$  values which were  $79\mu\text{M}$  for lymphocyte-gated cells and  $62\mu\text{M}$  for monocyte-gated cells. Also whilst the curves for the lymphocyte-gated cells were parallel, the monocyte-gated cells demonstrated a steeper curve with the shorter stimulation time (Hill slope = 1.8).

One possible explanation is receptor desensitisation with a longer stimulation time, although this has only been seen with the  $P2X_1$  and  $P2X_3$  receptors so far (North, RA *et al.*, 2000). A more likely reason for the effect is substrate depletion. As described in section 3.3.2., many cells express nucleotidase enzymes which break down ATP and BzATP (Zimmermann, H, 2000; Kukley, M *et al.*, 2004). With longer incubation times more of the agonist will be removed by the enzymes, thus requiring a higher concentration to produce a similar effect.

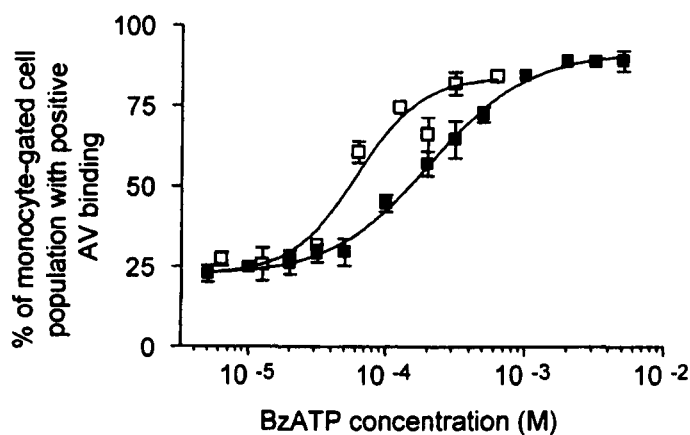
#### **4.3.3.3. AV binding in LPMCs**

Total AV-positive binding in both lymphocyte and monocyte-gated cell populations increased in a concentration-dependent manner for both agonists (Fig 4.11.d & e). BzATP was approximately twenty-five times more

## a) Lymphocyte-gated cells



## b) Monocyte-gated cells



■ 20min stimulation

□ 5min stimulation

c) Table of EC<sub>50</sub> values with 95% CI

	5min stimulation	20min stimulation
Lymphocyte-gated cells	79μM 49 - 126μM	246μM 168 - 359μM
Monocyte-gated cells	62μM 45 - 83μM	210μM 163 - 272μM

**Fig 4.12. Comparison of BzATP-stimulation of AV binding in PBMCs stimulated for 5 or 20 minutes**

Total AV-positive binding was measured by flow cytometry after 5 (n=3) or 20min (n=4) stimulation with BzATP in RPMI 1640. Cells staining positive for AV were expressed as a percent of the lymphocyte or monocyte cell population gated by FS/SS; results show the mean ± sem of separate experiments.

potent than ATP in monocyte-gated cells with  $EC_{50}$  values of  $83\mu\text{M}$  and  $2.1\text{mM}$  respectively. This reduced to sixteen times in lymphocyte-gated cells ( $EC_{50}$  values of  $131\mu\text{M}$  for BzATP and  $2.1\text{mM}$  for ATP).

ATP acted as a partial agonist in lymphocyte-gated cells producing only 80% of the BzATP response, but in monocyte-gated cells ATP produced a greater maximum response than BzATP. AV binding in BzATP-stimulated monocyte-gated LPMCs was unusual in that it was very low (maximum response only 47% of the gated population) and it also occurred over a very narrow concentration range typical of that seen with ATP in other cell types. This was reflected in the value for the Hill slope which was 5.6 for BzATP-stimulated monocyte-gated cells, but only 2.4 for lymphocyte-gated cells. Normal tissue macrophages are long-lived cells that provide an efficient scavenging host defence function without promoting mucosal inflammation. PS externalisation is a marker for macrophage phagocytosis, but for them to remain functioning within the tissue it is important that macrophages do not express PS readily on their external membrane surface and thus become a target of their own phagocytic process. Normal tissue macrophages may therefore be resistant to  $P2X_7$ -stimulated PS flip to enable them to live longer. This could be the reason for their low level of AV binding on stimulation with BzATP, although this is contradicted somewhat by the response to ATP.

#### **4.3.3.4. Summary of $P2X_7$ -stimulated AV binding**

All of the cell types displayed a concentration-dependent increase in total AV-binding, and for all of them BzATP was a more potent agonist than ATP. This shows that increased AV-binding, and hence PS-flip, is the result of  $P2X_7$  receptor-stimulation.

Very little work has been published on the kinetics of AV binding and this study is the first reported for primary human colonic LPMCs. Most of the published studies have used mouse cell lines. Murine macrophages have demonstrated increased AV binding with both  $100\mu\text{M}$  BzATP (Wilson, HL *et al.*, 2004) and  $5\text{mM}$  ATP (Pelegri, P *et al.*, 2004). Similarly, mouse T cells stimulated with  $150\mu\text{M}$  BzATP exhibited increased AV-binding in up to 35-

40% of the cells (Elliott, JI *et al.*, 2005), and a similar response was also shown by BALB/c thymocytes stimulated with 1mM ATP (Courageot, MP *et al.*, 2004; Le Stunff, H *et al.*, 2004).

Apoptotic cell death is generally considered to be a non-inflammatory process (Savill, J *et al.*, 2002). Susceptibility to apoptosis has been shown to be decreased in cells from inflamed tissue (Boirivant, M *et al.*, 1999), and several treatments for IBD act by promoting apoptotic cell death. The activity of the P2X<sub>7</sub> receptor is anomalous in that stimulation releases IL-1 $\beta$  which is pro-inflammatory, but also induces PS flip, a characteristic of apoptosis which is non-inflammatory. MacKenzie, AB *et al.*, (2005) has suggested that PS-flip associated with brief stimulation of the P2X<sub>7</sub> receptor is not part of the apoptotic process and has called it 'pseudoapoptosis'. They have proposed that P2X<sub>7</sub> couples to two distinct mechanisms.

Firstly, brief stimulation of P2X<sub>7</sub> receptors leads to influx of extracellular Ca<sup>2+</sup> into the cell resulting in PS-flip and loss of membrane asymmetry. This directly triggers actin filament disruption and zeiotic membrane blebbing. Increased intracellular Ca<sup>2+</sup> also enters the mitochondria, leading to mitochondrial swelling but no cytochrome c release. This process is completely reversible when intracellular Ca<sup>2+</sup> levels fall.

Alternatively, prolonged P2X<sub>7</sub> stimulation for more than 20-30 minutes leads to the release of cytochrome c from the mitochondria, which initiates apoptosome formation, activation of caspase enzymes and subsequent apoptotic cell death. Apoptotic membrane blebbing occurs via a calcium-independent pathway involving the ROCK-1 (RhoA-associated kinase) signalling cascade and is not reversible.

MacKenzie, AB *et al.*, (2005) also proposed that the rapid reversible cytoskeletal rearrangements may be linked to other similar effects of P2X<sub>7</sub> stimulation such as cell proliferation and giant cell formation, which also require plasma membrane disruption.

In the data presented here, P2X<sub>7</sub>-stimulated IL-1 $\beta$  release generally occurred at lower agonist concentrations than AV binding. IL-1 $\beta$  release has also been shown to be maximal after short stimulation times (MacKenzie, A *et al.*, 2001). This suggests that IL-1 $\beta$  release is associated with reversible PS-flip

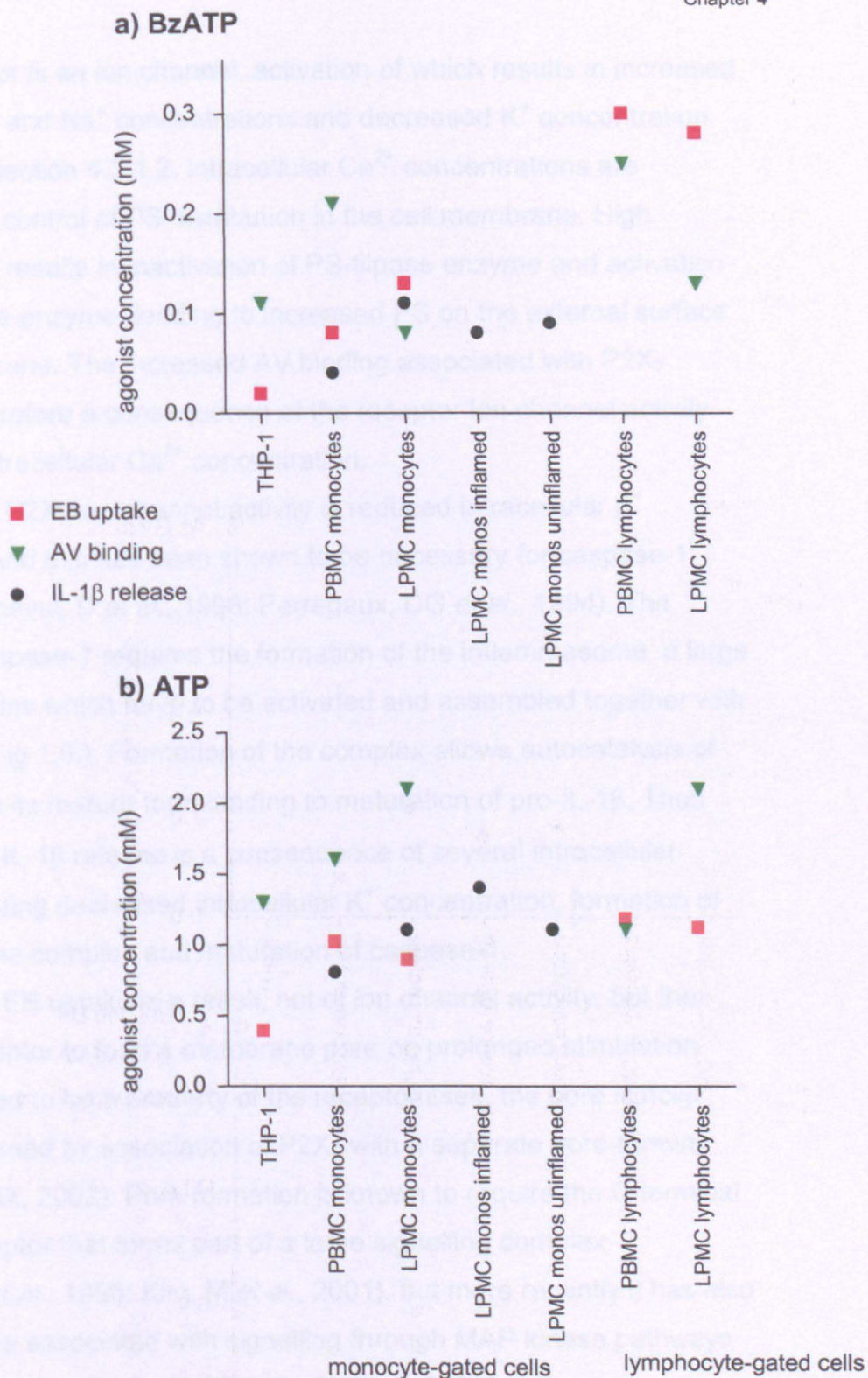
rather than irreversible apoptosis which requires 30 minutes stimulation of the P2X<sub>7</sub> receptor (MacKenzie, AB *et al.*, 2005). This implies that IL-1 $\beta$  release is a primary response to P2X<sub>7</sub> receptor stimulation whereas apoptosis is a secondary effect of prolonged stimulation. P2X<sub>7</sub> antagonism could therefore provide a useful treatment for IBD by reducing levels of IL-1 $\beta$  release. IL-1 $\beta$  has been shown to reduce monocyte apoptosis, so inhibition of IL-1 $\beta$  release might also encourage apoptotic cell death, further reducing the numbers of inflammatory cells (Mangan, DF *et al.*, 1991).

#### 4.4. THE FUNCTIONAL CHARACTERISATION OF THE P2X<sub>7</sub> RECEPTOR

The results presented in chapters 3 and 4 have shown that human colonic LPMCs express functional P2X<sub>7</sub> receptors in common with PBMCs and the monocyte cell line, THP-1 cells. This has been demonstrated by increases in pore formation, IL-1 $\beta$  release and PS flip on stimulation with the P2X<sub>7</sub> receptor agonists, BzATP and ATP. A pictorial comparison of the EC<sub>50</sub> values obtained for EB uptake, IL-1 $\beta$  release and AV binding is shown in Fig 4.13. Although the values for each agonist were all of the same order, it appears that those for AV binding were somewhat higher than those for IL-1 $\beta$  release and EB uptake, which were very similar. This was particularly true for ATP stimulation of monocyte cells.

EC<sub>50</sub> values, while being a measure of the half-maximal response, are not a measure of receptor occupancy, and in many tissues a maximal response can occur when an agonist occupies less than a tenth of available receptors (Jenkinson, DH, 2003). In many cases the response seen in the tissue is not a direct effect of receptor occupation but involves activation of a chain of signalling molecules. Each step in the chain of events can produce many more active molecules leading to amplification of the response. Therefore, a response involving many steps is likely to require less initial activation (and hence have a smaller EC<sub>50</sub> value) than one involving few steps.

The three responses of P2X<sub>7</sub> receptor binding studied here, namely IL-1 $\beta$  release, AV binding and EB uptake, are produced by different consequences of P2X<sub>7</sub> activation.



**Fig 4.13. A pictorial comparison of  $EC_{50}$  values for EB uptake, AV binding and IL-1 $\beta$  release in mononuclear cells stimulated with BzATP or ATP**

$EC_{50}$  values for AV binding appear to be higher than those for IL-1 $\beta$  release and EB uptake, particularly for ATP stimulation of monocyte cells (b). The  $EC_{50}$  values for EB uptake are very similar to those for IL-1 $\beta$  release except for BzATP stimulation of lymphocyte cells where the values were high.



The P2X<sub>7</sub> receptor is an ion channel, activation of which results in increased intracellular Ca<sup>2+</sup> and Na<sup>+</sup> concentrations and decreased K<sup>+</sup> concentration. As described in section 4.3.1.2, intracellular Ca<sup>2+</sup> concentrations are important for the control of PS distribution in the cell membrane. High intracellular Ca<sup>2+</sup> results in inactivation of PS-flipase enzyme and activation of the scramblase enzyme, leading to increased PS on the external surface of the cell membrane. The increased AV binding associated with P2X<sub>7</sub> stimulation is therefore a consequence of the receptor ion channel activity and increased intracellular Ca<sup>2+</sup> concentration.

Another result of P2X<sub>7</sub> ion channel activity is reduced intracellular K<sup>+</sup> concentrations, and this has been shown to be necessary for caspase-1 maturation (Cheneval, D *et al.*, 1998; Perregaux, DG *et al.*, 1994). The maturation of caspase-1 requires the formation of the inflammasome, a large complex of proteins which have to be activated and assembled together with pro-caspase-1 (Fig 1.6.). Formation of the complex allows autocatalysis of pro-caspase-1 to its mature form leading to maturation of pro-IL-1 $\beta$ . Thus P2X<sub>7</sub>-stimulated IL-1 $\beta$  release is a consequence of several intracellular processes, including decreased intracellular K<sup>+</sup> concentration, formation of the inflammasome complex and maturation of caspase-1.

P2X<sub>7</sub>-stimulated EB uptake is a result, not of ion channel activity, but the ability of the receptor to form a membrane pore on prolonged stimulation. Originally believed to be a property of the receptor itself, the pore is now thought to be formed by association of P2X<sub>7</sub> with a separate pore-forming protein (North, RA, 2002). Pore formation is known to require the C-terminal chain of the receptor that forms part of a large signalling complex (Surprenant, A *et al.*, 1996; Kim, M *et al.*, 2001), but more recently it has also been shown to be associated with signalling through MAP kinase pathways (Faria, RX *et al.*, 2005; Donnelly-Roberts, D *et al.*, 2004).

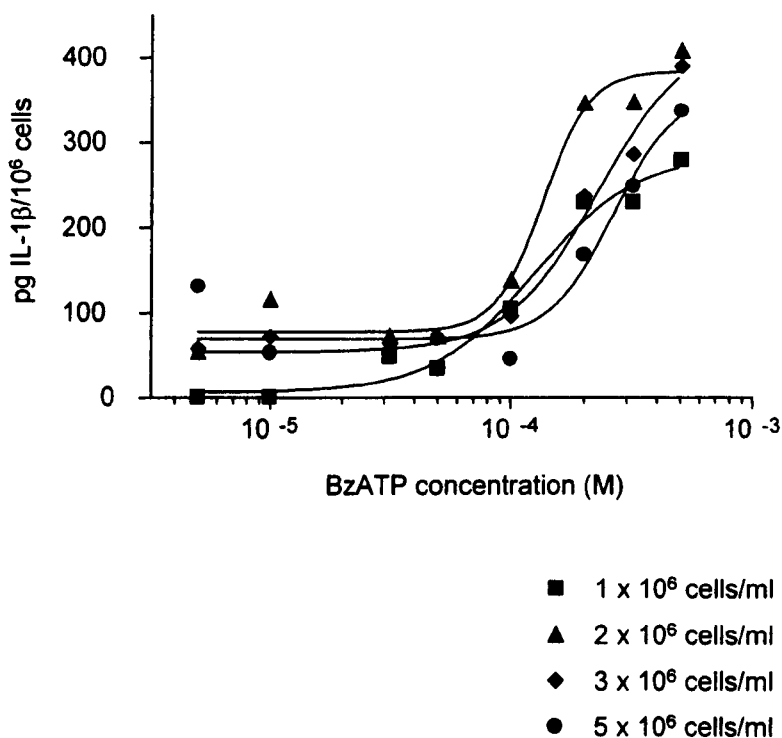
Since IL-1 $\beta$  release and EB uptake are associated with complex signalling pathways involving multiple steps, it is not surprising that the measured EC<sub>50</sub> values for these processes were generally lower than those for AV binding. EC<sub>50</sub> values, although a useful measure, are not an absolute value and only apply to the experimental conditions in which they are measured. In order to

enable comparison between the results from each set of experiments, the incubation conditions were kept as similar as possible for all three, the main difference being that IL-1 $\beta$  release was measured using  $1 \times 10^6$  cells/ml rather than  $2 \times 10^6$  cells/ml. This could potentially affect the EC<sub>50</sub> value since if more cells were present, then more agonist might be required to stimulate them. To check this, IL-1 $\beta$  release was measured from BzATP-stimulated LPMCs, using different concentrations of cells. The results (Fig 4.14.) showed that doubling of the cell number from  $1 \times 10^6$  cells/ml to  $2 \times 10^6$  cells/ml had a substantial effect on the total amount of IL-1 $\beta$  released, but very little effect on the EC<sub>50</sub> value. In support of this, the EB uptake experiments were carried out using cells at a concentration of  $2 \times 10^6$  cells/ml, the same as for AV binding, yet the resulting EC<sub>50</sub> values were very similar to those for IL-1 $\beta$  release.

The only other difference between the experimental conditions was that IL-1 $\beta$  release was carried out in the presence of 0.1% v/v FCS. Binding to FCS has been shown to reduce agonist potency (Michel, AD *et al.*, 2001), so that the presence of FCS in the experimental medium might be expected to increase the value of the EC<sub>50</sub>. In the presence of 1% v/v FCS, ATP concentration was only decreased by approximately 10% over 20 minutes (Michel, AD *et al.*, 2001). It is unlikely therefore that a concentration of FCS as low as 0.1% had any significant effect on agonist concentration.

The EC<sub>50</sub> data for the lymphocytes did not show such clear differences as that for the monocytes and may reflect different levels of receptor number or activity of the different pathways in the different cell types.

It is becoming apparent that the length of stimulation time is critical to the effects of P2X<sub>7</sub> stimulation, that P2X<sub>7</sub>-stimulated PS-flip is not necessarily a mark of apoptosis and that apoptotic cell death requires stimulation for 30 minutes or more (MacKenzie, AB *et al.*, 2005). The effect of stimulation time on IL-1 $\beta$  release and AV binding was therefore examined.



Cell number	EC <sub>50</sub> (BzATP μM)
1 x 10 <sup>6</sup>	125
2 x 10 <sup>6</sup>	135
3 x 10 <sup>6</sup>	226
5 x 10 <sup>6</sup>	262

**Fig 4.14. Effect of cell number on IL-1 $\beta$  release from LPMCs isolated from normal tissue**

LPMCs were resuspended at the concentrations shown and incubated with LPS for 3h prior to stimulation for 20min with BzATP in RPMI 1640 + 0.1% FCS. The EC<sub>50</sub> values obtained are shown in the table. Results are from a single experiment.

## CHAPTER 5. P2X<sub>7</sub> RECEPTOR STIMULATION: TIME COURSE OF RESPONSE

### 5.1. INTRODUCTION

Early studies of IL-1 $\beta$  release suggested that it resulted from processes that caused cell damage, and was therefore thought to be associated with cell death (Gery, I *et al.*, 1981; Hogquist, KA *et al.*, 1991b). In addition, Hogquist, KA *et al.*, (1991a) showed that induction of necrotic cell death was associated with the release of pro-IL-1 $\beta$ , but that mature IL-1 $\beta$  was released from cells undergoing apoptosis. Later studies however, demonstrated that the release of IL-1 $\beta$  was mediated by ATP (Griffiths, RJ *et al.*, 1995; Perregaux, DG *et al.*, 1994).

The cytotoxic ability of ATP has been known for some time (Arav, R & Friedberg, I, 1985; Mirabelli, F *et al.*, 1986; Kitagawa, T *et al.*, 1988). Many studies have shown that incubation for longer than 20 minutes in the presence of large (1-5mM) concentrations of extracellular ATP caused cell death; examples of these studies are listed in Table 5.1. In most of these studies the cells exhibited apoptotic changes, but ATP stimulation can also result in necrotic cell death (Zanovello, P *et al.*, 1990).

The 'cytotoxic ATP receptor' was subsequently identified as the P2X<sub>7</sub> receptor (Surprenant, A *et al.*, 1996; Chiozzi, P *et al.*, 1996). P2X<sub>7</sub> is a bifunctional receptor that behaves as a cation-selective channel or non-selective pore depending on the level of its activation. All cells sensitive to ATP-mediated cell death express P2X<sub>7</sub>, and the degree of sensitivity generally correlates with the level of expression (Gu, BJ *et al.*, 2000). The best evidence that P2X<sub>7</sub> is the main cytotoxic receptor for ATP has come from studies using cells selected for high or low expression of P2X<sub>7</sub> (Chiozzi, P *et al.*, 1996), or using cells isolated from P2X<sub>7</sub> knock-out mice (Brough, D *et al.*, 2002; Le Stunff, H *et al.*, 2004). Chiozzi, P *et al.*, (1996) selected macrophage clones that hyper-expressed the P2X<sub>7</sub> receptor and showed that they underwent a high rate of spontaneous cell death during culture. This cell death was greatly reduced by addition of the P2X<sub>7</sub> antagonist, oxidised ATP (oATP). Others have shown that microglia (Brough, D *et al.*,

Reference	cell type	agonist	stimulation time
Ferrari, D <i>et al.</i> , (2000)	human dendritic cells	5mM ATP	2 hours
Nihei, OK <i>et al.</i> , (2000a)	mouse dendritic cells	5mM ATP	30 minutes
Coutinho-Silva, R <i>et al.</i> , (1999)	D2SC/1 mouse dendritic cell line	5mM ATP	30 minutes
Blanchard, DK <i>et al.</i> , (1991)	human PBMC-derived macrophages	1.25mM ATP	6 hours
Le Feuvre, RA <i>et al.</i> , (2002)	mouse peritoneal macrophages	1 or 5mM ATP	30 minutes
Raymond, MN & Le Stunff, H, (2006)	RAW 264.7 mouse macrophages	3mM ATP	2 hours
Chiozzi, P <i>et al.</i> , (1997)	J774 mouse macrophages	3mM ATP	6 hours
Ferrari, D <i>et al.</i> , (1997a)	N9 & N13 microglial cell lines	3mM ATP	30 minutes
Brough, D <i>et al.</i> , (2002)	mouse microglia	1 or 5mM ATP	3 hours
Zheng, LM <i>et al.</i> , (1991)	rat thymocytes	1mM ATP	90 minutes
Sugiyama, T <i>et al.</i> , (2005)	rat retinal microvessels	3mM ATP	24 hours
Morelli, A <i>et al.</i> , (2003)	HEK293 cells expressing hP2X <sub>7</sub>	3mM ATP	20 minutes

**Table 5.1. Examples of published data for ATP-stimulated cell death**

2002) and thymocytes (Le Stunff, H *et al.*, 2004) isolated from P2X<sub>7</sub> knock-out mice did not release lactate dehydrogenase (LDH) in response to ATP, compared to cells from wild-type mice.

Sensitivity to ATP-mediated cell death varies between different cell types.

Mouse YAC-1 lymphoma cells are rapidly lysed by 0.2-0.5mM ATP (Zanovello, P *et al.*, 1990), whereas macrophage and microglial cells require stimulation with at least 1mM ATP for 30 minutes or more (Blanchard, DK *et al.*, 1991; Ferrari, D *et al.*, 1999). In contrast some cells, such as cytotoxic T lymphocytes or lymphokine-activated killer cells, are resistant to ATP-mediated death (Di Virgilio, F *et al.*, 1989).

The kinetics of P2X<sub>7</sub> receptor stimulation in isolated LPMCs has never been studied. Hickman, SE *et al.*, (1994) reported that the P2X<sub>7</sub> receptor is upregulated during development from monocyte to macrophage. They showed that human monocytes matured in culture with 30% v/v human serum demonstrated an increase in ATP-stimulated pore formation from 10% to 45% of the cell population after 7 days. LPMC macrophages might therefore be expected to be more susceptible to ATP-mediated cell death than PBMC monocytes. Similarly Gu, BJ *et al.*, (2000) showed that monocytes have a four- to five-fold greater expression of P2X<sub>7</sub> than lymphocytes suggesting that they should be more susceptible to ATP-mediated cell death than the lymphocyte population.

The kinetics of P2X<sub>7</sub> stimulation of LPMCs with ATP, and the more potent agonist BzATP, were investigated and compared to those of PBMCs and THP-1 cells. AV-FITC was used as a marker of PS-flip (and hence apoptosis) and PI as a marker of cell death. The kinetics of IL-1 $\beta$  release were also studied and compared to those for AV/PI binding.

## **5.2. TIME COURSE OF P2X<sub>7</sub>-STIMULATED AV-BINDING**

LPMCs isolated from normal tissue were used for these experiments. They would be expected to contain principally mature tissue macrophages with few peripheral blood monocytes, which are usually present in higher numbers in inflamed tissue. The effect of stimulation time was investigated at both maximal and half-maximal concentrations for the two agonists taken

from the concentration-response curves. At the start of these experiments, the aim was to use the same concentrations of the two agonists as far as possible for all three cell types. The EC<sub>50</sub> values were used as a guide for the half-maximum concentrations, and for all of the experiments 100µM BzATP and 1mM ATP were used. These were a compromise between the EC<sub>50</sub> values determined for IL-1β release (Table 4.1.) and those for AV binding (Table 4.2.). Maximum concentrations were chosen visually from the concentration-response curves, being the concentration at which the curve reached a plateau. For most of the cells the maximum ATP concentration used was 5mM, this was reduced to 3mM for IL-1β release in LPMCs. The maximum BzATP concentration used was 1mM with THP-1 cells but this was reduced to 300µM or 500µM for the other cell types.

The PBMC/LPMC lymphocyte and monocyte cell populations were gated using CD3 and CD14 or CD33 markers respectively as shown in Fig 2.2.a & b. For each gated cell population, values for AV+PI- and AV+PI+ binding were obtained as described in section 4.3.3 (Fig 4.10.). These were plotted separately to look at the effect of stimulation time on apoptosis and cell death.

### 5.2.1. Method

Cells were resuspended in RPMI 1640 at a concentration of  $2 \times 10^6$  cells/ml and incubated with BzATP or ATP, for 0, 1, 2, 3, 5, 10, 20, 30, 45 or 60min at 37°C in a total incubation volume of 100µl. Unstimulated controls were also prepared for each time point. Incubation was stopped by adding PBS (400µl), the cells were placed on ice and then centrifuged at 350 x g for 5min. THP-1 cells were resuspended in 250µl of cold binding buffer containing AV-FITC and PI, kept on ice and analysed by flow cytometry. For the PBMCs and LPMCs, cells were resuspended in 40µl of cold binding buffer containing CD3 and CD14 or CD33 and incubated for 30min on ice in the dark. Cold binding buffer (200µl) containing AV-FITC and PI was then added, and the samples analysed by flow cytometry.



## 5.2.2. Results and Discussion

### 5.2.2.1. Time course of AV binding in THP-1 cells

With 1mM BzATP (Fig 5.1.a), AV+PI- binding rose rapidly from 9% to 61% of the total cell population after stimulation for 1 minute, and then increased gradually to a maximum of 70% at 10 minutes. It then steadily decreased to 29% at 60 minutes, with an accompanying increase in AV+PI+ binding to 51% of the total cell population. Unstimulated control levels of both AV+PI- and AV+PI+ staining remained unchanged throughout, at approximately 5% and 9% respectively.

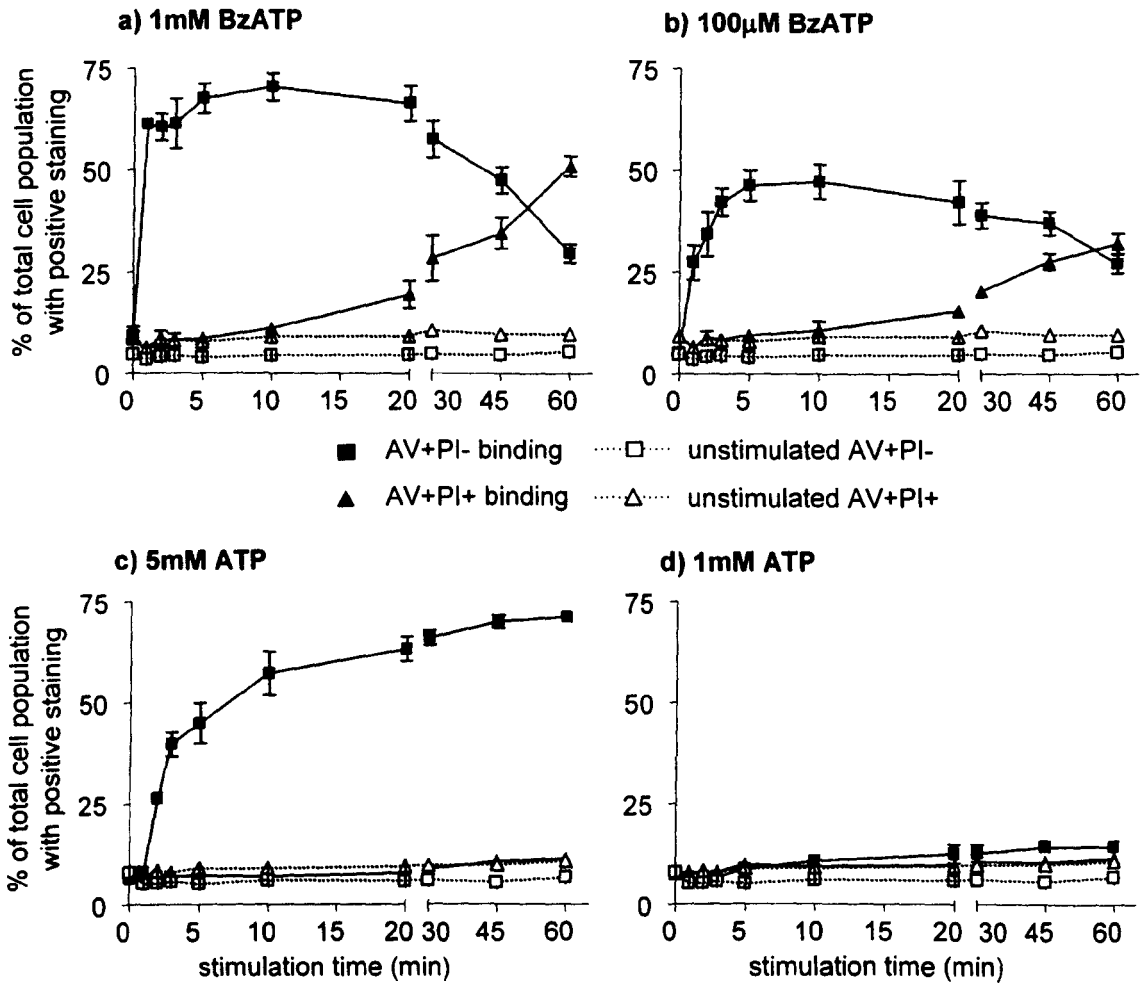
Similarly with 100 $\mu$ M BzATP (Fig 5.1.b), AV+PI- binding increased rapidly for the first 5 minutes, reached a peak at 10 minutes (47%), and then decreased to 27% at 60 minutes, with an associated increase in AV+PI+ binding (32%). The rate of AV/PI binding per minute was calculated by subtracting the value for each time point from the value for the following time point and dividing by the total time passed. For both concentrations of BzATP the rate of AV binding was highest at 0-1 minute (Fig 5.1.e). The rate for 1mM BzATP was approximately twice that for 100 $\mu$ M BzATP which is compatible with using maximal and half-maximal concentrations of agonist.

For both concentrations of BzATP, the increase in AV+PI+ staining matched the decrease in AV+PI- staining suggesting that the increased AV+PI+ cell population resulted directly from cells from the AV+PI- population. This was supported by the fact that the unstained AV-PI- population showed very little change after 10 minutes (Fig 5.2.a).

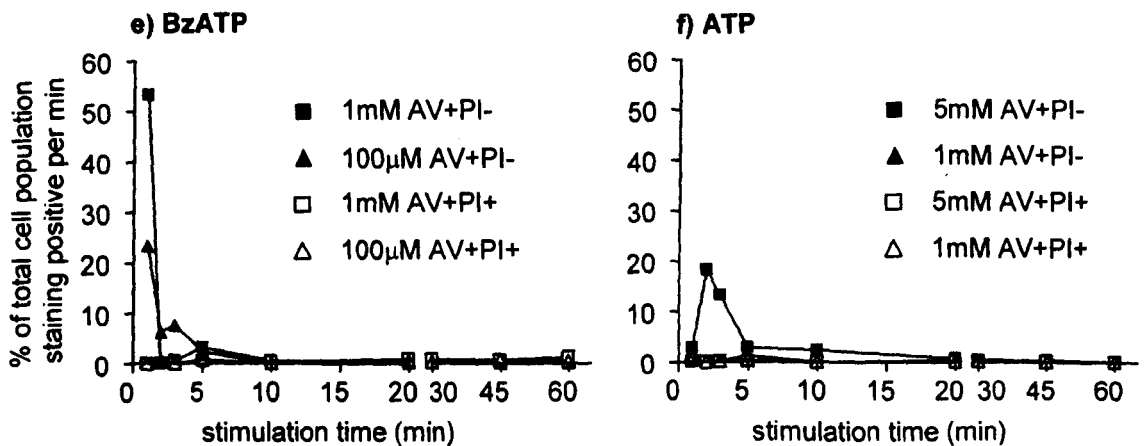
With 5mM ATP (Fig 5.1.c), AV+PI- binding increased rapidly with time over the first 5 minutes, and then continued to increase steadily to a maximum of 70% of the total cell population by 45 minutes. The highest AV binding rate occurred at 1-2 minutes (18% per min, Fig 5.1.f), but was less than half that for 1mM BzATP stimulation (53% per min). There was no increase in AV+PI+ binding, which was at or below the level of the controls.

With 1mM ATP, there was very little stimulation of AV binding, only 14% of the total cell population responding after 45 minutes (Fig 5.1.d), but this may have been due to inaccurate measurement of the EC<sub>50</sub>. The increase in the concentration-response curves for AV binding with ATP occurred over a very

**i. Percent of AV/PI binding**



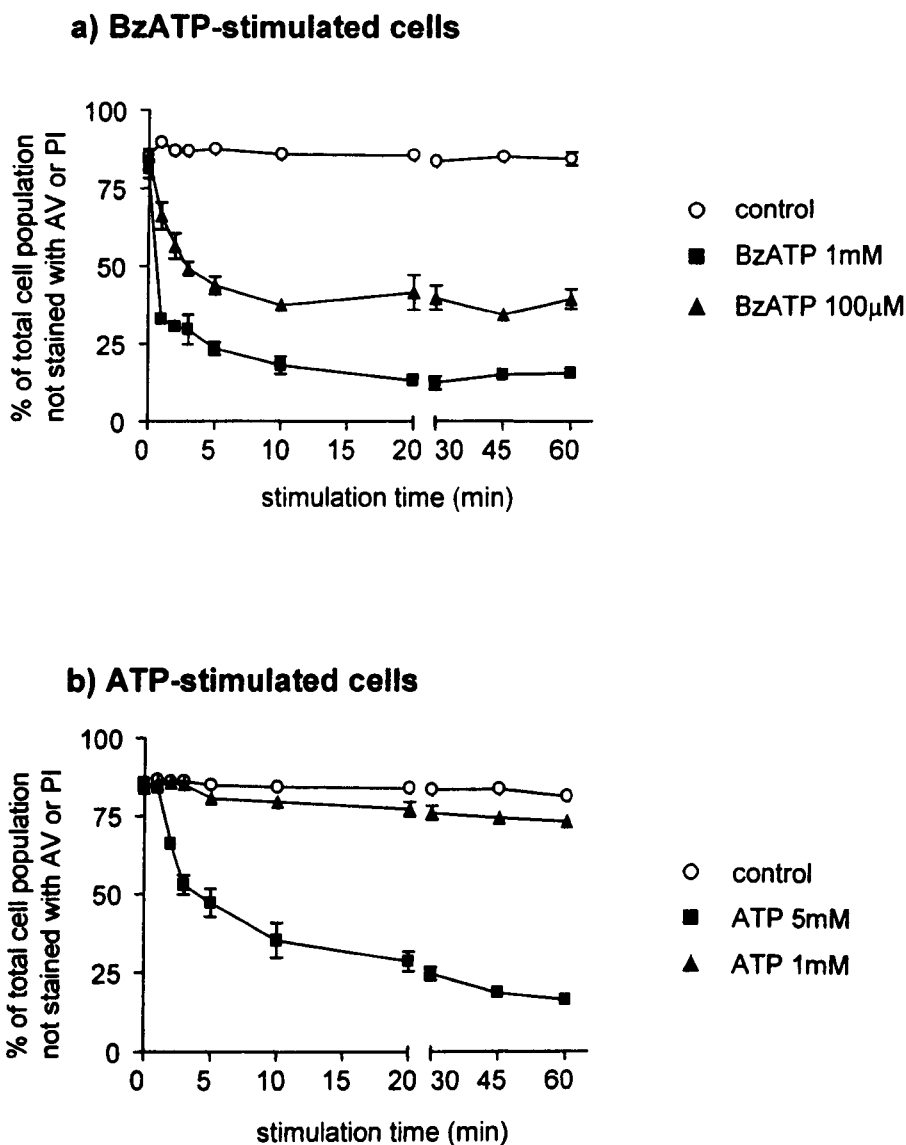
**ii. The rate of AV/PI binding**



**Fig 5.1. Time course of AV/PI binding in THP-1 cells**

i. AV/PI binding was measured by flow cytometry after 0-60min stimulation with BzATP or ATP in RPMI 1640 at maximal (a & c) or half-maximal (b & d) concentrations, taken from the concentration-response curves. Cells staining positive were expressed as a percent of the total cell population, and show the mean  $\pm$  sem of four separate experiments. Values for the unstimulated control cells are also shown.

ii. Rate of binding was calculated by subtracting values for percent of total cell population binding AV/PI at each time point from the value for the following time point and dividing by the total time passed.



**Fig 5.2. The effect of stimulation time on the unstained population of THP-1 cells**

AV/PI binding was measured by flow cytometry after 0-60min stimulation with BzATP or ATP in RPMI 1640 at maximal or half-maximal concentrations, taken from the concentration-response curves. The graphs show the percent of the total cell population which remained unstained, and show the mean  $\pm$  sem of four separate experiments.

narrow concentration range (Fig 4.11.a). Only two concentration points were on the slope of the curve and these generally had wide error bars due to variability of responses. This resulted in a wide margin of error for the curve fit and hence the value of the  $EC_{50}$ .

In THP-1 cells, BzATP stimulation clearly produced a faster response, a higher rate of PS flip and more cell death than ATP. It may be that ATP requires stimulation times longer than 60min to cause cell death. This is supported by the fact that in many of the examples shown in Table 5.1., the cells were stimulated for several hours to bring about cell death.

#### **5.2.2.2. Time course of AV binding in PBMCs**

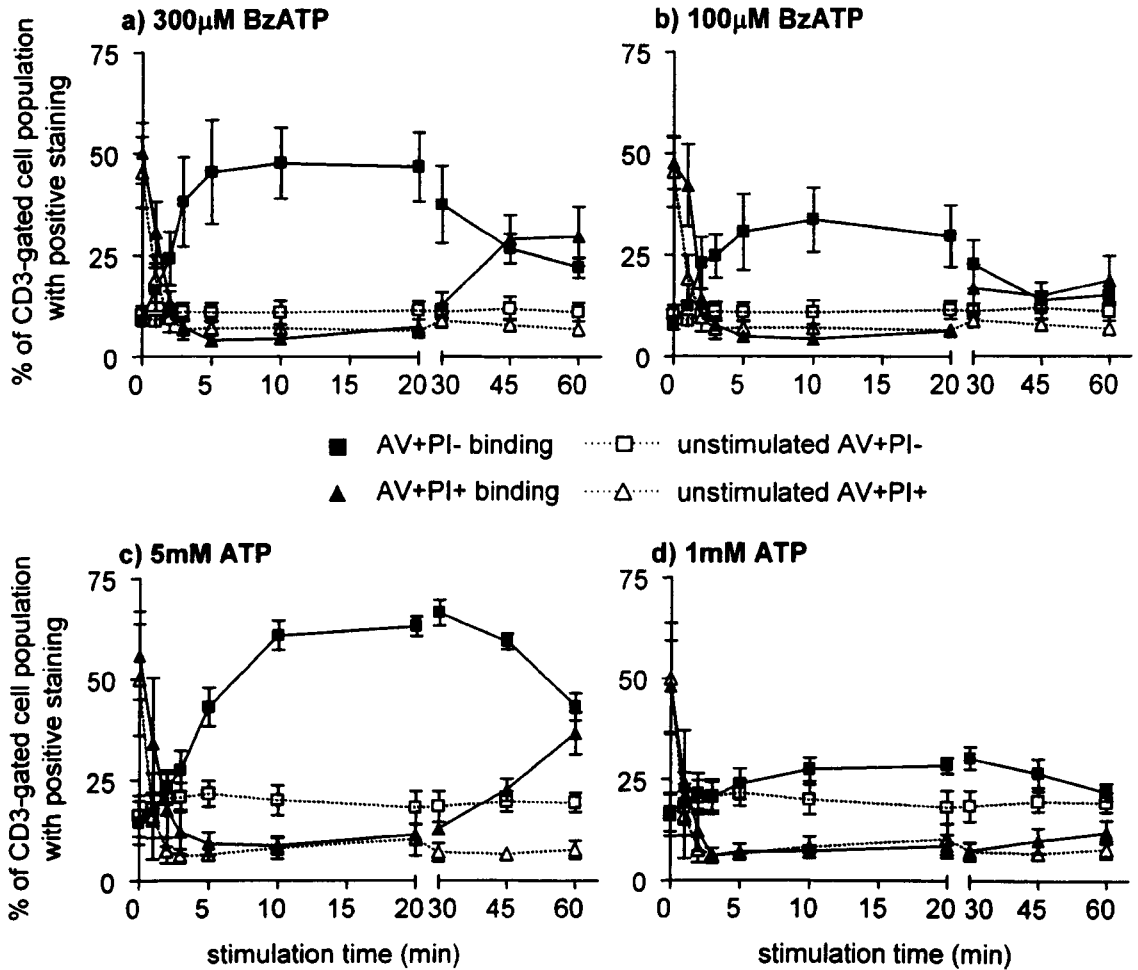
The maximal BzATP concentration used for the PBMC time course experiments was  $300\mu\text{M}$ . This was based on earlier results from the concentration-response curves in which the cells were stimulated for 5 minutes (Fig 4.12.). These curves produced lower BzATP concentrations for both the  $EC_{50}$  and the maximal response than those in which the cells were stimulated for 20 minutes. As a result fewer cells responded to stimulation, but the level of response was comparable to that seen in the concentration-response curves for this concentration of BzATP (Fig 4.11b & c)

##### ***i. Lymphocyte-gated cells***

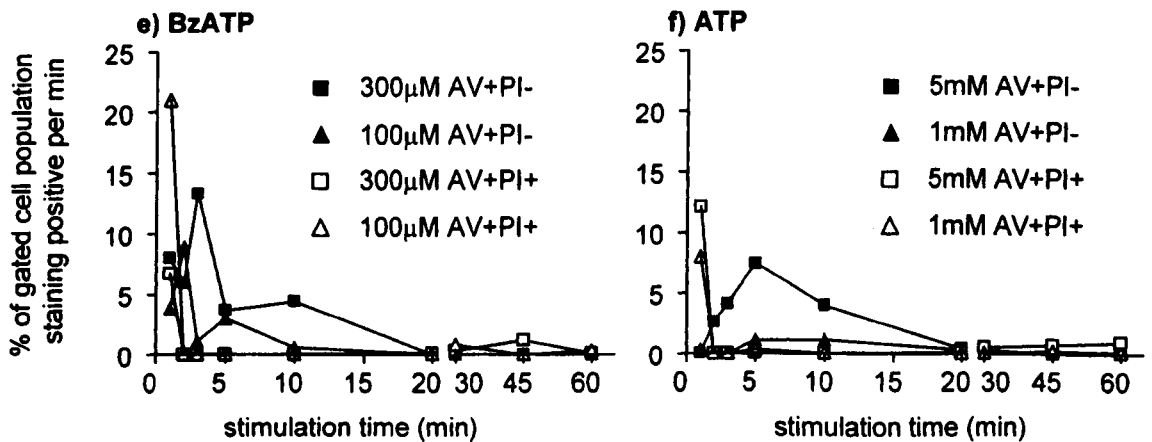
With  $300\mu\text{M}$  BzATP (Fig 5.3.a), AV+PI- binding increased rapidly from 9% to 46% of the lymphocyte-gated cell population over the first 5 minutes, peaked at 48% at 10 minutes and then decreased steadily to 22% at 60 minutes. The decrease was matched by an increase in AV+PI+ binding which was most marked between 30-45 minutes, rising to 30% at 60 minutes. With  $100\mu\text{M}$  BzATP (Fig 5.3.b), the pattern of AV/PI binding was very similar to that seen with  $300\mu\text{M}$  BzATP except that the percentage of cells staining was approximately half that seen at the higher concentration.

ATP-stimulation of the lymphocyte-gated cells at both concentrations (Fig 5.3.c & d) produced a similar pattern of AV/PI binding to that of BzATP, except that the subsequent decrease in AV+PI- staining did not occur until after 30 minutes stimulation, with a correspondingly slower increase in

## i. Percent of AV/PI binding



## ii. The rate of AV/PI binding



**Fig 5.3. Time course of AV/PI binding in lymphocyte-gated PBMCs**

i. AV/PI binding was measured by flow cytometry after 0-60min stimulation with BzATP or ATP in RPMI 1640 at maximal (a & c) or half-maximal (b & d) concentrations, taken from the concentration-response curves. Cells staining positive were expressed as a percent of the lymphocyte cell population gated by CD3-PE binding, and show the mean  $\pm$  sem of four separate experiments. Values for the unstimulated control cells are also shown.

ii. Rate of release was calculated by subtracting values for percent of gated cell population binding AV/PI at each time point from the value for the following time point and dividing by the total time passed.

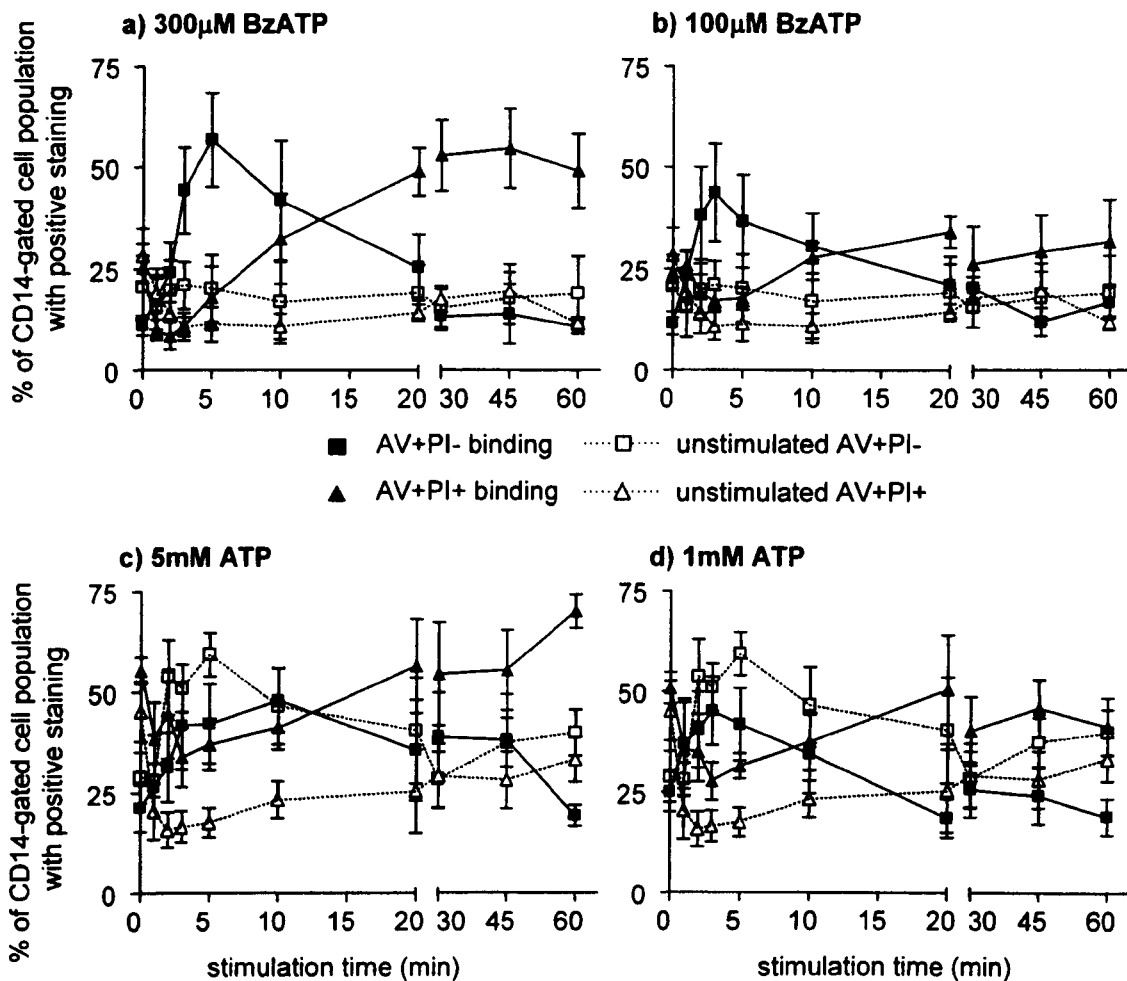
AV+PI+ staining. The maximum rate of binding with 5mM ATP (7% per min) was approximately half that seen with 300 $\mu$ M BzATP stimulation (13% per min, Fig 5.3.e & f) and occurred at 3-5min compared to 2-3min with BzATP. Unstimulated control cells demonstrated no change in AV/PI staining over the 60 minute period, remaining at approximately 11-19% AV+PI- staining and 7% AV+PI+ staining.

### **ii. Monocyte-gated cells**

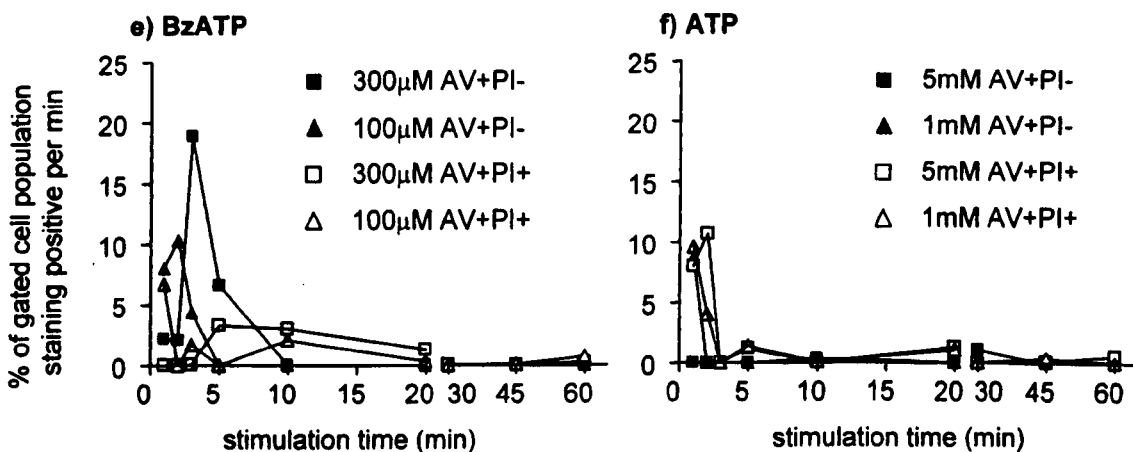
Like the lymphocyte-gated cells, PBMC monocyte-gated cells demonstrated rapid increases in AV+PI- staining following stimulation. However the increased staining also decreased rapidly, with an accompanying rise in AV+PI+ binding after short stimulation times (Fig 5.4.a-d). In cells stimulated with 300 $\mu$ M BzATP (Fig 5.4.a), AV+PI- binding rose from 11% to 57% of the gated cell population within 5 minutes, but then decreased to values below those of the unstimulated control by 30 minutes. This was accompanied by a rise in AV+PI+ binding to 45% after 20 minutes stimulation. Stimulation with 100 $\mu$ M BzATP gave similar binding patterns but with only half the number of cells staining (Fig 5.4.b). In PBMC monocytes, the maximum rate of binding for both agonists occurred over 2-5 minutes (Fig 5.4.e & f), but the maximum rate of response to BzATP was greater than that to ATP.

The incubations with BzATP and ATP were carried out at different times and the unstimulated controls demonstrated distinct staining patterns. In the experiments with BzATP, the level of control AV/PI staining during the 60 minute period remained at approximately 18% of the gated cell population for AV+PI- staining and 14% for AV+PI+ (Fig 5.4a & b, open symbols). In contrast the unstimulated controls from the set of experiments with ATP showed increased AV+PI- staining over the first 5 minutes up to 59% of the gated cell population, which then fell to the initial level of staining of approximately 30% (Fig 5.4c & d, open squares). The AV+PI+ staining (open triangles) increased steadily throughout the 60 minute incubation period from 16% to 33% of the gated cell population.

Although both sets of experiments were carried out following the same protocol, the different results suggest that some factor during the experiments using ATP caused the control cells to become apoptotic and



## ii. The rate of AV/PI binding

**Fig 5.4. Time course of AV/PI binding in monocyte-gated PBMCs**

i. AV/PI binding was measured by flow cytometry after 0-60min stimulation with BzATP or ATP in RPMI 1640 at maximal (a & c) or half-maximal (b & d) concentrations, taken from the concentration-response curves. Cells staining positive were expressed as a percent of the monocyte cell population gated by CD14-APC binding, and show the mean  $\pm$  sem of four separate experiments. Values for the unstimulated control cells are also shown.

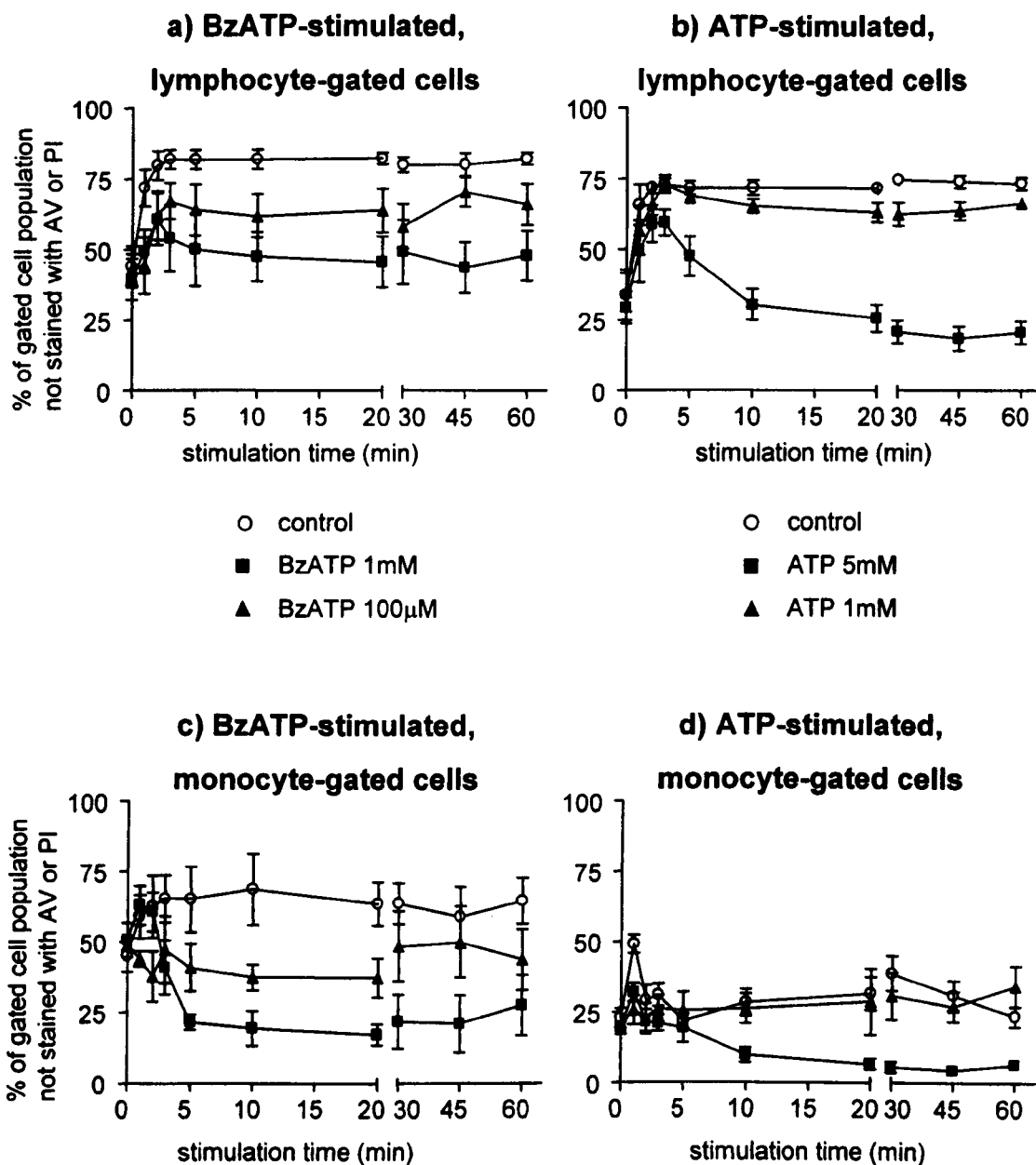
ii. Rate of release was calculated by subtracting values for percent of gated cell population binding AV/PI at each time point from the value for the following time point and dividing by the total time passed.

die. One possible reason might be contamination of the PBMC preparation with platelets. Platelets contain large amounts of ATP but are normally removed during the low speed centrifugation washing procedure. A change in the protocol, such as use of a different centrifuge, might cause some platelets to remain or even rupture. Any ATP released into the medium would then trigger responses in the unstimulated cells, the effect being masked in the stimulated cells by the large amounts of external ATP added to them. This could also explain the higher level of AV+PI- staining observed in the controls for the corresponding ATP-stimulated lymphocyte-gated cells (approximately 20%, compared to 11% in the BzATP-stimulated controls, Fig 5.3.c).

Another unusual feature of this set of experiments with PBMCs was that both the stimulated and unstimulated cells appeared to have a population of cells demonstrating high levels of PI staining at time 0 min, which decreased during the first 3 minutes of incubation. This decrease was mirrored by an increase in the unstained AV-PI- population over the same time period (Fig 5.5.). This phenomenon was only observed with the PBMC cell populations and therefore must have been caused either by the isolation procedure or by a unique feature of these cells. The apparent reversal of PI staining within the cell populations would not be possible if the cells were dead and suggests that the PI staining is due to another cause.

For the monocyte-gated cells, increases in AV+PI+ staining could be due to entry of the propidium ion through the P2X<sub>7</sub> pore. As described earlier (section 1.7.1.), the P2X<sub>7</sub> receptor has the ability to form a non-selective membrane pore that is permeable to small molecules. The monocyte pore is large, allowing permeation of molecules up to 900Da in size, and the propidium ion has a molecular weight of only 414Da. It is possible to surmise therefore that ATP released during the isolation procedure, maybe from dead cells or platelet contamination, could stimulate pore opening allowing entry of PI into the cells. Subsequent incubation and ATP removal by endonucleases would then allow the pore to close resulting in reduced staining with PI. Lymphocytes are reported to have a smaller pore than monocytes, approximately 300Da in size, so that any staining with PI should be an indication of cell death. However the size of the lymphocyte pores has not





**Fig 5.5. The effect of stimulation time on the unstained populations of lymphocyte- and monocyte-gated PBMCs**

AV/PI binding was measured by flow cytometry after 0-60min stimulation with BzATP or ATP in RPMI 1640 at maximal or half-maximal concentrations, taken from the concentration-response curves. The graphs show the percent of the respective gated cell populations which remained unstained, and show the mean  $\pm$  sem of four separate experiments.

been absolutely defined, and the propidium ion is only slightly larger than the estimated pore size, so it is possible that a similar effect may occur.

### **5.2.2.3. Time course of AV binding in LPMCs**

The maximal BzATP concentration used was 500 $\mu$ M for this set of experiments.

#### ***i. Lymphocyte-gated cells***

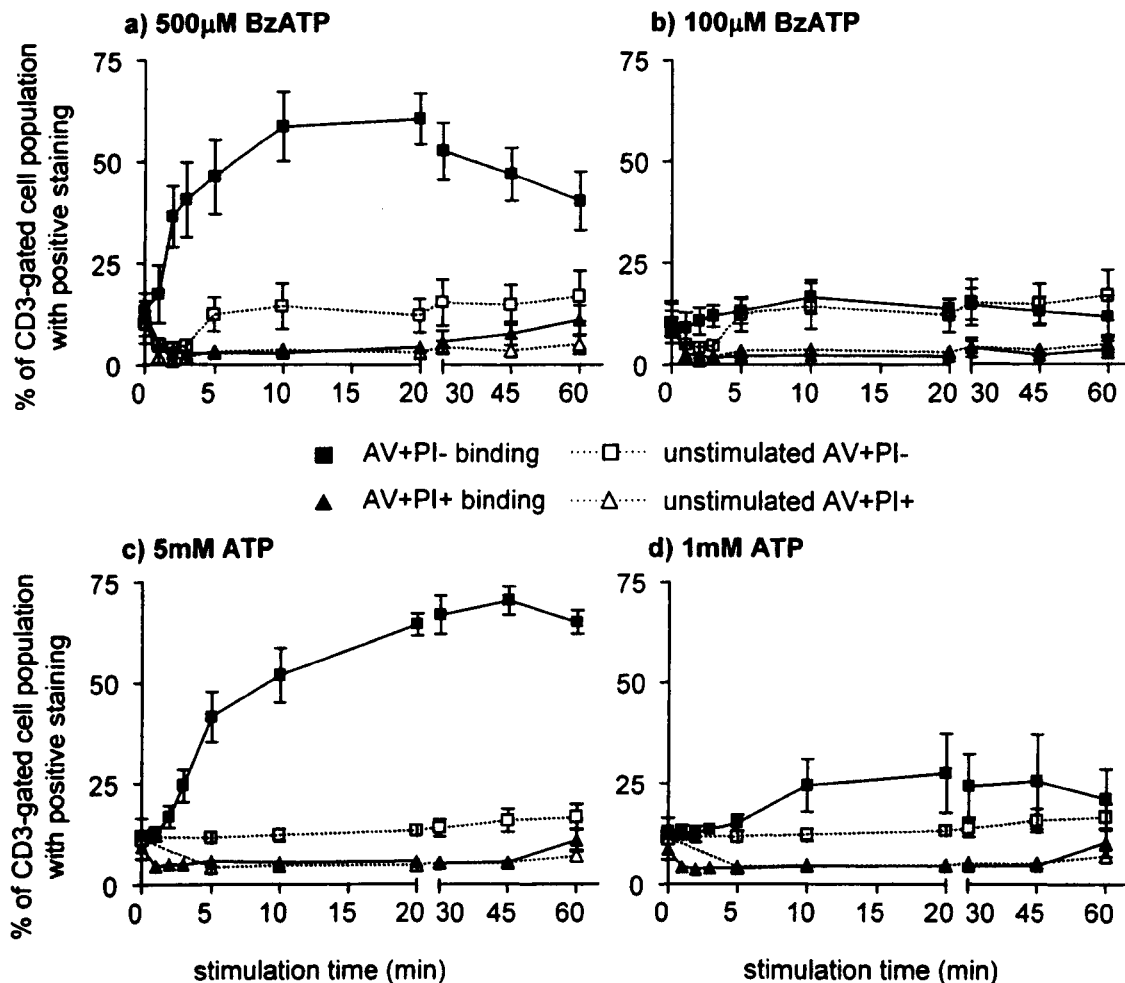
The pattern of AV/PI binding in lymphocyte-gated LPMCs stimulated with BzATP or ATP was similar to that seen in lymphocyte-gated PBMCs except that there was very little increase in AV+PI+ binding above control values (Fig 5.6.a-d). This suggests that LPMC lymphocytes are less susceptible to P2X<sub>7</sub>-stimulated cell death than PBMC lymphocytes. Maximum AV+PI- binding occurred at 10 minutes with BzATP stimulation and 20 minutes with ATP; the maximum levels reached (60-70%) were similar for both agonists. The maximum rate of binding with BzATP stimulation also occurred earlier (1-2min) than with ATP stimulation (3-5min) (Fig 5.6.e & f) and was twice that of ATP (20% per min for BzATP and 9% per min for ATP). There was little change in AV/PI staining in unstimulated control cells which remained at approximately 14% AV+PI- staining and 4% AV+PI+ staining.

#### ***ii. Monocyte-gated cells***

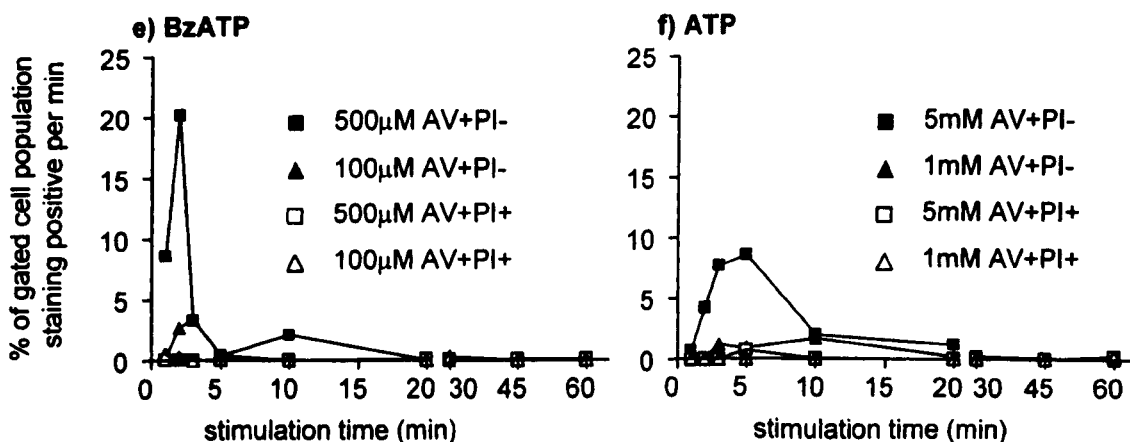
AV+PI- staining in LPMC monocyte-gated cells rose rapidly on stimulation with both BzATP and ATP, followed by a steady decrease and an associated increase in AV+PI+ staining (Fig 5.7.a-d). Both the maximum levels of AV binding and the maximum rates (Fig 5.7.e & f) were similar for both agonists but occurred slightly earlier with BzATP stimulation. As seen for PBMCs, the peak rates of reaction for each agonist with the monocyte-gated cells occurred earlier than with lymphocyte-gated cells.

It was noticeable that the pattern of AV+PI+ staining in unstimulated control cells matched that of the stimulated cells, demonstrating a rise from approximately 10% to 30% of the cell population over the incubation period. This implies that the LPMC monocytes spontaneously died during incubation and that stimulation with BzATP or ATP did not substantially increase cell death. However the observed decrease in the AV+PI- stained population of

## i. Percent of AV/PI binding



## ii. The rate of AV/PI binding

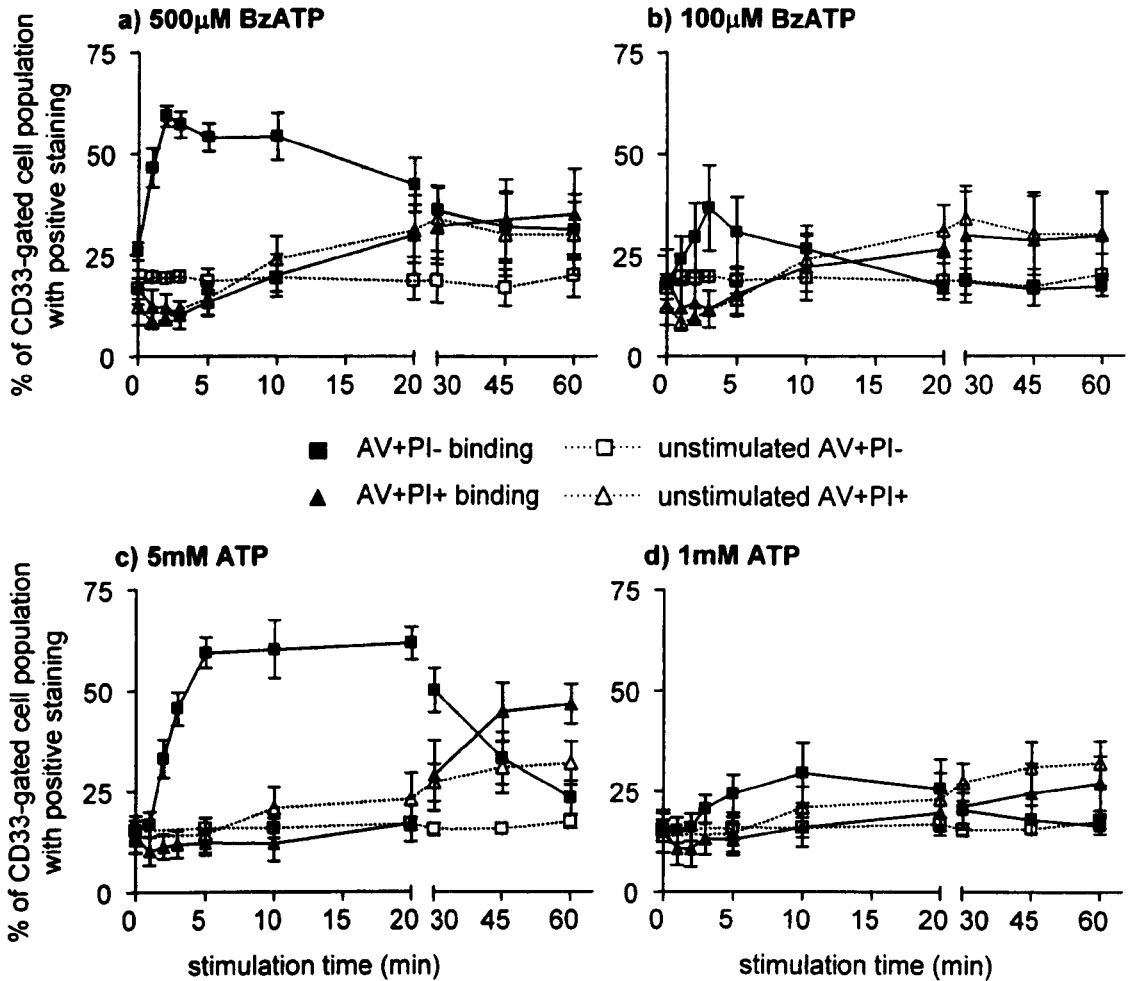


**Fig 5.6. Time course of AV/PI binding in lymphocyte-gated LPMCs**

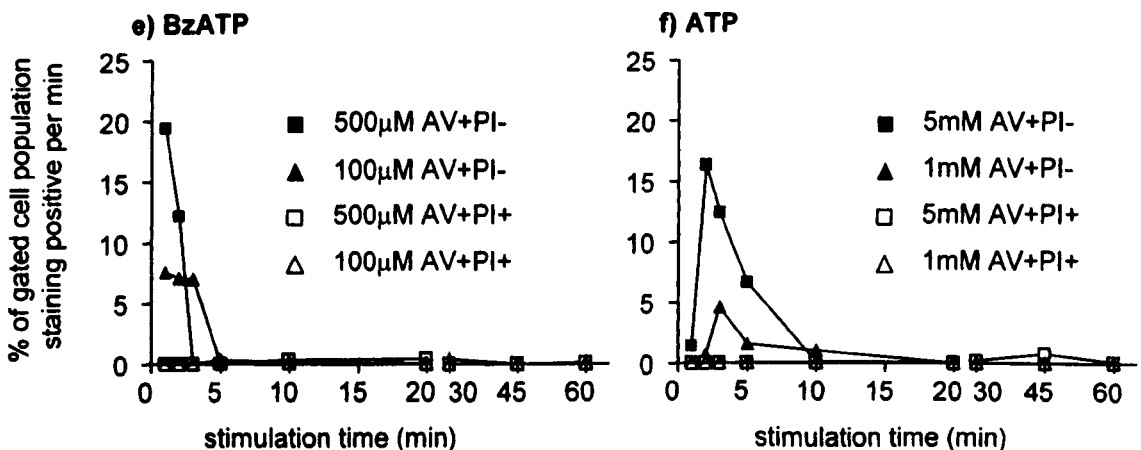
i. AV/PI binding was measured by flow cytometry after 0-60min stimulation with BzATP or ATP in RPMI 1640 at maximal (a & c) or half-maximal (b & d) concentrations, taken from the concentration-response curves. Cells staining positive were expressed as a percent of the lymphocyte cell population gated by CD3-PE binding, and show the mean  $\pm$  sem of four separate experiments. Values for the unstimulated control cells are also shown.

ii. Rate of release was calculated by subtracting values for percent of gated cell population binding AV/PI at each time point from the value for the following time point and dividing by the total time passed.

## i. Percent of AV/PI binding



## ii. The rate of AV/PI binding

**Fig 5.7. Time course of AV/PI binding in monocyte-gated LPMCs**

i. AV/PI binding was measured by flow cytometry after 0-60min stimulation with BzATP or ATP in RPMI 1640 at maximal (a & c) or half-maximal (b & d) concentrations, taken from the concentration-response curves. Cells staining positive were expressed as a percent of the monocyte cell population gated by CD33-APC binding, and show the mean  $\pm$  sem of four separate experiments. Values for the unstimulated control cells are also shown.

ii. Rate of release was calculated by subtracting values for percent of gated cell population binding AV/PI at each time point from the value for the following time point and dividing by the total time passed.

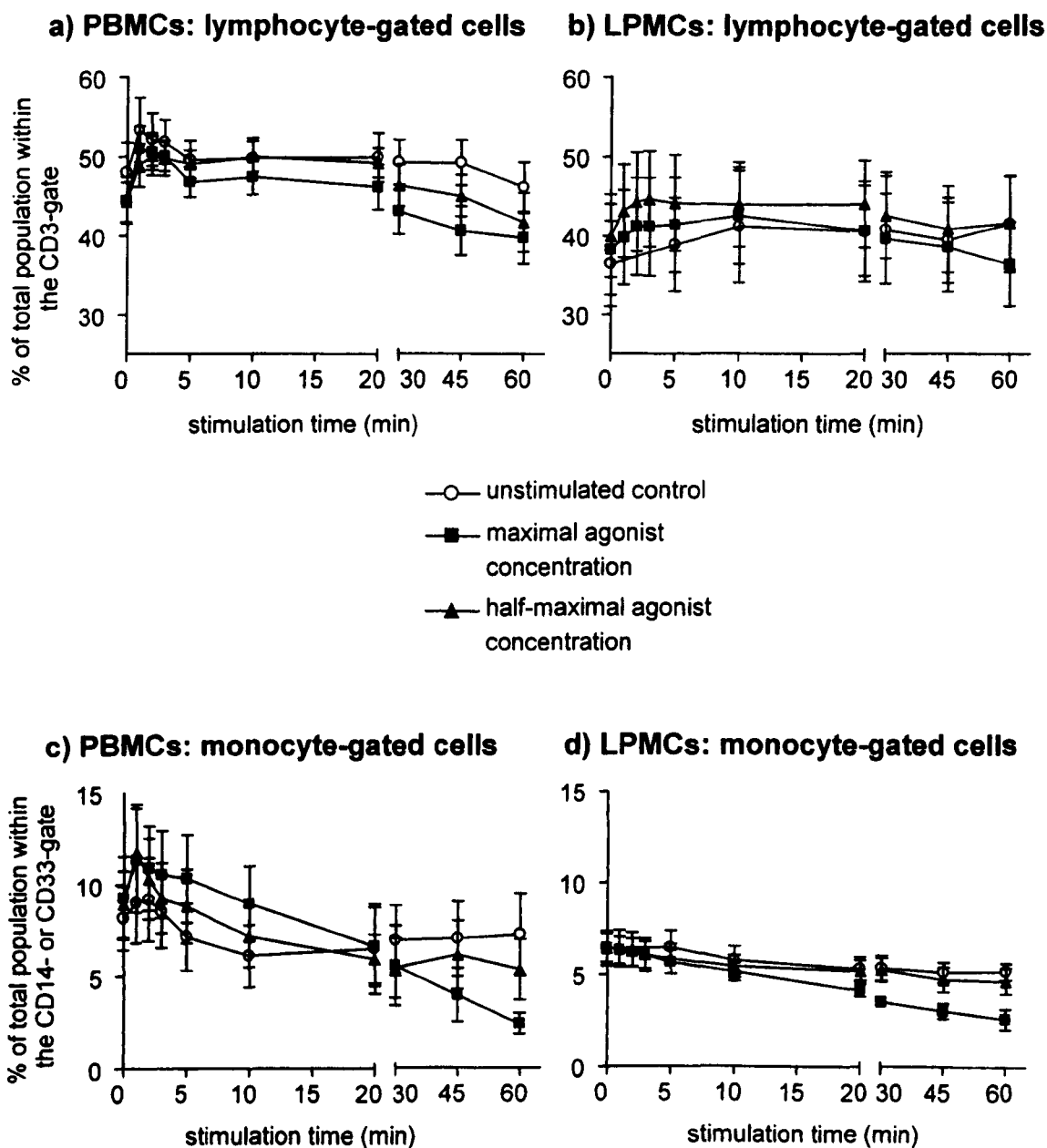
stimulated cells was likely to be a result of cells becoming positively stained for PI, and hence becoming part of the AV+PI+ population. This, together with the apparent spontaneous cell death seen in these cells, should have given rise to a bigger population of AV+PI+ cells. The fact that it didn't suggested that dead cells were being lost from the cell gate and hence not being counted.

This was confirmed by looking at the number of cells contained within the monocyte gate. Cell death is accompanied by shrinking and fragmentation resulting in non-inclusion within the cell gate. The number of cells contained within the gates was plotted for both PBMCs and LPMCs, and is shown in Fig 5.8. All of the gated cell populations showed a reduction in number during the incubation period, but the monocyte-gated cells were particularly affected. For the LPMC monocyte-gated cells, the number within the unstimulated control cell population fell from 6.5% to 5.2% of the total cell population, a drop of 20% over the 60 minute incubation period. However, for the LPMCs stimulated with maximal agonist concentration, the number contained within the monocyte gate fell to 2.8% of the total cell population, a decrease of 57%. This might therefore account for the apparent lack of increased AV+PI+ staining on stimulation with BzATP and ATP.

### 5.2.3. Summary of AV binding

The kinetics of AV binding, and hence PS externalisation on the cell membrane, appeared to be rapid in all the cell types following P2X<sub>7</sub> stimulation. One study has shown that PS was exposed on the surface of THP-1 or HEK cells within 1-2 seconds of exposure to BzATP (MacKenzie, A *et al.*, 2001). The results presented here did not measure such small time periods, but maximum AV+PI- binding (indicating PS flip) was achieved with BzATP stimulation over a period of 2-10 minutes with the greatest rate of binding per minute occurring between 0-3 minutes. For ATP the kinetics was slightly slower with maximum AV+PI- binding at 10-30 minutes and peak rate per minute at 1-5 minutes. In general, the maximum rate of BzATP-stimulated AV binding was twice that of ATP-stimulated binding.

For all the cell types the maximum level of AV+PI- binding was very similar for both agonists at their maximal concentrations, and was comparable to the



**Fig 5.8. Changes in the number of cells contained within the gates for the lymphocyte and monocyte populations of PBMCs and LPMCs**

The lymphocyte and monocyte populations were gated from the SS vs CD marker dotplots and the number of cells contained within each gate were expressed as a percent of the total cell population.

level of binding seen in the concentration response curves. As expected, 100 $\mu$ M BzATP produced a level of AV+PI- binding that was approximately half that produced by the maximal concentration of BzATP, but with ATP the response to 1mM was less than half that to 5mM. This was because of the difficulties of trying to standardise the agonist concentrations used for all parts of these experiments. The calculated EC<sub>50</sub> values for IL-1 $\beta$  release were generally lower than those for AV binding, and the half-maximal concentration selected for the ATP experiments was less than its EC<sub>50</sub> value for AV binding.

Stimulation with 1mM ATP has produced variable responses in other studies using mouse thymocytes. Both demonstrated maximal PS externalisation after approximately 15 minutes, but while the maximal response was 15% in one study (Le Stunff, H *et al.*, 2004), in the other it was 40% (Courageot, MP *et al.*, 2004), which is closer to the values seen with 5mM ATP in the results presented here.

Reducing the concentration of agonist did not affect the pattern of AV+PI- and AV+PI+ binding relative to each other. The overall effect of using half the maximal agonist concentration was to reduce the number of cells responding by approximately half. This was particularly clear for BzATP stimulation of PBMC monocytes (Fig 5.4.a & b).

The increase in AV binding occurred more rapidly in monocyte-gated cells than lymphocyte-gated cells, demonstrated by the peak rates of binding per minute which occurred at 0-2min for monocytes and 2-5min for lymphocytes. This was expected since monocytes have 4-5-fold greater expression of P2X<sub>7</sub> than lymphocytes (Gu, BJ *et al.*, 2000).

The findings of Hickman, SE *et al.*, (1994), who showed increased P2X<sub>7</sub> activity in macrophages compared to monocytes, suggested that macrophages should be more susceptible to cell death than monocytes. This appeared not to be the case since PBMC monocytes exhibited a more rapid increase in levels of AV+PI+ binding (indicating cell death) than LPMC monocytes. PBMCs were also stimulated with a lower maximal concentration of BzATP than the LPMCs yet demonstrated a greater response.

The innate susceptibility of the different populations to cell death can be looked at by considering the AV/PI staining patterns for the unstimulated control cells shown below.

<b>Cell type</b>	<b>AV+PI- staining (PS flip)</b>	<b>AV+PI+ staining (cell death)</b>
PBMC lymphocytes	approx 11-19%	approx 7%
LPMC lymphocytes	approx 14%	approx 4%
THP-1 cells	approx 5%	approx 9%
PBMC monocytes	approx 18-30%	approx 15% increasing to 33%
LPMC monocytes	approx 18%	approx 10% increasing to 30%

Lymphocytes exhibited a low level of spontaneous cell death (4-7% of cells) that remained relatively constant throughout the whole time course of the experiment. In contrast, monocytes not only displayed a greater level of basal AV+PI+ staining (9-15% of cells) than lymphocytes, but this also increased with time to 30% of the cell population. These levels were similar for both PBMC and LPMC monocyte gated cells.

Another indication of cell death is the decrease in the gated cell population, as was described in section 5.2.2.4.ii and Fig 5.8. The percent decreases in the respective cell populations are shown below:

	unstimulated control cells	maximally stimulated cells
PBMC lymphocyte-gated cells	12%	23%
LPMC lymphocyte-gated cells	5%	15%
PBMC monocyte-gated cells	35%	78%
LPMC monocyte-gated cells	20%	57%

It is clear that monocyte-gated cells were more susceptible to both spontaneous and stimulated cell death than lymphocyte-gated cells, and lost a much greater percentage of their respective cell populations. Isolated PBMCs also exhibited greater loss of cells than LPMCs for both cell types, suggesting that in fact they are more susceptible to cell death.

However, the reduction in cell number in the gated PBMC monocyte population is not solely due to cell death but also to loss of CD14, the marker used to identify the monocyte population. Bazil, V & Strominger, JL, (1991) demonstrated that monocytes shed CD14 following stimulation with activating agents such as PMA and LPS, and suggested that it was a



regulatory mechanism to reduce the monocyte response following initial activation. The flow cytometry dotplots for CD14 staining of PBMC monocytes clearly demonstrated a reduction in the CD14-positive population following BzATP (Fig 5.9.) and ATP stimulation.

As described in section 5.2.2.3.ii, increased AV+PI+ staining in monocyte-gated cells may be due to PI entry through the P2X<sub>7</sub> pore rather than cell death. It has been proposed that pore formation is a result of P2X<sub>7</sub> coupling to a pore-forming protein within the membrane (North, RA, 2002). This could explain the diversity of the PI+ staining in the three monocyte cell types studied here, that are likely to demonstrate differences in their membrane proteins and regulation of pore formation.

To attempt to dissociate increased PI staining due to uptake through the pore from that due to cell death, an experiment was carried out using a larger nucleic acid dye, TOTO-3.

### **5.3. USE OF TOTO-3 IODIDE TO INVESTIGATE CELL DEATH IN PBMCS**

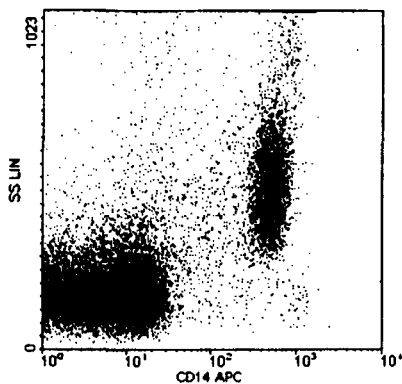
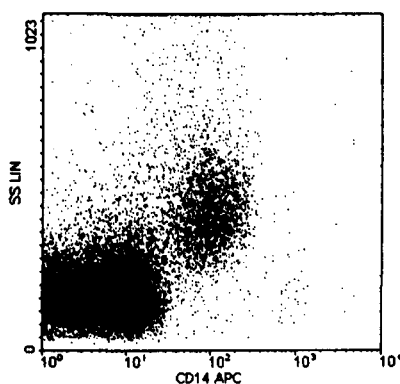
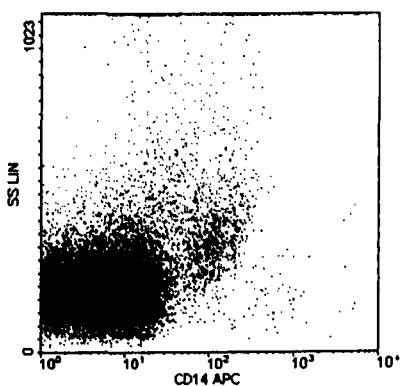
#### **5.3.1. Introduction**

TOTO-3 iodide (Molecular Probes, Invitrogen) is a dimeric cyanine dye which exhibits strong fluorescence on binding to DNA (Molecular Probes, 2000).

The aim of this experiment was to use a nucleic acid dye which was too large to enter through the pore of monocytes i.e. with a molecular weight greater than 900Da, and compare its uptake into PBMCS with that of the smaller dyes.

#### **5.3.2. Method**

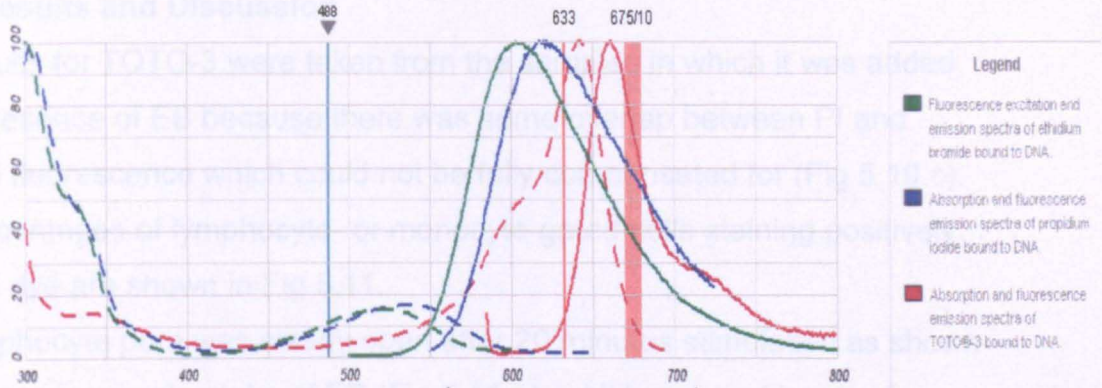
PBMCS from a single subject were stimulated with 1mM BzATP as previously described for 5, 20 or 60 minutes and then stained with AV-FITC and TOTO-3 (0.1 $\mu$ M) together with either EB or PI. PE-labelled CD3 or CD14 were added to identify the lymphocyte or monocyte populations respectively. EB and PI have similar excitation and emission wavelengths and cannot be measured together (Fig 5.10.a). Each test condition was therefore set up in quadruplicate with the following combinations of stains:

**a. unstimulated control cells****b. cells stimulated with 100 $\mu$ M BzATP****c. cells stimulated with 300 $\mu$ M BzATP**

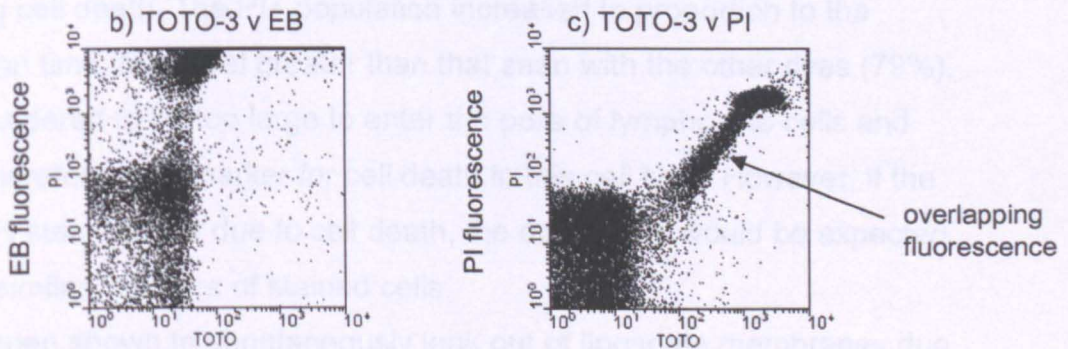
**Fig 5.9. Effect of BzATP stimulation on CD14 staining of PBMC monocytes**

The figures show flow cytometry dotplots of side scatter v CD14 staining of PBMCs. The population of monocytes visible in the unstimulated control cells clearly diminishes on stimulation with BzATP. A similar effect was observed following ATP stimulation

a) Excitation and emission spectra of TOTO-3 (red line), PI (blue line) and EB (green line). The wavelength of the filter for TOTO-3 detection is shown as a red band.



Molecular Probes Fluorescence Spectra Viewer,  
<http://probes.invitrogen.com/resources/spectraviewer/>



**Fig 5.10. Emission spectra and dotplots of TOTO-3 and PI or EB fluorescence**

Fig a shows the overlap between the emission spectra of EB, PI and TOTO-3. It is clear that some of the EB and PI emitted fluorescence falls within the TOTO-3 filter wavelength. The overlapping fluorescence can normally be compensated for as in the case of EB (fig b), but because the PI emission is greater than the EB emission it could not be fully compensated for. The cells which fluoresce in both channels appear as a linear band on the dotplot (fig c).

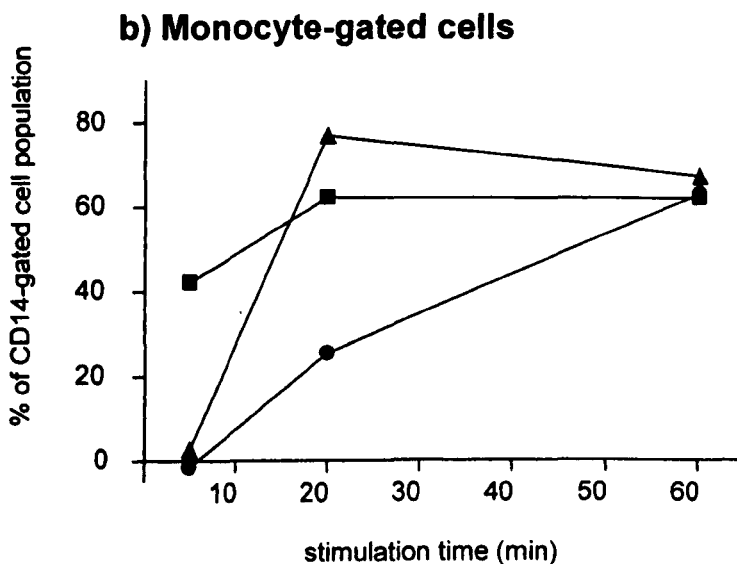
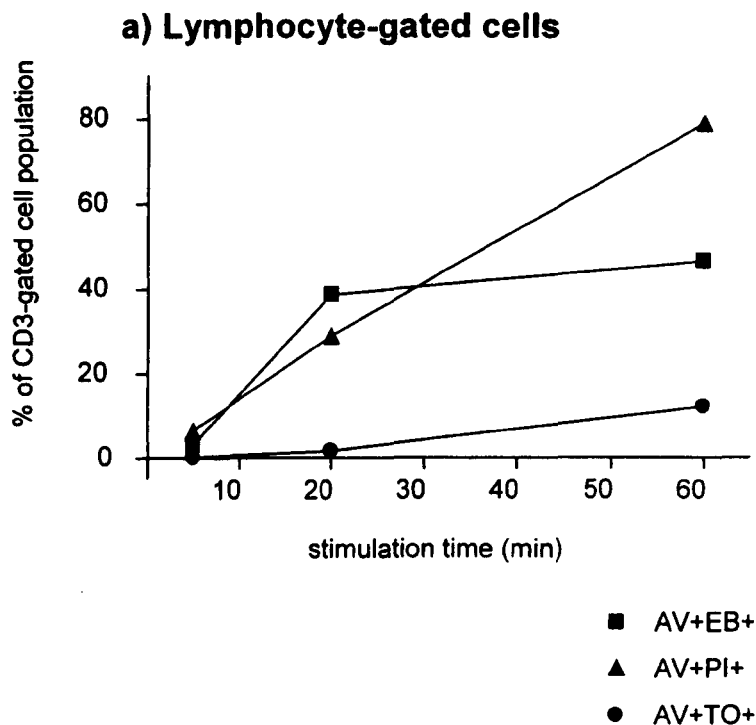
tube 1	tube 2	tube 3	tube 4
AV-FITC	AV-FITC	AV-FITC	AV-FITC
TOTO-3	TOTO-3	TOTO-3	TOTO-3
EB	EB	PI	PI
CD3-PE	CD14-PE	CD3-PE	CD14-PE

### 5.3.3. Results and Discussion

The results for TOTO-3 were taken from the samples in which it was added in the presence of EB because there was some overlap between PI and TOTO-3 fluorescence which could not be fully compensated for (Fig 5.10.c). The percentages of lymphocyte- or monocyte-gated cells staining positively for each dye are shown in Fig 5.11.

The lymphocyte pore was clearly open after 20 minutes stimulation as shown by the near-maximal uptake of EB (Fig 5.11.a), which reached levels of staining (approximately 40%) comparable to that seen in the concentration-response curves (Fig 3.3.b). The increase in the number of TOTO-3-stained cells was small, only 12% of the population after 60 minutes stimulation, reflecting cell death. The PI+ population increased in proportion to the stimulation time, to a level greater than that seen with the other dyes (79%). PI is considered to be too large to enter the pore of lymphocyte cells and should therefore be a marker for cell death in this cell type. However, if the level of PI staining was due to cell death, the other dyes would be expected to show similar numbers of stained cells.

EB has been shown to spontaneously leak out of liposome membranes due to its higher lipophilicity than that of propidium (Silvander, M & Edwards, K, 1996), a process that might also occur in cell membranes and therefore lead to reduced uptake. This may explain the lower level of EB uptake compared to PI uptake. The difference between the PI and the TOTO-3 staining could be a result of their molecular sizes. PI (MW 414) is only slightly larger than the pore size of lymphocytes (300Da) and therefore could enter the cell quite quickly once the membrane starts to break down. TOTO-3 however, is twice as large as PI and would require a greater degree of membrane disruption to enter the cells, and hence a longer stimulation time. It is also possible however that the high level of PI staining is an artefact of the overlapping fluorescence with TOTO-3 shown in Fig 5.10.c.



**Fig 5.11. Measurement of cell death in BzATP-stimulated PBMCs using EB, PI and TOTO-3**

PBMCs were incubated with 1mM BzATP for 5, 20 or 60min and then stained with AV-FITC and TOTO-3 with either EB (ethidium bromide) or PI (propidium iodide). Cell populations were identified with PE-labelled antibodies to CD3 or CD14. Each data set has been corrected for the unstimulated control value and show the results of a single experiment.

In monocyte-gated cells, the number of EB-stained cells was high after only 5 minutes stimulation (42%) and maximal after 20 minutes (62%) (Fig 5.11.b). This is because the pore in these cells is much larger (900Da), allowing the passage of the small EB ion (MW 314) with much shorter stimulation times. PI staining was low after stimulation for 5 minutes (3%) but was maximal after 20 minutes stimulation (77%). Following stimulation for 20 minutes, TOTO-3 was only taken up by 25% of the cell population, but after 60 minutes stimulation the uptake of all three dyes was the same (62-67%). The difference between the levels of staining for PI and TOTO-3 after 20 minutes stimulation suggests that some component of PI uptake in monocytes is probably due to entry through the pore with shorter stimulation times. After 60 minutes stimulation however, 60% of the cell population was clearly dead.

TOTO-3 was not an ideal dye for this experiment because the molecular weight of the ion is 846, slightly smaller than the estimated size of the monocyte pore. However it was the largest dye available that was suitable for excitation with the flow cytometer. It is possible therefore that some of the fluorescence with TOTO-3 in monocytes was also due to entry through the pore. Nevertheless the much lower level of TOTO-3 staining after 20 minutes stimulation compared to that of PI suggests that TOTO-3 is a better marker of cell death than PI in monocytes. The use of TOTO-3 however was inconclusive as a means of comparing pore entry with cell death. Despite this, the results confirmed that monocytes are much more susceptible than lymphocytes to cell death with prolonged P2X<sub>7</sub> stimulation, and suggest that lymphocytes might need longer than 60 minutes incubation with agonist to instigate cell death.

As an alternative indicator of cell death, the release of lactate dehydrogenase (LDH) was measured.

## **5.4. LDH RELEASE FROM P2X<sub>7</sub>-STIMULATED CELLS AS A MEASURE OF CELL DEATH**

### **5.4.1. Introduction**

LDH is an enzyme found in the cytoplasm of viable cells which catalyses the interconversion of pyruvate and lactate. Dead or damaged cells release LDH into the supernatant and this provides a marker of population viability and membrane integrity (Legrand, C *et al.*, 1993).

### **5.4.2. Method**

For PBMCs and LPMCs, the supernatants from the IL-1 $\beta$  experiments (section 5.5) which had been stored at -80°C were assayed for LDH release. For THP-1 cells, experiments were set up as described for IL-1 $\beta$  release (section 5.5.1.) and the supernatants stored at -80°C until assayed for LDH. Because the samples for IL-1 $\beta$  analysis had been pre-incubated with LPS, the LPS controls were also assayed for LDH release.

Samples were assayed using an LDH kit (In vitro Toxicology Assay Kit, Lactic Dehydrogenase based, Sigma-Aldrich) according to the manufacturer's instructions. Positive controls were prepared from freshly isolated cells lysed using the solution provided. 100 $\mu$ l of cell supernatant was pipetted into duplicate wells of a 96-well plate. Controls were prepared to match the experimental conditions; negative controls using RPMI 1640 and positive controls with lysed-cell supernatant. Assay mix was prepared by mixing equal volumes of LDH substrate, enzyme and dye solutions, and 50 $\mu$ l was added to each well. Plates were incubated for 20-30min at room temperature in the dark and the reaction was stopped by adding 15 $\mu$ l of 1M hydrochloric acid. Absorbance was read at 492/690nm using a Multiskan Ascent<sup>®</sup> microplate photometer (Thermo Electron Corporation). Results were corrected for the negative control and expressed as a percent of the positive control.

### 5.4.3. Results and Discussion

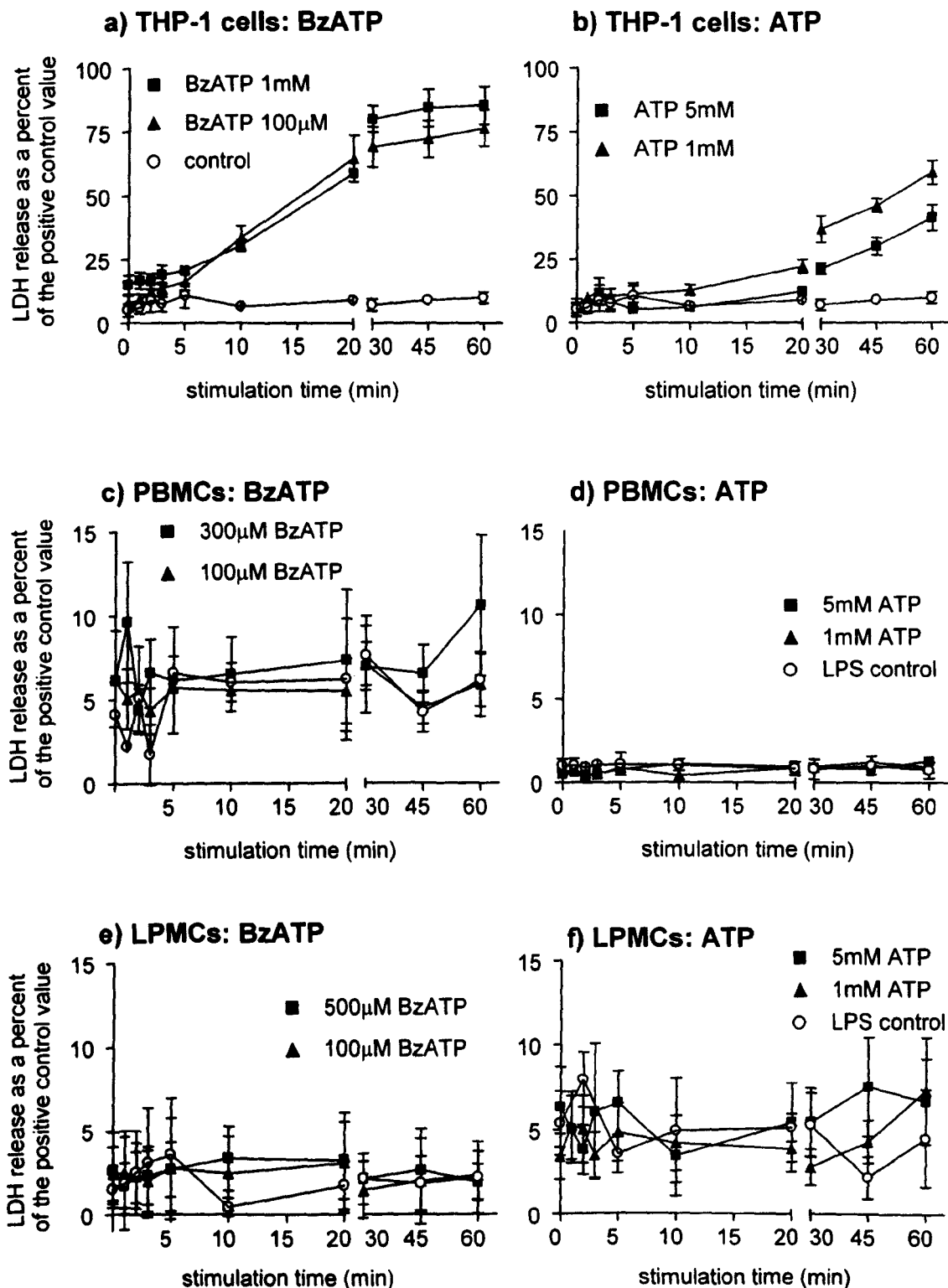
THP-1 cells displayed little LDH release for the first 5 minutes of stimulation after which time LDH levels rose steadily with ATP stimulation, and more rapidly with BzATP stimulation (Fig 5.12.a & b). For each agonist, the amount of LDH released was similar for both concentrations, although 1mM ATP released more LDH than 5mM ATP. This concurs with two other studies, using macrophages differentiated from PBMCs (Ferrari, D *et al.*, 1997b) and mouse thymocytes (Le Stunff, H *et al.*, 2004), that also showed LDH release to be maximal at 1mM ATP and reduced at higher concentrations. The fact that BzATP, a more potent P2X<sub>7</sub> agonist, induced both a greater and a more rapid release of LDH enzyme than ATP suggests that stimulation of the P2X<sub>7</sub> receptor results in cell death.

Following stimulation for up to 60 minutes, neither PBMCs nor LPMCs demonstrated a sizeable increase in LDH release (Fig 5.12.c-f). BzATP stimulation seemed to release more LDH from PBMCs than LPMCs but with ATP-stimulation this was reversed, and the secretion from ATP-stimulated PBMCs was very low. One possible reason for this might be that PBMCs are more efficient at breaking down ATP with endonuclease enzymes. This is supported by the earlier observation that stimulation of AV binding in PBMCs for 20 minutes required higher concentrations of agonist to achieve the same level of binding as stimulation for 5 minutes (Fig 4.12.). However, this is contradicted somewhat by the results for PI staining, which was greater for PBMCs than LPMCs with both agonists, but as indicated previously, high levels of PI staining can reflect uptake through the P2X<sub>7</sub> pore rather than cell death.

In both PBMCs and LPMCs neither agonist appeared to release more LDH than that produced by the LPS controls. Le Feuvre, RA *et al.*, (2002) showed that LPS priming of mouse peritoneal macrophages made the cells more susceptible to ATP-stimulated cell death, hence it is possible that LPS alone could cause some release of LDH.

For the mixed cell populations it is impossible to know which cells had released the LDH. However given the low PI<sup>+</sup> binding of lymphocytes, it is more likely that the monocytes are the source. The mixed cell populations





**Fig 5.12. The release of LDH enzyme from mononuclear cells stimulated with BzATP or ATP**

Samples were assayed according to the manufacturer's instructions. Results were corrected for the negative control and expressed as a percent of the positive control (THP-1 cells or PBMCs). The PBMC and LPMC samples assayed were the supernatants from the incubations for IL-1 $\beta$  release. These cells had been incubated with LPS prior to stimulation, therefore the LPS control samples were also assayed for LDH release.

were composed of approximately 40-50% lymphocytes and 5-10% monocytes, it is therefore possible to surmise that the secretion of LDH seen in the mixed cells, which was approximately 5% of the positive control, may have been due to the response of monocytes to LPS incubation and P2X<sub>7</sub>-stimulation.

This is supported by the high level of LDH release observed in the THP-1 monocyte cells following P2X<sub>7</sub> stimulation, and also by the results of TOTO-3 uptake in PBMCs. Those experiments demonstrated that following stimulation for 60 minutes, 60% of the monocyte cell population were permeable to TOTO-3 but only 10% of the lymphocyte population. This suggests a much greater disruption of the monocyte cell membrane compared to lymphocytes and hence a greater likelihood of the release of LDH, an even bigger molecule than TOTO-3, from the monocytes.

Most studies using non-monocyte cells have shown less than 10% of total LDH released into the supernatant after 60 minutes stimulation with ATP (Le Stunff, H *et al.*, 2004; Grahames, CB *et al.*, 1999; Gudipaty, L *et al.*, 2003).

However, other studies have found that stimulation for 60 minutes released approximately 27% of total LDH from macrophages stimulated with 1mM ATP and approximately 42% from dendritic cells with 5mM ATP (Ferrari, D *et al.*, 1997b; Ferrari, D *et al.*, 2000). In contrast, Murgia, M *et al.*, (1992) stimulated J774 macrophages with 3mM ATP and found that LDH was not released until 1 hour after addition of agonist (10%) but then increased steadily up to 60% at 6 hours. Similarly, Grahames, CB *et al.*, (1999) stimulated PMA-differentiated THP-1 cells with 5mM ATP for up to 4 hours and found that less than 10% of the total LDH was released.

The molecular weight of LDH is approximately 140,000 (Plummer, DT & Leathwood, PD, 1967) and such a large molecule is likely to be released only during the final stages of cell death when membrane disruption is high. It is probable therefore that stimulation for a period greater than 1 hour is required to observe significant amounts of LDH released from PBMCs and LPMCs.

## 5.5. TIME COURSE OF P2X<sub>7</sub>-STIMULATED IL-1 $\beta$ RELEASE

The kinetics of IL-1 $\beta$  release was measured in PBMCs and LPMCs.

Monocytes are the principal source of IL-1 $\beta$  so the kinetics of its release was compared to the kinetics of AV/PI binding in monocyte-gated cells.

### 5.5.1. Method

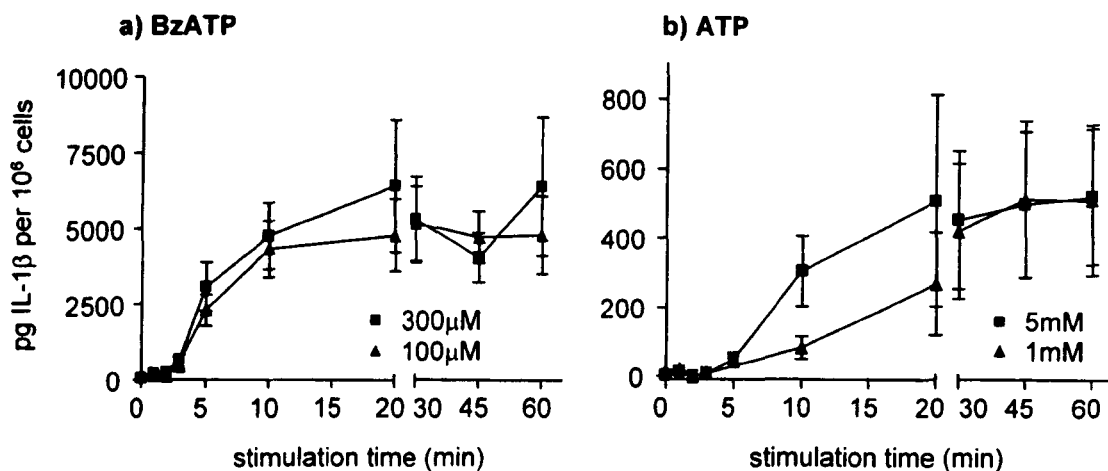
Cells were resuspended in RPMI 1640 containing 0.1% v/v FCS at a concentration of  $1 \times 10^6$  cells/ml, and incubated with LPS (1 $\mu$ g/ml) for approximately three hours at 37°C in 5% CO<sub>2</sub>. Cells were then incubated with BzATP or ATP for 0, 1, 2, 3, 5, 10, 20, 30, 45 or 60min at 37°C in a total incubation volume of 100 $\mu$ l. LPS controls (containing no BzATP or ATP) and RPMI medium controls were also prepared for each time point. Incubation was stopped by adding PBS (400 $\mu$ l), the cells were placed on ice and then centrifuged at 350 x g for 5min. Aliquots (450 $\mu$ l) of the supernatants were stored at -80°C for assay by sandwich ELISA, according to the manufacturer's instructions.

### 5.5.2. Results and Discussion

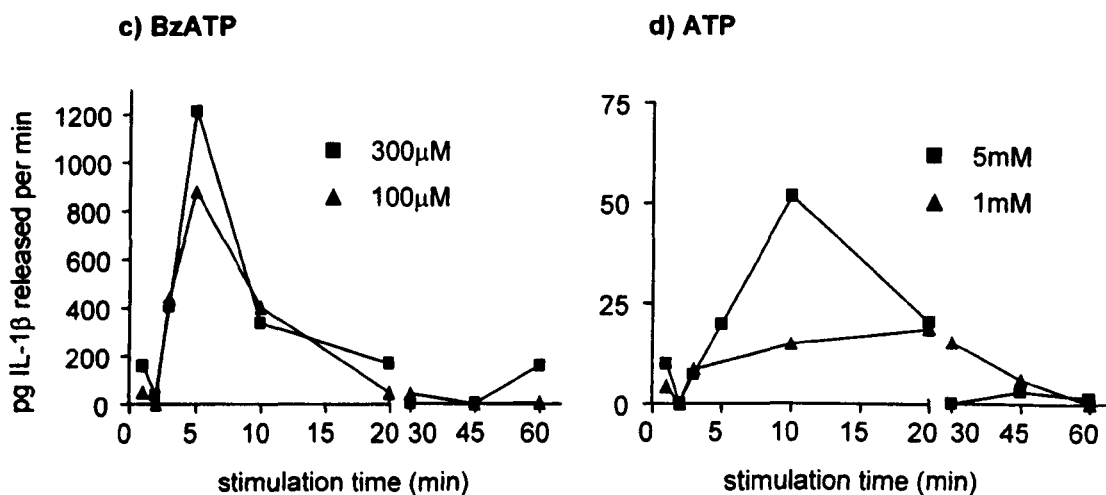
#### 5.5.2.1. Time course of IL-1 $\beta$ release from PBMCs

For PBMCs, the release of IL-1 $\beta$  was very similar with both concentrations of BzATP (Fig 5.13.a). After 2 minutes, a sharp rise in IL-1 $\beta$  release was observed reaching a plateau at approximately 20 minutes, after which time there was no further increase. Both concentrations of BzATP released large amounts of IL-1 $\beta$ , reaching a maximum of  $6403 \pm 2174$  pg/10<sup>6</sup> cells at 20 minutes with 300 $\mu$ M BzATP, and  $5186 \pm 1220$  pg/10<sup>6</sup> cells at 30 minutes with 100 $\mu$ M BzATP. These values are of a similar order but greater than the amount released in the concentration-response curve which peaked at  $2509 \pm 777$ pg/10<sup>6</sup> cells with 112 $\mu$ M BzATP and 20 minutes stimulation. The rate of release of IL-1 $\beta$  was calculated for each time point and this was maximal at 2-5 minutes (Fig 5.13.c). It is clear from the results that the half-maximal concentration used was too high and gave a near-maximal response. This is because the EC<sub>50</sub> for IL-1 $\beta$  release from PBMCs was much lower than that for AV binding, and it would have been better to use individualised

### i. Amount of IL-1 $\beta$ released into the supernatant



### ii. The rate of release of IL-1 $\beta$



**Fig 5.13. Time course of IL-1 $\beta$  release from PBMCs**

i. Cells were stimulated for up to 60min with BzATP or ATP in RPMI 1640 + 0.1% FCS at maximal or half-maximal concentrations, taken from the concentration-response curves. Results were corrected for the LPS control, and show the mean  $\pm$  sem of four separate experiments.

ii. Rate of release was calculated by subtracting values for pg IL-1 $\beta$  released at each time point from the value for the following time point and dividing by the total time passed.

concentrations for each, rather than trying to standardise the concentrations used in all parts of the experiment.

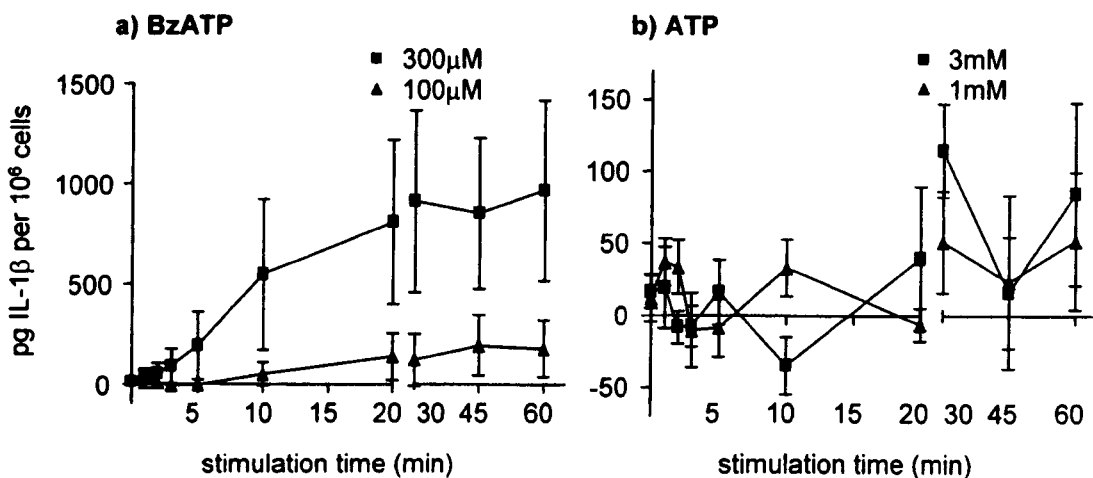
With 5mM ATP, the release of IL-1 $\beta$  also reached a plateau after 20 minutes although the amount released was much less than with BzATP (Fig 5.13.b). The rate of release was also slower than that of BzATP and reached a maximum at 5-10 minutes (Fig 5.13.d). With 1mM ATP the release of IL-1 $\beta$  was slower and did not reach a plateau until 45 minutes. Both concentrations of ATP however did ultimately release similar maximal amounts of IL-1 $\beta$ ;  $507 \pm 305$  pg/10<sup>6</sup> cells at 20 minutes with 5mM ATP and  $513 \pm 226$  pg/10<sup>6</sup> cells at 45 minutes with 1mM ATP. These values are considerably less than the maximum amount released in the concentration-response curve which was  $1767 \pm 508$ pg/10<sup>6</sup> cells with 2mM ATP and 20 minutes stimulation. The lack of IL-1 $\beta$  release from ATP-stimulated PBMCs was due to the use of K<sub>2</sub>ATP in this set of experiments. Because of a shortage of Na<sub>2</sub>ATP stock, K<sub>2</sub>ATP was used for the time course experiments and resulted in reduced release of IL-1 $\beta$ . This was confirmed by a test carried out with LPMCs and detailed in section 5.5.2.2. below.

#### **5.5.2.2. Time course of IL-1 $\beta$ release from LPMCs**

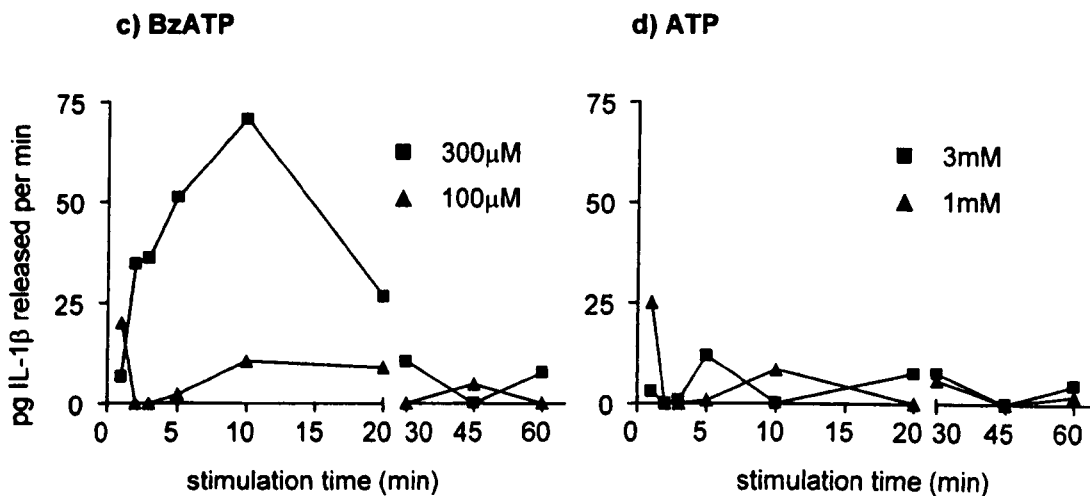
For LPMCs stimulated with 300 $\mu$ M BzATP IL-1 $\beta$  release increased steadily with time reaching a plateau at 30 minutes (Fig 5.14.a); the rate of release was greatest at 5-10 minutes (Fig 5.14.c). As to be expected the amount of IL-1 $\beta$  released ( $911 \pm 453$  pg/10<sup>6</sup> cells at 30 minutes) was much less than that seen with PBMCs, but was comparable with the amount released in the concentration-response curve for this concentration of BzATP ( $716 \pm 234$  pg/10<sup>6</sup> cells). The variability between subjects in the amount of IL-1 $\beta$  released was very high as demonstrated by the wide error bars. Following stimulation with 100 $\mu$ M BzATP there was very little release of IL-1 $\beta$ , only reaching a peak of  $194 \pm 149$ pg/10<sup>6</sup> cells at 45 minutes.

With ATP-stimulation there was very little IL-1 $\beta$  released at either concentration;  $114 \pm 33$  pg/10<sup>6</sup> cells at 30 minutes with 3mM ATP, and  $51 \pm 35$  pg/10<sup>6</sup> cells at 30 minutes with 1mM ATP (Fig 5.14.b). This is somewhat

### i. Amount of IL-1 $\beta$ released into the supernatant



### ii. The rate of release of IL-1 $\beta$



**Fig 5.14. Time course of IL-1 $\beta$  release from LPMCs**

i. Cells were stimulated for up to 60min with BzATP or ATP in RPMI 1640 + 0.1% FCS at maximal or half-maximal concentrations, taken from the concentration-response curves. Results were corrected for the LPS control, and show the mean  $\pm$  sem of four separate experiments.

ii. Rate of release was calculated by subtracting values for pg IL-1 $\beta$  released at each time point from the value for the following time point and dividing by the total time passed.

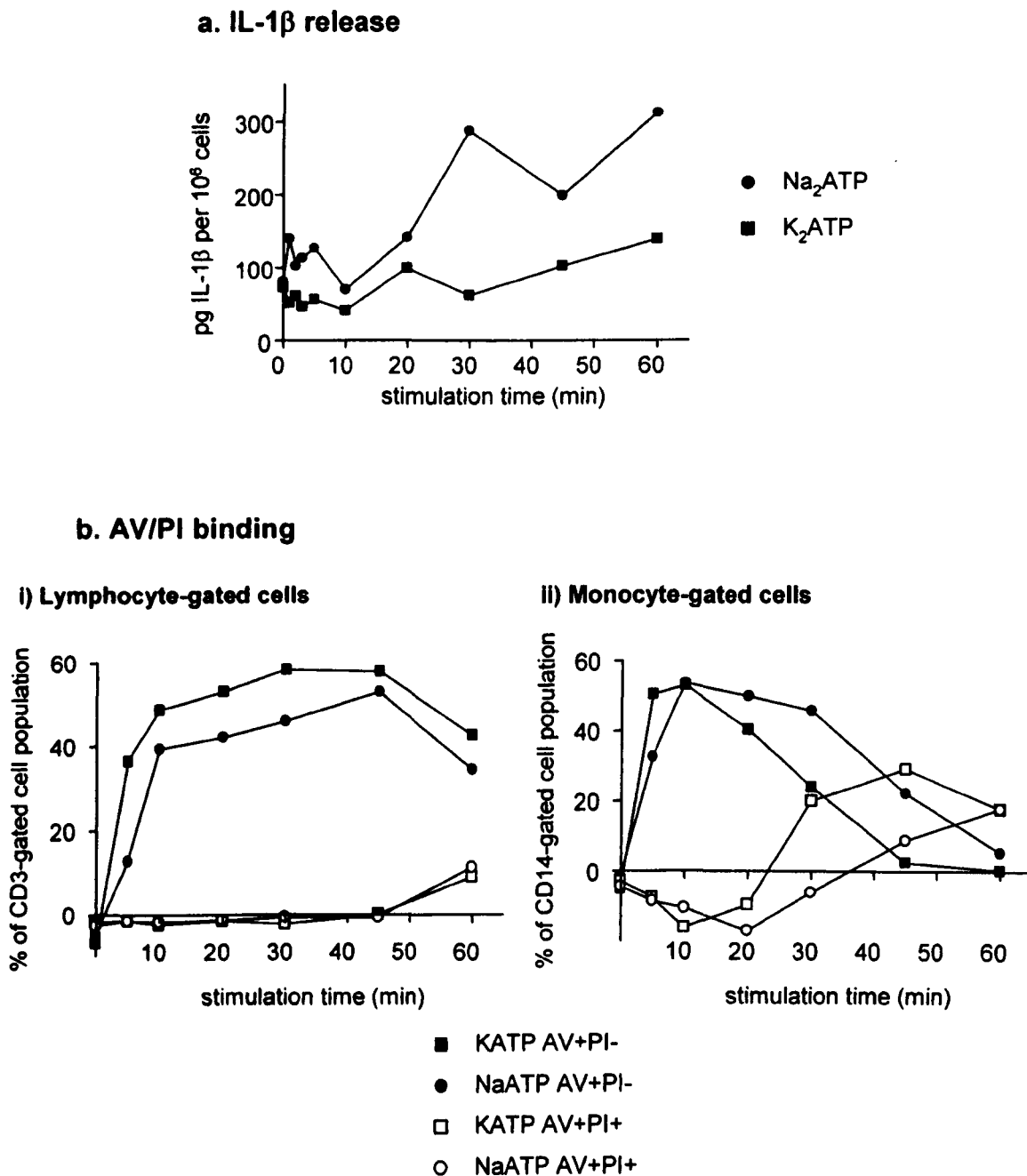
less than the amounts released in the concentration-response curve, which were  $362 \pm 215$  pg/ $10^6$  cells and  $110 \pm 49$  pg/ $10^6$  cells for the same concentrations of ATP with 20 minutes stimulation.

As stated above  $K_2ATP$  was used for this set of experiments and to test whether this was responsible for the reduced IL-1 $\beta$  levels, a comparison was made between  $K_2ATP$ - and  $Na_2ATP$ -stimulated IL-1 $\beta$  release from LPMCs from a single subject. The results (Fig 5.15.a) show that  $Na_2ATP$  released 2-3 times as much IL-1 $\beta$  as  $K_2ATP$ ; up to 300pg/ $10^6$  cells, a value comparable to that seen with the concentration-response curve.

Interestingly, the monocyte-gated cells, which are the source of IL-1 $\beta$ , demonstrated less AV+PI+ staining with  $Na_2ATP$  compared to  $K_2ATP$  (Fig 5.15.bii) showing that increased IL-1 $\beta$  release is not a result of cell death. For lymphocyte-gated cells, there was no difference in AV/PI staining with either type of ATP. These results concur with the findings of Perregaux, DG & Gabel, CA, (1998a) who showed that  $Na^+$  was required for IL-1 $\beta$  processing, and that this was not due to inactivation of the receptor but rather involved a downstream step in the process.

Stimulation of the  $P2X_7$  ion channel results in influx of  $Na^+$  and  $Ca^{2+}$  ions into the cell and efflux of  $K^+$  ions, but various studies have shown that receptor activity is markedly affected by ion concentrations. Physiological concentrations of both divalent and monovalent cations, and anions have been shown to inhibit agonist activity (Michel, AD *et al.*, 1999). It has been suggested that cations affect the availability of  $ATP^{4-}$ , the active form of the agonist, but a study by Virginio, C *et al.*, (1997) found that divalent cations reduced  $ATP^{4-}$  concentration by only 50% whereas dye uptake through the  $P2X_7$  pore was inhibited by more than 95%.

It is unlikely that the increased IL-1 $\beta$  release observed with  $Na_2ATP$  stimulation compared with  $K_2ATP$  is due solely to differences in the ion affinities for  $ATP^{4-}$ , since ion concentrations within the RPMI 1640 would be expected to outweigh any influence of the type of ATP used, and hence the amount of  $ATP^{4-}$  available for  $P2X_7$  receptor stimulation. Melchior, NC, (1954) demonstrated that both  $Na^+$  and  $K^+$  form complexes with  $ATP^{4-}$  and that these complexes are present at physiological pH ranges and ion



**Fig 5.15. Comparison of the effects of K<sub>2</sub>ATP or Na<sub>2</sub>ATP as a P2X<sub>7</sub> agonist in LPMCs**

LPMCs from a single subject were stimulated for up to 60min with 5mM K<sup>+</sup> or Na<sup>+</sup>ATP in RPMI 1640. Figure a shows IL-1 $\beta$  release corrected for the LPS control. Figure b shows AV/PI staining. Cells staining positive were expressed as a percent of the gated cell population; each data point has been corrected for the unstimulated control value.



concentrations. He proposed that combination with a metal ion specifically alters the molecular shape, and if a particular shape is favoured by an enzyme it could affect the rate of reaction.

More recently it has been shown that P2X<sub>7</sub> has binding sites for Na<sup>+</sup> and Cl<sup>-</sup> ions that appear to regulate its permeability to large molecules (Li, Q *et al.*, 2005). Chloride ions have also been shown to inhibit IL-1 $\beta$  release by attenuating inflammasome formation and hence maturation of caspase-1 (Verhoef, PA *et al.*, 2005).

Studies of ionic effects on receptor activity generally involve manipulation of the ion content of the medium, and it is interesting that simply using different forms of ATP in the same medium also had an effect on IL-1 $\beta$  release and AV/PI binding. This suggests that either the ionic complexes with ATP are important, as suggested above (Melchior, NC, 1954), or that the P2X<sub>7</sub> receptor is exquisitely sensitive to ionic balances in the medium so that adding Na<sub>2</sub>ATP or K<sub>2</sub>ATP can alter receptor activity.

It seems therefore that the outcome of P2X<sub>7</sub> stimulation is governed by complex interactions between availability of ATP<sup>4-</sup> and interactions of ions with the receptor, and with intracellular pathways associated with pore formation, PS flip and IL-1 $\beta$  release.

### 5.5.3. Summary of IL-1 $\beta$ release

The kinetics of IL-1 $\beta$  release was similar for both PBMCs and LPMCs with maximum secretion generally occurring after 20-30 minutes. Various studies have shown similar IL-1 $\beta$  release with ATP stimulation. Several, using a range of cell types, have demonstrated maximum release of IL-1 $\beta$  at 30 minutes following stimulation with 1-5mM ATP (Mehta, VB *et al.*, 2001; Andrei, C *et al.*, 2004; Colomar, A *et al.*, 2003; Kahlenberg, JM & Dubyak, GR, 2004b; Gudipaty, L *et al.*, 2003). Other studies using human monocytes or macrophages have found peak IL-1 $\beta$  release times of 1-2 hours (Chessell, IP *et al.*, 2001; Ferrari, D *et al.*, 1997b; Wewers, MD *et al.*, 1997).

Wewers, MD *et al.*, (1997) compared ATP-induced release of IL-1 $\beta$  from monocytes and macrophages and showed that macrophage release was slower than that of monocytes. They found that 30 minutes after stimulation,

58% of maximum IL-1 $\beta$  release had occurred from monocytes but only 23% from macrophages. The absolute amount released was also higher from monocytes (23 ng/10<sup>6</sup> cells) than macrophages (9 ng/10<sup>6</sup> cells) and they suggested that macrophages have a tighter control on IL-1 $\beta$  release than monocytes. The results presented here also showed that PBMCs released much larger amounts of IL-1 $\beta$  than LPMCs and the rate of release was quicker; maximum rate with BzATP at 2-5min for PBMCs and 5-10min for LPMCs. The release of IL-1 $\beta$  occurred more slowly than PS-flip in the monocyte-gated cells. Maximum levels of AV+PI- staining were achieved by 5-10 minutes with maximum rates of binding at 1-3 minutes.

## **5.6. SUMMARY OF THE EFFECT OF P2X<sub>7</sub>-RECEPTOR STIMULATION TIME ON CELL DEATH AND IL-1 $\beta$ RELEASE**

Stimulation of the P2X<sub>7</sub> receptor for up to one hour in LPMCs and PBMCs resulted in a rapid increase in PS-flip (indicated by increased AV binding), followed by cell death (indicated by increased PI uptake) with longer stimulation times. Monocyte-gated cells were more susceptible to cell death than lymphocyte-gated cells; in addition, peripheral blood cells appeared to be more susceptible than colonic LPMCs. Approximately half of the AV+PI- population of lymphocytes from PBMCs became AV+PI+, whereas only around 10% of the equivalent LPMC population became AV+PI+. In contrast, all of the AV+PI- population of PBMC monocytes became AV+PI+ and most of the LPMC population, although this occurred much more quickly in the PBMCs. Whether the increased uptake of PI in monocytes was due solely to cell death or to uptake through the P2X<sub>7</sub> pore could not be confirmed conclusively using a larger nucleic acid dye or by measuring LDH release.

Increased AV binding occurred more rapidly following P2X<sub>7</sub> stimulation than IL-1 $\beta$  release. This was particularly true for BzATP-stimulation for which AV binding reached a plateau after 5-10 minutes compared to 20-30 minutes for IL-1 $\beta$  release. This reflects the fact that secretion of IL-1 $\beta$  is a more complicated process requiring formation of the complex inflammasome

structure, whereas PS-flip is a result of altered enzyme activity due to rapid  $\text{Ca}^{2+}$  influx through the ion channel. The two concentrations of ATP used throughout the experiments were the same for all the cell types and it was noticeable that whilst 1mM ATP produced only 50% of the level of AV binding stimulated by 5mM ATP, the amount of IL-1 $\beta$  released was almost the same. This is probably because the complex pathway of IL-1 $\beta$  release results in amplification of the response (section 4.3) and hence can be initiated by lower concentrations of agonist.

Recently MacKenzie, AB *et al.*, (2005) proposed that P2X<sub>7</sub>-induced PS-flip is an initial upstream effector in a P2X<sub>7</sub> signalling pathway. Using HEK293 cells expressing P2X<sub>7</sub> they showed that stimulation of the receptor caused influx of extracellular  $\text{Ca}^{2+}$ , mitochondrial swelling and release of  $\text{Ca}^{2+}$  from the mitochondria. High intracellular  $\text{Ca}^{2+}$  resulted in PS-flip with subsequent cytoskeletal disruption and membrane blebbing. The physiological function of the blebbing is not known but may be connected with the microvesicular release of IL-1 $\beta$  demonstrated by the same group (MacKenzie, A *et al.*, 2001), who showed that the vesicles all exhibited positive AV staining. If PS-flip is an early step in IL-1 $\beta$  release, it would need to occur more rapidly than IL-1 $\beta$  processing, as was demonstrated here.

A signalling role has also been proposed for P2X<sub>7</sub>-mediated PS-flip in lymphocytes (Elliott, JI *et al.*, 2005). Changes in membrane PS distribution stimulated shedding of CD62L, a protein involved in homing to lymph nodes, and also inhibited P-glycoprotein, a protein that has also been implicated in lymphocyte homing. The authors proposed that the P2X<sub>7</sub> signalling pathway may mediate the 'danger response' of T cells, promoting T cell migration to non-lymphoid sites. It is therefore becoming clear that PS externalisation on the cell membrane is not always a sign of apoptotic cell death.

MacKenzie, A *et al.*, (2001) has shown that if P2X<sub>7</sub> activation was limited to less than 10 minutes, PS-flip completely reversed within 3 hours and there was no cell death in the subsequent 24 hours. In contrast, a 30 minute

stimulation of the P2X<sub>7</sub> receptor with BzATP resulted in irreversible PS-flip and loss of all cells within 24 hours. The effect of short and long stimulation times on the reversibility of PS-flip in human colonic LPMCs was therefore investigated.

## CHAPTER 6. P2X<sub>7</sub> RECEPTOR STIMULATION: REVERSIBILITY OF RESPONSE

### 6.1. INTRODUCTION

Studies have shown that prolonged stimulation of the P2X<sub>7</sub> receptor leads to cell death by necrotic or apoptotic mechanisms (Hogquist, KA *et al.*, 1991a; Di Virgilio, F *et al.*, 1996; Ferrari, D *et al.*, 1997a; Virginio, C *et al.*, 1999a). P2X<sub>7</sub>-stimulated necrotic death was initially considered to be a result of pore formation and subsequent loss of cell contents. Pore formation occurs after prolonged or repeated ATP stimulation, but it is now known that provided the ATP is removed within 10-15 minutes most cell types can reseal the pore and recover (Morelli, A *et al.*, 2001; Chessell, IP *et al.*, 1997; Virginio, C *et al.*, 1999a). Pore formation is not unique to the P2X<sub>7</sub> receptor. Other P2X receptors, namely P2X<sub>2</sub>, P2X<sub>4</sub> and the heteromeric P2X<sub>2</sub>/P2X<sub>3</sub> receptors, have been shown to form similar pores, but stimulation of these receptors never results in cell death (Virginio, C *et al.*, 1999b; Khakh, BS *et al.*, 1999). P2X<sub>7</sub> has been regarded as an apoptotic receptor because some characteristics of P2X<sub>7</sub> stimulation are similar to morphological features of apoptotic cell death, namely PS exposure on the outer plasma membrane and rapid membrane blebbing (Nihei, OK *et al.*, 2000a; Le Stunff, H *et al.*, 2004; Tsukimoto, M *et al.*, 2005). These features are generally irreversible when associated with apoptosis but this is not the case for P2X<sub>7</sub> stimulation. MacKenzie, A *et al.*, (2001) used AV-binding to study BzATP-stimulated PS exposure in HEK cells expressing the rat P2X<sub>7</sub> receptor and detected AV-positive cells as early as 1-2 seconds after application of BzATP. This rapid PS-flip was quickly followed by shedding of microvesicles from the plasma membrane, which contained IL-1 $\beta$  and also bound AV on their surface. They also found that, if P2X<sub>7</sub> activation was limited to less than 10 minutes, PS translocation was completely reversed with no cell death. In contrast, stimulation for 30 minutes resulted in irreversible PS flip and loss of all cells after 24 hours. Another study in the same cells demonstrated reversible membrane blebbing when ATP stimulation was removed after 20 minutes (Morelli, A *et al.*, 2003). This study also showed that bleb formation could be

prevented by preincubation with the P2X<sub>7</sub> inhibitor, oxidised ATP. Reversible vesicle formation has also been demonstrated in human skin fibroblasts (Solini, A *et al.*, 1999). When the cells were stimulated with BzATP a large number of perinuclear microvesicles were formed whose function was unknown, but which completely disappeared within 15-30 minutes of BzATP removal.

In more recent studies, MacKenzie, AB *et al.*, (2005) has proposed that P2X<sub>7</sub> activation is linked to two signalling pathways, depending on the length of exposure to stimulation. They showed that high intracellular Ca<sup>2+</sup> levels resulting from P2X<sub>7</sub> stimulation led to PS-flip, cytoskeletal protein disruption and membrane blebbing which were fully reversible provided receptor activation was less than 20-30 minutes. However prolonged P2X<sub>7</sub> stimulation resulted in apoptotic cell death due to cytochrome c release from the mitochondria and a caspase-3/ROCK-1 signalling cascade. It would thus appear that some of the effects resulting from P2X<sub>7</sub> stimulation can be reversed provided the stimulation time is short, and that stimulation of the receptor does not necessarily result in apoptotic cell death.

The reversibility of PS-flip following P2X<sub>7</sub>-stimulation was therefore studied in LPMCs, PBMCs and THP-1 cells, using BzATP stimulation for 5 or 30 minutes. AV-binding was used as a measure of PS-flip and staining with PI as a measure of cell death. Since PS-flip is not a definitive method for measuring apoptotic cell death following P2X<sub>7</sub> stimulation, induction of apoptosis was also measured using cell cycle analysis and Hoechst staining. Likewise, because increased PI staining may be due to uptake through the P2X<sub>7</sub> pore, LDH release was used as another measure of cell death. The effect of different stimulation times on the release of IL-1 $\beta$  was also measured.

## **6.2. METHODS**

### **6.2.1. Reversibility of P2X<sub>7</sub>-induced AV binding**

Cells were resuspended in RPMI 1640 at a concentration of  $2 \times 10^6$  cells/ml and incubated with 500 $\mu$ M BzATP for 5 or 30min at 37°C in a total incubation

volume of 100µl. Based on previous results, this concentration of BzATP should give a maximal, or close to maximal response in all the cell types. Incubation was stopped by adding PBS (400µl), the cells were placed on ice and then centrifuged at 350 x g for 5min. Cells were resuspended in 40µl of warm AV-binding buffer and incubated at 37°C for 0, 5, 10, 20, 40 or 60min. Unstimulated controls were also prepared for each time point. Incubation was stopped by placing the cells on ice. To the THP-1 cells, 200µl of cold binding buffer containing AV-FITC and PI was added, the cells were kept on ice and analysed by flow cytometry. For the PBMCs and LPMCs, CD3 and CD14/CD33 were added and the cells incubated for 30min on ice in the dark. Cold binding buffer (200µl) containing AV-FITC and PI was then added, and the samples analysed by flow cytometry.

### **6.2.2. Release of LDH from cells stimulated for 5 or 30 minutes**

LDH release was determined as described in section 5.4.2. The samples assayed were the supernatants from the cells set up for Hoechst staining (section 6.2.4.) and stored at -20°C.

### **6.2.3. P2X<sub>7</sub>-stimulated apoptosis determined by cell cycle analysis**

Cell cycle analysis measures the DNA content of a cell and as well as identifying cells in the G<sub>0</sub>/1 and G<sub>2</sub>/M phases, it can also be used to identify apoptotic cells. The fixing process, as well as preserving the cells, also makes them permeable and accessible to a fluorochrome. In apoptotic cells DNA is cleaved by endonucleases, creating fragments which may be washed out of the cells during the hydration and staining process. Apoptotic cells therefore have less DNA than cells in the G<sub>0</sub>/1 phase, and are known as the 'sub-G<sub>0</sub>/1 cell population' (Darzynkiewicz, Z *et al.*, 1999).

Cells were resuspended in RPMI 1640 at a concentration of  $2.5 \times 10^6$  cells/ml and incubated with 500µM BzATP for 5 or 30min at 37°C in a total incubation volume of 100µl. Incubation was stopped by adding PBS (400µl), the cells were placed on ice and then centrifuged at 350 x g for 5min. Cells were resuspended in 40µl of warm RPMI 1640 and incubated at 37°C for 0, 20, 40, 60min, 3h, 6h and 24h. Unstimulated controls were also prepared for

each time point. Incubation was stopped by placing the cells on ice and adding 960µl of ice-cold PBS with 0.1% bovine serum albumin (BSA, Sigma-Aldrich). Cells were mixed well by repeated pipetting and 2.3ml ice-cold absolute ethanol was added to fix the cells. After thorough mixing, samples were stored at -20°C. To process for flow cytometry; after bringing to room temperature, cells were centrifuged at 350 x g for 5min and washed twice with 1ml PBS with 0.1% BSA with gentle pipetting to resuspend. The cells were then incubated in 500µl of PI/RNASE staining buffer (BD Pharmingen, Cowley, Oxford) for 15min at 37°C in the dark and analysed on the flow cytometer. The RNase enzyme breaks down any double-stranded RNA present in the cells which would intercalate the PI and give a false positive result.

The flow cytometry data was analysed as described in section 2.4.2.

#### **6.2.4. P2X<sub>7</sub>-stimulated apoptosis determined by Hoechst staining**

Hoechst 33342 (Molecular Probes) is a cell-permeable bisbenzimidazole dye that fluoresces bright blue on binding to DNA (Molecular Probes, 2005). Apoptotic cells appear with visibly fragmented nuclei and brighter than average staining due to condensation of chromatin.

Cells were resuspended in RPMI 1640 at a concentration of  $2 \times 10^6$  cells/ml and incubated with BzATP as described in section 6.2.3. At the end of the incubation time, 400µl ice-cold PBS was added; cells were centrifuged at 350 x g for 5min and fixed in 50µl of 4% paraformaldehyde. The cells were stored at 4°C until stained.

The cells were washed once with 200µl PBS and then spun onto glass slides using a Shandon/Lipshaw cytospin centrifuge (ThermoShandon, Pittsburgh, USA) at 60 x g for 10 min. After air-drying, the slides were stained with Hoechst 33342 (10µg/ml in PBS) for 10 min at room temperature in the dark. The slides were washed gently in distilled water and left to dry in the dark before mounting with a coverslip using PBS/glycerol (50/50 v/v). The coverslips were sealed with nail varnish and the slides stored in the dark at 4°C until counted. Cell fluorescence was monitored with a fluorescent microscope (Leica DMIRB) using a blue filter (488nm). A total of 200 cells



were counted on each slide from randomly selected fields and the number of apoptotic cells calculated as a percentage of total cells. Cells with brightly staining visibly fragmented nuclei were scored as apoptotic.

### **6.2.5. IL-1 $\beta$ release from cells stimulated for 5 or 30 minutes**

For IL-1 $\beta$  release, the concentration of BzATP was reduced to 200 $\mu$ M since IL-1 $\beta$  release occurs at lower agonist concentrations than AV binding. Cells were resuspended in RPMI 1640 containing 0.1% v/v FCS at a concentration of  $1 \times 10^6$  cells/ml, and incubated with LPS (1 $\mu$ g/ml) for approximately three hours at 37°C in 5% CO<sub>2</sub>. BzATP was then added and incubated for 5 or 30min at 37°C in a total incubation volume of 100 $\mu$ l. Incubation was stopped by adding PBS (400 $\mu$ l), the cells were placed on ice and then centrifuged at 350 x g for 5min. Cells were resuspended in 100 $\mu$ l of warm RPMI 1640 with 0.1% v/v FCS containing 1 $\mu$ g/ml LPS and incubated at 37°C for 0, 20, 40, 60min, 3h, 18h and 24h. Unstimulated controls were also prepared for each time point. Incubation was stopped by adding cold PBS (400 $\mu$ l) and placing the cells on ice. The cells were centrifuged at 350 x g for 5min and aliquots (450 $\mu$ l) of the supernatants were stored at -80°C for assay by sandwich ELISA, according to the manufacturer's instructions. The cells remaining in the tubes were lysed by incubation with 250 $\mu$ l of 0.1% saponin in RPMI 1640 for 30min at 37°C. The cells were centrifuged at 350 x g for 5min and aliquots (250 $\mu$ l) of the supernatants were stored at -80°C for IL-1 $\beta$  assay.

## **6.3. RESULTS AND DISCUSSION**

### **6.3.1. Reversibility of P2X<sub>7</sub>-induced AV binding**

For AV binding, the individual PBMC and LPMC cell populations were gated using CD markers as described in section 5.2.

#### **6.3.1.1. Reversibility of AV binding in THP-1 cells**

Following 5 minutes stimulation with 500 $\mu$ M BzATP,  $85 \pm 1.0\%$  of the cell population exhibited positive AV binding but there was very little AV+PI+ staining ( $10 \pm 0.6\%$ ), indicating a high level of PS-flip but not cell death (Fig

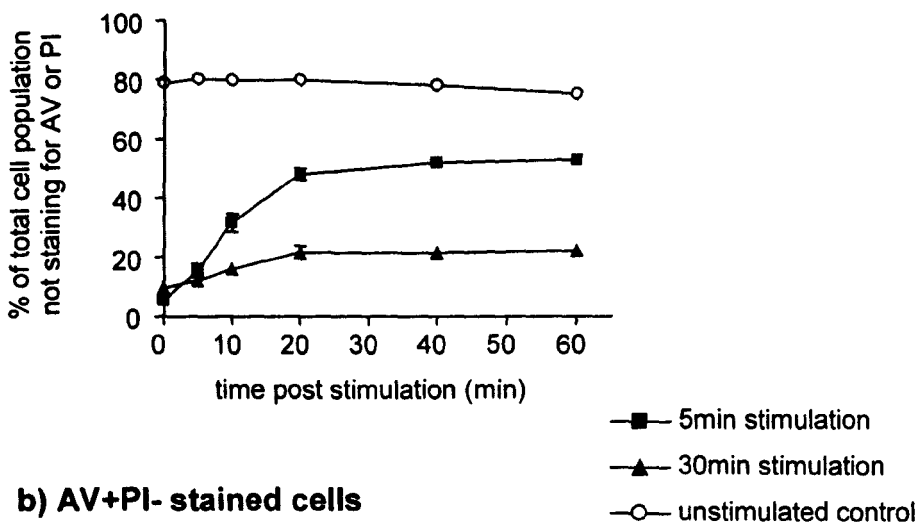
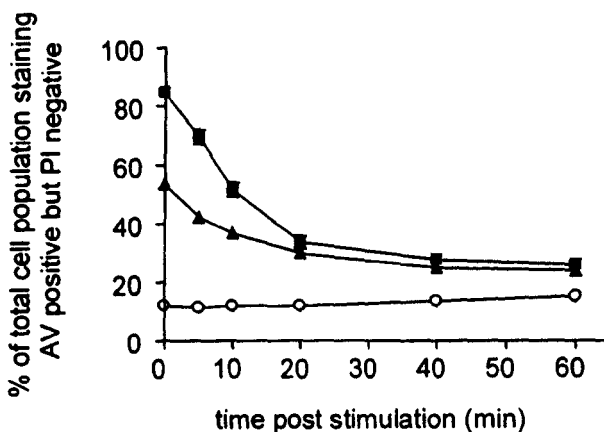
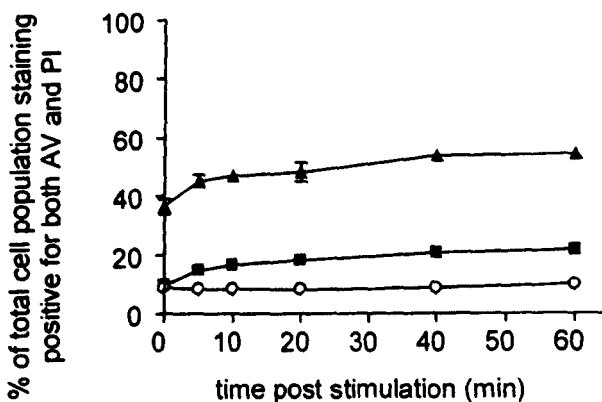
6.1.b & c, solid squares). After removal of agonist, the AV+PI- staining decreased rapidly to  $34 \pm 2.2\%$  at 20 minutes post-stimulation, finally falling to  $26 \pm 0.5\%$  after 60 minutes. Most of the AV+PI- population of cells reversed the PS-flip and lost the annexin staining, as shown by the increase in the unstained AV-PI- population (Fig 6.1.a) which rose from  $5.5 \pm 0.7\%$  to  $48 \pm 2.0\%$  of the total cell population after 20 minutes incubation. Only a small proportion of the AV+PI- cells became PI-positive; the AV+PI+ population rising from  $10 \pm 0.6\%$  to  $21 \pm 1.1\%$  after 60 minutes incubation. In contrast, after 30 minutes stimulation with BzATP  $54 \pm 1.9\%$  of the population exhibited AV+PI- staining but  $36 \pm 2.5\%$  were AV+PI+ (Fig 6.1.b & c, solid triangles). Furthermore, following removal of agonist, 60% of the AV+PI- population became AV+PI+, with only 40% reversing the PS-flip and becoming unstained.

Unstimulated control cells demonstrated little or no changes in levels of AV/PI staining, with 80% of the cell population unstained, approximately 12% staining positive for annexin and only 9% with AV+PI+ staining.

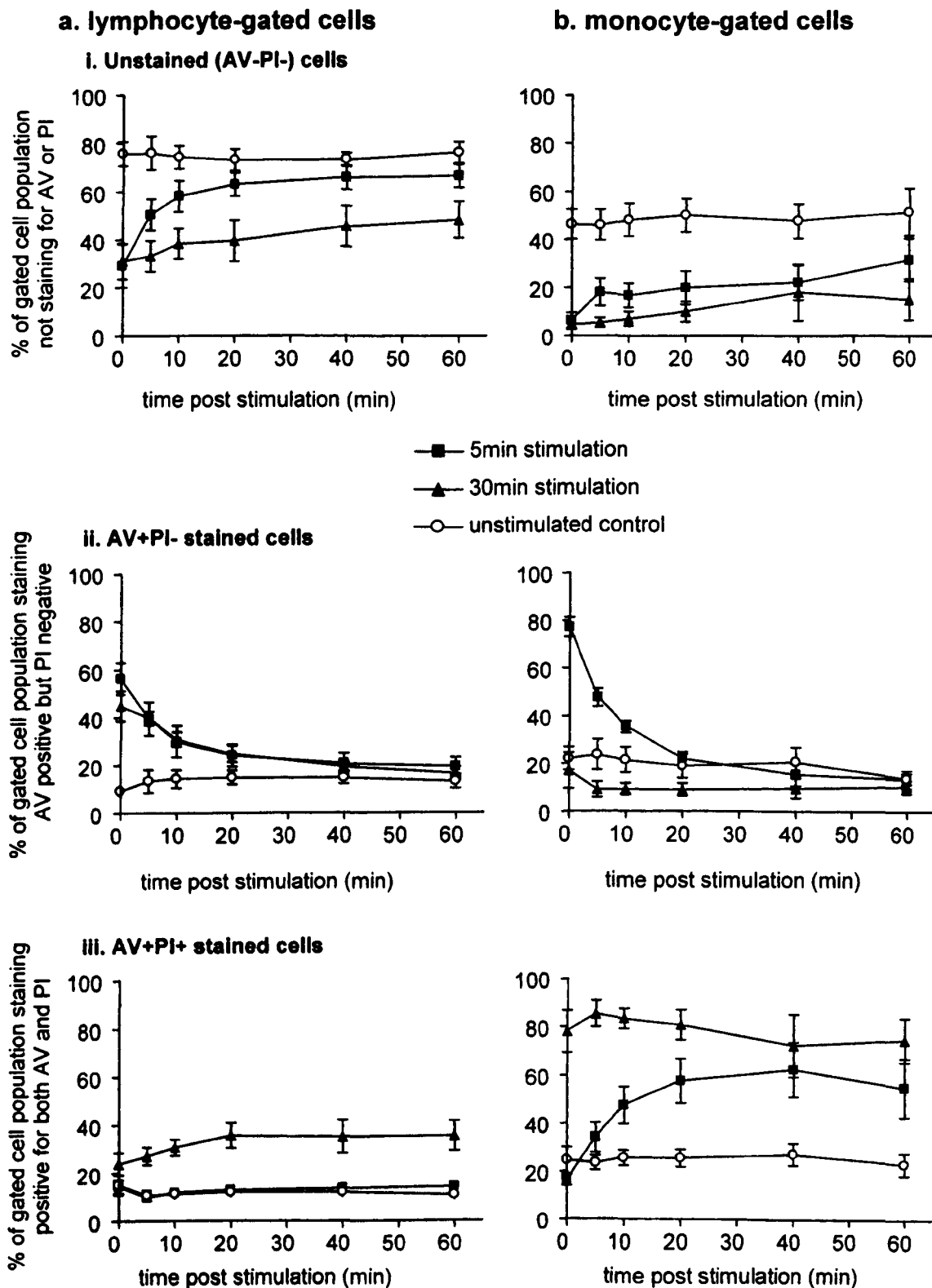
It is clear from these results that in THP-1 cells brief stimulation of the P2X<sub>7</sub> receptor for 5 minutes resulted in almost complete reversal of PS-flip. Stimulation for 30 minutes however, resulted in committed PS-flip in 80% of the population, with 50% of the population also staining positively for PI.

### **6.3.1.2. Reversibility of AV binding in PBMCs**

Following 5 minutes stimulation with BzATP,  $56 \pm 6.6\%$  of the lymphocyte-gated cell population exhibited AV+PI- staining compared with  $77 \pm 4.1\%$  of the monocyte-gated population (Fig 6.2.ii, solid squares). However, whereas 94% of the lymphocyte AV+PI- population reversed the PS-flip and became AV-PI- (Fig 6.2.ai), 76% of the monocyte AV+PI- population became AV+PI+ (Fig 6.2.biii) and only 24% reversed the PS flip (Fig 6.2.bi). This suggests that, based on positive PI staining, the monocyte-gated cells were much more susceptible to P2X<sub>7</sub>-stimulated cell death than the lymphocytes. After 30minutes stimulation,  $24 \pm 4.5\%$  of the lymphocyte-gated cells were stained AV+PI+ and this increased to  $35 \pm 6.4\%$  after 60 minutes incubation post-stimulation (Fig 6.2.aiii, solid triangles). However 58% of the AV+PI- population of cells still recovered and reversed the PS-flip. In contrast,

**a) Unstained (AV-PI-) cells****b) AV+PI- stained cells****c) AV+PI+ stained cells****Fig 6.1. Reversibility of AV binding in THP-1 cells**

Cells were stimulated for 5 or 30min with 500 $\mu$ M BzATP, then resuspended in AV binding buffer and incubated for up to 60min. AV/PI binding was measured by flow cytometry and cells with positive staining were expressed as a percent of the total cell population. Values for unstimulated control cells are also shown. Figures show the mean  $\pm$  sem of four separate experiments.



**Fig 6.2. Reversibility of AV binding in PBMCs**

Cells were stimulated for 5 or 30 minutes with 500 $\mu$ M BzATP, then resuspended in AV binding buffer and incubated for up to 60min. AV/PI binding was measured by flow cytometry and cells with positive staining were expressed as a percent of the lymphocyte or monocyte cell population gated with CD3 or CD14 respectively. Values for unstimulated control cells are also shown. Figures show the mean  $\pm$  sem of three separate experiments.

approximately 80% of the monocyte-gated cells were stained AV+PI+ at the end of the 30 minute stimulation period (Fig 6.2.biii), with little change throughout the subsequent incubation. The AV+PI- population of cells was less than the unstimulated control population.

The unstimulated control cells demonstrated higher levels of AV and PI staining in the monocyte-gated cells compared to lymphocyte-gated cells. Only approximately 45% of the monocyte-gated control cell population remained unstained (AV-PI-) throughout the experiment (Fig 6.2.bi, open circles), compared to 75% of the lymphocyte-gated cells (Fig 6.2.ai).

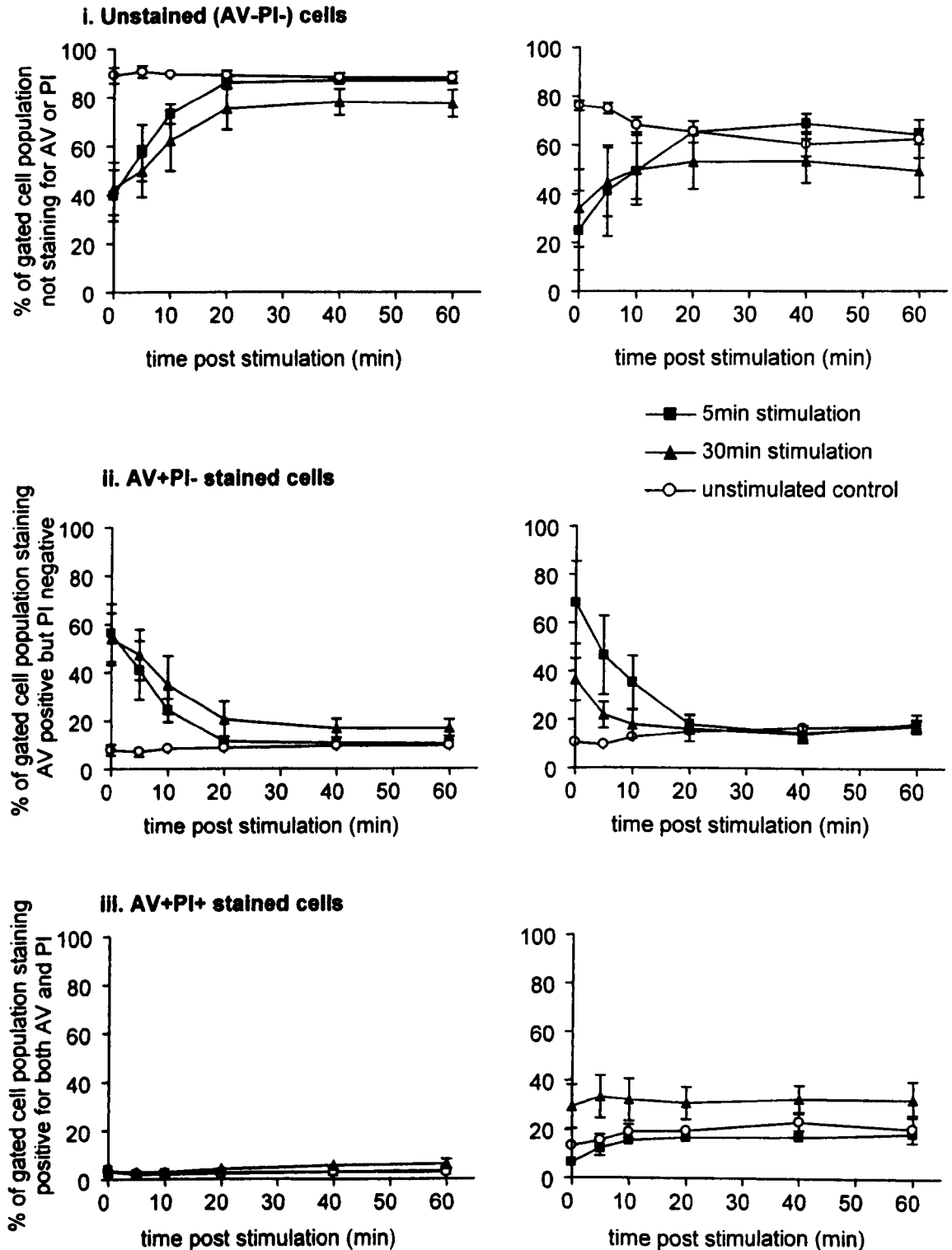
Hence, using PI staining as a marker of cell death, these results suggest that PBMC monocyte-gated cells are very susceptible to P2X<sub>7</sub>-stimulated cell death, and also demonstrate a greater level of spontaneous cell death than PBMC lymphocytes.

### **6.3.1.3. Reversibility of AV binding in LPMCs**

The lymphocyte-gated LPMCs demonstrated a similar pattern of staining after stimulation with BzATP for both 5 and 30 minutes (Fig 6.3.a). There was almost complete reversal of PS-flip in both cases and, unlike the PBMC lymphocytes, there was no increase in AV+PI+ staining even after 30 minutes stimulation (Fig 6.3.a.iii). Approximately 90% of the unstimulated control lymphocyte population remained unstained throughout the incubation, with approximately 9% AV+PI- and 3% AV+PI+. This showed that the cells were very stable and not subject to spontaneous cell death over the 60 minute incubation period.

The monocyte-gated LPMCs also appeared to be less susceptible to P2X<sub>7</sub>-stimulated PI staining than PBMC monocytes. After 5 minutes stimulation 68 ± 17% of the LPMC monocyte-gated cells were AV+PI-, falling to 18 ± 3.9% after 20 minutes incubation (Fig 6.3.bii). However, whereas 76% of the AV-positive PBMC monocytes became AV+PI+, only 20% of the AV+PI- LPMC monocyte-gated cells became PI-positive (Fig 6.3.biii) and 80% reversed the PS flip and became unstained (AV-PI-, Fig 6.3.bi).

After 30 minutes stimulation, approximately 30-35% of the LPMC monocyte-gated cells were AV+PI+ (Fig 6.3.biii), but this was much less than the level of 80% seen with the PBMC monocytes (Fig 6.2.biii). Furthermore, the

**a. lymphocyte-gated cells****b. monocyte-gated cells****Fig 6.3. Reversibility of AV binding in LPMCs**

Cells were stimulated for 5 or 30 minutes with  $500\mu\text{M}$  BzATP, then resuspended in AV binding buffer and incubated for up to 60min. AV/PI binding was measured by flow cytometry and cells with positive staining were expressed as a percent of the lymphocyte or monocyte cell population gated with CD3 or CD33 respectively. Values for unstimulated control cells are also shown. Figures show the mean  $\pm$  sem of three separate experiments.

decrease in the AV+PI- population during the subsequent incubation (Fig 6.3.bii) was matched by an increase in the unstained AV-PI- population (Fig 6.3.bi) and there was no increase in the AV+PI+ population. This suggests that some P2X<sub>7</sub>-stimulated cell death (based on increased PI staining) occurred during the 30 minute stimulation period, but there was no further increase once the agonist was removed and the cells which had been displaying PS flip recovered. Unstimulated control LPMC monocyte-gated cells also demonstrated less spontaneous cell death than the PBMC monocytes, with 60-80% of cells remaining unstained throughout the incubation.

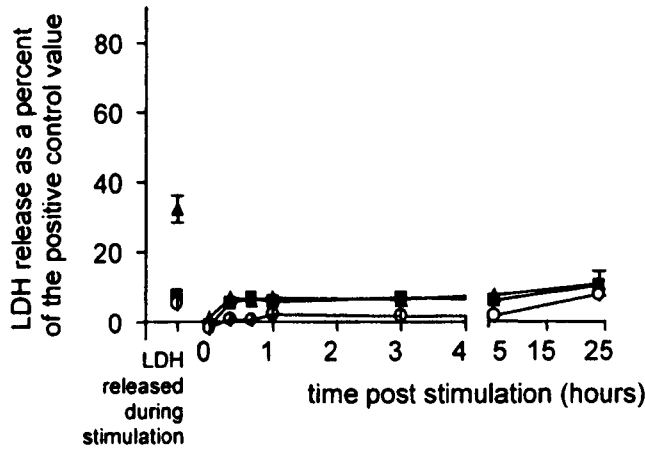
These results showed that following stimulation of the P2X<sub>7</sub> receptor for 5 minutes, all of the cells displayed increased AV-positive staining which was reversible, except for PBMC monocyte-gated cells which demonstrated an increase in AV+PI+ staining. Following 30 minutes stimulation with BzATP, all of the cells except for LPMC lymphocytes demonstrated increased levels of AV+PI+ staining which increased only a little once the agonist had been removed.

Based on the results for PI staining therefore, PBMC monocytes appear to be highly susceptible to cell death from P2X<sub>7</sub> stimulation, but also spontaneously probably as a result of cell handling and the experimental procedures. THP-1 cells and LPMC monocytes were much less susceptible to P2X<sub>7</sub>-stimulated cell death, and the lymphocyte-gated cells were even less so. This was particularly true of LPMC lymphocyte-gate cells in which there was no increase in AV+PI+ staining for either stimulation time.

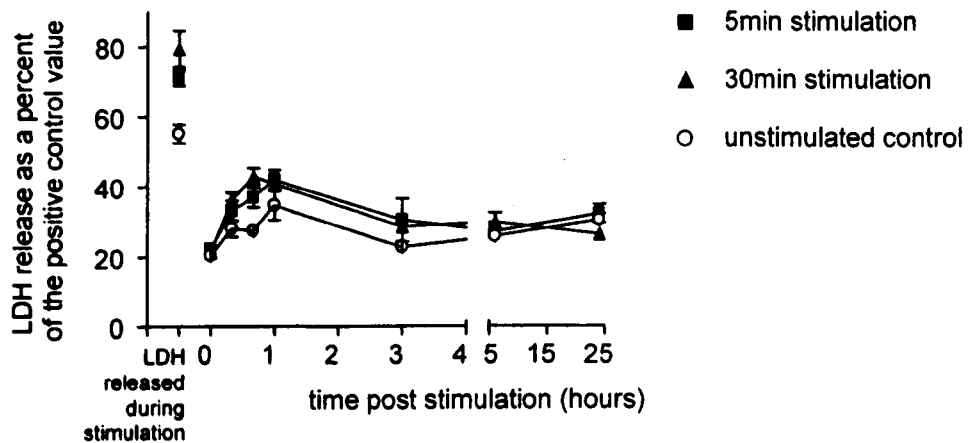
### **6.3.2. Release of LDH from cells stimulated for 5 or 30 minutes**

The release of LDH was used to confirm whether prolonged stimulation of the P2X<sub>7</sub> receptor with BzATP resulted in cell death. Supernatants were collected from the cells set up for Hoechst staining and levels of LDH were measured. The amount of LDH released by the cells was expressed as a percent of the total amount released by lysed freshly isolated cells, and the results are shown in Fig 6.4.

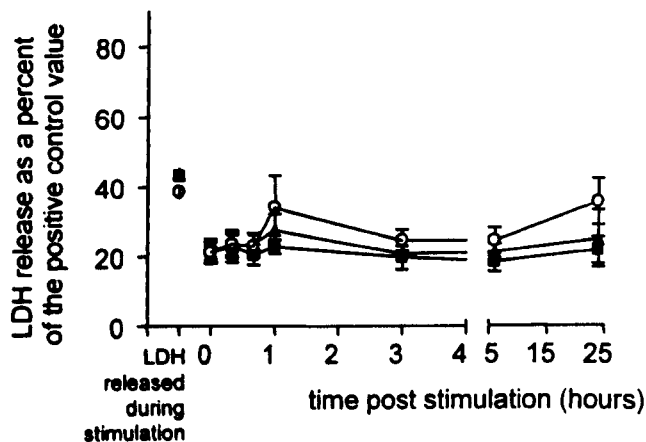
## a) THP-1 cells



## b) PBMCs



## c) LPMCs



**Fig 6.4. Release of LDH from mononuclear cells stimulated with 500 $\mu$ M BzATP for 5 or 30 minutes**

The amount of LDH released into the supernatant after 5 or 30min stimulation with BzATP is shown. 'LDH released during stimulation' indicates the amount of LDH released during the stimulation period with BzATP; the time 0 symbols represent the amount of LDH present immediately after resuspension in RPMI 1640. The results have been expressed as a percent of the positive control and represent the mean  $\pm$  sem of three separate experiments.



LDH release increased for up to 1 hour post stimulation in all the cell types but there was no further increase after that time. The observed increase was very small (approximately 6%) for THP-1 cells and LPMCs, but more marked (approximately 20%) for PBMCs. The overall amount of LDH release was also greater from PBMCs than either LPMCs or THP-1 cells. For all the cell types however, there was little difference between the levels released by the BzATP-stimulated cells and the unstimulated controls.

In contrast, there were differences in the amount of LDH released into the supernatants during the stimulation period. In THP-1 cells, LDH release during the 5 minute stimulation period was similar to that of the unstimulated control (Fig 6.4.a). After 30 minutes stimulation however, LDH release increased four-fold to  $32 \pm 3.9\%$  of the positive control value, a figure comparable to the level of AV+PI+ staining (36%, Fig 6.1.b). This confirms that stimulation of THP-1 cells for 30 minutes results in the death of approximately 30% of the cell population. During the subsequent incubation following removal of agonist, LDH release was the same for both the 5- and 30-minute stimulated cells, suggesting that it was not directly related to duration of P2X<sub>7</sub> stimulation.

PBMCs released a large amount of LDH during both stimulation periods (70-80% of the positive control value), but the release was also high in the unstimulated control cells (55%). This was probably due to handling procedures, e.g. centrifugation, and confirms that PBMCs are very susceptible to cell death. This was also indicated by their elevated levels of AV+PI+ staining, particularly in the monocyte-gated cells, following stimulation with BzATP.

LPMCs released less LDH during stimulation than PBMCs and there was very little difference between the amounts released by the BzATP-stimulated cells and the unstimulated controls. The levels of LDH release during the subsequent incubation were also at or below the level of the unstimulated controls.

Because LDH release from the different PBMC and LPMC cell populations cannot be distinguished, it is not possible to directly correlate the release with the AV+PI+ staining for each cell type. However the data for the THP-1 cells implies that AV+PI+ staining is an indication of cell death, and the

results for AV+PI+ staining for the PBMCs and LPMCs suggest that the source of the LDH is more likely to be the monocytes.

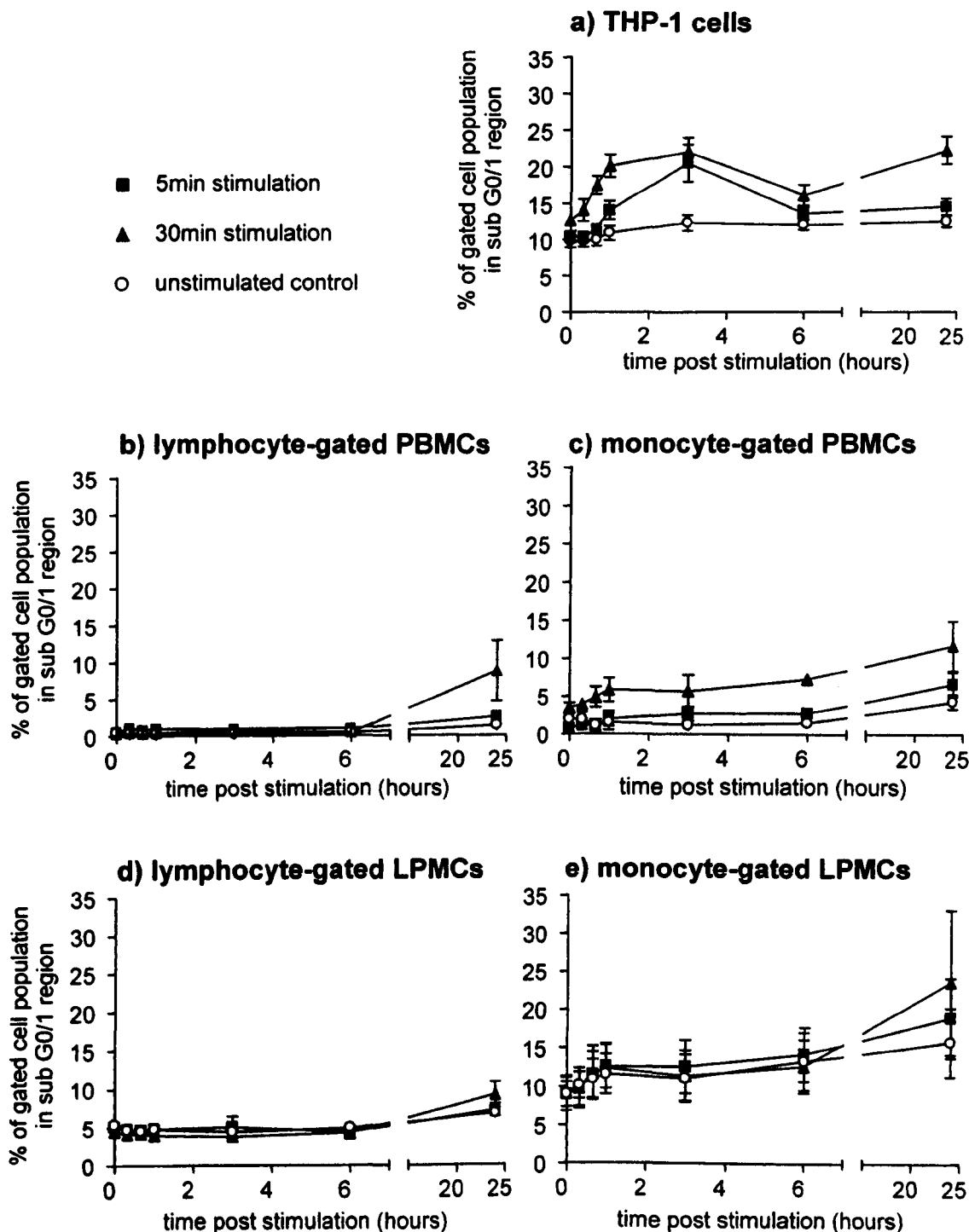
The results for LDH release and AV binding together therefore indicate that prolonged stimulation of the P2X<sub>7</sub> receptor does lead to cell death, particularly in monocyte cells, but the effect appears to occur only in the presence of the agonist. Le Stunff, H *et al.*, (2004) demonstrated a similar effect in mouse thymocytes and showed that LDH release was reduced by 40% when ATP (1mM) was removed after 30 minutes incubation compared to sustained incubation for 5 hours.

### **6.3.3. P2X<sub>7</sub>-stimulated apoptosis determined by cell cycle analysis**

During apoptosis, DNA is broken down by endonucleases within the cell creating fragments which can be removed by washing. Cell cycle analysis measures the number of cells with less than a normal complement of DNA (sub-G<sub>0</sub>/1 fraction) and therefore gives an indication of the number of apoptotic cells within the population. The method was used to determine whether P2X<sub>7</sub>-stimulation for 5 or 30 minutes induces apoptosis. The PBMC and LPMC cell populations were gated using the FS/SS dotplots. Because the cells had been fixed prior to analysis, the LPMC monocyte and neutrophil populations were indistinguishable and were therefore included in a single gate. However, since neutrophils do not express P2X<sub>7</sub> receptors on their cell surface (Gu, BJ *et al.*, 2000), any responses could be attributed to the monocyte cells. Neutrophils are known to undergo spontaneous apoptosis (Scheel-Toellner, D *et al.*, 2004) but this was accounted for by the values for the unstimulated controls. The results are shown in Fig 6.5.

THP-1 cells demonstrated the greatest increase in number of apoptotic cells, from approximately 10% of the cell population to approximately 20% over the first 3 hours post-stimulation (Fig 6.5.a). There was little difference however between the numbers of apoptotic cells for both 5- and 30-minute stimulation times.

Lymphocyte-gated PBMCs demonstrated no induction of apoptosis at all following 5 minutes stimulation with BzATP (Fig 6.5.b), and only a small rise ( $8.9 \pm 4.1\%$  of gated cell population) after 30 minutes stimulation and 24 hours incubation. The monocyte-gated PBMCs stimulated with BzATP for 30



**Fig 6.5. Induction of apoptosis in BzATP-stimulated mononuclear cells, determined by cell cycle analysis**

After 5 or 30min stimulation with 500 $\mu$ M BzATP cells were resuspended in RPMI 1640 and incubated for up to 24h. Cells were processed for DNA analysis as described in section 6.2.3 and 2.4.2. For the mixed cell populations, the single-cell gate was combined with a gate identifying lymphocyte and monocyte cells (based on FS/SS plot). Cell fluorescence was analysed using Cylchred (Terry Hoy, Cardiff University) and the graph represents the percent of gated cells in the sub-G0/1 peak. Results show the mean  $\pm$  sem of three separate experiments. The higher percentage of apoptotic cells in the monocyte-gated LPMC unstimulated control was probably due to the neutrophil population, which could not be separated by gating.

minutes had increased numbers of apoptotic cells at all time points compared to the unstimulated controls, but the increase was small (Fig 6.5.c).

There was no increase in the percentage of apoptotic cells in the LPMC lymphocyte-gated cell population following BzATP stimulation for 5 or 30 minutes, and only a small rise in the monocyte-gated cell population following 24 hours incubation (Fig 6.5.d & e). LPMCs had a higher percentage of apoptotic cells in the unstimulated controls than the PBMCs suggesting a higher level of spontaneous apoptosis, but this was probably due to the neutrophil population.

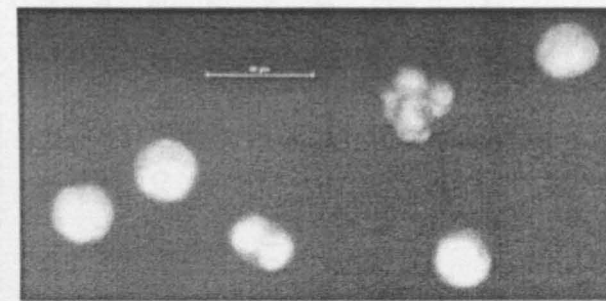
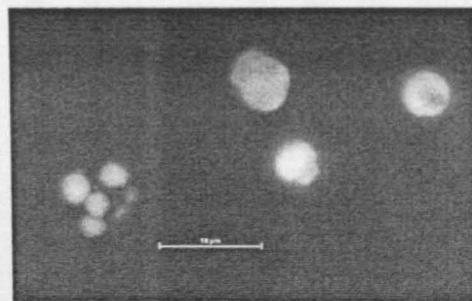
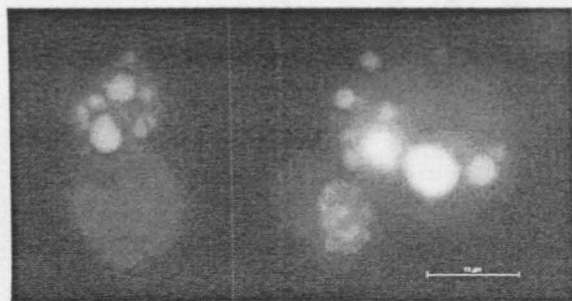
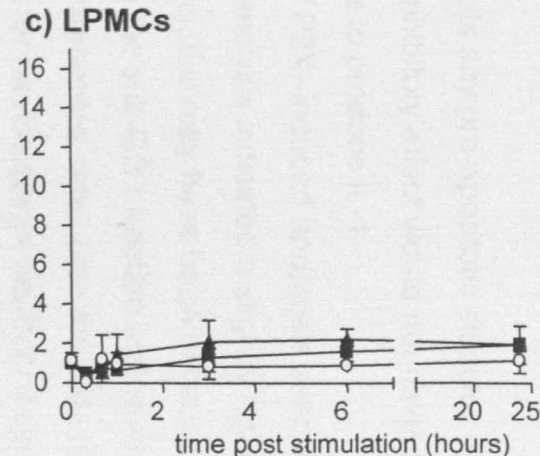
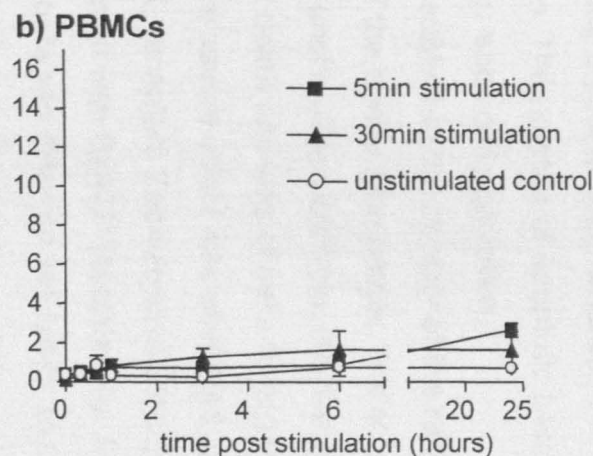
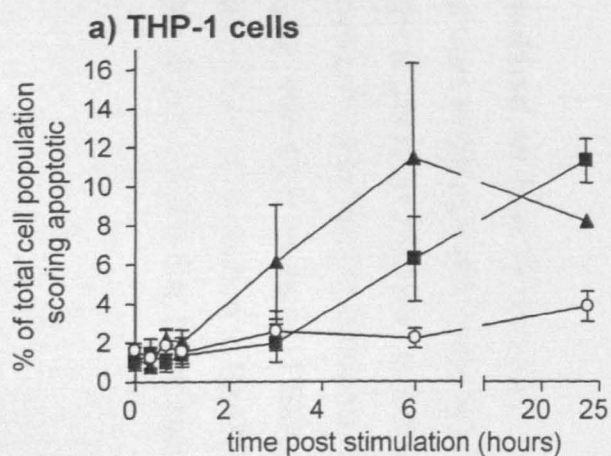
The results for cell cycle analysis indicated that prolonged P2X<sub>7</sub> stimulation induced some apoptosis in human PBMCs, particularly the monocyte-gated cells, and in THP-1 cells, but had little effect on human LPMCs.

#### **6.3.4. P2X<sub>7</sub>-stimulated apoptosis determined by Hoechst staining**

Induction of apoptosis by P2X<sub>7</sub> stimulation was also measured using the nucleic acid dye, Hoechst 33342. The cell-permeable dye fluoresces on binding to DNA allowing visual detection of nuclei. During apoptosis the nuclear chromatin condenses making the staining brighter, and subsequent fragmentation of the nucleus itself allows clear identification of apoptotic cells. The number of visible apoptotic cells was expressed as a percent of the total cell count and the results are shown in Fig 6.6.

The results indicate that P2X<sub>7</sub> receptor activation induced very little apoptosis during the 24 hours following BzATP stimulation of the cells. The greatest effect was seen with THP-1 cells in which 6-12% of the cells became apoptotic during the first 6 hours post stimulation, but there was little difference between the cells stimulated for 5 or 30 minutes. This concurred with the results for cell cycle analysis in which 10-20% of the cells appeared apoptotic following both 5 and 30 minutes stimulation.

With PBMCs and LPMCs there were very few apoptotic cells, only approximately 1-2% of the cell population. It has been shown that apoptotic cell death in monocytes is inhibited by stimulation with LPS or cytokines such as IL-1 $\beta$  and TNF- $\alpha$  (Mangan, DF *et al.*, 1991). It may be therefore that

Scale bar = 10 $\mu$ m

**Fig 6.6. Induction of apoptosis in BzATP-stimulated mononuclear cells, determined by Hoechst staining**

After 5 or 30min stimulation with 500 $\mu$ M BzATP cells were resuspended in RPMI 1640 and incubated for up to 24h. Cells were processed for Hoechst staining as described in section 6.2.4. The graphs show the number of apoptotic cells scored, expressed as a percent of the total cells counted. Results show the mean  $\pm$  sem of three separate experiments. Values for the unstimulated controls are also shown.

P2X<sub>7</sub>-stimulated IL-1 $\beta$  release inhibits any pro-apoptotic stimulus also associated with the receptor. This inhibitory effect would not have occurred with THP-1 cells which were unable to produce IL-1.

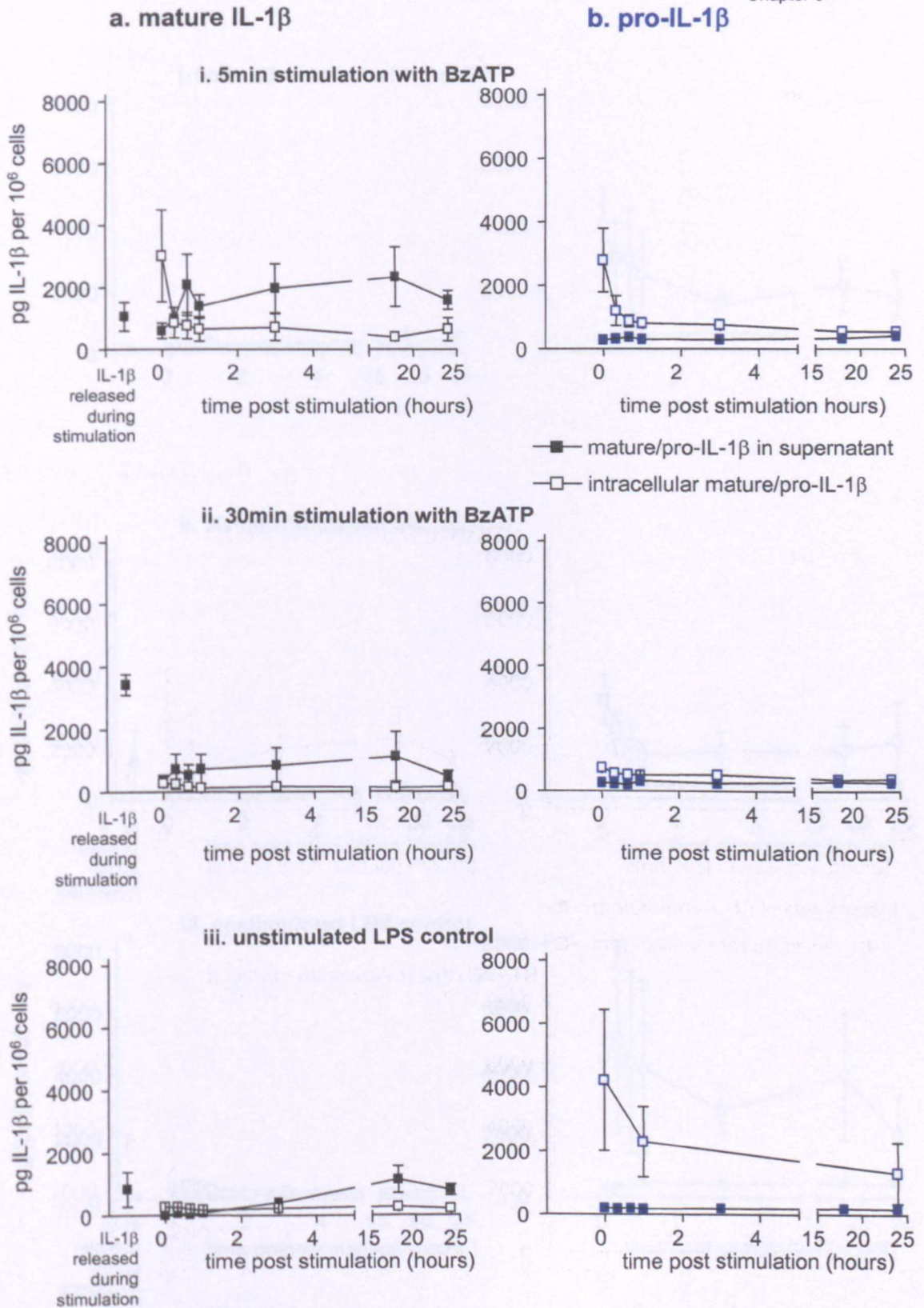
These results confirmed the lack of P2X<sub>7</sub>-induced apoptosis seen with cell cycle analysis. Although cell cycle analysis indicated a slightly higher level of apoptotic cells than Hoechst staining, this may have been because the data analysis also included cell debris in the sub-G0/1 fraction and would therefore include some dead and fragmented cells. Also the data for cell cycle analysis was divided into that for monocytes (+ neutrophils in the case of LPMCs) and lymphocytes by gating on the dotplot, whereas the Hoechst data was for the total cell population. The number of apoptotic cells would therefore be proportionally higher for each cell population.

For all of the cell types, it was noticeable that the density of the cells on the 24-hour slides was less than that of the earlier time points. The effect was not so marked on the slides for the unstimulated controls, indicating that P2X<sub>7</sub> stimulation led to necrotic cell death and loss of cells during the incubation period. A similar but more severe effect was observed in a study using HEK cells expressing rat P2X<sub>7</sub> receptors that demonstrated complete loss of all cells 6 hours post-stimulation with BzATP (MacKenzie, A *et al.*, 2001). This illustrates the variation between different cell types and also different species; BzATP has been shown to be a more potent agonist at rat P2X<sub>7</sub> receptors than at human ones (Hibell, AD *et al.*, 2000).

### **6.3.5. IL-1 $\beta$ release from cells stimulated for 5 or 30 minutes**

The release of mature and pro-IL-1 $\beta$  from PBMCs and LPMCs following BzATP-stimulation for 5 or 30 minutes is shown in Figs 6.7. and 6.8. The graphs show that whilst both cell types contained similar amounts of intracellular pro-IL-1 $\beta$  (Fig 6.7.b & 6.8.b, blue open squares) PBMC monocytes released more of the mature form in response to BzATP stimulation than the LPMC monocytes (Fig 6.7.a & 6.8.a, black solid squares).

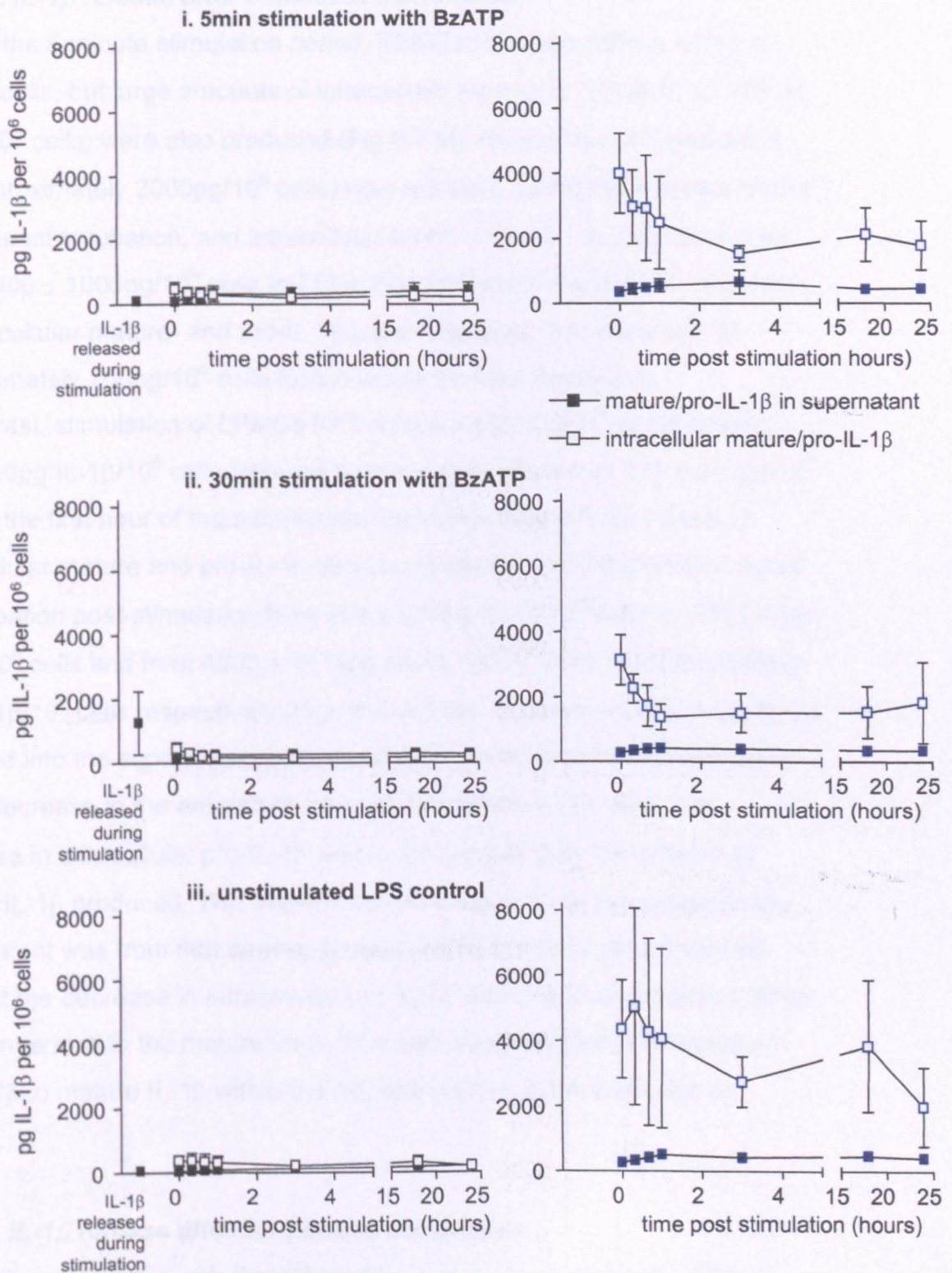




**Fig 6.7. Release of IL-1 $\beta$  from PBMCs stimulated with BzATP for 5 or 30 minutes**

After 5 or 30min stimulation with 200 $\mu$ M BzATP, cells were resuspended in RPMI 1640 containing 0.1% FCS with 1 $\mu$ M LPS and incubated for up to 24h. IL-1 $\beta$  released into the supernatant was measured by ELISA (closed symbols). After removal of the supernatant, cells were treated with 0.1% saponin to release intracellular IL-1 $\beta$  (open symbols). Mature IL-1 $\beta$  release is shown in the figures on the left and pro-IL-1 $\beta$  release on the right. Results show the mean  $\pm$  sem of three separate experiments.



a. mature IL-1 $\beta$ b. pro-IL-1 $\beta$ 

**Fig 6.8. Release of IL-1 $\beta$  from LPMCs stimulated with BzATP for 5 or 30 minutes**

After 5 or 30min stimulation with 200 $\mu$ M BzATP, cells were resuspended in RPMI 1640 containing 0.1% FCS with 1 $\mu$ M LPS and incubated for up to 24h. IL-1 $\beta$  released into the supernatant was measured by ELISA (closed symbols). After removal of the supernatant, cells were treated with 0.1% saponin to release intracellular IL-1 $\beta$  (open symbols). Mature IL-1 $\beta$  release is shown in the figures on the left and pro-IL-1 $\beta$  release on the right. Results show the mean  $\pm$  sem of three separate experiments.



### **6.3.5.1. IL-1 $\beta$ release after 5 minutes stimulation**

During the 5 minute stimulation period, PBMCs released  $1055 \pm 454$ pg IL-1 $\beta$ /10<sup>6</sup> cells, but large amounts of intracellular mature IL-1 $\beta$  ( $3011 \pm 1487$  pg IL-1 $\beta$ /10<sup>6</sup> cells) were also produced (Fig 6.7.ai). Most of this intracellular IL-1 $\beta$  (approximately 2000pg/10<sup>6</sup> cells) was released during the first hour of the subsequent incubation, and intracellular levels of pro-IL-1 $\beta$  also decreased from  $2800 \pm 1005$ pg/10<sup>6</sup> cells to  $756 \pm 27$ pg/10<sup>6</sup> cells (Fig 6.7.bi). The levels of intracellular mature- and pro-IL-1 $\beta$  (open squares) then remained at approximately 700pg/10<sup>6</sup> cells for the whole 24 hour incubation.

In contrast, stimulation of LPMCs for 5 minutes with BzATP released only  $100 \pm 50$ pg IL-1 $\beta$ /10<sup>6</sup> cells followed by the further release of  $321 \pm 245$ pg/10<sup>6</sup> cells in the first hour of the subsequent incubation (Fig 6.8.ai). Levels of intracellular mature and pro-IL-1 $\beta$  decreased steadily during the first 3 hours of incubation post-stimulation from  $454 \pm 231$ pg IL-1 $\beta$ /10<sup>6</sup> cells to  $186 \pm 47$ pg IL-1 $\beta$ /10<sup>6</sup> cells and from  $4003 \pm 1219$ pg pro-IL-1 $\beta$ /10<sup>6</sup> cells to  $1525 \pm 248$ pg pro-IL-1 $\beta$ /10<sup>6</sup> cells respectively (Figs 6.8.ai & bi). The amount of mature IL-1 $\beta$  released into the supernatant post stimulation corresponded approximately to the decrease in the amount of intracellular mature IL-1 $\beta$ . Also, the decrease in intracellular pro-IL-1 $\beta$  was much greater than the amount of mature IL-1 $\beta$  produced. This implies that the mature IL-1 $\beta$  released into the supernatant was from that already present within the cell post-stimulation and that the decrease in intracellular pro-IL-1 $\beta$  was due to degradation rather than conversion to the mature form. This also suggests that conversion of pro-IL-1 $\beta$  to mature IL-1 $\beta$  within the cell only occurs in the presence of agonist.

### **6.3.5.2. IL-1 $\beta$ release after 30 minutes stimulation**

Stimulation of PBMCs with BzATP for 30 minutes released  $3435 \pm 329$ pg mature IL-1 $\beta$ /10<sup>6</sup> cells into the supernatant but there was very little intracellular pro-IL-1 $\beta$  ( $756 \pm 157$ pg/10<sup>6</sup> cells) or mature IL-1 $\beta$  ( $283 \pm 124$ pg/10<sup>6</sup> cells) present within the cells following stimulation (Fig 6.7.a.ii &

bii). During the subsequent incubation further release of mature IL-1 $\beta$  reached a maximum of approximately 700pg/10<sup>6</sup> cells during the first hour. LPMCs stimulated for 30 minutes released 1255  $\pm$  995pg mature IL-1 $\beta$ /10<sup>6</sup> cells into the supernatant (Fig 6.8.aii). However the amount released by LPMCs from different patients was highly variable; one subject releasing over 3000pg IL-1 $\beta$ /10<sup>6</sup> cells whilst the other two released only 120 and 406pg/10<sup>6</sup> cells, amounts similar to those produced after 5 minutes stimulation. The subject who produced over 3000pg IL-1 $\beta$ /10<sup>6</sup> cells had received radical radiotherapy for prostate cancer and although there was no obvious inflammation of the tissue, radiotherapy has been shown to increase levels of eicosanoid inflammatory mediators in the bowel (Cole, AT *et al.*, 1993). During the subsequent incubation period, 254  $\pm$  158pg IL-1 $\beta$ /10<sup>6</sup> cells were released from the LPMCs within the first hour, an amount similar to that released following 5 minutes stimulation. Levels of intracellular mature and pro-IL-1 $\beta$  were also very similar to those seen after stimulation for 5 minutes.

Neither PBMCs nor LPMCs released substantial amounts of pro-IL-1 $\beta$  into the supernatant during incubation post-stimulation (Fig 6.7.b & 6.8.b, solid symbols). This shows that the decrease in intracellular pro-IL-1 $\beta$  was due to degradation within the cell and not release due to cell death and breakdown. It is clear from these results that LPMCs stimulated with BzATP did not produce or release large amounts of mature IL-1 $\beta$  regardless of the length of stimulation, and that high levels of pro-IL-1 $\beta$  produced within the cell in response to LPS were quickly degraded. This is confirmed by the results for the LPS control (Fig 6.8.aiii & biii) in which large amounts of pro-IL-1 $\beta$  were produced intracellularly but very little of this was converted to mature IL-1 $\beta$  or secreted.

In contrast, PBMCs produced large amounts of mature IL-1 $\beta$  and this was rapidly released in response to P2X<sub>7</sub> stimulation. LPS alone also released mature IL-1 $\beta$  from PBMCs but required a long incubation time. After 18 hours incubation the amount of IL-1 $\beta$  released by cells incubated with LPS alone was 1147  $\pm$  433pg IL-1 $\beta$ /10<sup>6</sup> cells (Fig 6.7.aiii), almost 10-fold more than that

produced by LPMCs, and confirming the sensitivity of PBMC monocytes to bacterial endotoxin compared to that of LPMC macrophages.

PBMCs generally secreted approximately 10 times more mature IL-1 $\beta$  than LPMCs, but stimulation for 30 minutes as opposed to 5 minutes did not increase the total amount of cytokine released. The longer stimulation time resulted in a greater release of IL-1 $\beta$  during the BzATP stimulation period but less during the subsequent incubation in the absence of agonist. Two studies have showed that a long stimulation time with ATP is not required for maximum IL-1 $\beta$  release. Kahlenberg, JM *et al.*, (2004b) and Colomar, A *et al.*, (2003) in studies using Bac1 macrophages and mouse Schwann cells respectively, stimulated the cells with ATP for 30 minutes or for 5 minutes followed by 25 minutes incubation in the absence of agonist, and found that similar amounts of IL-1 $\beta$  were released under both conditions.

#### **6.4. SUMMARY OF THE REVERSIBILITY OF THE EFFECTS OF P2X<sub>7</sub> STIMULATION**

The results for AV/PI binding showed that, in all the cell types, brief (5min) stimulation of the P2X<sub>7</sub> receptor induced increased AV binding that was not associated with cell death and that reversed when the stimulus was removed. The only exception was PBMC monocytes in which 40% of the cell population did not reverse the AV binding and also stained positive for PI. This confirmed the susceptibility of these cells to P2X<sub>7</sub>-stimulated cell death as seen in the previous chapter (section 5.2), based on PI staining as a measure of cell death. It always has to be considered however with PI staining in monocytes, that some component is due to uptake through the pore rather than cell death.

Prolonged stimulation for 30 minutes led to high levels of AV+PI+ staining in THP-1 cells (50%) and PBMC monocytes (80%), but only a small increase in LPMC monocytes above control levels. Lymphocyte-gated cells from both PBMCs and LPMCs largely reversed the AV-positive binding following 5 or 30 minutes stimulation; only PBMC lymphocytes stimulated for 30 minutes demonstrated irreversible PS flip and an increase in PI staining.

Using LDH release as a measure of cell death suggested that the P2X<sub>7</sub> receptor mediated cell death during stimulation but there was little effect following removal of the agonist. This agreed with the results for PI staining which were elevated post stimulation and remained at the same level during subsequent incubation. Only THP-1 cells demonstrated a marked difference in the amount of LDH released following 5 or 30 minutes stimulation, and only PBMCs exhibited any substantial release after 5 minutes stimulation with BzATP. LPMCs released very little LDH above control values highlighting their lack of response to P2X<sub>7</sub> stimulation. These results concurred with those for AV/PI binding in that PBMCs were most affected by P2X<sub>7</sub> stimulation.

P2X<sub>7</sub> did not appear to induce significant apoptotic cell death, measured by cell cycle analysis or Hoechst staining, in any of the cell populations, however it is possible that the cells were not stimulated for long enough to demonstrate an effect. Stimulation for 30 minutes has been shown in several studies to be sufficient for induction of apoptosis (MacKenzie, A *et al.*, 2001; Nihei, OK *et al.*, 2000a; Coutinho-Silva, R *et al.*, 1999), but the susceptibility of cells to P2X<sub>7</sub>-mediated death will obviously vary with cell type and the incubation conditions. Many studies of apoptotic cell death induced by P2X<sub>7</sub> stimulated the cells for several hours or with very high concentrations of agonist (Ferrari, D *et al.*, 1999; Le Stunff, H *et al.*, 2004; Lepine, S *et al.*, 2006).

It is possible that isolating the separate monocyte and lymphocyte populations and treating them separately might have shown an effect, particularly with the PBMC monocytes. In fact, this was implied by the differences in the results for Hoechst staining, which were expressed as a percent of the total cell population, and cell cycle analysis, in which the two cell populations were gated and counted separately. For THP-1 cells both methods of analysis gave an approximate increase of 10% apoptotic cells above control values. For the PBMCs and LPMCs however, whilst Hoechst staining measured an approximate increase of 2% apoptotic cells, cell cycle analysis gave an increase in apoptotic cell number of approximately 7% above control values. Lymphocytes have been shown throughout all of the experiments to be less responsive to P2X<sub>7</sub> stimulation than monocytes, and

because they make up the largest fraction of the PBMC and LPMC cell populations they may have masked a greater response in the monocyte cells.

Overall these results suggest that, in the cell types studied here, the P2X<sub>7</sub> receptor mediates necrotic cell death, and monocyte cells are more susceptible than lymphocyte cells, probably because of their higher levels of receptor expression. P2X<sub>7</sub> appears not to induce apoptotic cell death but this may require stimulation for longer than 30 minutes in these cells.

#### **6.4.1. P2X<sub>7</sub>-mediated cell death**

Mechanisms of P2X<sub>7</sub>-mediated cell death are still not understood but have been studied by many groups who have shown that cell lysis and induction of apoptosis occur via different signalling pathways.

P2X<sub>7</sub> mediated cell lysis has been shown to involve signalling via the ERK1/2 MAPK pathway (Auger, R *et al.*, 2005). MAPKs (mitogen-activated protein kinases) compose a family of protein kinases that regulate cellular activities ranging from gene expression, mitosis, movement, metabolism and apoptosis (Johnson, GL & Lapadat, R, 2002). There are three well characterised families of MAPKs; ERKs (extracellular signal-regulated kinases, mainly involved in cell growth and proliferation), JNKs (c-jun NH<sub>2</sub>-terminal kinases, activated by cellular stress) and p38 enzymes (activated by inflammatory cytokines, endotoxins or osmotic shock, and associated with apoptosis). ATP stimulation of murine thymocytes has been shown to activate all three types of MAPK enzymes but only the ERK1/2 pathway was associated with necrotic death and cell lysis (Auger, R *et al.*, 2005), and activation involved the N-terminal chain of the receptor (Amstrup, J *et al.*, 2003).

The ERK1/2 signalling pathway is not involved in PS exposure or pore formation, which are regulated by the C-terminal chain (Auger, R *et al.*, 2005). Other studies using THP-1 cells have shown that p38 MAPKs are involved in pore formation (Donnelly-Roberts, D *et al.*, 2004).

Two of the principal characteristics of apoptotic cell death are cell shrinking and membrane blebbing. Chloride ions entering cells via the P2X<sub>7</sub>-stimulated pore have been associated with cell shrinkage and apoptotic death in DT40

B cells transfected with the rat P2X<sub>7</sub> receptor (Tsukimoto, M *et al.*, 2005).

The same group later showed that chloride ions had no effect on ERK1/2 activity confirming that these enzymes are not involved in cell shrinking and apoptotic cell death (Tsukimoto, M *et al.*, 2006).

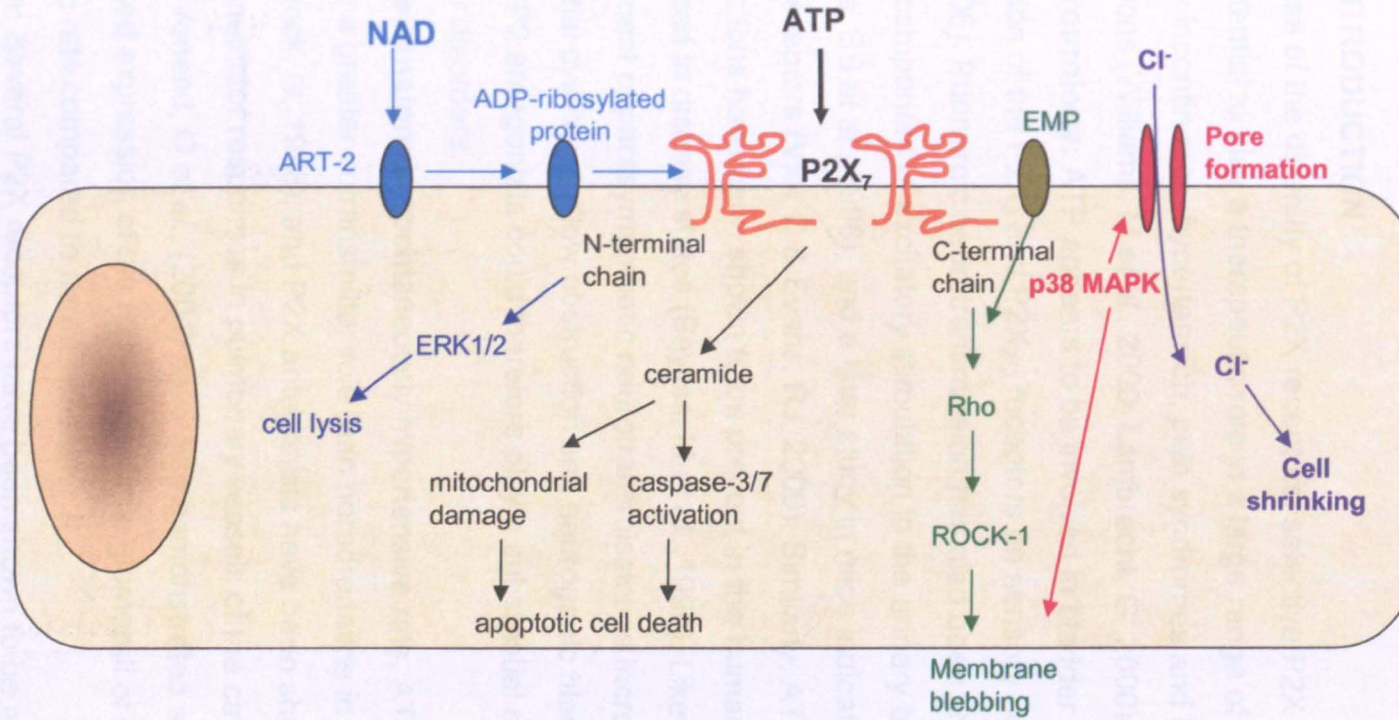
Several groups have studied membrane blebbing associated with P2X<sub>7</sub> stimulation and have shown it to be associated with activation of p38 MAPK and ROCK -1 (Rho-effector kinase-1) (Pfeiffer, ZA *et al.*, 2004; Morelli, A *et al.*, 2003; Verhoef, PA *et al.*, 2003). The ROCK enzymes are important regulators of cell growth, migration and apoptosis through control of actin cytoskeletal assembly (Noma, K *et al.*, 2006). Wilson, HL *et al.*, (2002) demonstrated that the C-terminus of the P2X<sub>7</sub> receptor is required for blebbing and that it mediates the process via an interaction with members of the epithelial membrane protein family (EMP-1, -2, -3, and PMP-22).

Many of the changes associated with apoptotic cell death are brought about by effector caspase enzymes. ATP stimulation of RAW 264.7 macrophages was shown to lead to the synthesis and accumulation of ceramide, an apoptogenic sphingolipid, which was required for activation of caspase-3 and caspase-7 (Raymond, MN *et al.*, 2006). A similar P2X<sub>7</sub>-stimulated accumulation of ceramide was found in thymocytes, but in these cells there was resulting mitochondrial damage rather than caspase activation (Lepine, S *et al.*, 2006).

The natural agonist for the P2X<sub>7</sub> receptor is ATP but T cell death has also been shown to be induced by NAD (nicotine adenine dinucleotide) acting at the receptor (Seman, M *et al.*, 2003; Kawamura, H *et al.*, 2005). NAD is a substrate for ART-2 (ADP-ribosyl transferase), an ectoenzyme that transfers ADP-ribosyl groups onto cell surface proteins. Binding of the ADP-ribosyl group to P2X<sub>7</sub> has been shown to induce rapid cell death within seconds (Kawamura, H *et al.*, 2005). Death was characterised by increased AV binding and pore formation, and was instigated by low NAD concentrations (<10 $\mu$ M). It was proposed that NAD-induced cell death provides a safeguard mechanism, operating during conditions of trauma when NAD would be released from cells. T cells activated by APCs shed ART-2 and are therefore

not susceptible to NAD-induced cell death, but bystander T cells would bind NAD and die, thus preventing the activation of an irrelevant response.

It is clear therefore that P2X<sub>7</sub>-mediated cell death involves a delicate balance between many complicated cellular pathways (Fig 6.9.). The P2X<sub>7</sub> receptor appears to cause cell death by pore formation and cell lysis in certain susceptible cell types, but requires prolonged stimulation for induction of apoptosis. The two types of cell death involve different signalling pathways and do not appear to be associated with IL-1 $\beta$  release. This was demonstrated by the short stimulation time required for IL-1 $\beta$  release and the lack of pro-IL-1 $\beta$  secretion despite high intracellular concentrations. A study on ROCK-dependent membrane blebbing has also shown that it is dissociated from IL-1 $\beta$  release (Verhoef, PA *et al.*, 2003).



**Fig 6.9. P2X<sub>7</sub>-stimulated mechanisms of cell death**

Stimulation of the P2X<sub>7</sub> receptor by ATP leads to activation of different MAP kinase enzymes, some of which are involved in cell lysis (ERK1/2) and some in pore formation and apoptosis (p38 MAPK). Other aspects of apoptotic cell death such as cell shrinking and caspase activation have been shown to involve chloride ions and production of ceramide, an apoptogenic sphingolipid. The N-terminal chain of the receptor has been shown to be involved in cell lysis whereas the C-terminal chain is important for pore formation and membrane blebbing. In T cells, P2X<sub>7</sub> has also been shown to be activated by NAD via ADP-ribosylation of surface proteins.

ART-2: ADP-ribosyl transferase; NAD: nicotine adenine dinucleotide; EMP: epithelial membrane protein

Rho: a GTPase enzyme important in migration and stress fibre formation; ROCK-1: Rho-effector kinase 1



## CHAPTER 7. THE EFFECTS OF P2X<sub>7</sub> ANTAGONISTS ON IL-1 $\beta$ RELEASE FROM HUMAN COLONIC TISSUE AND FROM ISOLATED LPMCs

### 7.1. INTRODUCTION

Because of the diversity of P2X receptors, selective P2X antagonists have the potential to play a therapeutic role in a large range of diseases such as urinary incontinence, hypertension, pain syndromes and inflammatory conditions (Williams, M *et al.*, 2000; Lambrecht, G, 2000).

**i. Neurourology:** ATP appears to be involved in bladder sensation via activation of the P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors on sensory neurons (Ford, AP *et al.*, 2006). Purinergic neurotransmission has also been shown to represent a major component of excitatory stimulation in the urinary bladder of rats (Hegde, SS *et al.*, 1998), and a later study in mice indicated that this involved P2X<sub>1</sub> receptors (Vial, C & Evans, RJ, 2000). Similarly, ATP-stimulated contractions have been shown to be present in the human bladder, and to be enhanced in disease states (Bayliss, M *et al.*, 1999). Likewise, the purinergic component of parasympathetic neurotransmission is increased up to 40% in interstitial cystitis, outflow obstruction and neurogenic bladder (Burnstock, G, 1998). P2 antagonists could therefore play a substantial role in treating bladder disorders.

**ii. Hypertension:** In spontaneously hypertensive rats, ATP has been shown to have a greater cotransmitter role than noradrenaline in blood vessels (Burnstock, G, 1998), and P2X antagonists have been shown to reduce vasoconstrictor responses in pulmonary vessels of the cat (Neely, CF *et al.*, 1996). Vonend, O *et al.*, (2004) have also demonstrated significantly increased expression of the P2X<sub>7</sub> receptor in glomeruli of hypertensive or diabetic rats compared to normal animals.

**iii. Pain:** Several P2X receptors have been shown to be associated with pain. ATP injections have been known for many years to produce pain, but its role has only become clear since P2X subunit cloning demonstrated selective expression of P2X<sub>3</sub> at high levels in nociceptive sensory neurons (Burnstock, G, 2006a). P2X<sub>3</sub> has been shown to form functional receptors

both on its own and in combination with the P2X<sub>2</sub> receptor (Tsuda, M *et al.*, 1999; Burgard, EC *et al.*, 1999). The two receptors are found in a wide range of organs including skin, tongue, tooth pulp, bladder and intestine (Burnstock, G, 2006a).

Microglial cells of the central nervous system are thought to play a role in neuropathic pain. Following injury to a peripheral nerve, microglia in the spinal cord become activated and upregulate expression of the P2X<sub>4</sub> receptor, leading to release of trophic factors, increased neuron signalling and pain hypersensitivity (Trang, T *et al.*, 2006).

Antagonism of the P2X<sub>7</sub> receptor has been shown to relieve inflammatory pain in rats and lead to a reduction in receptor expression in peripheral nerve endings and in endothelial cells (Dell'Antonio, G *et al.*, 2002). Similarly, chronic inflammatory and neuropathic pain was completely abolished in P2X<sub>7</sub> knockout mice (Chessell, IP *et al.*, 2005).

Sensory nerve endings in the gastrointestinal tract express P2X receptors and antagonists could be used to reduce abdominal pain in IBS patients (Galligan, JJ, 2004). In a similar way, P2X agonists or antagonists acting at enteric motor neurons could be used to treat IBS symptoms of constipation or diarrhoea respectively, by altering intestinal motility and secretion.

**iv. Inflammation:** P2X<sub>7</sub> plays a central role in the maturation and release of IL-1 $\beta$ . Macrophages from P2X<sub>7</sub> knockout mice do not release IL-1 $\beta$  in response to ATP and demonstrate reduced incidence and severity of collagen-induced arthritis (Labasi, JM *et al.*, 2002). IL-1 $\beta$  is a principal cytokine in the inflammatory processes of IBD and some current treatments already act by reducing cytokine release (Carter, MJ *et al.*, 2004). 5-Aminosalicylic acid has been shown to inhibit the production of IL-1 $\beta$  in colonic tissue from IBD patients (Mahida, YR *et al.*, 1991) and corticosteroids act by inhibiting its transcription (Carter, MJ *et al.*, 2004). Selective P2X<sub>7</sub> antagonists could therefore be important therapeutic agents in the control of inflammation.

### 7.1.1. Current P2X<sub>7</sub> antagonists

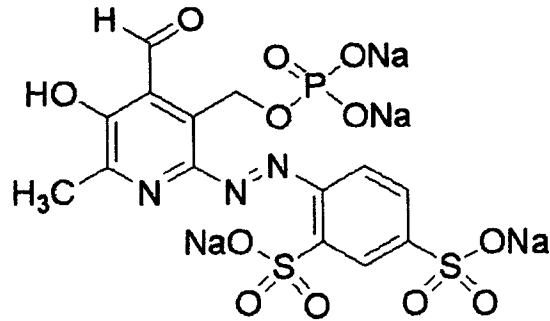
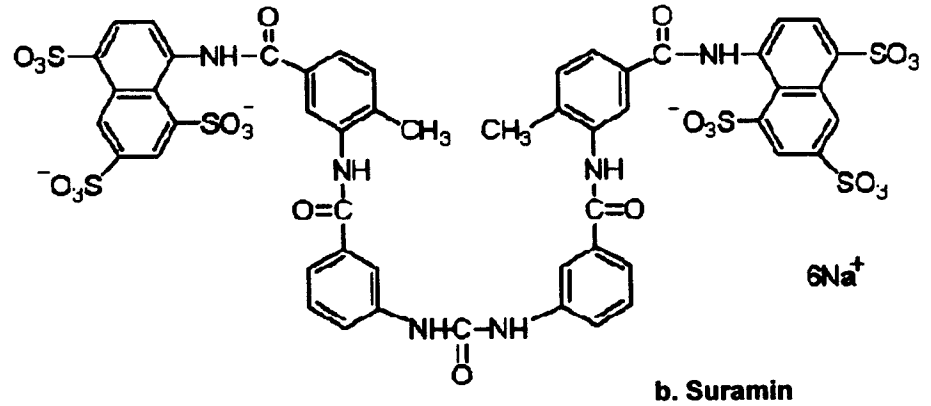
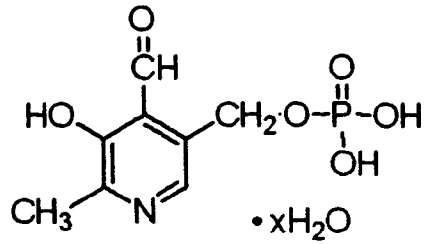
Unfortunately a lack of selective antagonists that are stable and potent, and that can be administered orally, means that there have been hardly any *in vivo* studies with P2 receptor antagonists. Very few selective P2X antagonists exist. Pyridoxal-5-phosphate and suramin (Fig 7.1.a & b) are general P2 antagonists which do not discriminate between P2X and P2Y receptors. Analogues of these compounds, PPADS (pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid)) and NF023 (a suramin-based compound) (Fig 7.1.c & d), have been developed that show moderate selectivity for P2X receptors but still retain some P2Y activity. Currently, there are two widely used antagonists for the P2X<sub>7</sub> receptor; periodate-oxidized ATP (oATP) and KN62 (Fig 7.2.a & b). Some of the known P2X<sub>7</sub>-mediated effects that they have been shown to inhibit are listed in Table 7.1.

#### 7.1.1.1. oATP

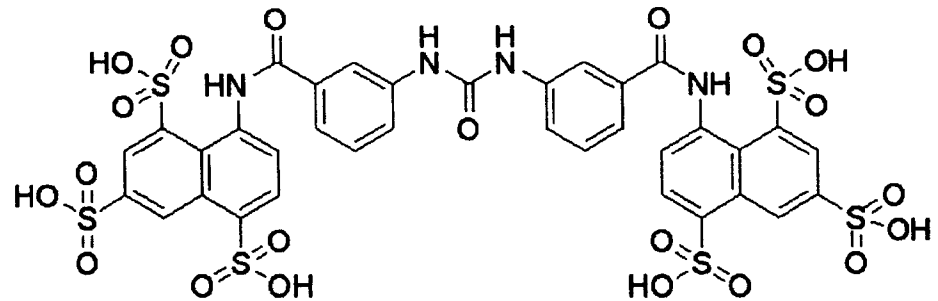
Periodate oxidation of ATP produces 2',3'-dialdehyde ATP (oATP), which has long been used to affinity label nucleotide sites in enzymes. oATP reacts with accessible lysine residues within the nucleotide binding site forming covalent bonds, and has been used to label the ATP binding site in a variety of purified proteins (Colman, RF, 1983). Murgia, M *et al.*, (1993) subsequently showed that oATP at low concentrations (100µM) was also an irreversible antagonist of the P2X<sub>7</sub> receptor in mouse macrophages, although it required a prolonged incubation. They demonstrated complete blockage of P2X<sub>7</sub>-mediated pore formation and ethidium bromide uptake, but showed that mobilisation of Ca<sup>2+</sup> from intracellular stores, a process mediated by P2Y receptors, was unaffected.

oATP has since been widely used to antagonise ATP stimulation of P2X<sub>7</sub>, particularly in studies of its proinflammatory responses and IL-1β release (Mehta, VB *et al.*, 2001; Ferrari, D *et al.*, 1997b; Ferrari, D *et al.*, 1997c). Other studies investigating the role of P2X<sub>7</sub> in pain have shown that oATP not only inhibited inflammatory pain and reduced proinflammatory chemokine secretion, but there was also decreased P2X<sub>7</sub> expression in nerves and

a. Pyridoxal-5-phosphate



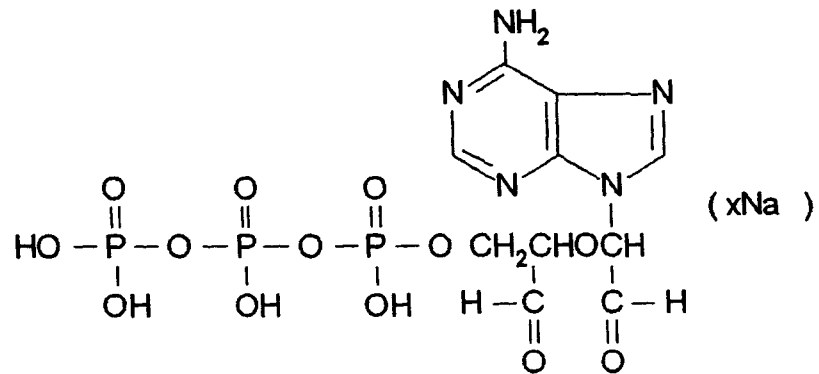
c. PPADS



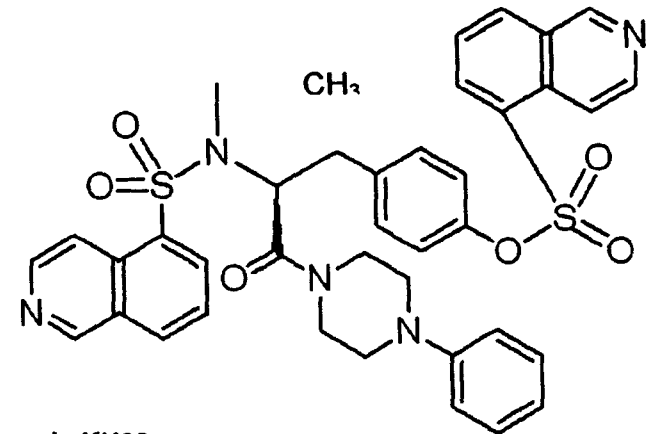
d. NF023

**Fig 7.1. P2 receptor antagonists**

Pyridoxal-5-phosphate (a) and suramin (b) are general P2 antagonists which do not discriminate between P2X and P2Y receptors. PPADS (c) and NF023 (d) show moderate selectivity for P2X receptors but still retain some P2Y activity.



a. oATP



b. KN62

### Fig 7.2. P2X<sub>7</sub> receptor antagonists

oATP and KN62 are widely used antagonists for the P2X<sub>7</sub> receptor and have been shown to inhibit many P2X<sub>7</sub>-mediated effects including ion channel activity, IL-1 $\beta$  release, ethidium bromide uptake, CD23 and L-selectin shedding and phospholipase D activity.

Authors	Cell type	Antagonist	Agonist	P2X <sub>7</sub> -mediated effect
Humphreys, BD & Dubyak, GR, (1996)	THP-1 cells treated with LPS & IFN- $\gamma$	300 $\mu$ M oATP for 3h or 5 $\mu$ M KN62 for 10min	100 $\mu$ M BzATP for 15min	phospholipase D activity
Lammas, DA <i>et al.</i> , (1997)	monocyte-derived macrophages	300 $\mu$ M oATP for 2h	3mM ATP for 10min	Lucifer-yellow uptake
Murgia, M <i>et al.</i> , (1993)	J774 macrophages	300 $\mu$ M oATP for up to 3h	3mM ATP for 10min	Lucifer-yellow and EB uptake
Wiley, JS <i>et al.</i> , (1994)	PBMC lymphocytes from B-CLL patients	300 $\mu$ M oATP for 1h	0.1mM ATP	Ba <sup>2+</sup> influx
Gu, B <i>et al.</i> , (1998)	PBMC lymphocytes from B-CLL patients	300 $\mu$ M oATP for 1h	100 $\mu$ M BzATP for up to 15min	CD23 and L-selectin shedding
Gargett, CE & Wiley, JS, (1997b)	PBMC lymphocytes from B-CLL patients	1-1000nM KN62 for 5min	500 $\mu$ M ATP for 5-15min	Ba <sup>2+</sup> influx, EB uptake, phospholipase D activity, L-selectin shedding
Michel, AD <i>et al.</i> , (2000)	HEK293 cells expressing human rP2X <sub>7</sub>	1-100mM oATP or 10-1000nM KN62 for 30min	0.3-20 $\mu$ M BzATP for 20min	YO-PRO-1 uptake
Chessell, IP <i>et al.</i> , (1998)	HEK293 cells expressing human rP2X <sub>7</sub>	10-100nM KN62 for 10min	1-200 $\mu$ M BzATP for 2sec	ion flow (inward current)
Humphreys, BD <i>et al.</i> , (1998b)	HEK293 cells expressing human rP2X <sub>7</sub>	0.03-1 $\mu$ M KN62 for 10min	1mM ATP for up to 4min	EB uptake

**Table 7.1. Examples of P2X<sub>7</sub> receptor-mediated effects that have been inhibited by oATP and KN62**

endothelial cells close to the site of inflammation (Dell'Antonio, G *et al.*, 2002; Fulgenzi, A *et al.*, 2005).

Recently a study of three human cell lines not expressing the P2X<sub>7</sub> receptor has demonstrated an inhibitory effect of oATP, suggesting that P2X<sub>7</sub> is not its only target. Using human HUVEC, HEK293 and 1321N1 astrocyte cells, Beigi, RD *et al.*, (2003) showed that oATP decreased IL-8 secretion stimulated by TNF- $\alpha$ , IL-1 $\beta$  or LPS. They proposed that oATP may interfere directly with activation of cytokine or TLR receptors, or that it may enter the cells and inhibit downstream signalling from the cytokine receptors. It has been proposed therefore that oATP should only be used to study P2X<sub>7</sub> receptor function under selected experimental conditions; namely when the stimulant is a known P2X<sub>7</sub> agonist such as ATP or BzATP, and the 'read-out' is a known P2X<sub>7</sub>-dependent response (Di Virgilio, F, 2003).

#### **7.1.1.2. KN62**

The isoquinoline derivative KN62 (1-[N,O-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) is a selective inhibitor of calmodulin-dependent protein kinase II (Tokumitsu, H *et al.*, 1990), but has also been reported to be a potent antagonist of P2X<sub>7</sub> at nanomolar concentrations (Gargett, CE *et al.*, 1997b; Chessell, IP *et al.*, 1998; Humphreys, BD *et al.*, 1998b). Gargett, CE *et al.*, (1997b) showed that the P2X<sub>7</sub> antagonism was independent of the kinase inhibition since KN04, a structural analogue of KN62 which has no effect on calmodulin-dependent protein kinase II, was almost equally potent at inhibiting P2X<sub>7</sub>-mediated effects.

Attempts have been made to find more potent and selective P2X<sub>7</sub> antagonists by modifying the structure of KN62. Baraldi, PG *et al.*, (2000) synthesised a conformationally restrained analogue, in which the N-methyl group on the tyrosine molecule was constrained into a ring structure, but found that it led to a complete loss of antagonist activity. The same group also showed that the isoquinoline-5-sulphonyl moiety is essential for activity, and by modifying the piperazine residue they obtained compounds more potent than KN62 (Baraldi, PG *et al.*, 2003).

Ravi, RG *et al.*, (2001) showed that the methyl group on the nitrogen of the tyrosine is not essential for activity but that opening the piperaziny ring abolished antagonism. By systematically modifying three positions on the KN62 molecule they also produced compounds slightly more potent than KN62 itself.

Michel, AD *et al.*, (2000) have suggested that KN62 acts as an allosteric inhibitor of the P2X<sub>7</sub> receptor. They studied BzATP-stimulated YO-PRO-1 uptake in HEK293 cells expressing the human P2X<sub>7</sub> receptor, and compared antagonistic effects of oATP, PPADS, pyridoxal-5-phosphate (P5P), KN04 and KN62. They found that oATP, PPADS and P5P acted as irreversible antagonists but KN04 and KN62 behaved as non-competitive antagonists. They also showed that PPADS and P5P were able to prevent the antagonistic effects of oATP, but that KN04 and KN62 did not. Since oATP is structurally similar to ATP, they proposed that oATP, PPADs and P5P act by binding to the ATP-binding site on P2X<sub>7</sub>, but that KN04 and KN62 interact at an allosteric site on the receptor.

### 7.1.1.3. Other P2X<sub>7</sub> antagonists

**Brilliant Blue G** has been reported to inhibit ATP-evoked inward currents in HEK293 cells expressing the rat or human P2X<sub>7</sub> receptor (Jiang, LH *et al.*, 2000a). However, while being a highly selective antagonist with nanomolar affinity at rat P2X<sub>7</sub> receptors, it is less effective at human receptors. Brilliant Blue G also inhibits P2X<sub>4</sub> receptors, but with greater potency at human than at rat receptors (Jiang, LH *et al.*, 2000a). Consequently for rat receptors Brilliant Blue G has 1000-fold selectivity for P2X<sub>7</sub> compared with P2X<sub>4</sub>, but for human P2X<sub>7</sub>, selectivity is only approximately 15-fold. P2X<sub>7</sub> and P2X<sub>4</sub> receptors are commonly expressed in the same cells, therefore Brilliant Blue G provides a useful tool for selectively antagonising rat P2X<sub>7</sub> receptors but not the human receptor.

**Chelerythrine** is a protein kinase C inhibitor which has also been shown to block actions of the P2X<sub>7</sub> receptor. Using human peripheral blood B-lymphocytes stimulated with ATP, Shemon, AN *et al.*, (2004) demonstrated inhibition of both cation fluxes and phospholipase D activity. They suggested



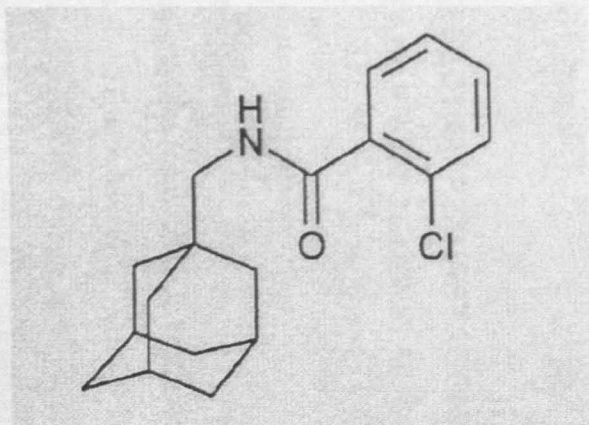
that chelerythrine acts in a non-competitive manner, binding at a site on the P2X<sub>7</sub> receptor other than the ATP-binding site.

**Decavanadate** (H<sub>2</sub>V<sub>10</sub>O<sub>28</sub><sup>-4</sup>) is a polymeric molecule, formed in solution from ionic species of oxides of vanadium, which has been shown to act as a reversible and competitive antagonist of the P2X<sub>7</sub> receptor (Michel, AD *et al.*, 2006). Decavanadate inhibited ATP-stimulated uptake of ethidium in HEK293 cells expressing the human, rat or mouse recombinant P2X<sub>7</sub> receptors. A similar inhibition was also seen in BzATP-stimulated THP-1 cells which express an endogenous P2X<sub>7</sub> receptor. Decavanadate also competitively blocked the antagonistic effects of oATP and PPADS, suggesting that they act at the same site. The interaction of decavanadate and KN62 however was non-competitive, implying that they act at different sites on the P2X<sub>7</sub> receptor and reinforcing the suggestion that KN62 is an allosteric antagonist of P2X<sub>7</sub>.

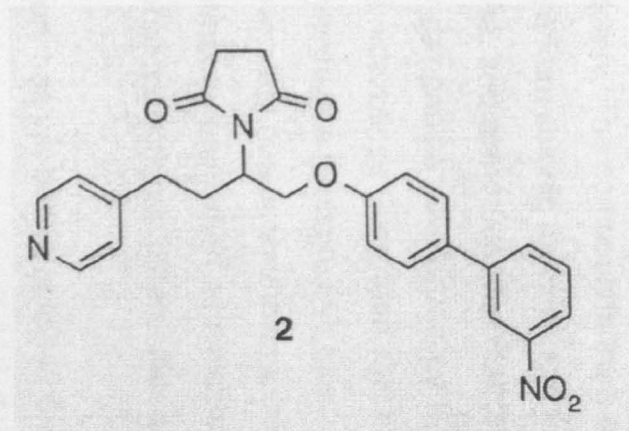
### 7.1.2. Novel P2X<sub>7</sub> antagonists

Several drug companies have produced novel compounds with P2X<sub>7</sub> antagonist properties. High throughput screening of large numbers of compounds enables the companies to develop those with the most promising properties and potential as candidate drugs. A typical screen for measuring P2X<sub>7</sub> receptor inhibition is the reduction of pore formation and ethidium bromide uptake in cells expressing the receptor (e.g. THP-1 cells) and stimulated with BzATP. The properties of the candidate drugs to be considered are potency, molecular weight, lipophilicity and clearance (Baxter, A *et al.*, 2003). Potency of a compound can be estimated by its pA<sub>2</sub> value, which is the negative log of the concentration of antagonist that shifts the agonist concentration/effect curve two-fold (Neubig, RR *et al.*, 2003). An ideal drug candidate should have a pA<sub>2</sub> value > 7. The compound should also not be too large (MW < 450) and should be lipophilic, to enable binding to membrane receptors (Baxter, A *et al.*, 2003).

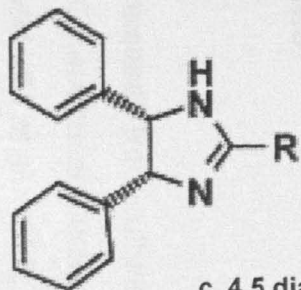
Using these criteria, AstraZeneca have produced two sets of compounds, one based on the adamantane amide structure (Baxter, A *et al.*, 2003) and the other on cyclic imides (Alcaraz, L *et al.*, 2003) (Fig 7.3.a & b). One of



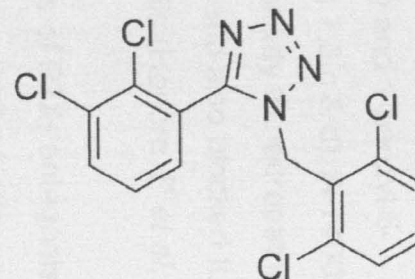
**a. adamantane based structure**  
(Baxter, A *et al.*, 2003)



**b. cyclic imide based structure**  
(Alcaraz, L *et al.*, 2003)



**c. 4,5 diarylimidazole based structure**  
(Merriman, GH *et al.*, 2005)



**d. 1-benzyl-5-phenyltetrazole based structure**  
(Nelson, DW *et al.*, 2006)

**Fig 7.3. Novel P2X<sub>7</sub> receptor antagonists**

Several drug companies have produced novel compounds with P2X<sub>7</sub> antagonist properties. AstraZeneca have produced two sets of compounds, one based on the adamantane amide structure (a) and the other on cyclic imides (b). Aventis have produced antagonists based on 4,5-diarylimidazolines (c), and Abbott Laboratories based on 1-benzyl-5-phenyltetrazole (d).

their P2X<sub>7</sub> antagonists, AZD9056, has recently been advanced into Phase II clinical trials for rheumatoid arthritis (Gever, JR *et al.*, 2006). Two novel P2X<sub>7</sub> antagonist compounds, AZ10573295 and AZ10603690, were provided by AstraZeneca for use in this project. AZ10573295 is a more potent antagonist with a pA<sub>2</sub> value of 8.0, whereas AZ10603690 has a pA<sub>2</sub> value of <5.5 and is less potent. The compounds were studied for their ability to inhibit IL-1 $\beta$  release from colonic biopsies and from LPMCs isolated from inflamed colonic mucosa, and compared with that of oATP.

Other companies have produced P2X<sub>7</sub> antagonists based on 4,5-diarylimidazolines (Merriman, GH *et al.*, 2005) and 1-benzyl-5-phenyltetrazole (Nelson, DW *et al.*, 2006) (Fig 7.3.c & d). A-740003 (Abbott Laboratories) has been shown to reduce sensitivity to neuropathic or inflammatory pain when administered to rats, and also blocked IL-1 $\beta$  release and pore formation in differentiated THP-1 cells (Honore, P *et al.*, 2006).

No work has yet been published on the effects of P2X<sub>7</sub> antagonists on colonic mucosal inflammatory cells. IL-1 $\beta$  is the principal inflammatory cytokine in IBD. If P2X<sub>7</sub> antagonists can reduce IL-1 $\beta$  release from colonic LPMCs this would provide a new therapeutic target for the treatment of IBD. Both established and novel P2X<sub>7</sub> antagonists were therefore investigated for their effects on IL-1 $\beta$  release from colonic tissue biopsies and isolated colonic LPMCs.

## **7.2. EFFECT OF P2X<sub>7</sub> ANTAGONISTS ON SPONTANEOUS IL-1 $\beta$ RELEASE FROM COLONIC MUCOSAL BIOPSIES**

### **7.2.1. Introduction**

Studies have shown that IL-1 $\beta$  is spontaneously released from cultured biopsies taken from colonic mucosa of CD and UC patients at much higher levels than that seen with normal mucosa (Reimund, JM *et al.*, 1996; Dionne, S *et al.*, 1998; Ligumsky, M *et al.*, 1990). Furthermore, this release has been shown to be reduced by incubation with various drugs such as 5-

aminosalicylic acid and dexamethasone (Mahida, YR *et al.*, 1991), and oxpentifylline (Reimund, J-M *et al.*, 1997).

P2X<sub>7</sub> antagonist studies were carried out on biopsies from both normal (from cancer patients, sampled >5cm from the tumour) and inflamed/uninflamed tissue from IBD patients. The effects of established P2X<sub>7</sub> inhibitors oATP and KN-62 were investigated, as well as those of the novel AstraZeneca P2X<sub>7</sub> antagonist AZ10603690.

### **7.2.2. Method**

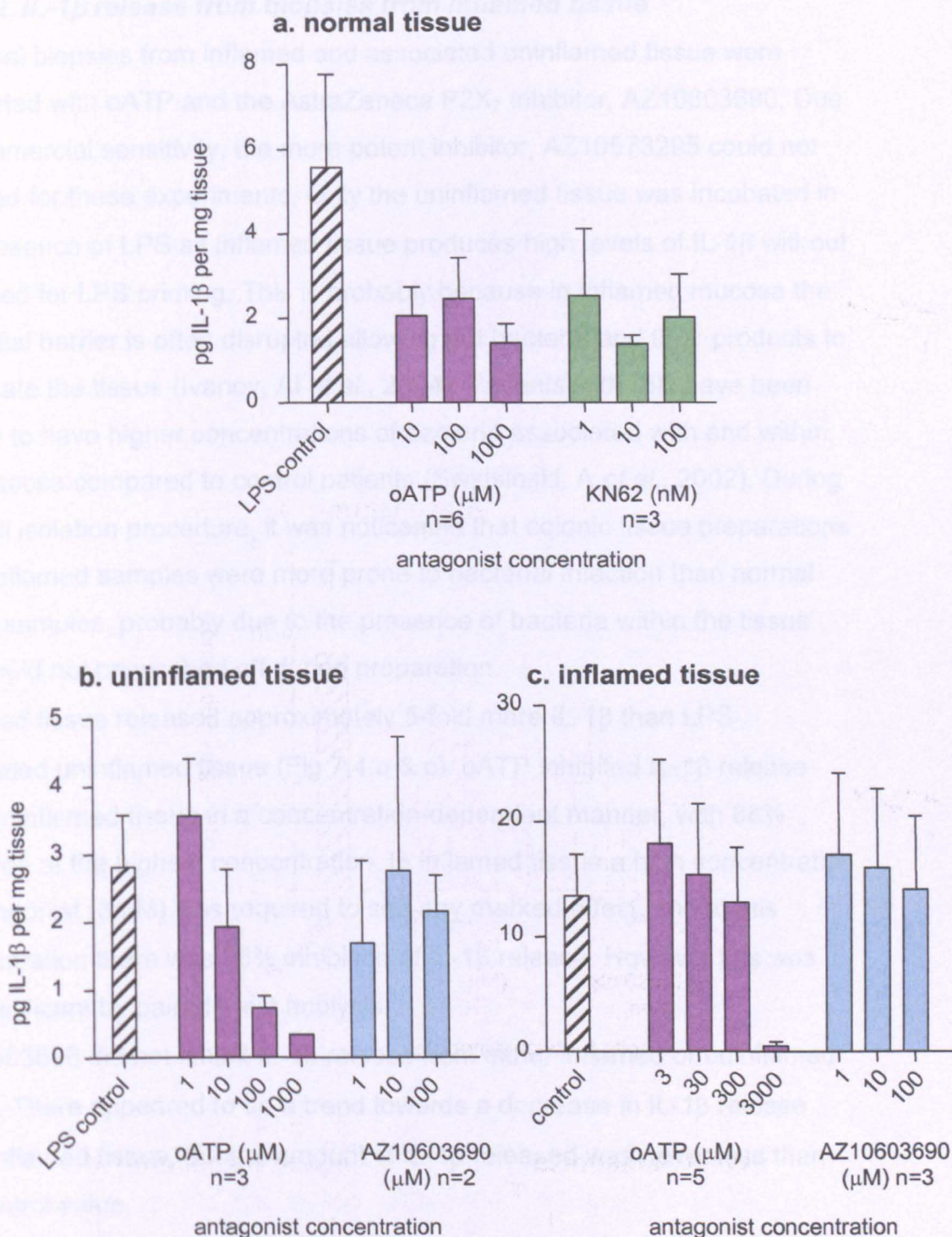
Small pieces (approx 10mg) were cut from a washed strip of colonic mucosa, weighed and placed on steel mesh in an organ culture dish (Falcon™, BD Pharmingen) containing 1ml of RPMI medium (Eastwood, GL & Trier, JS, 1973). The dishes were placed in a sealed chamber equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub> and incubated at 37°C for 1h, after which time the medium was changed for fresh medium with or without antagonists. The chamber was re-sealed, re-equilibrated and incubated at 37°C for 24h. After this time, supernatants were removed and stored at -80°C for IL-1β assay. Because normal and uninflamed tissues release very little IL-1β, these biopsies were incubated in the presence of LPS (10μg/ml):

### **7.2.3. Results**

The effect of P2X<sub>7</sub> receptor antagonists on IL-1β release from colonic mucosal biopsies maintained in organ culture is shown in Fig 7.4.

#### **7.2.3.1. IL-1β release from biopsies from normal tissue**

Normal mucosa was incubated with oATP and KN62 in the presence of LPS. All concentrations of the inhibitors reduced the amount of IL-1β released compared to the LPS control, but the effect did not appear to be concentration-related (Fig 7.4.a). The amounts of IL-1β released were very small, ranging from approximately 1.5-2.5pg/mg tissue.



**Fig 7.4. Effect of P2X<sub>7</sub> antagonists on IL-1 $\beta$  release from colonic mucosal biopsies maintained in organ culture**

Small tissue biopsies (10 - 20mg) were cultured for 24h in RPMI medium containing 10% FCS in the presence of P2X<sub>7</sub> antagonists. The amount of IL-1 $\beta$  released into the supernatant was measured by ELISA. LPS (10 $\mu$ g/ml) was also added to the incubation medium of the normal and uninflamed tissue. Results show the mean  $\pm$  sem of separate experiments.



### **7.2.3.2. IL-1 $\beta$ release from biopsies from inflamed tissue**

Mucosal biopsies from inflamed and associated uninflamed tissue were incubated with oATP and the AstraZeneca P2X<sub>7</sub> inhibitor, AZ10603690. Due to commercial sensitivity, the more potent inhibitor, AZ10573295 could not be used for these experiments. Only the uninflamed tissue was incubated in the presence of LPS as inflamed tissue produces high levels of IL-1 $\beta$  without the need for LPS priming. This is probably because in inflamed mucosa the epithelial barrier is often disrupted allowing gut bacteria and their products to penetrate the tissue (Ivanov, Al *et al.*, 2004). Patients with IBD have been shown to have higher concentrations of bacteria associated with and within the mucosa compared to control patients (Swidsinski, A *et al.*, 2002). During the cell isolation procedure, it was noticeable that colonic tissue preparations from inflamed samples were more prone to bacterial infection than normal tissue samples, probably due to the presence of bacteria within the tissue that could not be washed off during preparation.

Inflamed tissue released approximately 5-fold more IL-1 $\beta$  than LPS-stimulated uninflamed tissue (Fig 7.4.b & c). oATP inhibited IL-1 $\beta$  release from uninflamed tissue in a concentration-dependent manner, with 88% inhibition at the highest concentration. In inflamed tissue a high concentration of antagonist (3mM) was required to see any marked effect, and at this concentration there was 96% inhibition of IL-1 $\beta$  release. However this was not significant by paired t test analysis.

AZ10603690 did not inhibit IL-1 $\beta$  release from either inflamed or uninflamed tissue. There appeared to be a trend towards a decrease in IL-1 $\beta$  release from inflamed tissue, but the amount of IL-1 $\beta$  released was never less than the control value.

### **7.2.4. Discussion**

The results demonstrated that the release of IL-1 $\beta$  is markedly elevated in inflamed colonic mucosal tissue as has been shown previously (Ligumsky, M *et al.*, 1990; Reimund, JM *et al.*, 1996), however the P2X<sub>7</sub> antagonist, AZ10603690 appeared to have no effect on its release. This may have been because AZ10603690 is a weak antagonist (pA<sub>2</sub> < 5.5), requiring higher

concentrations to produce an effect, and the more potent antagonist, AZ10573295 ( $pA_2$  8.0), might have reduced IL-1 $\beta$  release. Another possibility is that AZ10603690 was unable to penetrate the tissue to access the inflammatory cells on which the P2X<sub>7</sub> receptor is expressed. Experiments using isolated cells should overcome this.

Alternatively, organ culture may not be a suitable system for testing P2X<sub>7</sub> activity. The method has been used successfully to demonstrate inhibition of IL-1 $\beta$  release by 5-aminosalicylic acid (Mahida, YR *et al.*, 1991), but P2X<sub>7</sub>-mediated IL-1 $\beta$  release requires stimulation with ATP. The experiments relied on incubation with LPS to promote IL-1 $\beta$  release which may or may not involve ATP.

LPS has been shown to induce the release of ATP from RAW 264.7 macrophages (Sperlagh, B *et al.*, 1998), and from N13 microglial cells and monocyte-derived human macrophages (Ferrari, D *et al.*, 1997c). In contrast, a study of BAC1.2F5 macrophages found no increase in ATP release on stimulation with LPS (Beigi, RD & Dubyak, GR, 2000). It may be therefore, that the P2X<sub>7</sub> inhibitor AZ10603690 did not inhibit IL-1 $\beta$  release because ATP stimulation was not involved. The apparent inhibition by oATP could have been due to its non-specific effects described in section 7.1.1.1. which might have been mediated through the epithelial cells rather than the inflammatory cells.

IL-1 $\beta$  released from both normal and inflamed intestine is produced by the mononuclear cells of the lamina propria (Youngman, KR *et al.*, 1993). Colonic epithelial cells have been shown to express both IL-1 $\beta$  and caspase-1, but caspase-1 is only expressed in the inactive pro-form (Jarry, A *et al.*, 1999). A similar study by McAlindon, ME *et al.*, (1998b) demonstrated that colonic macrophages isolated from normal tissue synthesised only pro-caspase-1, whereas those from IBD tissue produced active caspase-1 and hence mature IL-1 $\beta$ . It is possible therefore that under certain circumstances, epithelial cells could also produce mature caspase-1 leading to maturation of IL-1 $\beta$ .

Epithelial cells from normal colonic mucosa have been shown to barely express TLR4, the receptor which detects bacterial LPS, but the receptor is

strongly upregulated in both UC and CD (Cario, E *et al.*, 2000). Under normal circumstances therefore, the intestinal epithelium is unresponsive to bacteria and their products, is not a source of IL-1 $\beta$ , and has been shown to produce IL-1receptor antagonist (IL-1ra), in keeping with an anti-inflammatory role (Daig, R *et al.*, 2000). Under inflammatory conditions however it is possible that the control mechanisms are defective, and although the majority of IL-1 $\beta$  is likely to be produced by macrophages, there may be an epithelial component.

### **7.3. EFFECT OF P2X<sub>7</sub> ANTAGONISTS ON P2X<sub>7</sub>-STIMULATED IL-1 $\beta$ RELEASE FROM ISOLATED LPMCs**

Antagonism of IL-1 $\beta$  release was studied in LPMCs isolated from normal tissue from cancer patients (sampled >5cm from the tumour), and from tissue from IBD patients. Wherever possible, tissue samples from both inflamed and uninfamed sections of the colon were obtained from the IBD patients.

#### **7.3.1. Method**

LPMCs isolated from normal or uninfamed colonic mucosa were resuspended in RPMI 1640 containing 0.1% v/v FCS at a concentration of  $1.1 \times 10^6$  cells/ml and incubated with LPS (1 $\mu$ g/ml) for approximately three hours at 37°C in 5% CO<sub>2</sub>. Cells isolated from inflamed tissue produce IL-1 $\beta$  spontaneously and did not need LPS-priming (Mahida, YR *et al.*, 1989b). Antagonist was pre-incubated with the cells for up to 30 minutes at 37°C prior to addition of agonist. Agonist was then added and incubation continued for up to 20 minutes in a final total incubation volume of 100 $\mu$ l. Incubation was stopped by adding PBS (400 $\mu$ l) and the cells were placed on ice. Following centrifugation at 350 x g for 5min, aliquots (450 $\mu$ l) of the supernatants were stored at -80°C for IL-1 $\beta$  assay. The cells remaining in the tubes were incubated with 250 $\mu$ l of 0.1% saponin in RPMI 1640 for 30min at 37°C. The cells were centrifuged at 350 x g for 5min and aliquots (250 $\mu$ l) of the supernatants were stored at -80°C for IL-1 $\beta$  assay.



### 7.3.2. Effect of P2X<sub>7</sub> antagonists on IL-1 $\beta$ release from LPMCs isolated from normal tissue

Inhibition of IL-1 $\beta$  release from LPMCs isolated from normal tissue and primed with LPS was carried out using the established P2X<sub>7</sub> antagonists, oATP and KN62. The concentrations of the two antagonists used were based on those from other published studies. oATP has been widely used at a concentration of 300 $\mu$ M to inhibit P2X<sub>7</sub> activity (Table 7.1.) and was used over the concentration range 3 $\mu$ M to 5mM. KN62 has been reported to have a pA<sub>2</sub> value of  $8.1 \pm 0.09$  for uptake of YO-PRO-1 in HEK293 cells and was used over the concentration range 1nM to 300nM (Michel, AD *et al.*, 2000).

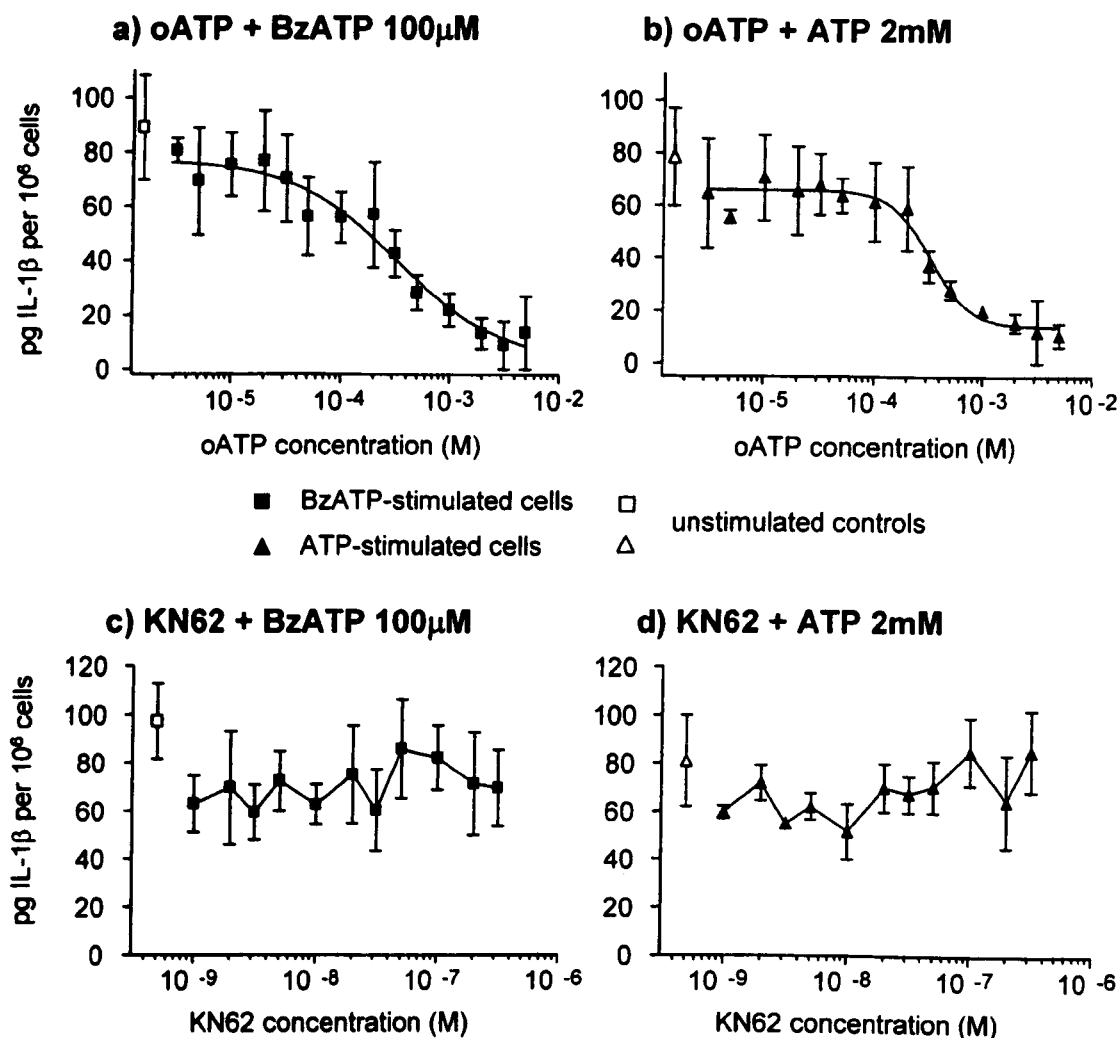
#### 7.3.2.1. Results

The results (Fig 7.5.) clearly show that whilst oATP antagonised both BzATP- and ATP-stimulated IL-1 $\beta$  release, KN62 had no effect. oATP inhibited IL-1 $\beta$  release in a dose-dependent manner for both BzATP and ATP stimulated cells. The IC<sub>50</sub> values were the same for both agonists (325 $\mu$ M oATP) but the shape of the inhibition curves was different, the BzATP-curve being shallower than that of ATP. This was reflected in the values obtained for the Hill slopes ( $-0.9 \pm 0.7$  for BzATP and  $-2.4 \pm 1.6$  for ATP) and in the 95% confident intervals for the IC<sub>50</sub> which were much wider for BzATP (60-1751 $\mu$ M oATP) than for ATP (183-575 $\mu$ M oATP). The overall reduction in IL-1 $\beta$  release was the same (approximately 80%) for both agonists.

Even though the cells were incubated in the presence of LPS, the amount of IL-1 $\beta$  released by both BzATP and ATP was very small, reaching a maximum of only  $80 \pm 4.4\text{pg}/10^6$  cells. This might be due to reduced expression of P2X<sub>7</sub> receptors in these cells but also reflects the low expression of TLR4 receptors and/or CD14 that has been demonstrated in colonic LPMCs (Austin, AS *et al.*, 2005; Ortega-Cava, CF *et al.*, 2003).

#### 7.3.2.2. Discussion

Various other studies, have demonstrated a similar inhibition of IL-1 $\beta$  release by oATP in a number of different cell types. Mehta, VB *et al.*, (2001) showed that 300 $\mu$ M oATP added 30 minutes prior to stimulation with 5mM ATP



### e) Pharmacological parameters for oATP inhibition of IL-1 $\beta$ release

Agonist	IC <sub>50</sub> value with 95% confidence intervals	Hill slope $\pm$ sem	Max & min pg IL-1 $\beta$ released (mean $\pm$ sem)	% inhibition of IL-1 $\beta$ release
BzATP	325 $\mu$ M 60 - 1751 $\mu$ M	-0.9 $\pm$ 0.7	81 $\pm$ 4 13 $\pm$ 13	84%
ATP	325 $\mu$ M 183 - 575 $\mu$ M	-2.4 $\pm$ 1.6	71 $\pm$ 16 11 $\pm$ 4	80%

### Fig 7.5. Effect of P2X<sub>7</sub> antagonists on IL-1 $\beta$ release from LPMCs isolated from normal tissue

LPS-primed cells in RPMI 1640 + 0.1% FCS were incubated with antagonist for 30min prior to stimulation for 10min with BzATP or ATP. IL-1 $\beta$  released into the supernatant was measured by ELISA. Results show the mean  $\pm$  sem of four experiments. Open symbols represent the amount of IL-1 $\beta$  released in the absence of antagonist.

completely abolished IL-1 $\beta$  release from human monocytes. Similar results have been shown in microglial cells (Ferrari, D *et al.*, 1997c), mature dendritic cells (Ferrari, D *et al.*, 2000), and in macrophages differentiated from PBMC monocytes (Ferrari, D *et al.*, 1997b). All of these studies used 300 $\mu$ M oATP added 2 hours prior to stimulation with 1-5mM ATP, and all demonstrated a total block of IL-1 $\beta$  release.

In contrast to the data presented here, some studies have shown a dose dependent inhibition of IL-1 $\beta$  release by both oATP and KN62. PMA-treated THP-1 cells incubated with KN62 for 30 minutes prior to stimulation with 5mM ATP demonstrated a decrease in IL-1 $\beta$  release of 60% with 300nM KN62 and 85% with 1 $\mu$ M KN62 (Grahames, CB *et al.*, 1999). Freshly isolated human monocytes also exhibited 88% inhibition of IL-1 $\beta$  release with 1 $\mu$ M KN62. In the same study, a 2 hour incubation with oATP inhibited ATP-stimulated release by 25% with 30 $\mu$ M oATP increasing to 70% inhibition with 100 $\mu$ M oATP. Other studies using human PBMCs have demonstrated a dose-dependent decrease in IL-1 $\beta$  release with KN62 and oATP (Elssner, A *et al.*, 2004), and reduced IL-18 secretion with KN62 in PBMCs stimulated with LPS and ATP for 2 hours (Muhl, H *et al.*, 2003).

The reason for the lack of response to KN62 by the colonic LPMCs is not known. One explanation may be morphological differences between the cell types. The principal IL-1 $\beta$ -producing cells of LPMCs are the mature tissue macrophages, whereas the published studies using KN62 quoted above were all performed on monocytes. All of the experiments carried out in this project have indicated that PBMC monocytes are much more responsive to P2X<sub>7</sub> stimulation than LPMC macrophages from normal tissue. This implies therefore that PBMC monocytes are altered during maturation into tissue macrophages, probably changing the expression of receptors and other membrane proteins to make the cells more quiescent. As described in section 7.1.1.2, KN62 is thought to be an allosteric receptor, binding to a different site on the P2X<sub>7</sub> receptor than the ATP-binding site (Michel, AD *et al.*, 2000). It may be therefore that in mature macrophages this site has somehow been altered and is not available for KN62-binding. Studies have shown the existence of many different point mutations of the P2X<sub>7</sub> receptor

affecting its properties and expression (Denlinger, LC *et al.*, 2003; Wiley, JS *et al.*, 2003; Le Stunff, H *et al.*, 2004; Shemon, AN *et al.*, 2006), and it is possible that structural changes may occur during the maturation process that affect the site of KN62 binding. Alternatively, tissue macrophages may contain membrane proteins that bind or break down KN62 and make it unavailable for P2X<sub>7</sub> antagonism.

### **7.3.3. Effect of P2X<sub>7</sub> antagonists on IL-1 $\beta$ release from LPMCs isolated from inflamed tissue**

Based on the histopathology reports, the inflamed tissue was characterised into severe acutely inflamed tissue and moderately or chronically inflamed tissue, and the results were grouped accordingly. The results were expressed as pg IL-1 $\beta$ /10<sup>6</sup> cells to illustrate the variation in amounts of IL-1 $\beta$  released with different degrees of inflammation and are shown in Table 7.2.

#### **7.3.3.1. Results**

Isolated LPMCs from inflamed and associated uninflamed tissue were incubated with oATP and the AstraZeneca P2X<sub>7</sub> inhibitor AZ10603690, as were used with inflamed mucosal tissue biopsies. The more potent inhibitor, AZ10573295 was also used in this set of experiments.

oATP decreased IL-1 $\beta$  release by 85% or more in LPMCs from all three tissue types (Fig 7.6.a, c, e). The IC<sub>50</sub> values ranged from 119-185 $\mu$ M and showed a tendency to decrease with increased severity of inflammation (IC<sub>50</sub> value in LPMCs from normal tissue was 325 $\mu$ M). One possible explanation for this could be the greater number of PBMC monocytes usually seen in inflamed tissue which might respond differently to oATP. To see if this was the case, earlier data from incubations of PBMCs with oATP and 200 $\mu$ M BzATP was analysed and is shown in Fig 7.7. The data is the result of only two experiments but the IC<sub>50</sub> value obtained was 102 $\mu$ M, close to that seen with LPMCs from severely inflamed tissue (119 $\mu$ M). This suggests a difference between the responses of P2X<sub>7</sub> receptors in circulating monocytes compared to those of mature tissue macrophages. A similar effect was

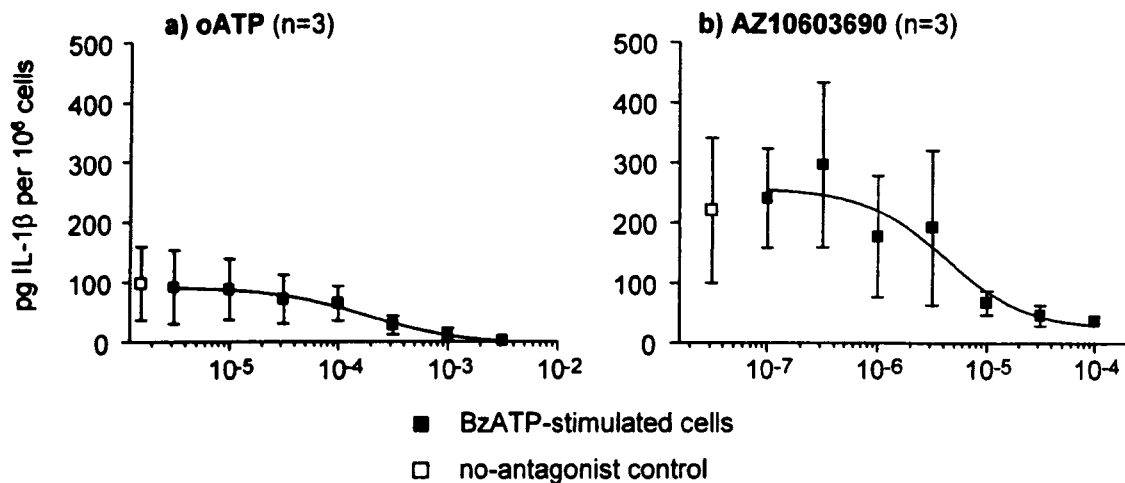
Antagonist	Tissue type	IC <sub>50</sub> value with 95% confidence intervals	Hill slope ± sem	Max & min pg IL-1β released (mean ± sem)	% inhibition of IL-1β release
oATP	uninflamed	185μM 2.6 - 13040μM	-1.0 ± 1.9	91 ± 62 1.4 ± 1.4	98%
	moderately inflamed	138μM 18 - 1048μM	-1.4 ± 1.7	277 ± 94 41 ± 28	85%
	severely inflamed	119μM n = 1	-0.8	2027 97	95%
AZ10603690	uninflamed	4.1μM 0.2 - 92μM	-1.2 ± 1.9	297 ± 138 37 ± 2.7	88%
	moderately inflamed	—	—	149 ± 52 88 ± 55	41%
	severely inflamed	4.2μM 0.5 - 34μM	-1.4 ± 1.8	1719 ± 984 270 ± 93	84%
AZ10573295	uninflamed	10.3nM 2.4 - 45nM	-1.4 ± 1.3	287 ± 99 68 ± 29	76%
	moderately inflamed	18.5nM 0.1 - 5516nM	-0.7 ± 1.4	143 ± 45 57 ± 13	60%
	severely inflamed	13.5nM 2.1 - 85nM	-1.6 ± 1.9	2730 ± 1421 124 ± 21	95%

**Table 7.2. Effect of P2X<sub>7</sub> antagonists on IL-1β release from LPMCs isolated from inflamed tissue**

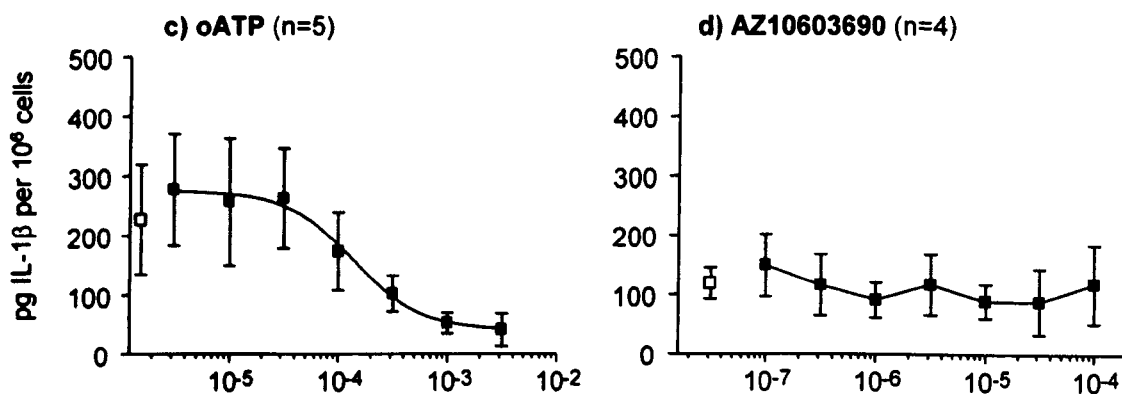
The table shows the EC<sub>50</sub> values, values for the Hill slope, the maximum and minimum amounts of IL-1β released, and the percent inhibition achieved with each antagonist in each tissue type. Inflamed and uninflamed LPMCs were isolated from tissue from IBD patients, from actively inflamed and uninflamed sections respectively. The results for the inflamed tissue were divided into severely inflamed and moderately inflamed, based on the histopathology reports.

All of the antagonists inhibited IL-1β release, with over 84% inhibition of release from severely inflamed tissue. The IC<sub>50</sub> values for each antagonist were similar for cells from all the tissue types. oATP appeared to demonstrate a decrease in IC<sub>50</sub> value with increased inflammation.

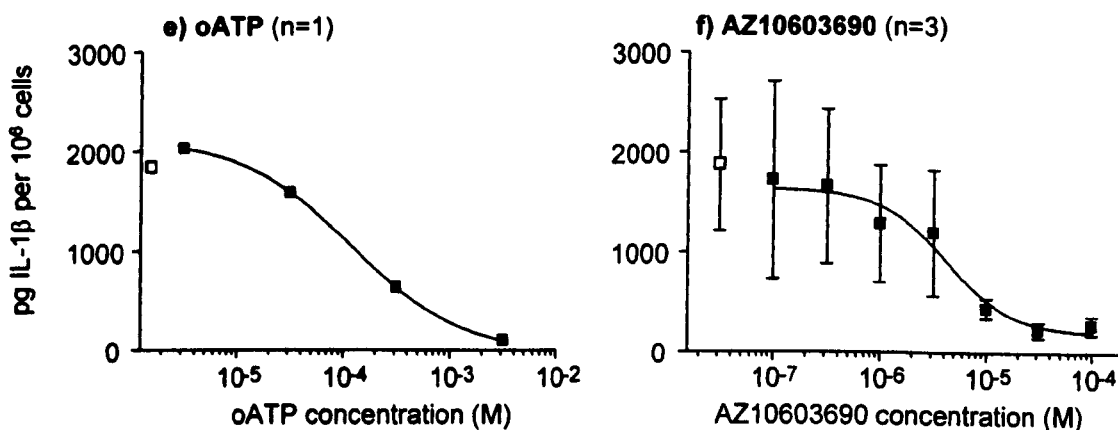
### i. LPMCs isolated from uninflamed tissue



### ii. LPMCs isolated from moderately inflamed tissue

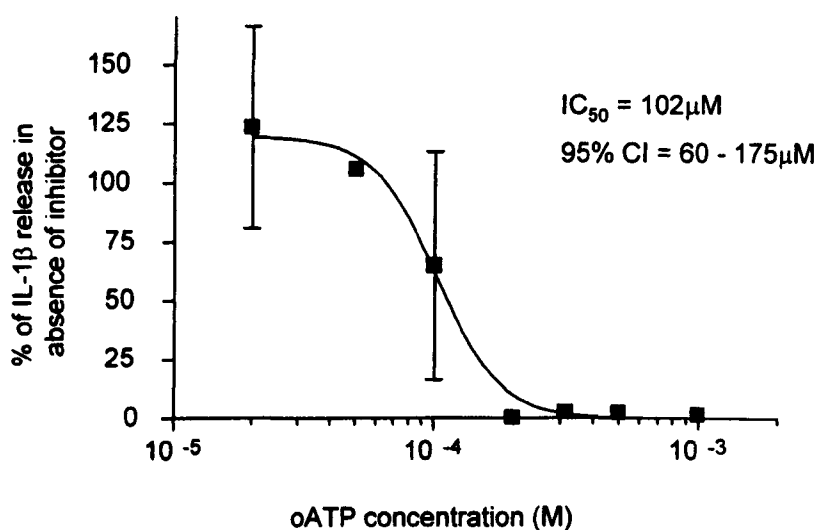


### iii. LPMCs isolated from severely inflamed tissue



## Fig 7.6. Effect of P2X<sub>7</sub> antagonists, oATP and AZ10603690, on IL-1 $\beta$ release from LPMCs isolated from inflamed tissue

Figures on the left represent cells treated with oATP and on the right with AZ10603690. Cells resuspended in RPMI 1640 + 0.1% FCS were incubated with antagonist for 10min prior to stimulation for 20min with 80 $\mu$ M BzATP. The amount of IL-1 $\beta$  released into the supernatant was measured by ELISA. Results show the mean  $\pm$  sem of separate experiments.



**Fig 7.7. Effect of oATP on IL-1 $\beta$  release from PBMCs stimulated with 200 $\mu$ M BzATP**

Cells were primed with LPS for approximately 4h and then incubated with oATP for 30min prior to stimulation with BzATP for 10min. Results were expressed as a percent of the IL-1 $\beta$  released in the absence of inhibitor and show the mean  $\pm$  sem of 2 experiments.

observed with the agonist concentration response curves for the release of IL-1 $\beta$ , in which the EC<sub>50</sub> value for PBMCs was lower than that for LPMCs, particularly with BzATP (39 $\mu$ M for PBMCs compared to 80 $\mu$ M for LPMCs isolated from inflamed tissue and 112 $\mu$ M for LPMCs isolated from normal tissue, Table 4.1.). This provides further evidence of changes in the characteristics of the P2X<sub>7</sub> receptor of circulating monocytes on maturation to tissue macrophages, indicating that the cells become less responsive and require higher concentrations of agonist to stimulate the receptor.

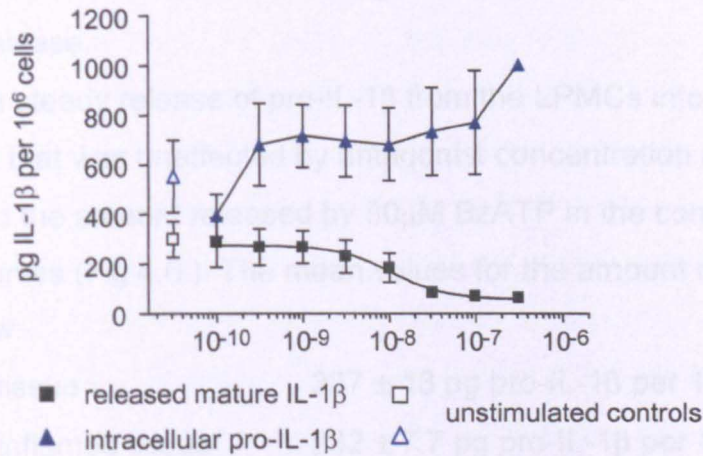
The AstraZeneca compound, AZ10603690 (Fig 7.6.b, d, f) reduced IL-1 $\beta$  release from LPMCs isolated from severely inflamed tissue and LPS-stimulated release from cells from uninflamed tissue by over 80%, but was ineffective in LPMCs from moderately inflamed tissue. IC<sub>50</sub> values for this compound were approximately 4 $\mu$ M (i.e. 10<sup>-5.4</sup>M), which closely matched its reported pA<sub>2</sub> value (<5.5). This gives confidence that the reported effects were P2X<sub>7</sub> receptor antagonist specific and not a result of non-specific effects of the chemical. An IC<sub>50</sub> value of 4 $\mu$ M makes AZ10603690 a useful P2X<sub>7</sub> inhibitor but not a suitable candidate for drug development.

AZ10573295 inhibited IL-1 $\beta$  release (Fig 7.8., solid squares) by 95% from LPMCs isolated from severely inflamed tissue, but was slightly less effective in cells from moderately inflamed or uninflamed tissue (60-76% decrease). However it is a much more potent antagonist than either oATP or AZ10603690, the IC<sub>50</sub> values for AZ10573295 being approximately 10-20nM, compared with values in the micromolar range for the other two antagonists. Like AZ10603690, the IC<sub>50</sub> values (10<sup>-8</sup>-10<sup>-7.7</sup>M) were comparable to its pA<sub>2</sub> value (8.0) showing that the effects were P2X<sub>7</sub>-specific. In LPMCs from severely inflamed tissue, AZ10573295 reduced the amount of IL-1 $\beta$  released from 2730pg/10<sup>6</sup> cells to 124pg/10<sup>6</sup> cells. AZ10573295 is thus a potent inhibitor of IL-1 $\beta$  release, showing that P2X<sub>7</sub> antagonism by this class of compound could provide a useful treatment for IBD, particularly acute inflammatory episodes.

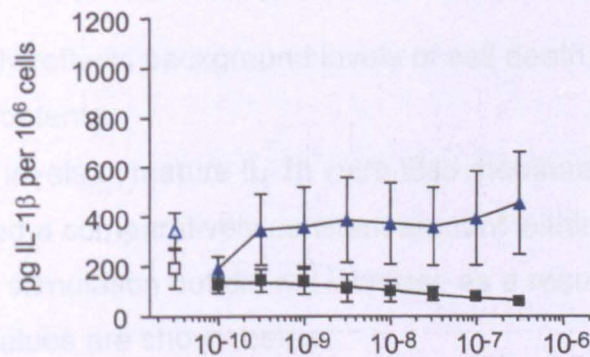
Levels of the IL-1 $\beta$  precursor, pro-IL-1 $\beta$  were also measured. Intracellular pro-IL-1 $\beta$  (Fig 7.8., blue triangles) increased with increasing antagonist concentration in LPMCs isolated from tissues from all three inflamed



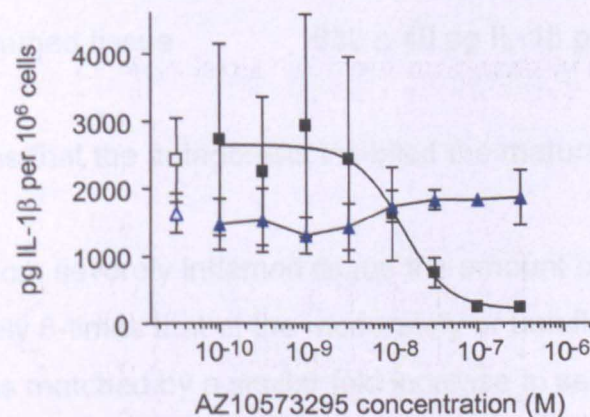
**a. LPMCs isolated from uninflamed tissue (n=3)**



**b. LPMCs isolated from moderately inflamed tissue (n=4)**



**c. LPMCs isolated from severely inflamed tissue (n=2)**



**Fig 7.8. Effect of AZ10573295 on IL-1 $\beta$  release from LPMCs isolated from inflamed tissue**

Cells resuspended in RPMI 1640 + 0.1% FCS were incubated with antagonist for 10min prior to stimulation for 20min with 80 $\mu$ M BzATP. The amount of IL-1 $\beta$  released into the supernatant was measured by ELISA. Results show the mean  $\pm$  sem of separate experiments. For the severely inflamed tissue (fig c) where there were only 2 subjects, the error bars show the responses of each individual and demonstrate the variation in the absolute amounts of IL-1 $\beta$  released.

conditions. This indicates that the antagonist is inhibiting IL-1 $\beta$  maturation as well as its release.

There was a steady release of pro-IL-1 $\beta$  from the LPMCs into the supernatant that was unaffected by antagonist concentration and was equivalent to the amount released by 80 $\mu$ M BzATP in the concentration-response curves (Fig 4.6.). The mean values for the amount released are shown below:

uninflamed tissue	327 $\pm$ 13 pg pro-IL-1 $\beta$ per 10 <sup>6</sup> cells
moderately inflamed tissue	232 $\pm$ 7.7 pg pro-IL-1 $\beta$ per 10 <sup>6</sup> cells
severely inflamed tissue	830 $\pm$ 80 pg pro-IL-1 $\beta$ per 10 <sup>6</sup> cells

This probably reflects background levels of cell death, breakdown and spillage of contents.

Intracellular levels of mature IL-1 $\beta$  were also measured and likewise demonstrated a comparatively constant amount within the cells that was not released on stimulation but did not increase as a result of antagonist activity.

The mean values are shown below:

uninflamed tissue	85 $\pm$ 8.1 pg IL-1 $\beta$ per 10 <sup>6</sup> cells
moderately inflamed tissue	75 $\pm$ 3.3 pg IL-1 $\beta$ per 10 <sup>6</sup> cells
severely inflamed tissue	632 $\pm$ 40 pg IL-1 $\beta$ per 10 <sup>6</sup> cells

This confirms that the antagonists inhibited the maturation of IL-1 $\beta$  as well as its release.

In LPMCs from severely inflamed tissue the amount of intracellular IL-1 $\beta$  was approximately 8-times that of the moderately or uninflamed tissue. This however was matched by a similar fold increase in secreted IL-1 $\beta$  (2730  $\pm$  1421pg/10<sup>6</sup> cells from severely inflamed tissue compared to 287  $\pm$  99pg/10<sup>6</sup> cells from uninflamed tissue) and reflected generally higher levels of production and release of IL-1 $\beta$  in the severely inflamed condition. In these cells the levels of both secreted and intracellular pro-IL-1 $\beta$  were also elevated compared to the other tissues.

### **7.3.3.2. Discussion**

The results showed that oATP and both AstraZeneca compounds demonstrated a similar pattern of antagonist responses to BzATP stimulation of LPMCs isolated from all the tissue types. Pre-incubation of LPMCs from uninflamed tissue with LPS produced levels of IL-1 $\beta$  release of 100-300pg/10<sup>6</sup> cells; higher than the amount released from cells isolated from normal tissue (80pg/10<sup>6</sup> cells), and equivalent to that released spontaneously from moderately inflamed tissue. In severe inflammatory conditions the amount of IL-1 $\beta$  was increased 10-fold. This is probably due to the presence of increased numbers of circulatory monocytes, cells which produce much higher levels of cytokine than tissue macrophages, but could also be a result of increased expression of P2X<sub>7</sub> receptors in the inflammatory state. The results show that P2X<sub>7</sub> activity is related to the severity of inflammation and therefore make it an important target for drug development.

### **7.3.4. Summary and Conclusion**

BzATP-stimulated release of IL-1 $\beta$  from isolated LPMCs has been shown to be inhibited by P2X<sub>7</sub> antagonists. The antagonists were particularly effective at reducing IL-1 $\beta$  secretion in the severely inflamed condition. This means that the development of specific P2X<sub>7</sub> antagonists could provide a useful tool for reducing levels of the inflammatory cytokine IL-1 $\beta$  in IBD tissue, particularly in acute episodes, and hence alleviate the degree of inflammation and associated damage to the tissue.

There is only one treatment currently available that acts by reducing IL-1 activity. Anakinra (Kineret, Amgen) is a recombinant form of the naturally occurring IL-1 receptor antagonist (IL-1Ra) and is used to treat moderate-to-severe rheumatoid arthritis (Furst, DE *et al.*, 2006). It appears to be most useful in treatment of periodic fever syndromes such as Muckle-Wells syndrome, neonatal onset multisystem inflammatory disease (NOMID) and adult onset Still's disease. However, in order to be effective, anakinra requires daily injections suggesting that it has very short-term effects (Burger, D *et al.*, 2006).

Other potential treatments currently undergoing clinical trial are IL-1 Trap, a soluble form of the IL-1 receptor used to bind and neutralise IL-1 (Gabay, C, 2003), and pralnacasan, an oral caspase-1 inhibitor (Randle, JC *et al.*, 2001). Both are being trialled for use in rheumatoid arthritis.

None of these treatments are so far being considered for IBD therapy although pralnacasan has been shown to prevent dextran sulphate sodium-induced colitis in mice. The results obtained here however, have demonstrated that P2X<sub>7</sub> antagonists can reduce IL-1 $\beta$  release from inflammatory cells and could provide a useful alternative treatment for IBD.

## CHAPTER 8. DISCUSSION AND FUTURE WORK

### 8.1. SUMMARY

This project set out to characterize the properties of the P2X<sub>7</sub> receptor in human colonic LPMCs, and to determine whether it would make a useful target for the treatment of inflammation in IBD. Its properties were compared to those of PBMCs, the source of recruited cells in inflamed tissue, and THP-1 cells, a human monocyte cell line known to constitutively express P2X<sub>7</sub>.

The initial step was to demonstrate the presence of functional P2X<sub>7</sub> receptors in LPMCs from human colonic tissue. One of the defining properties of the P2X<sub>7</sub> receptor is its ability to form a non-selective membrane pore permeable to small molecules on prolonged or repeated stimulation. The presence of the pore can be measured by the uptake of small fluorescent dyes such as ethidium bromide, which intercalate double-stranded DNA or RNA with a 20- to 30-fold increase in fluorescence (Haugland, RP, 2002). P2X<sub>7</sub> is also unique in that it is the only P2 receptor at which the ATP analogue BzATP is a more potent agonist than ATP; at all other P2 receptors BzATP is equipotent with or less potent than ATP (North, RA *et al.*, 2000). Using these two properties, the results presented in chapter 3 demonstrated that human colonic LPMCs express functional P2X<sub>7</sub> receptors with characteristics similar to those of PBMCs and THP-1 cells.

Stimulation of the P2X<sub>7</sub> receptor results in the release of IL-1 $\beta$  (Ferrari, D *et al.*, 1997b; Perregaux, DG *et al.*, 1994), a potent inflammatory cytokine shown to be present at higher levels in IBD tissue (Ligumsky, M *et al.*, 1990). It also causes increased exposure of phosphatidylserine on the outer surface of the cell membrane, known as PS flip, which has often been considered a marker of apoptotic cell death (Chiozzi, P *et al.*, 1996; Hogquist, KA *et al.*, 1991a). Both IL-1 $\beta$  release and apoptosis are important targets for anti-inflammatory drugs. Aminosalicylates and corticosteroids reduce cytokine production by inhibiting their transcription (Carter, MJ *et al.*, 2004), and several of the standard treatments for IBD act by inducing apoptosis,

including corticosteroids (Carter, MJ *et al.*, 2004), thiopurines (Tiede, I *et al.*, 2003) and the anti-TNF drug infliximab (ten Hove, T *et al.*, 2002). Selective P2X<sub>7</sub>-receptor antagonists could provide an alternate mechanism for controlling these processes, and hence potential treatment for inflammatory diseases such as IBD. For that reason the characteristics of P2X<sub>7</sub>-stimulated IL-1 $\beta$  release and PS flip (measured using AV-binding) were investigated.

### 8.1.1. Characteristics of P2X<sub>7</sub>-stimulated IL-1 $\beta$ release

The results presented in chapter 4 demonstrated that IL-1 $\beta$  was released from both PBMCs and LPMCs in response to P2X<sub>7</sub> stimulation. LPMCs isolated from normal colonic tissue secreted very low levels of IL-1 $\beta$ , but this increased dramatically in cells from inflamed tissue. This is likely to be for two reasons.

Firstly, resident tissue macrophages are non-inflammatory with the ability to phagocytose and kill microorganisms, but do not produce pro-inflammatory cytokines in response to phagocytosis (Smith, PD *et al.*, 2005). Macrophages have been shown to have a lower rate of constitutive caspase-1 activation than monocytes and hence reduced ability to process IL-1 $\beta$  (Kahlenberg, JM & Dubyak, GR, 2004a). Colonic mucosal macrophages also lack surface CD14, the receptor for bacterial LPS (Smith, PD *et al.*, 2001) and have no APC function (Rugtveit, J *et al.*, 1997). However, the presence of inflammatory stimuli, such as invading pathogens, results in maturation of dendritic cells and T cells and the release of inflammatory cytokines such as IFN- $\gamma$ . IFN- $\gamma$  promotes classical activation of macrophages, which have all the capabilities of antigen presentation and are major producers of inflammatory cytokines such as IL-1 $\beta$ .

Secondly, in active IBD there is an increase in the mucosal macrophage population thought to be derived from circulating monocytes (Rugtveit, J *et al.*, 1994; Allison, MC *et al.*, 1988). The results in chapter 4 (Table 4.1.) demonstrated that PBMC monocytes are capable of secreting much higher levels of IL-1 $\beta$  than normal tissue macrophages ( $2509 \pm 777\text{pg}/10^6$  cells compared to  $748 \pm 322\text{pg}/10^6$  cells) following BzATP stimulation. An influx of these cells into the tissue would therefore automatically elevate production of

IL-1 $\beta$ . The macrophage population from inflamed tissue has also been shown to exhibit greater heterogeneity than that of normal mucosa (Mahida, YR *et al.*, 1989a; Allison, MC *et al.*, 1991). Mahida, YR *et al.*, (1989a) demonstrated the presence of two macrophage populations not present in normal tissue; epithelioid cells which have a reduced phagocytic but greater secretory capacity, and a population of CD16<sup>+</sup> macrophages whose function was unknown but may have been lymph-homing dendritic cells that could also secrete inflammatory cytokines (Randolph, GJ *et al.*, 2002). All of these factors combined would lead to greatly increased secretion of IL-1 $\beta$  from inflamed tissue.

The results in chapter 5 showed that maximal IL-1 $\beta$  release was achieved after 20 minutes stimulation of the P2X<sub>7</sub> receptor and that stimulation for longer times had no further effect. The release of IL-1 $\beta$  was greater (5 fold) from PBMCs than LPMCs, and more rapid. The maximum rate of release (pg IL-1 $\beta$ /min) occurred at 5 minutes in PBMCs compared to 10 minutes for LPMCs, and the release of IL-1 $\beta$  reached a maximum at approximately 10 minutes stimulation compared to 20 minutes for LPMCs.

As demonstrated by the EC<sub>50</sub> values (Table 4.1 and 4.2), release of IL-1 $\beta$  generally required lower agonist concentrations than did stimulation of PS flip, but PS flip occurred more rapidly (maximum rate at approximately 2-3 minutes compared to 5-10 minutes for IL-1 $\beta$  release). This is not surprising since it has been suggested that P2X<sub>7</sub>-induced PS flip is an initial upstream effector in a P2X<sub>7</sub> signalling pathway, linked to cytoskeletal rearrangements and reversible membrane blebbing that may or may not be linked to microvesicular release of IL-1 $\beta$  (MacKenzie, AB *et al.*, 2005; MacKenzie, A *et al.*, 2001).

In chapter 6 it was demonstrated that 5 minutes stimulation with BzATP was sufficient to initiate IL-1 $\beta$  release, which then reached a maximum during the first hour of the subsequent incubation. Stimulating the cells for 30 minutes did not increase the total amount of IL-1 $\beta$  released; instead, more IL-1 $\beta$  was released during the stimulation period but less during the ensuing incubation.

As seen in chapter 5, PBMCs demonstrated a greater capacity for IL-1 $\beta$  release than LPMCs. Following stimulation for 5 minutes, they secreted more IL-1 $\beta$  than LPMCs but also contained higher levels of intracellular mature IL-1 $\beta$ . In contrast, LPMCs contained high levels of pro-IL-1 $\beta$  with very little mature IL-1 $\beta$  either intracellular or released. Stimulation for 30 minutes resulted in high levels of IL-1 $\beta$  secretion from PBMCs with little pro- or mature IL-1 $\beta$  remaining intracellularly. LPMCs however, although secreting some mature IL-1 $\beta$  during the 30 minutes, still contained high levels of pro-IL-1 $\beta$ .

The maximum amounts of IL-1 $\beta$  released from cells isolated from IBD patients matched the severity of their inflammation, making P2X<sub>7</sub> inhibition a very attractive target in the treatment of IBD. BzATP-stimulated release of IL-1 $\beta$  from LPMCs was shown to be inhibited by P2X<sub>7</sub> antagonists (chapter 7). The antagonism was particularly marked in cells from severely inflamed tissue and suggests that the development of specific P2X<sub>7</sub> antagonists could provide a useful approach for reducing levels of IL-1 $\beta$  in IBD, and hence alleviate the degree of inflammation and associated damage to the tissue. Unfortunately P2X<sub>7</sub> antagonists did not appear to inhibit IL-1 $\beta$  release from colonic mucosal biopsies maintained in organ culture. However, it is possible that organ culture is not a good model system for P2X<sub>7</sub> antagonism in vivo. P2X<sub>7</sub>-mediated IL-1 $\beta$  release requires stimulation of the receptor with ATP but the organ culture experiments used LPS to promote IL-1 $\beta$  production which may not involve the release of ATP.

The variable nature of cytokine secretion between patients seen in these results confirmed the nature of IBD, in that characteristics of the disease vary widely between subjects, with many factors involved in the nature of its presentation. There is a need therefore for treatments that can target the inflammatory processes in different ways since one that is effective in one patient may not be in another. New treatments already available that target IL-1 $\beta$  are anakinra (IL-1 receptor antagonist), IL-1 Trap (soluble IL-1 receptor)



and pralnacasan (caspase-1 inhibitor). Inhibition of P2X<sub>7</sub>-stimulated IL-1 $\beta$  release offers another means for such treatment.

### **8.1.2. Characteristics of P2X<sub>7</sub>-stimulated PS flip and cell death**

The results in chapter 4 demonstrated that all of the cell types displayed a P2X<sub>7</sub>-mediated increase in total AV-binding, and hence PS flip. Since PS flip has often been considered to be linked to apoptosis, this initially suggested that stimulation of the P2X<sub>7</sub> receptor leads to apoptosis in these cells.

MacKenzie, AB *et al.*, (2005) however has proposed that PS flip associated with brief stimulation of the P2X<sub>7</sub> receptor is not part of the apoptotic process but is completely reversible, and has called it 'pseudoapoptosis'. In contrast, they showed that prolonged P2X<sub>7</sub> stimulation for more than 20-30 minutes led to subsequent apoptotic cell death.

These differences prompted an investigation into the effect of P2X<sub>7</sub> receptor stimulation time on apoptosis and cell death in LPMCs. The results presented in chapter 5 showed the effect of increasing stimulation times of the P2X<sub>7</sub> receptor at maximal and half-maximal agonist concentrations. PS flip, measured by AV binding, was rapid in all cell types, with maximum rates of binding per minute occurring between 0-3 minutes with BzATP stimulation and 1-5 minutes with ATP stimulation. Monocytes generally demonstrated more rapid PS flip than lymphocytes, and they were also more susceptible to cell death, as measured by PI staining. Further investigation of cell death using a larger nucleic acid stain, TOTO-3, and LDH release suggested that some of the observed PI staining in monocytes was probably due to uptake of the dye through the P2X<sub>7</sub> pore rather than cell death. However they also confirmed that monocytes were more liable to die following P2X<sub>7</sub>-stimulation than lymphocytes, and also that PBMCs were more susceptible than LPMCs.

The reversibility of PS flip was then investigated using 5 or 30 minutes stimulation with BzATP. The results in chapter 6 showed that brief stimulation of the P2X<sub>7</sub> receptor induced PS flip (measured by AV binding) that was completely reversible in all the cell types except for PBMC

monocytes, in which 40% of the cells demonstrated irreversible PS flip. Following stimulation for 30 minutes however, all of the cells demonstrated irreversible PS flip and cell death, as measured by PI staining, except for LPMC lymphocytes which still reversed the AV binding. The combined results of AV/PI staining and LDH release confirmed the findings of chapter 5; namely that monocytes were more susceptible to cell death following P2X<sub>7</sub> stimulation than lymphocytes, and that PBMCs were more susceptible than LPMCs. This is not surprising since monocytes have been shown to have a four- to five-fold greater expression of P2X<sub>7</sub> than lymphocytes (Gu, BJ *et al.*, 2000). The pattern of LDH release suggested that P2X<sub>7</sub>-stimulated cell death was necrotic, occurring only in the presence of the agonist.

Cell cycle analysis and Hoechst staining indicated little induction of apoptosis following P2X<sub>7</sub> stimulation, but the 24-hour Hoechst-stained slides contained visibly less cells than the earlier time points, compatible with necrotic cell death. These results suggested therefore, that although P2X<sub>7</sub> stimulation was associated with cell death, this was not apoptotic cell death and P2X<sub>7</sub>-stimulated PS flip was not a marker for apoptosis.

## **8.2. CONCLUSIONS AND FUTURE WORK**

The results presented in this thesis have shown that P2X<sub>7</sub>-stimulated IL-1 $\beta$  release generally occurred at lower agonist concentrations than AV binding, and only required short stimulation times. This suggests that IL-1 $\beta$  release is associated with reversible PS flip rather than the irreversible flip associated with cell death, which generally requires at least 30 minutes stimulation of the P2X<sub>7</sub> receptor (MacKenzie, AB *et al.*, 2005).

The role of P2X<sub>7</sub> may therefore be two-fold. With minimal cell stress and/or damage, brief stimulation of the receptor caused by localised release of ATP would lead to IL-1 $\beta$  release and promotion of an inflammatory reaction. The action of nucleotidase enzymes would then quickly remove the ATP with resulting resolution and repair. However in the event of major damage to cells leading to the prolonged release of ATP and hence prolonged P2X<sub>7</sub> stimulation, it acts as a danger signal and promotes cell death to ensure that the inflammatory response is stopped before it causes harm to the tissue.

Pyroptosis is a form of pro-inflammatory cell death initially used to describe death associated with *Salmonella* and *Shigella* infection of host macrophages (Cookson, BT & Brennan, MA, 2001; Hilbi, H *et al.*, 1997). It was proposed as a mechanism of programmed cell death for the removal of potentially dangerous cells, such as infected cells, where recruitment of additional cells or cellular functions are required (Cookson, BT & Brennan, MA, 2001). Pyroptosis is dependent on caspase-1 activity and leads to membrane breakdown and pro-IL-1 $\beta$  processing.

A study using peritoneal macrophages showed that activation of caspase-1 following P2X<sub>7</sub> stimulation elicited rapid cell death preceded by IL-1 $\beta$  release that was analogous to pyroptosis (Brough, D *et al.*, 2007). More recently a unique structure called the pyroptosome has been proposed, composed of oligomerised ASC dimers (a component of the inflammasome, Fig 1.6.). Formation of the pyroptosome in THP-1 cells was driven by potassium efflux, a feature of P2X<sub>7</sub> stimulation, and resulted in rapid activation of caspase-1 and release of inflammatory cytokines (Fernandes-Alnemri, T *et al.*, 2007). Pyroptotic death of infected macrophages has also been shown to involve pore formation that required actin cytoskeleton rearrangements, also features of P2X<sub>7</sub> stimulation (Fink, SL & Cookson, BT, 2006). It is possible therefore that P2X<sub>7</sub> is not an apoptotic receptor as was initially believed, but a pyroptotic receptor, stimulation of which results in IL-1 $\beta$  release followed by cell death.

IL-1 $\beta$  is a highly inflammatory cytokine whose release needs to be tightly regulated. The requirement for P2X<sub>7</sub> as a second signal for IL-1 $\beta$  production forms a fail-safe mechanism to ensure that activation of such a potent inflammatory response only occurs when absolutely necessary. A study of murine macrophages demonstrated that P2X<sub>7</sub>-mediated activation of caspase-1 required prestimulation with LPS (Kahlenberg, JM *et al.*, 2005). This ensures that activation of P2X<sub>7</sub> by ATP will only result in the maturation of IL-1 $\beta$  if an inflammatory signal such as bacterial LPS is also present. Having such a potent receptor on the cell surface also requires tight control of its activity, and P2X<sub>7</sub> has been shown to be inhibited by physiological concentrations of various ions including Ca<sup>2+</sup> and Mg<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup> and Cl<sup>-</sup>,

(Michel, AD *et al.*, 1999; Virginio, C *et al.*, 1997). High levels of ATP are therefore needed for its stimulation, ensuring that the receptor is only activated under extreme conditions of cell stress or damage.

All of the investigations carried out in this project have shown that PBMCs, particularly monocytes, are more responsive to P2X<sub>7</sub> stimulation than LPMCs. In contrast, a study by Hickman, SE *et al.*, (1994) showed that P2X<sub>7</sub> expression increased as monocytes matured into macrophages. The macrophages they used however were matured in culture from blood monocytes incubated in medium containing 30% v/v FCS. They are likely therefore to have retained their monocyte properties, including expression of CD14, and to have characteristics of classically activated macrophages able to mount an inflammatory response. Conversely, normal tissue macrophages mature under the influence of other tissue cells and their secretory products, and become efficient scavenging cells with non-inflammatory characteristics. A study by Spottl, T *et al.*, (2001) demonstrated that incubation of peripheral blood monocytes with intestinal epithelial cell lines resulted in down-regulation of CD14 expression from 86% of the cell population to 11% after 7 days co-culture. It would be interesting to perform a similar experiment studying expression levels of the P2X<sub>7</sub> receptor.

Upregulation of the P2X<sub>7</sub> receptor during inflammation may in part be a result of phagocytosis of invading pathogenic bacteria. The C-terminal chain of the P2X<sub>7</sub> receptor has been shown to contain a region homologous to the LPS binding site of LPS-binding protein (Denlinger, LC *et al.*, 2001). The authors suggested that internalised LPS may play a regulatory role in receptor trafficking, and this is supported by the fact that point mutations involved in trafficking lie within the LPS-binding region (Denlinger, LC *et al.*, 2003). It would therefore be interesting to look at the distribution of the P2X<sub>7</sub> receptor in PBMCs and in LPMCs from normal, uninfamed and inflamed tissue using anti-P2X<sub>7</sub> antibodies. Slater, M *et al.*, (2004) showed that P2X<sub>7</sub> expression was associated with development of cancer in the prostate gland. The receptor appeared in a stage-specific manner beginning in the nucleus, progressing to the cytoplasm and finally gathering on the apical membrane of

the epithelial cells. It may be that a similar increase in P2X<sub>7</sub> expression occurs in colonic macrophages, related to increased inflammation. If the level of P2X<sub>7</sub> expression could be shown to correlate with the severity of inflammation this would confirm the usefulness of P2X<sub>7</sub> antagonists as a treatment for IBD.

It would also be interesting to discover whether increased receptor expression could be induced artificially by incubation of LPMCs from normal tissue with LPS or other inflammatory mediators, or by co-culture with bacteria such as *E. coli*. In association with P2X<sub>7</sub> expression, the levels of CD14 and TLR4 expression within the different tissue types would also indicate the cells' ability to respond to bacteria within the gut. This would confirm whether the principal modulator of P2X<sub>7</sub> expression was bacterial LPS or whether other inflammatory signals were required.

The role of P2X<sub>7</sub> in LPMC cell death could also be investigated further, in particular additional experiments to see whether P2X<sub>7</sub>-stimulated PS flip is associated with apoptosis. Since T cells from IBD tissue are resistant to apoptosis (Neurath, MF *et al.*, 2001), expansion of the studies of AV/PI binding to include cells from uninfamed and inflamed tissue would be expected to show different staining patterns to those from normal tissue. If there was no change, it would support the results from the cell cycle analysis and Hoechst staining indicating that P2X<sub>7</sub> does not play a significant role in apoptotic cell death.

The novel P2X<sub>7</sub> antagonists provided by AstraZeneca could be investigated to see whether they bind to the ATP binding site, like oATP, or to an allosteric site on the receptor, like KN62. Michel, AD *et al.*, (2000) pre-incubated cells with PPADS or KN62 prior to antagonism of BzATP stimulation with oATP. They found that PPADS attenuated the antagonistic effect of oATP but KN62 had no effect and this led them to surmise that KN62 was an allosteric antagonist acting at a site distinct from that recognised by oATP and PPADS. Similar experiments with the AZ antagonists could show whether they also bind to an allosteric site or whether they compete with the agonist for the same site. Many point

mutations have been identified in the P2X<sub>7</sub> receptor, modifying its efficacy, actions or expression. If the AZ antagonists bind to an allosteric site it is possible that mutations within that site could negate their effects and hence their value as P2X<sub>7</sub> inhibitors.

Ultimately, the most interesting future work would be to administer the AstraZeneca P2X<sub>7</sub> inhibitor, AZD9056 (currently being trialled for treatment of rheumatoid arthritis), to IBD patients. Measurement of pre- and post-treatment colonic biopsy IL-1 $\beta$  and plasma IL-18 would provide useful information, but the principal target would be the relief of inflammatory symptoms in the patient and to show that P2X<sub>7</sub> inhibitors are useful treatments for IBD.

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