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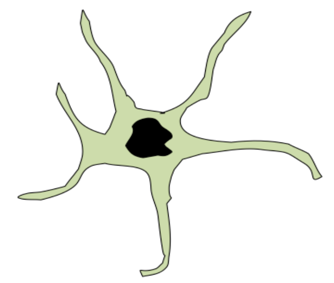
The Alternative Activation of Dendritic Cells



Lucy Helen Jones

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DECLARATION

I, the undersigned, hereby declare that the contents of this thesis have been composed by myself and that the work described herein is entirely my own unless acknowledged otherwise.

Lucy Helen Jones Date

CONTRIBUTIONS

The majority of experiments detailed in chapters 3 and 4 of this thesis were carried out in collaboration with, at the levels of conception, planning, execution or analysis, Dr Peter C. Cook. Particular credit should be given for the data in Fig. 4.12 and 4.13 in which I only provided technical assistance. Prof. Judith E. Allen, Dr Stephen J. Jenkins, Dr Dominik Ruckerl, Dr Rachel J. Lundie, Dr Alex L.T. Phythian-Adams, Miss Lauren M. Webb and Dr Jess G. Borger provided useful advice on experimental design and/or data comprehension.

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ABSTRACT

The alternative activation of macrophage populations by Interleukin-4 (IL-4) is well characterised. Alternatively activated macrophages (AAM) express high levels of the arginine converting enzyme arginase-1, and express a plethora of IL-4 driven molecules including the resistin like molecule alpha (RELMα) and the chitinase like molecule Ym1/2. Dendritic cells (DCs) are the professional antigen presenting cells (APC) of the immune system, responsible for the detection of invading pathogens, secretion of cytokines and the subsequent activation of T-cells. This thesis addresses whether IL-4 is able to 'alternatively activate' DCs both *in vitro* and *in vivo,* in a manner similar to that of AAM.

The impact of IL-4 on DC and macrophage activation was compared and contrasted, and it was confirmed for the first time that IL-4 can alternatively activate DCs, inducing high level expression of a range of alternative activation associated markers including RELMα, Ym1/2, CCL24 and dectin-1, with the exception of arginase. DCs were significantly more capable at the *in vivo* priming of T-cell responses in the context of both Th1 and Th2 polarising antigens than similarly exposed macrophages, confirming their superior capacity as APC.

The requirements for DC IL-4Rα expression were assessed, and IL-4 responsiveness was found to be required for the optimal induction of Th1 responses. Conversely, selective loss of only one facet of the IL-4 response, namely RELMα expression, limited the ability of IL-4 exposed DCs to induce the regulatory cytokine IL-10 both *in vitro* and *in vivo*. Furthermore, alternatively activated DCs (AADCs) were found in the spleen following 8 weeks of infection with the parasitic trematode *Schistosoma mansoni,* highlighting a role for DC alternative activation in a disease setting.

IL-4 was shown to induce expression of the vitamin A converting enzyme aldehyde dehydrogenase, and the product of such activity, retinoic acid (RA), was found to promote the expression of RELMα in IL-4 exposed DCs. Aldehyde dehydrogenase activity was found to inversely correlate with DC expression of Ym1/2 and inhibition of RA signalling limited IL-4 driven RELMα and promoted Ym1/2.

The impact of various stimuli on IL-4 driven alternative activation was assessed. Toll like receptor ligands (LPS, CpG, P3C) and complex antigens (zymosan, *P. acnes*, SEA) were found to differentially modulate IL-4 driven RELMα and Ym1. The ability of P3C to completely abrogate IL-4 driven RELMα but only limit IL-4 driven Ym1 was shown to be dependent on TLR2, whereas the zymosan effect was TLR2 independent.

A novel role for pERK in modulating IL-4 dependent alternative activation of DCs was discovered. pERK differentially modulated facets of IL-4 dependent alternative activation, inhibiting RELMα, promoting CCL24 and having no impact upon Ym1/2.

SCHEMATIC GLOSSARY

Image	Description
	Wild type bone marrow derived dendritic cell
	Gene deficient bone marrow derived dendritic cell
	Plasmacytoid dendritic cell
	Bone marrow derived macrophage
	Thioglycollate elicited macrophage
	$CD4^+$ T-lymphocytes
	Peritoneal exudate cells
	Spleen
	Popliteal lymph nodes
	Pooled spleen and lymph nodes
	Liver
	Mesenteric lymph nodes and Gut
	C57BL/6 mouse
	BALB/c mouse
	CFSE labeled T-lymphocytes
	Ovalbumin
	FITC conjugated dextran
	Coverslip
	Tissue culture well

ABBREVIATIONS

12/15LOX: 12/15 (S) Lipoxygenase

AADC: Alternatively activated DC

AAM: Alternatively activated macrophage

ADH: Alcohol dehydrogenase

ADRP: Adipose differentiation related protein

AKT: Ak Thymoma / protein kinase B

ALDH: Aldehyde dehydrogenase

AP1: Activator protein 1

APC: Antigen presenting cell

Arg1: Arginase 1

BATF3: Basic Leucine Zipper transcription factor, ATF-like 3

BEC: (S-(2-boronoethyl)-l-cysteine)

BM: Bone marrow

BMDC: Bone marrow derived dendritic cell

BMM: Bone marrow derived macrophage

BTK: Bruton’s tyrosine kianse

CARD: Caspase recruitment domain

CCL: CC Chemokine Ligand

CCR: CC Chemokine receptor

CD: Complementarity Determining

cDC: Conventional DC

CDP: Common DC precursor

CLR: C-type lectin Receptor

COX: Cyclooxygenase

CpG: CpG oligodeoxynucleotide 1826

CRBP:Cellular retinol binding protein

Csf1R: Colony stimulating factor 1 receptor

CTL: C-type lectin

CTLA-4: Cytotoxic T-lymphocyte antigen 4

CXCR: CXC cheomokine receptor

DAMP: Danger associated molecular pattern

DC-SIGN: DC specific ICAM 3 grabbing integrein

DC: Dendritic cell

Dectin1/*Clec7a*: Dendritic cell C-type lectin 1

DNA: Deoxyribonucleic acid

DSS: Dextran sulphate sodium

DUSP: Dual Specificity phosphatase

dZym: Zymosan depleted of contaminating TLR2 ligands

ELISA: Enzyme linked immunosorbent assay

ER: Endoplasmic reticulum

ERK: Extracellular signal related kinase

Ets2: E twenty six 2

FACS: Fluorescence activated cell sorting

FLDC: FLT3L differentiated BMDC

FLT3L: Fms Like tyrosine kinase 3 ligand

Foxp3: Forkhead box protein 3

g: gram

GALT: Gut associated lymphoid tissue

GATA: Gut associated lymphoid tissue

GC: Germinal centre

GM-CSF: Granulocyte macrophage-colony stimulating factor

GMDC: GMCSF differentiated BMDC

GMP: Granulocyte macrophage progenitor

Grb2: Growth factor receptor-bound protein 2

HMGB: High mobility group box protein

ICOS: Inducible costimulator

Id2: Inhibitor of DNA binding 2

IELs: Intra-epithelial lymphocytes

IFN*:* Interferon

IGSF: Immunoglobulin super family

IKKβ: IκB Kinase

IL : Interleukin

ILC: Innate lymphoid cell

iNOS: Inducible nitric oxide synthase

i.p: Intra-peritoneal

IRAK: IL-1 receptor associated kinase

IRF: Immune regulatory factor

IRS: Insulin receptor substrate

ITAM: Immunoreceptor tyrosine based activation motif

iTreg: peripherally induced FoxP3 expressing Treg

i.v.: Intra-venous

JAK: Janus associated kinase

JNK: cJun NH_2 terminal kinase

LN: Lymph Node

LO: Lymphoid organ

LP: Lamina propria

LPS: Lipopolysaccharide

LRAT: Lecitihin retinyl acyl transferase

M-CSF: Macrophage- colony stimulating factor

M1: Classically activated macrophages

M2: Non-classically activated macrophages

MAdCAM1: Mucosal addressin cell adhesion molecule 1

MAPK: Mitogen activated protein kinase

MDP: macrophage-DC precursor

MEK: Dual specificity mitogen activated protein kinase kinase

MGL: Macrophage galactose CTL

MHC: Major Histocompatibility Complex

MKK: MAPK kinase

MKKK: MAPK kinase kinase

MKPs: MAPK phosphatises

mLN: Mesenteric lymph node

MMP: Matrix metalloproteinase

MR/*Mrc1*: Mannose Receptor

MyD88: Myeloid differentiation factor 88

Mφ: Macrophage

NFκB: Nuclear factor κ B

NLR: NOD like receptors

NLRP3: NLR family, pyrin domain containing 3

NO: Nitric oxide

NOD: Nucleotide oligomerisation domain

nTreg: natural Treg

^{o}C: Degrees centrigrade

ODC: Ornithine decarboxylase

P3C: Pam3CSK4

Pa: Heat Killed *Propionibacterium acnes*

PAMP: Pathogen associated molecular pattern

PBS: Phosphate buffered saline

PD-L: Programmed death Ligand

PD: Programmed death

pDC: Plasmacytoid DC

pERK: Phosphorylated ERK

PGE_2: Prostaglandin E2

PI3K: phosphoinositide 3 kinase

PKC: Protein Kinase C

pLN: Popliteal LN

PP: Peyers patch

PRR: pattern recognition receptor

qPCR: Quantitative polymerase chain reaction\

RA: All-trans retinoic acid

RA: Retinoic acid

RALDH: Retinaldehyde dehydrogenase

RANK: Receptor activator of NFκB

RAR: Retinoic acid receptor

RARE: Retinoic acid response elements

RBP: Retinol binding protein

RELMα/*Retnla*: Resistin like molecule alpha

RIG: Retinoic acid inducible gene

RLR: RIG like receptors

RNA: ribonucleic acid

ROS/RNOS: Reactive (Nitrogen) oxygen species

RXR: Retinoid X receptor

SARM: Sterile-alpha and armadillo motif containing protein

SDR: Short chain dehydrogenase reductase

SEA: Soluble egg antigen of *Schistosoma mansoni*

SH2: Src homology domain containing 2

SI: Small intestinal

sMR: soluble MR

SOS: Son of sevenless

St: *Salmonella typhimurium*

STAT: Signal transducer and activator of transcription

STRA6: Stimulated by retinoic acid 6

Syk: Spleen tyrosine kinase

TAK: TGFβ activated kinase

TCR: T-cell receptor

TF: Transcription factor

Tfh: T follicular helper cell

TGFβ: Transforming growth factor beta

Th: T helper cell

Th3: TGFβ dependent, FoxP3 independent Treg

Thio: Thioglycollate treated

ThioM: Thioglycollate elicited macrophage

Tip: TNFα and iNOS producing

TIR: Toll IL-1R homology domain

TIRAP: Toll-IL-1receptor domain containing adaptor

TLR: Toll like receptor

TNF: Tumour necrosis factor

TNFRSF: TNF receptor super family

Tr1: Foxp3 independent IL-10 dependent Treg

TRAF: TNFR associated factor 6

TRAM: TRIF related adaptor molecule

Treg: T regulatory cell

TRIF: Toll-IL-1receptor domain containing adaptor IFNβ

TRIM: Trif related adaptor molecule

Ts: T suppressor

TTR: Transthyretin

VAD: Vitamin A deficient

VEGF: Vascular endothelial growth factor

WT: Wild type

Ym1/2: Chitinase like molecule 3 like 3 /3 like 4

Zym: Zymosan

Table of Contents

Chapter 1. General Introduction 1

1. INTRODUCTION 1

1.1 DENDRITIC CELLS 1

1.1.2 Differentiating DCs *in vitro* 4

1.2 DC RECOGNITION OF NON-SELF AND DANGER 5

1.2.1 Toll like receptors 6

1.2.2 MAPK signalling 6

1.2.3 Other PRRs 7

1.3 DCs AS ANTIGEN PRESENTING CELLS 8

1.3.1 Contact dependent signals between DCs and T-cells 9

1.3.2 $CD4^+$ T helper cell subsets 10

1.3.3 Th2 diversity 13

1.4 SCHISTOSOMA MANSONI INFECTION 14

1.4.1 Immune recognition of schistosome egg antigens 15

1.5 IMMUNE RECOGNITION OF BACTERIA 16

1.6 MACROPHAGES AND ALTERNATIVE ACTIVATION 16

1.6.1 Arginase 18

1.6.2 Ym1 19

1.6.3 Resistin like molecule alpha 20

1.6.4 Mannose receptor 23

1.6.5 Dectin-1 23

1.6.6. AAM associated chemokines 24

1.6.7 Metabolism and AAM 25

1.6.8 Other cytokines implicated in the induction of AAM 27

1.6.9 Alternative activation in non-macrophage populations 28

1.7 IL-4R SIGNALLING 29

1.7.1 IL-4 induction of DC IL-12 31

1.8 VITAMIN A METABOLISM 32

1.8.1 Retinoic acid signalling 34

1.8.2 Intestinal homing 34

1.8.3 $CD103^+$ DCs, RA production and RA effects on T-cells 35

1.8.4 Induction of RALDH2 in DCs 36

1.8.5 Immune mediators implicated in induction and regulation of DC RALDH 38
1.8.6 Vitamin A and Th1/Th2 39
1.8.7 Vitamin A and Schistosomiasis 40
1.9 THESIS AIMS 42
1.10 FIGURES 43
Figure 1.1. Murine DC and macrophage ontogeny 43
Figure 1.2 TLR signalling 44
Figure 1.3. MAPK signalling 45
Figure 1.4. Co-stimulatory/-inhibitory molecules 46
Figure 1.5. T-helper cell subsets 47
Figure 1.6. Diversity in molecules expressed by CAM and AAM 48
Figure 1.7. Arginine metabolism 49
Figure 1.8. IL-4Rα signalling 50
Figure 1.9. Retinol metabolism 51
Figure 1.10. DC imprinting of gut homing function in T-cells 52
Chapter 2. Materials and Methods 53
2.1 MICE, INFECTIONS AND *IN VIVO* EXPERIMENTS 53
2.1.1 Mice 53
2.1.2 *S. mansoni* infection 53
2.1.3 Intra-peritoneal injections 53
2.1.4 Thioglycollate elicited peritoneal macrophages 54
2.1.5 Sub-cutaneous injections into the feet 54
2.2 CELL ISOLATIONS 54
2.2.1 Innate cell isolation 54
2.2.2 Splenic DC purification 55
2.2.3 T-cell isolation for DC:T-cell co-culture 55
2.3 I*N VITRO* BONE MARROW DIFFERENTIATION 55
2.3.1 Bone marrow preparation 55
2.3.2 Heat inactivation of foetal calf serum 56
2.3.3 rGM-CSF BMDC culture 56
2.3.4 Fms tyrosine like kinase-3 ligand BMDC culture 57
2.3.5 rMCSF BMM culture 57
2.4 *IN VITRO* STIMULATIONS 57
2.4.1 Preparation of antigens 57

2.4.2 Prolonged stimulus 58
2.4.3 Short term stimulus 59
2.4.4 αCD40 stimulation 59
2.4.5 DC:T-cell co-culture 59
2.4.6 OT-II: DC cultures 59
2.5 ANALYSIS TECHNIQUES 60
2.5.1 Flow cytometry 60
2.5.2 The aldefluor assay 61
2.5.3 Enzyme linked immunosorbent assay (ELISA) 61
2.5.4 Griess reaction for NO_2 62
2.5.5 Arginase activity assay 62
2.5.6 Microscopy 63
2.5.7 RNA isolation and qPCR 63
2.6 MICROARRAY 64
2.6.1 Microarray sample preparation 64
2.6.2 Microarray post hybridization analysis 64
2.7 STATISTICAL ANALYSIS 64
2.8 FIGURES AND TABLES 66
Figure 2.1 Gating strategy for FLT3L cultures 66
Figure 2.2 Optimisation of M-CSF BMM culture 67
Figure 2.3 IL-4 dose response to optimise BMDC alternative activation 68
Figure 2.4 Optimisation of the timing of IL-4 treatment 69
Figure 2.5.Golgistop does not enhance intracellular levels of RELMα or Ym1/2 70
Table 2.1 Flow cytometry antibodies 71
Table 2.2 ELISA antibodies 72
Table 2.3 qPCR primers 73
Chapter 3. Can Dendritic Cells be Alternatively Activated? 74
3.1 INTRODUCTION 74
3.2 SPECIFIC AIMS 76
3.3 RESULTS 76
3.3.1 DC express IL-4Rα, which is downregulated following exposure to IL-4 *in vitro* 76
3.3.2 IL-4 up-regulates DC expression of RELMα and Ym1/2 76
3.3.3 IL-4 driven RELMα is located throughout the cytoplasm of BMDCs 77

3.3.4 IL-4 upregulates multiple transcripts associated with AAM, but not *Arg1* 77
3.3.5. Arginase-1 is not a marker of an alternatively activated DC (AADC) 78
3.3.6 FLT3L derived DCs do not become alternatively activated in response to IL-4 78
3.3.7 *Ex vivo* splenic DC can be alternatively activated by IL-4 79
3.3.8 DCs can be alternatively activated *in vivo* 80
3.3.9 Markers of alternative activation are upregulated following *S mansoni* infection 81
3.3.10 Parasitic helminth infection induces alternative activation of splenic DCs 82
3.3.11 Defining a population of AAMs for direct comparison to AADCs 83
3.3.12 BMDC and BMM are distinct cells types 83
3.3.13 GMDC express higher levels of costimulatory molecules and secrete more cytokine than BMM 84
3.3.14 GMDC are significantly better than BMM at priming both Th1 and Th2 responses *in vivo* 85
3.3.15 BMM are less competent than GMDC at polarising $CD4^+$ T-cells towards Th2 *in vitro* 86
3.4 SUMMARY 87
3.5 DISCUSSION 88
3.5.1 IL-4 and GM-CSF dose 88
3.5.2 IL-4 down modulation of the IL-4Rα 88
3.5.3 Differential expression of RELMα and Ym1/2 89
3.5.4 What are the vacuoles in IL-4 treated GMDCs? 90
3.5.5 DC arginase expression and activity 91
3.5.6 AADC secretion of chemokines 92
3.5.7 IL-4 Vs IL-13 in DC alternative activation 93
3.5.8 Why aren't FLDCs alternatively activated by IL-4? 94
3.5.9 Comparing the alternative activation of BMM and ThioM 94
3.5.10 APC function of BMM Vs BMDC 95
3.6 FIGURES 97
Figure 3.1 IL-4 stimulated BMDCs do not dramatically alter their activation phenotype 97
Figure 3.2 IL-4 upregulates RELMα and Ym1/2 in BMDCs via the IL-4Rα 98

Figure 3.3 IL-4 treated DCs produce RELMα protein throughout their cytoplasm 99
Figure 3.4 IL-4 up-regulates multiple markers associated with AAM, but not *Arg1* 100
Figure 3.5 Arginase-1 is not a marker of an alternatively activated DC (AADC) 101
Figure 3.6 FLDCs do not become alternatively activated in response to IL-4 102
Figure 3.7 IL-4 alternatively activates splenic ex vivo DCs *in vitro* 103
Figure 3.8 IL-4 alternatively activates DCs *in vivo* 104
Figure 3.9 Markers of alternative activation are upregulated following *S. mansoni* infection 105
Figure 3.10 Parasitic helminth infection induces alternative activation of Splenic DCs 106
Figure 3.11 BMM produce more RELMα than Ym1/2, the opposite is true of ThioM 107
Figure 3.12 BMDC and BMM are different cells types 108
Figure 3.13 GMDC express higher levels of co-stimulatory molecules and secrete more cytokine than BMM 109
Figure 3.14 GMDC are significantly better than BMM at priming both Th1 and Th2 responses *in vivo* 110
Figure 3.15 BMM are less competent than GMDC at polarising $CD4^+$ T-cells towards Th2 *in vitro* 111

Chapter 4. What is the Impact of IL-4 and Alternative Activation on DC Function? 112

4.1 INTRODUCTION 112
4.2 SPECIFIC AIMS 114
4.3 RESULTS 114
4.3.1 TLR ligation upregulates DC co-stimulatory molecule expression 114
4.3.2 Concurrent exposure to IL-4 at the time of TLR ligation boosts IL-12 and reduces IL-10 secretion by DCs 114
4.3.3 TLR agonists limit IL-4 induced alternative activation 115

4.3.4 Complex bacterial, but not helminth antigens modulate DC co-stimulatory molecule expression and cytokine production in a manner similar to defined TLR ligands 117
4.3.5 Complex bacterial and helminth antigens modulate DC alternative activation in a manner similar to defined TLR ligands 117
4.3.6 IL-4 alters the response of DCs to CD40 ligation 118
4.3.7 IL-4Rα$^{-/-}$ DCs are less able to induce IFNγ and IL-17 responses 118
4.3.8 DCs unable to respond to IL-4 are less able to support early Th2 polarisation *in vitro* 119
4.3.9 DC expression of RELMα during Th2 priming regulates IFNγ and promotes IL-10 and IL-13 production 120
4.3.10 DC derived RELMα promotes IL-4 driven IL-10, and inhibits T-cell IL-4 121
4.3.11 rRELMα does not activate GMDCs 122
4.3.12 WT, *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs are similarly capable at antigen uptake 122
4.3.13 Antigen processing and presentation is unimpaired in *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs 123
4.3.14 IL-4 treatment of RELMα deficient DCs significantly alters transcription of more genes than IL-4 treatment of WT DC 123
4.4 SUMMARY 127
4.5 DISCUSSION 128
4.5.1.TLR and bacterial modulation of DC co-stimulatory molecule expression 128
4.5.2 IL-4 regulation of bacterially induced DC function 128
4.5.3 IL-4 and SEA regulation of DC function 130
4.5.4 RELMα function 133
4.5.5 Antigen processing and presentation by WT, *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs 136
4.5.6 Microarray targets 136
4.6 FIGURES AND TABLES 142
Figure 4.1 TLR stimulated GMDCs upregulate co-stimulatory molecules 142
Figure 4.2 IL-4 alters the cytokine profile of DCs activated by TLR ligation 143
Figure 4.3 TLR agonists limit IL-4 driven alternative activation 144
Figure 4.4 Complex bacterial but not helminth antigens modulate DC co-stimulatory molecule expression and cytokine production 145

Figure 4.5 Complex bacterial and helminth antigens modulate DC alternative activation 146
Figure 4.6 IL-4 alters the response of DCs following CD40 ligation 147
Figure 4.7 *Il4ra*$^{-/-}$ DCs are less able to induce IFNγ and IL-17 responses 148
Figure 4.8 DC expression of IL-4Rα promotes early IL-4 driven T-cell IL-4, IL-10 and IL-13 149
Figure 4.9 DC expression of RELMα during Th2 priming regulates IFNγ and promotes IL-10 and IL-13 production 150
Figure 4.10 DC derived RELMα promotes IL-4 driven IL-10 but limits T-cell IL-4 151
Figure 4.11 rRELMα does not activate GMDCs 152
Figure 4.12. WT, *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs are equally capable at antigen uptake 153
Figure 4.13. Antigen processing and presentation is unimpaired in *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs 154
Figure 4.14. IL-4 treatment of RELMα deficient DCs significantly alters transcription of more genes than IL-4 treatment of WT DC 155
Table 4.3.1 Genes significantly down ≤0.5 fold in IL-4 treated WT and *Retnla* DC 157
Table 4.3.2 Genes significantly down ≤0.5 fold only in IL-4 treated *Retnla*$^{-/-}$ DC 158
Table 4.3.3 Genes significantly up ≥ 2 fold in IL-4 treated WT and *Retnla*$^{-/-}$ DC 159
Table 4.3.4 Genes significantly up ≥ 2 fold only in IL-4 treated *Retnla*$^{-/-}$ DC 160
Table 4.3.5 Genes significantly up ≥ 2 fold only in IL-4 treated WT DC 161
Figure 4.15. Model of IL-4 and bacterial agonist mediated changes in DC function 162
Figure 4.16. Models of IL-4 and helminth mediated changes in DC function 163
Chapter 5. How do IL-4 and Vitamin A Metabolites Alter DC Function? 164
5.1 INTRODUCTION 164
5.2 SPECIFIC AIMS 166
5.3 RESULTS 166

5.3.1 *Aldh1a2* and *Ak2* are significantly upregulated in IL-4 treated DCs 166
5.3.2 IL-4 induces *Aldh1a2* expression and aldehyde dehydrogenase activity in GMDC and FLT3L DCs treated with GM-CSF 166
5.3.3 *Schistosoma mansoni* infection does not enhance DC aldehyde dehydrogenase activity, but highlights a potential requirement for growth factors 167
5.3.4 RA does not enhance GMDC aldehyde dehydrogenase activity in the absence of IL-4 168
5.3.5 DC RALDH activity inversely correlates with expression of DC alternative activation markers, with the exception of *Retnla* 169
Figure 5.3.6 RA and IL-4 synergise to enhance DC RELMα but do not impact Ym1/2 170
5.3.7 RA does not synergise with IL-4 to enhance alternative activation in BMM 171
5.3.8 RAR signalling regulates IL-4 driven DC alternative activation 171
5.3.9 RARα signalling inhibits IL-4 driven RELMα and promotes IL-4 driven Ym1/2 172
5.3.10 RAR signalling modulates IL-4 driven aldehdye dehydrogenase activity in GMDC 173
5.3.11 RA and IL-4 alter DC co-stimulatory molecule and cytokine secretion 173
5.3.12 RA and IL-4 alter T-cell polarisation in the context of DC 174
5.3.13 The absence of RARα alters DC co-stimulatory molecule expression and cytokine secretion 176
5.3.14 The absence of RARα alters DC ability to polarise T-cell responses 177
5.4 SUMMARY 179
5.5 DISCUSSION 180
5.5.1 How do RA and IL-4 alter DC phenotype? 180
5.5.2 Why didn't we detect enhanced RELMα secretion? 181
5.5.3 Is DC expression of RALDH dependent upon colony stimulating factors? 182
5.5.4 What is the *in vitro* retinoid source? 183
5.5.5 The role of RA in T-cell polarisation by DCs 184
5.6 FIGURES AND TABLES 188
Figure 5.1 Genes involved in metabolic pathways are significantly upregulated

by IL-4 in WT GMDC 188
Table 5.3.1 Metabolic pathway genes significantly increased by IL-4 in WT GMDC 189
Figure 5.2 IL-4 induces *Aldh1a2* expression and aldehyde dehydrogenase activity in GMDC and FLDCs treated with GM-CSF 190
Figure 5.3. *Schistosoma mansoni* infection does not enhance DC aldefluor activity, but highlights a potential requirement for growth factors 191
Figure 5.4. RA does not enhance GMDC aldefluor activity in the absence of IL-4 192
Figure 5.5 IL-4 driven DC RALDH activity correlates with reduced expression of markers of alternative activation but not *Retnla* 193
Figure 5.6 RA enhances IL-4 driven RELMα in GMDC 194
Figure 5.7 RA does not enhance IL-4 driven RELMα in BMM 195
Figure 5.8 RAR signalling regulates IL-4 driven DC alternative activation 196
Figure 5.9 RARα signalling inhibits IL-4 driven RELMα and promotes IL-4 driven Ym1/2 197
Figure 5.10 RAR signalling modulates IL-4 driven aldehdye dehydrogenase activity in GMDC 198
Figure 5.11 Retinoic acid and IL-4 alter DC co-stimulatory molecule and cytokine secretion 199
Figure 5.12 RA and IL-4 alter DC APC capacity 200
Figure 5.13 RARα alters DC co-stimulatory molecule expression and cytokine secretion 201
Figure. 5.14 The absence of RARα alters DC ability to polarise T-cell responses 202
Figure 5.15 The impact of IL-4 and RA signalling in DCs 203

Chapter 6. How do IL-4 and TLR2 Signalling Pathways Integrate to Influence DC Activation? 204

6.1 INTRODUCTION 204
6.2 SPECIFIC AIMS 206
6.3 RESULTS 206
6.3.1 TLR2 agonists enhance IL-4 induced aldehyde dehydrogenase activity in GMDCs 206
6.3.2 TLR2 agonists activate GMDCs, IL-4 regulates zymosan directed secretion of IL-10 and IL-12 207

6.3.3 P3C and zymosan limit IL-4 driven RELMα and Ym1/2 to different extents 208
6.3.4 Zymosan inhibits arginase and promotes iNOS in GMDC 209
6.3.5 P3C and zymosan modulate chemokine production by IL-4 treated GMDCs 210
6.3.6 IL-4 and TLR2 counter-regulate one another in GMDCs 210
6.3.7 Tlr2 limits IL-4 driven RELMα in GMDC, and is responsible for P3C inhibition of alternative activation 211
6.3.8 IL-4 inhibition of zymosan driven phospho-ERK1/2 is dependent upon TLR2 212
6.3.9 IL-4 differentially modulates components of the MAPK signalling cascade in WT and RELMα deficient DCs 213
6.3.10 ERK signalling inhibits IL-4 driven RELMα in GMDC 214
6.4 SUMMARY 215
6.5 DISCUSSION 216
6.6.1 The TLR2 and IL-4 dependence of RALDH2 216
6.6.2 TLR2 and IL-4 modulation of DC cytokine production 216
6.6.3 Interactions between IL-4, TLR2 and ERK signalling 217
6.6.4 Interactions between IL-4, RELMα and ERK 219
6.6 FIGURES 223
Figure 6.1 TLR2 agonists enhance IL-4 driven aldehyde dehydrogenase activity in GMDCs 223
Figure 6.2 TLR2 agonists activate GMDCs, IL-4 regulates zymosan directed secretion of IL-10 and IL-12 224
Figure 6.3 P3C and Zymosan limit IL-4 driven RELMα and Ym1/2 to different extents 225
Figure 6.4 Zymosan inhibits arginase and promotes iNOS in GMDC 226
Figure 6.5 P3C and zymosan modulate chemokine production by IL-4 treated GMDCs 227
Figure 6.6 IL-4 and TLR2 counter-regulate one another 228
Figure 6.7 TLR2 limits IL-4 driven RELMα in GMDC, and is responsible for P3C inhibition of alternative activation 229
Figure 6.8 IL-4 inhibition of zymosan driven phospho-ERK1/2 is dependent upon TLR2 230

Figure 6.9 IL-4 differentially modulates components of the MAPK signalling cascade in WT and RELMα deficient DCs 231
Figure 6.10 ERK signalling inhibits IL-4 driven RELMα in GMDC 232
Figure 6.11 Model of how TLR2, IL-4 and RA signalling integrate in DCs 233
Chapter 7. Concluding remarks and future directions 234
Bibliography 237
Appendix 1 266

Chapter 1. General Introduction

1. INTRODUCTION

"A properly functioning adaptive immune system signifies the best features of life. It is diverse beyond compare, tolerant without fail, and capable of behaving appropriately with a myriad of infections and other challenges. Dendritic cells are required to explain how this remarkable system is energized and directed"

Ralph M Steinman 1943-2011

Mammals have evolved myriad mechanisms to avoid, delay and eradicate infection. The mammalian immune system is a complex multi-cellular machine tasked with blocking pathogen entry (barrier sites, skin, mucosa, antimicrobial peptides), detecting pathogen presence (innate immunity), responding appropriately to the pathogen and remembering the pathogen such that a faster, stronger, more targeted response can be mounted upon re-exposure (adaptive immunity).

1.1 DENDRITIC CELLS

Dendritic cells (DCs) are a vital link between the innate and adaptive immune system, due to their capacity for antigen uptake, migration and presentation of acquired antigen to activate naïve T-cells of the adaptive immune system (Steinman 2012). Identifications of humans with DC deficiencies and the posthumous award of the Nobel prize for physiology or medicine to Ralph Steinman for his 1978 discovery of DCs earlier this year highlights the importance of these cells in the maintenance of human health (Collin, Bigley *et al.* 2011). Since their discovery in 1978, DC were predominantly (if possibly inaccurately) defined by expression of the integrin CD11c and high levels of major histocompatibility complex (MHC) expression (Jung, Unutmaz *et al.* 2002; van Rijt, Jung *et al.* 2005; Geissmann, Gordon *et al.* 2010; Phythian-Adams, Cook *et al.* 2010; Steinman 2012).

However, it is now appreciated, that there are multiple distinct and overlapping DC subsets found *in vivo* (Fig. 1.1) and that can also be generated *in vitro* from bone marrow or peripheral blood precursors. The naming of DC types can become quite complicated due to the large number of surface markers and locations

used to define the various populations, in this introduction we will aim to explain the current understanding of murine DC subsets (Fig. 1.1) and briefly relate this to the equivalent human DC populations currently described. Conventional/classical DCs (cDC) can be defined broadly as non-lymphoid tissue or lymphoid tissue-resident. Both cDC types have two main functions, 1) maintenance of self tolerance and 2) induction of specific immune responses against invading pathogens (Merad and Manz 2009).

Lymphoid tissue resident cDCs of the spleen can be classified into two subsets, $CD4^{+}CD8\alpha^{-}CD11b^{+}$ DCs and $CD8\alpha^{+}CD4^{-}CD11b^{+}$ (Naik 2008; Merad and Manz 2009). $CD8\alpha^{+}$ DCs are specialised in antigen presentation via MHC-I whereas the $CD4^{+}CD8\alpha^{-}$ DCs present antigen to T-cells via MHC-II (see below section 1.3 DCs as APCs). $CD8^{-}$ DCs are the most numerous resident cDC in the spleen (Naik 2008). In addition to the $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DCs of the spleen, LNs contain populations of migratory DCs that enter via the afferent lymphatics, draining from the local tissue (Merad and Manz 2009).

Non-lymphoid tissue resident cDCs are present in most tissues in the steady state. Tissue resident steady state dermal, lung parenchyma, liver, kidney and pancreatic islet DCs can be divided based on expression of CD103, CD11b and CX_3CR1, with $CD103^{+}$ DCs being $CD11b^{-}CX_3CR1^{-}$ (Helft, Ginhoux *et al.* 2010). With the exception of the lung, $CD103^{+}$ DCs represent ~20% of the steady state population at each tissue site (Merad and Manz 2009). DCs of the small intestinal (SI) lamina propria (LP) and peyers patch (PP) are $MHC\text{-}II^{+}CD11c^{hi}$, and can also be separated into 3 different populations, based upon expression of CD11b, CD103 and CX_3CR1 (Merad and Manz 2009). $CD103^{+}CD11b^{-}$ DCs are found in the PP; LP DCs are $CD103^{+}CD11b^{+}$ or $CD103^{-}CD11b^{+}CX_3CR1^{+}$ (Bogunovic, Ginhoux *et al.* 2009; Helft, Ginhoux *et al.* 2010). Resident $CD8^{+}$ DCs from lymphoid tissue have been shown to develop from a pre-cursor shared by the resident non-lymphoid tissue $CD103^{+}$ DCs(Hashimoto, Miller *et al.* 2011). Both the $CD8^{+}$ and $CD103^{+}CD11b^{-}$ DC populations are dependent upon the transcription factors Batf3, IRF8 and Id2 (Aliberti, Schulz *et al.* 2003; Hacker, Kirsch *et al.* 2003; Hildner, Edelson *et al.* 2008; Ginhoux, Liu *et al.* 2009; Edelson, Wumesh *et al.* 2010).

At sites of tissue injury or microbial infection, a population of inflammatory DCs can develop transiently, these cells derive from circulating Ly6C^{hi} monocytes and are characterised as Ly6C^{+}CD11b^{hi}CD11c^{int} (Dominguez and Ardavin 2010). TNFα and iNOS producing (TipDCs) are an example of inflammatory DC population, TipDCs were found in the spleen of mice infected with *Listeria monocytogenes* (Serbina, Salazar-Mather *et al.* 2003) and it has been questioned whether these are indeed DCs or classically activated macrophages (Dominguez and Ardavin 2010).

Plasmacytoid DCs (pDCs), are a class of pre-DC, that develop in the bone marrow (BM) and blood and can circulate in a CD62L dependent manner (Naik 2008). Upon viral activation via TLR7 and TLR9 pDCs secrete high levels of type-1 interferon (IFN) (Liu 2005; Naik 2008). pDCs are more lymphocytic in appearance than conventional DCs (cDCs), only becoming dendritic upon activation (Liu 2005; Naik 2008). pDCs circulate in the blood and in the steady state can be found in the bone marrow, spleen, thymus, liver and LNs (Merad and Manz 2009). Murine pDCs are defined as being MHC-II^{+}CD11b^{-} CD11c^{lo}B220^{+}Ly6C/Gr1^{+}PDCA1^{+}SiglecH^{+}TLR7^{+}TLR9^{+}, human pDCs are functionally closely equivalent to murine pDCs but are defined by a different set of surface markers, they are CD11c^{-}BDCA2^{+}BDCA4^{+}IL-3R^{+}CD4^{+}CD45RA^{+} TLR7^{+}TLR9^{+} (Merad and Manz 2009; Collin, Bigley *et al.* 2011).

The accurate characterisation of human DCs has been hindered by the difficulty in isolating such cells from living subjects, and as such the circulating blood DCs are the best characterised, with both pDC and cDC cells having being located (Merad and Manz 2009). An human equivalent of the murine CD8^{+} lymphoid/CD103^{+} non-lymphoid DCs has been characterised as expressing the C-type lectin *Clec9a*, which was also found to be expressed on the murine DC subsets (Caminschi, Proietto *et al.* 2008; Sancho, Mourao-Sa *et al.* 2008; Collin, Bigley *et al.* 2011). Human monocyte derived DCs are a CD14^{+}CD11b^{+}CX$_{3}$CR1^{+}CD209^{+} subset that secrete high levels of the proinflammatory cytokine TNFα. In humans, mutations in GATA binding protein 2 (GATA2) or IRF8 result in DC deficiency, surprisingly these deficiencies were first described in 2011 (Dickinson, Griffin *et al.* 2011; Hambleton, Salem *et al.* 2011).

In 2012, multiple studies have begun to address the requirement for a DC specific marker that can be used to unequivocally say ‘this cell is a DC’. The initial results of high throughput transcriptional profiling and characterisation of myriad sorted cell populations has been the discovery of a defining conventional DC (cDC) specific transcription factor zDC (*Zbtb46*) (Meredith, Liu *et al.* 2012; Meredith, Liu *et al.* 2012; Miller, Brown *et al.* 2012; Satpathy, Wumesh *et al.* 2012).

1.1.2 Differentiating DCs *in vitro*

Due to the scarcity of DCs *in vivo* (<1% of total hematopoietic cells during steady state at sites of immune priming (Merad and Manz 2009)) and the technical difficulties in isolation of these sensitive cells *ex vivo* without mechanical/enzymatic activation meant that a large proportion of the DC research to date had to focus on study of *in vitro* derived DCs. Granulocyte macrophage colony stimulating factor (GM-CSF; *Csf2*) is used routinely for the *in vitro* production of cDCs from murine bone marrow (GMDCs), resulting in populations that are $CD11c^{+}CD11b^{+}MHC\text{-}II^{+}CD8^{-}$ (Lutz, Kukutsch *et al.* 1999). GM-CSF is also used to differentiate DCs from human monocytes isolated from blood. However, in this context, IL-4 is used in combination with GM-CSF to differentiate human monocyte derived DCs (moDC) *in vitro* (Sallusto and Lanzavecchia 1994). Interestingly, it has never been shown that monocytes differentiate into DCs *in situ* in humans (Collin, Bigley *et al.* 2011). In some studies, IL-4 is also used in combination with GM-CSF to differentiate murine GMDC, a study directly comparing murine GMDC differentiated with or without IL-4 found that post differentiation the same number of DCs were present in either culture, with a slight enhancement in the level of CD11c on the cells differentiated in the presence of IL-4 (Sriram, Biswas *et al.* 2007).

The CD8 negativity of murine GMDCs resulted in them being correlated with *in vivo* resident steady state $CD8^{-}$ cDCs (Naik 2008). It has been suggested that GMDCs have a phenotype more akin to Tip inflammatory monocyte derived DCs (Serbina, Salazar-Mather *et al.* 2003; Xu, Zhan *et al.* 2007). In fact GM-CSF has been implicated in driving inflammatory monocyte DC populations *in vivo* (Naik, Metcalf *et al.* 2006). Earlier this year a paper was published which disputed this finding, claiming that GM-CSF was dispensable for inflammatory DCs, but required for the regulation of non-lymphoid tissue DCs (Greter, Helft *et al.* 2012). An overall consensus on the requirement for GM-CSF in DC development has not yet been

reached. However, the use of GM-CSF *in vitro* does result in the differentiation of a population of cells able to secrete high levels of cytokine, and to efficiently activate T-cell secretion of proinflammatory cytokines *in vivo,* which could be considered inflammatory properties. cDC differentiated with GM-CSF from BM were confirmed to express the DC specific transcription factor *Zbtb46 (Satpathy, Wumesh et al. 2012)* eschewing the notion that GM-CSF DCs are a type of macrophage.

Although GM-CSF was known to be important for DC differentiation *in vitro* relatively early during DC history (Inaba, Inaba *et al.* 1992; Inaba, Steinman *et al.* 1992; Sallusto and Lanzavecchia 1994), it had also been shown that DCs were still present in mice lacking GM-CSF or GM-CSF receptor expression (Vremec, Lieschke *et al.* 1997). *Fms* like tyrosine kinase 3 ligand (Flt3L) was found to be the 'missing factor' for DC development *in vivo (Maraskovsky, Brasel et al. 1996; D'Amico and Wu 2003)* and a protocol has since been developed for the *in vitro* production of BMDCs using Flt3L (FLDC) (Naik, Proietto *et al.* 2005). Culture with Flt3L produces 3 populations of DCs: a $B220^{+}$ population of pDCs, a $CD11c^{hi}CD11b^{lo}CD24^{hi}Sirp\alpha^{-}$ population that corresponds to the *in vivo* steady state $CD8^{+}$ subset of cDCs, and a $CD11c^{lo}CD11b^{hi}CD24^{lo}Sirp\alpha^{+}$ population that corresponds with the *in vivo* steady state $CD8^{-}$ subset of cDCs (Naik, Proietto *et al.* 2005). The use of *in vitro* DC populations derived from bone marrow has allowed the field of DC biology to advance substantially, as without such techniques the rarity of this specialised cell population would hinder such expansive research.

1.2 DC RECOGNITION OF NON-SELF AND DANGER

DCs express a broad range of pattern recognition receptors (PRRs) that enable them to recognise and respond to non-self products, known as pathogen associated molecular patterns (PAMPs) (Janeway 1989; Kapsenberg 2003). PRR are also able to sense endogenously produced danger associated molecules (DAMPs) such as high mobility group box 1 proteins (HMGB1), nucleic acids and heat shock proteins released by damaged/necrotic cells (Matzinger 2002; Nace, Evankovich *et al.* 2011) these 'danger signals' have also been referred to as alarmins (Bianchi 2007).

1.2.1 Toll like receptors

The toll-like receptor (TLR) family is the most well characterised PRR family, named for their similarity to Toll, a fungal recognition protein of Drosophila (Takeda, Kaisho *et al.* 2003) . The ten human and twelve murine TLRs recognise a broad range of PAMPs and DAMPs, highlighted in Fig. 1.2. TLRs are located both intra- and extra-cellularly. For example, TLR3, TLR7, and TLR9, which recognise nucleic acids, are mainly located on the endoplasmic reticulum (ER) membrane (Takeuchi and Akira 2010). Transcriptional activation occurs following binding of ligands to their cognate TLRs, the activation of specific genes is dependent upon which TLRs are engaged, and the toll/IL-1R homology (TIR) domain containing adaptor proteins that are recruited (Jenkins and Mansell 2010) (Fig. 1.2). There are five adaptor molecules that can be recruited (MyD88, TRIF, TIRAP/MAL, TRAM and SARM) (Jenkins and Mansell 2010). TLR signal transduction can be MyD88 dependent (all TLRs except TLR3) or MyD88 independent/TRIF dependent (TLR3 and TLR4) (Shaded region in Fig. 1.2). Following TLR ligation, MyD88 interacts with IL-1R-associated kinase (IRAK) family members, which dissociate from MyD88 to allow interaction with TNFR-associated factor 6 (TRAF6). TRAF6 becomes ubiquitinated which leads to the phosphorylation of IκB Kinase (IKK)-β and MAP kinase kinase 6. The ultimate outcome of this is the activation of inflammatory cytokine genes either via NF-κB or the transcription factor complex AP-1 (Takeuchi and Akira 2010) (Jenkins and Mansell 2010). Ligation of dsRNA to TLR3 recruits the adaptor molecule TRIF leading the activation of IRF3 and IRF7, which then induce type-1 interferon production. Other intracellular TLRs responding to nucleic acids can also induce downstream activation of IRF7 (Takeuchi and Akira 2010).

1.2.2 MAPK signalling

Downstream of various growth hormones, TLRs and cytokine receptors, intracellular mitogen activated protein kinases (MAPK) are activated. There are three main families of MAPK in immune cells: the extracellular signal-regulated protein kinases (ERK), the p38 MAPK, and the c-Jun NH_2-terminal kinase (JNK) (Zhang and Dong 2005). All of the MAPK have a Threonine-X-Tyrosine (TXY) motif, and phosphorlyation of both the threonine and tyrosine residues is required for their activation (Zhang and Dong 2005). Activation of all three MAPK families occurs via a triple kinase pathway, a MAPK-Kinase-Kinase (MAPKKK) activates a downstream MAPK-kinase (MAPKK) that then activates the target MAPK. MAPKKs are highly

specific for their target kinases, (Fig. 1.3) (Zhang and Dong 2005). GTPases such as Ras and the Rho family members Rac and Cdc42 are involved in transferring phosphate groups to mediate intracellular activation of receptors following ligand binding (Scita, Tenca *et al.* 2000; Yang, Shin *et al.* 2012). Following activation of any of the MAPK pathways, the phosphorylated MAPK enters the nucleus where it is able to bind to recognition sequences in DNA and regulate transcription or repression of target genes. Signalling by MAPKs is terminated by MAP kinase phosphatases (MKPs) also known as dual specificity phosphatases (DUSPS), which function in a complex negative regulatory network to control the duration, magnitude and timing of MAPK activation in response to physiological signals (Caunt and Keyse 2012).

1.2.3 Other PRRs

C-type lectin receptors (CLRs) are a family of carbohydrate binding lectins that are involved in altering gene expression downstream of pathogen products, either directly or by modulating TLR signalling (Geijtenbeek and Gringhuis 2009). Group I CLRs are members of the mannose receptor family, group II CLRs are asialoglycoprotein receptor family members such as DC-associated C-type lectin 1 (dectin-1)(Takeuchi and Akira 2010). CLRs recognise glucan, mannose and fucose carbohydrate structures present in fungi, bacteria, viruses, mycobacteria and helminths(Takeuchi and Akira 2010). CLR signalling is transduced either via association with immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor molecules or via direct kinase/phosphatase activation (Rogers, Slack *et al.* 2005), both of which can result in activation or modulation of NF-κB (Geijtenbeek and Gringhuis 2009). CLR signalling can occur both dependent on, or independently of, crosstalk with other PRRs. For example, DC-specific ICAM3 grabbing integrin (DC-SIGN) can modulate TLR-induced gene expression but has not been shown to induce gene expression changes independently of other PRRs (Gringhuis, den Dunnen *et al.* 2007; Gringhuis, den Dunnen *et al.* 2009). Similarly to DC-SIGN, the group II CLR dectin-1 is able to modulate TLR signalling. However, this important CLR can also direct gene transcription independently of other PRRs, via the activation of spleen tyrosine kinase (Syk) (Rogers, Slack *et al.* 2005).

Retinoic acid inducible gene-1 (RIG) like receptors (RLR) and nucleotide-binding oligomerisation domain (NOD) like receptors (NLR) are intracellular PRRs

(Kawai and Akira 2009). RLRs detect RNA species present in cytoplasm (e.g. from viral infection) and elicit type-1 interferon production (Kawai and Akira 2009). There are three RLR family members RIG-1, MDA5 and LGP2; RIG-1 and MDA5 interact with other proteins using their caspase recruitment domain (CARD) like regions and sense ssRNA and other viruses (Kawai and Akira 2009). The NLR family of proteins all contain 3 main domains: a nucleotide binding domain, a C-terminal leucine rich repeat for agonist binding, and an N-terminal region for homotypic protein-protein interactions, which enables scaffolds of proteins to form inflammasomes. There are multiple NLR family members including NOD-1, NOD-2 and NLRP3. The NODs are involved in the detection of intracellular bacterial products such as muramyl dipeptide (MDP) (Kawai and Akira 2009). NLRP3 is an important component of the inflammasome (Dagenais, Skeldon *et al.* 2011).

Due to the diversity of PAMPs the number of recognised PRR families will undoubtedly expand over time as researchers discover new mechanisms via which mammaliam cells have evolved to detect both pathogens and danger.

1.3 DCs AS ANTIGEN PRESENTING CELLS

The ability to present antigen to activate naïve T-cells is one of the defining features of a DC. MHC are glycoprotein complexes used by cells to present peptides for recognition by T cells. MHC class I molecules (MHC-I) can be found on the surface of all nucleated cells and present peptides to $CD8^+$ T-cells. MHC class II molecules (MHC-II) are expressed by DC, B cells, macrophages and particular types of thymic epithelial cells (Li, Gregg *et al.* 2005). Furthermore, in infection settings, MHC-II can be upregulated on other cell types including basophils (Perrigoue, Saenz *et al.* 2009; Sokol, Chu *et al.* 2009) and eosinophils (Shi 2004).

Antigens can be found in two predominant cellular domains; free in the cytosol or within membrane bound vesicles (phago/endosomes). Antigens present within the cytosol are commonly referred to as endogenous (having been synthesised within the cell) while those found within vesicles are commonly called exogenous (having origin outside of the cell) (Cresswell 2005). In its classical form, processing and presentation of endogenous and exogenous antigens was perceived as being two separate processes leading to the presentation of endogenous antigens on MHC I molecules to $CD8^+$ T-cells and exogenous antigens on MHC-II to

CD4$^+$ T-cells (Cresswell 2005). Targeting of endogenous antigens to virus specific CD8$^+$ T-cells would ensure that only cells definitively virally infected are killed (Heath and Carbone 2001). It is now appreciated that cross presentation of antigens occurs as DCs can present exogenous peptides on MHC-I, and cytosolic peptides on MHC-II (Li, Gregg *et al.* 2005; Trombetta and Mellman 2005; Nair, Amsen *et al.* 2011).

1.3.1 Contact dependent signals between DCs and T-cells

DC priming of T-cell responses requires three interactions: 1) a cognate interaction between T-cell receptor (TCR) and peptide complexed MHC molecule; 2) co-stimulation provided by contact dependent interactions between molecules expressed on both DC and the T-cell surfaces; 3) the secretion of cytokines (Kapsenberg 2003). DC express multiple surface receptors (Fig. 1.4) that enable them to interact with cognate receptors expressed on T-cells. Such receptors are generally termed ‘co-stimulatory’ or ‘co-inhibitory’ molecules, dependent upon whether they are required in addition to initial TCR:MHC recognition to provide ‘signal 2’ for full activation of naïve T-cells (stimulatory) or to provide negative signals to T-cells to control the amplitude of an on-going immune response (inhibitory). Co-stimulatory/inhibitory molecules belong broadly to two protein families, the TNF receptor superfamily (TNFRSF) (CD40 (Caux, Massacrier *et al.* 1994), OX40L (Fillatreau and Gray 2003; Jenkins, Perona-Wright *et al.* 2007), RANK (Andrew, Ruffing *et al.* 2001)) or the immunoglobulin superfamily (IGSF) (CD28 (Linsley and Ledbetter 1993), CTLA-4 (Kearney, Walunas *et al.* 1995), PD-1 (Ishida, Agata *et al.* 1992), CD80/CD86 (Greenwald, Freeman *et al.* 2005), ICOS (Hutloff, Dittrich *et al.* 1999; Greenwald, Freeman *et al.* 2005)). Co-stimulatory molecules such as CD80, CD86 and CD40 (amongst others, see red text Fig. 1.4) are required to provide signal 2 to activate T-cells.

Co-inhibitory molecules (see blue text Fig. 1.4) are not required during the initial phase of DC:T-cell interaction but become upregulated at a later stage. For example, cytotoxic lymphocyte antigen-4 (CTLA-4) is expressed on activated T-cells and is able to outcompete CD28 for binding to CD80/CD86, resulting in suppression of T-cell activation via recruitment of phosphatases that inhibit AKT (Parry, Chemnitz *et al.* 2005). It was shown in 2011 that CTLA-4 can capture the ligands CD80 and CD86 from the surface of opposing cells via transendocytosis, thus reducing the availability of ligands for co-stimulatory binding by CD28 (Qureshi,

Zheng *et al.* 2011). Another CD28 family member, programmed cell death 1 (PD-1), also mediates T-cell inhibition following interaction with its ligand PD-L1 (Parry, Chemnitz *et al.* 2005). The expression of co-stimulatory or co-inhibitory receptors by DCs highlights their importance in the orchestration of immune responses, both at the initiation and resolution phase (Kapsenberg 2003).

1.3.2 CD4$^+$ T helper cell subsets

The MHC-II molecules expressed by specialised antigen presenting cells such as DCs, are required for the activation of CD4$^+$ T-cells, as they possess TCRs that are both MHC-II dependent (the TCR cannot recognise it's cognate peptide in the absence of MHC-II presentation) and restricted (the TCRs will only recognise self MHC-II molecules). CD4$^+$ T-cells are also called T-helper (Th) cells, on account of their ability to help B-cells via provision of cytokines. A fundamental immunological concept developed over the last 25 years is that of the counter-regulatory nature of CD4$^+$ T-cell populations. A seminal study published in 1986 described for the first time the characteristic pattern of 'lymphokine' produced by two distinct groups of helper T-cell, defined as Th1 and Th2 (Mosmann, Cherwinski *et al.* 1986). Within the next 10 years, a wealth of research was undertaken looking at T-helper cell diversity within normal and disease states (Abbas, Murphy *et al.* 1996). An important area of research focused on inbred strains of mice that were found to be either resistant (CBA) or susceptible (BALB/c) to *Leishmania major* infection based on their propensity toward Th1 (resistant) or Th2 (susceptible) immune responses (Reiner and Locksley 1995).

In the context of a viral or bacterial initiated immune response IL-12 drives differentiation of a population of IFNγ secreting Th1 cells (Macatonia, Hosken *et al.* 1995; Hilkens, Kalinski *et al.* 1997). Antigen stimulated DCs have been shown to polarise their microtubule organizing centre (MTOC) toward the interacting T-cell enabling the delivery of IL-12 to the immune synapse (region of contact between DC and naïve T-cell during priming) (Pulecio, Petrovic *et al.* 2010).

Helminth infection and allergy drive the differentiation of an IL-4, IL-5, IL-9, IL-10 and IL-13 secreting population of Th2 cells (Pulendran and Artis 2012). However, the requirements for DC induction of Th2 responses remains much less well defined than that of IL-12 for Th1 cells (MacDonald and Maizels 2008). It has

recently been shown by our lab and others that DCs are both necessary and sufficient for the induction of Th2 responses *in vivo* (Hammad, Plantinga *et al.* 2010; Ohnmacht, Schwartz *et al.* 2010; Phythian-Adams, Cook *et al.* 2010). Nevertheless, the identity of the DC specific factor required for Th2 promotion and polarisation is still unknown, though multiple molecules have been proposed, and subsequently ruled out. For example, DCs do not produce IL-4 (MacDonald and Maizels 2008), and IL-4 deficient DCs can capably promote Th2 polarisation both in vitro and in vivo (MacDonald and Pearce 2002). Similarly, IL-$10^{-/-}$ DCs are capable at inducing a Th2 response when transferred into wild type mice (Perona-Wright, Jenkins *et al.* 2006). However, CD40/CD154 (MacDonald, Patton *et al.* 2002; MacDonald, Straw *et al.* 2002; Jenkins, Perona-Wright *et al.* 2008) has been shown to be required for full Th2 induction. Furthermore, OX40/OX40L interactions are required for sustained Th2 survival (Jenkins, Perona-Wright *et al.* 2007).

The discovery that transcription factors Tbet (Szabo, Kim *et al.* 2000) and GATA-3 (Zheng and Flavell 1997) are necessary and sufficient to determine the lineage choice (Th1/Th2) made by a naïve $CD4^+$ T-helper cells (Th0) enabled the two populations to be identified based on more than their cytokine profiles alone. It was further shown that GATA-3 and IL-12 signalling are mutually antagonistic (Ouyang, Ranganath *et al.* 1998) and that Tbet represses IL-4 and IL-5 production in Th2 clones (Szabo, Kim *et al.* 2000). These reports proved to strengthen the emerging paradigm that Th cells can counter-regulate one another. More recently, the range and diversity of known 'Th' cell types has expanded (Fig. 1.5) as has the number of cell types shown to influence Th cell differentiation. For example, most recently, type 2 innate lymphoid cells (ILCs) have been described to be a potent source of Th2 cytokines (Fallon, Ballantyne *et al.* 2006; Neill, Wong *et al.* 2010; Saenz, Siracusa *et al.* 2010; Mjosberg, Bernink *et al.* 2012).

IL-17 secreting T-cells were associated with autoimmunity, a key factor leading to their discovery was the finding that autoimmune disease states thought to be mediated by Th1 cells were exacerbated in the absence of the Th1 cytokine IFNγ (Harrington, Hatton *et al.* 2005; Langrish, Chen *et al.* 2005). Th17 cells were named when it was realised that the IL-17 secreting cells differentiated down a separate lineage to Th1 or Th2 cells, and this differentiation was inhibited by IFNγ (Harrington, Hatton *et al.* 2005; Langrish, Chen *et al.* 2005). The defining

transcription factor of Th17 cells was determined as being the orphan nuclear receptor gamma T (RORγt) (Ivanov, McKenzie *et al.* 2006). IL-6 and TGFβ are required for differentiation of Th17 cells and IL-23 has been shown to be involved in their maintenance (Ivanov, McKenzie *et al.* 2006).

The concept of immune regulation was discussed within the immunological community prior to the discovery that Th subsets existed. In the 1970's research focused on a population of $CD8^+$ T-suppressor (Ts) cells (Gershon and Kondo 1970), this theory fell out of favour when the proposed components that were described as mediating suppression could not be isolated at the molecular level (Basten and Fazekas de St Groth 2008). Regulation by T-cells was thrown back into immunological favour in the mid 1990's (Sakaguchi, Sakaguchi *et al.* 1995). It was noticed that ~10% of peripheral $CD4^+$ cells in non-immunised adult mice expressed the IL-2Rα chain (CD25), antibody mediated depletion of $CD25^+$ cells from $CD4^+$ LN and spleen preparations prior to reconstitution of athymic (nu/nu, nude) mice resulted in development of spontaneous autoimmune disease (Sakaguchi, Sakaguchi *et al.* 1995). Furthermore, nu/nu animals reconstituted with $CD4^+CD25^-$ cells were found have a heightened immune response against self and non-self proteins following allogeneic skin grafting, and this response could be ameliorated by provision of $CD25^+$ T-cells (Sakaguchi, Sakaguchi *et al.* 1995). These cells became known as T-regulatory cells (Treg), further research highlighted the dependence of these cells on the expression of the transcription factor forkhead box protein 3 (Foxp3) (Fontenot, Gavin *et al.* 2003; Hori, Nomura *et al.* 2003). $Foxp3^{-/-}$ mice were found to have a specific deficit in thymic development of $CD4^+CD25^+$ T-cells and over expression of Foxp3 in $CD4^+CD25^-$ T-cells converted them to a regulatory phenotype (Fontenot, Gavin *et al.* 2003; Hori, Nomura *et al.* 2003). IL-2 was later found to be required for maintenance of Treg homeostasis and survival *in vivo* but was not required for Treg development (Fontenot, Rasmussen *et al.* 2005). Thymically-derived Tregs are known as natural or nTregs, another subtype of Tregs exists known as the adaptive or inducible Tregs (iTreg), iTregs are produced *de novo* in the periphery from $CD4^+CD25^-$ cells. iTregs can be $Foxp3^+$ or $Foxp3^-$, the best characterised of the $Foxp3^-$ Tregs are the IL-10 producing Tr1 cells (Awasthi, Carrier *et al.* 2007).

TGF-β was initially found to be responsible for the conversion of $CD4^{+}Foxp3^{-}$ T-cells into suppressive $Foxp3^{+}$ iTregs at peripheral sites (Chen, Jin *et al.* 2003; Cobbold, Castejon *et al.* 2004). $Foxp3^{+}$ iTregs are induced in mesenteric LNs (mLN) during induction of oral tolerance (Coombes, Siddiqui *et al.* 2007), and differentiate in the steady state in response to food antigens and microbiota in the lamina propria of the gut (Sun, Hall *et al.* 2007). A role for retinoic acid in the induction of iTregs at mucosal sites has been shown (Coombes, Siddiqui *et al.* 2007) (see section 1.8 Vitamin A).

Follicular B helper T cells (Tfh) are a population T-cells that are required for providing help to B-cells (Crotty 2011). Tfh secrete IL-21, are defined by expression of PD-1, CXCR5 and ICOS, (Vinuesa, Tangye *et al.* 2005) and are required for the formation of germinal centres (GC). Their development is dependent upon the transcription factor Bcl6 (Nurieva, Chung *et al.* 2009). IL-21 and IL-6 are both redundantly required for induction of Tfh phenotype (Eto, Lao *et al.* 2011

) and ICOS has more recently been shown to initiate Bcl6 expression and drive T cells to a follicular rather than effector phenotype (Choi, Kageyama *et al.* 2011).

The newest reported player in the Th cell family, and something of a black sheep are the Th9 population of cells which differentiate in response to TGFβ and IL-4 and are associated with high level secretion of IL-9 (Dardalhon, Awasthi *et al.* 2008; Veldhoen, Uyttenhove *et al.* 2008). Th9 cells have recently been associated with expression of the transcription factors PU.1 (Chang, Sehra *et al.* 2010; Goswami and Kaplan 2012) and IRF4 (Staudt, Bothur *et al.* 2010), however, in the absence of a 'defining' transcription factor it has to be questioned whether Th9 are in fact a separate Th subset.

1.3.3 Th2 diversity

Th2 cells were originally described as secreting IL-4, IL-5, IL-9, IL-13 and IL-10 and expressing the transcription factor GATA3, and being STAT5 and STAT6 dependent (Pulendran and Artis 2012). However, there is in fact marked heterogeneity in the profile of cytokines secreted by individual Th2 cells, originally shown by single cell PCR studies (Kelso, Groves *et al.* 1999). It is currently unknown whether populations of Th2 cells induced *in vivo* secrete all the Th2

associated cytokines simultaneously, or a select pattern of expression dependent upon the cellular and cytokine environment in which they are differentiating.

1.4 SCHISTOSOMA MANSONI INFECTION

Worldwide, more than 200 million people are chronically infected with parasites of the genus *Schistosoma* (Steinmann, Keiser *et al.* 2006). In sub-Saharan Africa, more than 280,000 people are estimated to die each year due to pathologies associated with schistosome infection (Pearce and MacDonald 2002). Following release from their snail intermediate host, *S. mansoni* cercariae initiate mammalian infection; after penetration of the skin and development into schistosomula, they migrate via the vasculature to the hepatic-portal system where they reside (Allen and Wynn 2011). Adult worms mature, pair up and, approximately 4-6 weeks after infection, the female begins to releases eggs (Wilson, Draskau *et al.* 1978; Wilson, Mentink-Kane *et al.* 2007). Schistosome eggs cause much of the pathology/morbidity associated with disease as following release, they are swept by the flow of blood into the liver. In immunocompetent hosts collagen rich granulomas develop around the tissue trapped eggs, sequestering egg products (Pearce and MacDonald 2002). Granulomas are composed of a mixed population of immune cells including $CD4^+$ T-cells (a population on which granuloma formation is dependent (Phillips, DiConza *et al.* 1977)), macrophages and eosinophils (Pearce and MacDonald 2002) (Wilson, Mentink-Kane *et al.* 2007). The granulomatous response causes much of the morbidity associated with infection, as it leads to hepatic fibrosis and portal hypertension (Wilson, Mentink-Kane *et al.* 2007). Furthermore, granuloma encased eggs cause extensive damage as they pass from the vasculature into the intestines *en route* out of the host. The onset of egg release correlates with the emergence of a strong Th2 response, which peaks approximately 8 weeks following infection (Pearce and MacDonald 2002). A role for $CD4^+CD25^+Foxp3^+$ T-cells in controlling the size of colonic granulomas in intestinal schistosomiasis has been shown (Layland, Rad *et al.* 2007; Turner, Jenkins *et al.* 2011). $LysM^{Cre}IL\text{-}4R\alpha^{-/flox}$ mice that are unable to respond to IL-4/IL-13 in their neutrophil and macrophage compartments show 100% mortality following *S. mansoni* infection (Herbert, Holscher *et al.* 2004). The $LysM^{Cre}IL\text{-}4R\alpha^{-/flox}$ animals had increased systemic Th1 cytokines, liver damage and excessive gut inflammation, due to a failure to heal wounds quickly, which resorted in sepsis (Herbert, Holscher *et al.* 2004). Infection of WT mice depleted of neutrophils did not

have the same outcome, implicating AAM as a crucial cell type required for surviving schistosomiasis, particularly in protecting against sepsis (Herbert, Holscher *et al.* 2004).

1.4.1 Immune recognition of schistosome egg antigens

DCs can sense, capture and process antigens derived from schistosome eggs (Cervi, MacDonald *et al.* 2004; van Liempt, van Vliet *et al.* 2007; Everts, Perona-Wright *et al.* 2009; Everts, Hussaarts *et al.* 2012). DCs pulsed with soluble egg antigen (SEA) from *S. mansoni* prime strong Th2 immune responses (MacDonald, Straw *et al.* 2001). However, they fail to conventionally mature in response to SEA, retaining immature DC levels of expression of co-stimulatory molecules, and producing no measurable cytokines (MacDonald, Straw *et al.* 2001; Perona-Wright, Jenkins *et al.* 2006; van Liempt, van Vliet *et al.* 2007). DCs have been shown to take up SEA, or components of SEA, via multiple CLRs including DC-SIGN, MGL and MR (van Liempt, van Vliet *et al.* 2007; Everts, Hussaarts *et al.* 2012). Elements of SEA have been reported to signal through various TLRs, including TLR3 (Aksoy, Zouain *et al.* 2005), TLR2 (van der Kleij, Latz *et al.* 2002) (Gao, Zhang *et al.* 2012), and TLR4, though the requirements for MyD88, TLR2 and TLR4 signalling for SEA driven DC initiation of Th2 responses has been questioned (Kane, Jung *et al.* 2008). A glycoprotein component of SEA, Omega-1 (Dunne, Jones *et al.* 1991), has been reported to be one of the factors that enables the initiation of Th2 responses by SEA exposed DCs (Everts, Perona-Wright *et al.* 2009; Steinfelder, Andersen *et al.* 2009; Everts, Hussaarts *et al.* 2012). Both recombinant Omega-1 and Omega-1 purified from SEA had RNAse activity and could initiate Th2 polarisation via modulation of human and murine DCs (Everts, Perona-Wright *et al.* 2009; Steinfelder, Andersen *et al.* 2009; Everts, Hussaarts *et al.* 2012). It was hypothesised that SEA/Omega-1 exposure may create a setting of weak TCR signal strength analogous to that seen with low-dose antigen (Steinfelder, Andersen *et al.* 2009), which has been proposed to result in Th2 induction (Paul and Zhu 2010). Considering the usage of SEA as a Th2 polarising antigen preparation it is surprising that the impact that simultaneous exposure to SEA and IL-4 has on APC function has not been previously reported.

1.5 IMMUNE RECOGNITION OF BACTERIA

In the work described in this thesis, both gram positive (*Propionibacterium acnes;* Pa) and gram negative (*Salmonella typhimurium;* St) bacterial species have been used to stimulate DCs and macrophages prior to assessment of activation status and ability to prime Th1 and Th17 responses following transfer *in vivo.* Pa is known to instruct immune activation via TLR2 (Kim, Ochoa *et al.* 2002) and in acne lesions where the predominant bacterial species is Pa, TLR2 and TLR4 are induced (Jugeau, Tenaud *et al.* 2005). Furthermore, a specific role for TLR2 in mediating activation driven by Pa is highlighted by the finding that Pa was able to drive IL-6 production from cells in the absence of TLR1 and TLR6, and the use of anti-TLR4 antibodies did not block the ability of Pa to drive IL-12p40 and IL-8 secretion (Kim, Ochoa *et al.* 2002). Recognition of St by macrophages is mediated entirely via TLRs as BMM lacking both MyD88 and TRIF did not respond to heat killed St (Arpaia, Godec *et al.* 2011). TLR2, TLR4 and TLR5 mediate the majority of St recognition and an additional role for TLR9 was confirmed using TLR2, TLR4 and TLR9 double and triple knock out mouse strains (Arpaia, Godec *et al.* 2011). St flagellin is recognised by TLR5 (Feuillet, Medjane *et al.* 2006). However, murine BMM do not express TLR5 (Arpaia, Godec *et al.* 2011). Recognition of St by the intracellular TLRs TLR7 or TLR3 was also suggested, as the use of BafilomycinA1 (an inhibitor of endosome acidification) blocked the remaining TNFα induced by St in TLR2xTLR4xTLR9 triple knock out cells (Arpaia, Godec *et al.* 2011). In addition to complex bacterial antigen such as Pa and St, defined TLR agonists are also used within this thesis to aid understanding of the complex interplay between PAMPs and PRRs.

1.6 MACROPHAGES AND ALTERNATIVE ACTIVATION

The archetypal Th2 cytokine, IL-4 has been shown to have diverse effects upon macrophage biology, a topic which will be discussed in detail within this section. Macrophages are professional phagocytes of the innate immune system (Murray and Wynn 2011), the majority of macrophages have their origins in monocytes of the haematopoietic system (see Fig. 1.1), however some macrophage subsets, such as Kupffer cells of the liver, have embryonic origins having developed at the yolk sac stage of development and persisted in the adult (Schulz, Gomez Perdiguero *et al.* 2012). Generation of macrophages and monocytes is dependent upon the growth factor receptor Csf1r (M-CSFR) (Geissmann, Manz *et al.* 2010).

Monocytes are released into the circulation from the bone marrow following development via granulocyte macrophage and macrophage DC progenitors (GMP to MDP to monocyte; Fig. 1.1) (Hashimoto, Miller *et al.* 2011). Monocytes extravasate through the endothelium to seed all tissues and replenish the pool of tissue resident macrophages (Murray and Wynn 2011). Macrophages are often distinguished from DCs by their expression of surface markers such as F4/80 (*Emr1;* expressed on most murine tissue macrophages*),* CD11b (*Itgam;* expressed on all myeloid lineage cells including neutrophils), and CD68 (*Cd68;* expressed on all macrophages) (Murray and Wynn 2011). Macrophages can also be defined based on their location, with specialised subsets being present within different tissue sites. For example, alveolar macrophages in the lung (Balhara and Gounni 2012), and Kupffer cells of the liver (Crispe 2009) .

Over the past 30 years it has become indisputable that the local cytokine milieu impacts the activation and phenotype of all immune cells, not just T-cells. A great deal of research has focused on the activation status of the macrophage, an important area given the roles that macrophages play in multiple disease states. Classically, macrophages were thought of as pro-inflammatory, antimicrobial cells, driven by interferon gamma (IFNγ), able to destroy harmful intracellular pathogens. Thus, macrophages induced within Th1 environments are referred to as 'classically' activated macrophages (CAM or M1). In contrast, those found within Th2 environments are referred to as alternatively activated macrophages (AAM or M2) (Gordon and Martinez 2010). There exists a large literature detailing a myriad molecules associated with the phenotype and function of what is now known to be an extremely heterogeneous population of cells (Fig. 1.6). In an attempt to further classify the macrophage phenotype, Mantovani and colleagues introduced the nomenclature M2a, M2b or M2c (Mantovani, Sica *et al.* 2004). 'M2' designated these cells as being non-classically activated, with a, b, and c referring to the polarising signal influencing the detail of each phenotype (IL-4 and IL-13, immune complex, or IL-10, respectively) (Mantovani, Sica *et al.* 2004). It has also been proposed that macrophage activation is a spectrum that encompasses the three main areas of macrophage function: host defence, wound healing and immune regulation (Mosser and Edwards 2008). Thinking of macrophage heterogeneity as a continuous spectrum rather than a linear process (either M1 or M2) may more

accurately describe the wide range of macrophage phenotypes. The IL-4R dependent, IL-4 and IL-13 driven M2a/AAM has been investigated in great depth over the past 20 years, and a wealth of literature exists detailing the association of numerous molecules with this cell type (Fig. 1.6) and the relevance of these cells in both health and disease states (Loke, Nair *et al.* 2002; Herbert, Holscher *et al.* 2004; Ruckerl, Hessmann *et al.* 2006; Mylonas, Nair *et al.* 2009; Nair, Du *et al.* 2009; Pesce, Ramalingam *et al.* 2009; Pesce, Ramalingam *et al.* 2009; O'Brien, Lyons *et al.* 2010). In this thesis AAM have been defined as IL-4R dependent cells in line with the original description of this cell type (Stein, Keshav *et al.* 1992). The main markers associated with AAM are discussed in more detail below.

1.6.1 Arginase

One of the defining characteristics of an IL-4 exposed macrophage is expression of arginase-1 (*Arg1) (Modolell, Corraliza et al. 1995).* Arginase-1 is an enzyme that catabolises the conversion of arginine into polyamines and proline (Hesse, Modolell *et al.* 2001), molecules that promotes cell growth, collagen synthesis and fibrosis. Arginase-1 is known to compete with inducible nitric oxide synthase (iNOS; *Nos2*), an IFNγ induced enzyme that controls the production of nitric oxide from arginine in CAM (Fig. 1.7) (Munder, Eichmann *et al.* 1998; Munder, Eichmann *et al.* 1999).

In the past few years, the relevance of arginase in the pathology of Th2 mediated disease states has been highlighted by a number of research groups. Intra-peritoneal (i.p.) sensitisation, followed by intra venous (i.v.) challenge with *S. masoni* eggs in IL-4, IL-4/IL-13 or IL-4/IL-10 doubly deficient animals showed a correlation between reduced arginase activity, eosinophillia and pathology (lung granuloma volume) (Hesse, Modolell *et al.* 2001). Arg-1 expression localises to granulomatous tissues (liver and intestines) of *S. mansoni* infected mice (Hesse, Modolell *et al.* 2001). Blocking ornithine decarboxylase, an enzyme responsible for the production of polyamines downstream of arginase-1 (Bronte and Zanovello 2005), resulted in enhanced fibrosis in *S. mansoni* infected livers (Hesse, Modolell *et al.* 2001). Leishmania infected BMM also produce arginase-1(Kropf, Fuentes *et al.* 2005), and in the context of non-healing Leishmania lesions, arginase depletes L-arginine from the local environment, limiting the ability of T-cells to proliferate and produce IFNγ (Modolell, Choi *et al.* 2009). Interestingly, studies using two different

strains of conditional macrophage specific arginase-1 deficient mice showed that during *S. mansoni* infection, mice died at an accelerated rate with enhanced liver fibrosis (Pesce, Ramalingam *et al.* 2009). *Arg1* deficient macrophages failed to inhibit T-cell proliferation *in vitro*, in contrast to WT AAM (Pesce, Ramalingam *et al.* 2009). In correlation with the study using Leishmania, this was found to be due to the arginase activity of WT macrophages depleting L-arginine from the culture medium, as restoration of $CD4^+$ T-cell activity could be achieved by addition of exogenous L-arginine (Pesce, Ramalingam *et al.* 2009).

Due to the discovery of arginase as an AAM associated molecule as early as 1995 (Modolell, Corraliza *et al.* 1995), there is a broader published literature on its immune function in disease states as compared to other more recently described AAM markers.

1.6.2 Ym1

A more recently described intracellular marker for murine AAMs is Ym1, a chitinase family member initially found to be highly transcribed in murine spleen and lung (Jin, Copeland *et al.* 1998). Since it has no apparent chitinase activity, Ym1 is often classified as a 'chitinase-like' molecule (Webb, McKenzie *et al.* 2001). Ym1 is encoded by the gene *Chi3l3,* a very closely related gene *Chi3l4* encodes Ym2, an isomer of Ym1 (Webb, McKenzie *et al.* 2001). The protein products of *Chi3l3* and *Chi3l4* genes have 91% identity (Webb, McKenzie *et al.* 2001; Raes, De Baetselier *et al.* 2002) and as such are indistinguishable using antibodies. Thus, in the absence of a method to distinguish between these closely related molecules, protein studies within this thesis will refer to Ym1/2.

Chi3l3 and *Chi3l4* are not found in the human genome, however a family of human chitinases with roles in innate inflammation do exist and many have murine homologs, for example Acidic mammalian chitinase (AMCase/*CHIA*) and BRP-39/YKL-40 (*Chi3l1)* (Lee, Da Silva *et al.* 2011).

High levels of Ym1 crystals have been reported to be present in the bronchoalveolar lavage fluid (BALF) of inflamed and allergic murine lungs (Guo, Johnson *et al.* 2000; Webb, McKenzie *et al.* 2001) dependent upon IL-4R signalling (Webb, McKenzie *et al.* 2001), and in peritoneal exudate cells (PEC) in response to

Trichinella spiralis infection (Chang, Hung *et al.* 2001). Ym1 was also found to be highly expressed in nematode (Loke, Nair *et al.* 2002) and trypanosome (Raes, De Baetselier *et al.* 2002) elicited macrophages. Furthermore, such macrophage specific upregulation of Ym1/2 could be induced *in vitro* and *ex vivo* by IL-4 or IL-13 (Raes, De Baetselier *et al.* 2002; Welch, Escoubet-Lozach *et al.* 2002; Nair, Cochrane *et al.* 2003). Ym1/2 was originally anticipated to function via the binding of chitin containing pathogens (Nair, Gallagher *et al.* 2005). However, the only reports of a functional role for Ym1/2 show that it promotes type-2 cytokine production in allergic mice (Cai, Kumar *et al.* 2009) and in response to treatment with statins (Arora, Chen *et al.* 2006). Ym1/2 was found to limit the production of IL-5 and IL-13 by inhibiting 12/15(S) lipoxygenase (12/15LOX) (Cai, Kumar *et al.* 2009), a lipid peroxidating enzyme. Furthermore, 12/15LOX also catalyses, in an IL-4 dependent manner, the conversion of arachidonic and linoleic acid into activating ligands for the AAM associated genetic sensor of fatty acids, PPARγ (Huang, Welch *et al.* 1999; Odegaard, Ricardo-Gonzalez *et al.* 2007).

On account of the high level secretion of Ym1/2 by AAM, we believe a role for Ym1/2 in Th2 immune responses is undoubted, however further work is required to characterise the cell-type specific function of this protein within the immune system.

1.6.3 Resistin like molecule alpha

Another IL-4 induced molecule produced by AAM and discovered at a similar time to Ym1/2 is resistin-like molecule alpha (RELMα) a cysteine rich secreted protein of the resistin family (Banerjee and Lazar 2001). RELMα is also known as ‘found in inflammatory zone 1’ (FIZZ1), due to its original identification in bronchial epithelial cells in the context of allergic pulmonary inflammation (Holcomb, Kabakoff *et al.* 2000) and as hypoxia-induced mitogenic factor (HIMF) (Teng, Li *et al.* 2003). Initial reports suggested that RELMα was only expressed in the lung (Banerjee and Lazar 2001), tongue, mammary and white adipose tissues of mice (Steppan, Brown *et al.* 2001). The adipose tissue RELMα expression was found to associate with the stromal vascular fraction of WAT and was found to decrease upon high-fat diet feeding (Rajala, Lin *et al.* 2002), an outcome that has since been linked to AAM (see below ‘Metabolism and AAM’). In 2002 RELMα was found to be the second most highly upregulated transcript in nematode elicited peritoneal macrophages, second

only to Ym1 (Loke, Nair *et al.* 2002), and such expression was dependent on IL-4. In the same year, a similar report using *Trypanosoma brucei brucei* infection also made the correlation between AAM and expression of RELMα (Raes, De Baetselier *et al.* 2002). Since then, macrophage RELMα expression has also been shown to occur during other parasitic helminth infections including *Brugia malayi, Litomosoides sigmodontis* and *Nippostrongylus brasiliensis* (Nair, Cochrane *et al.* 2003; Nair, Gallagher *et al.* 2005; Jenkins, Ruckerl *et al.* 2011; Ruckerl, Jenkins *et al.* 2012).

The first reports of a functional role for RELMα did not involve work focusing on macrophages (Liu, Dhanasekaran *et al.* 2004; Wagner, Hellberg *et al.* 2004). However, the functions attributed to RELMα and the signalling cascades downstream of RELMα reception are nonetheless enlightening. RELMα can elicit α-smooth muscle actin and collagen expression by myofibroblasts (Liu, Dhanasekaran *et al.* 2004). Surprisingly, given the known Th2 dependence of RELMα in AAM, RELMα was expressed in the lung following i.p. lipopolysaccharide (LPS) injection and was also induced in the murine colon following induction of DSS-induced experimental colitis (Tong, Zheng *et al.* 2006; Munitz, Waddell *et al.* 2008). Munitz *et al* also showed that rRELMα causes increased expression of pro-inflammatory cytokines IL-6 and TNFα by bone marrow derived macrophages co-cultured with LPS, suggesting that RELMα may polarise a pro-, rather than anti-inflammatory response (Munitz, Waddell *et al.* 2008).

A series of studies focusing on RELMα primarily in the lung revealed that it can be induced by hypoxia (Teng, Li *et al.* 2003) and that RELMα expression co-localises with hypoxia induced factor 2α (HIF2α), but not HIF1α (Wagner, Hellberg *et al.* 2004). In correlation with upregulation during hypoxia, rRELMα is pro-angiogenic and enhances vascular endothelial growth factor (VEGF), and monocyte chemotactic protein-1 expression in the lung (Teng, Li *et al.* 2003; Tong, Zheng *et al.* 2006; Yamaji-Kegan, Su *et al.* 2006). Interestingly, given the reports of AAM expression of RELMα, i.v. injection of rRELMα resulted in increased $CD68^+$ monocyte/macrophage recruitment to the lungs (Yamaji-Kegan, Su *et al.* 2006). An aetiological consequence of RELMα presence in the lung is the induction of vascular remodelling associated with pulmonary hypertension (PH) (Angelini, Su *et al.* 2009). The same authors also showed that an induced model of PH (instillation of rRELMα)

resulted in recruitment of bone marrow derived mesenchymal-like cells to the pulmonary vasculature (Angelini, Su *et al.* 2010). A role for rRELMα in inducing migration of bone marrow derived cells had previously been shown to be dependent upon Bruton's tyrosine kinase (BTK) signalling; using a transwell migration assay it was shown that rRELMα induced the migration of bone marrow cells (Su, Zhou *et al.* 2007). BTK was identified as a binding partner for rRELMα in BMM and following treatment of BMM with rRELMα, BTK was phosphorylated within a few minutes (Su, Zhou *et al.* 2007).

Multiple reports have shown that rRELMa activates the PI3K/AKT/NFκB signalling pathways within various cell types (Teng, Li *et al.* 2003; Tong, Zheng *et al.* 2006; Tong, Zheng *et al.* 2006) and is able to mobilise intracellular calcium stores from smooth muscle cells (Fan, Su *et al.* 2009).

In 2009, two reports using *Retnla*$^{-/-}$ mice demonstrated that RELMα functions as a negative regulator of helminth induced Th2 immunity (Nair, Du *et al.* 2009; Pesce, Ramalingam *et al.* 2009). The Nair study showed that *S. mansoni* egg challenged *Retnla*$^{-/-}$ mice secreted enhanced antigen specific IL-4, IL-5 and IL-13 following restimulation of mediastinal LNs, which drain the site of granuloma formation in this model. Furthermore, when used in an *in vitro* OT-II culture system, *Retnla*$^{-/-}$ BMM stimulated enhanced IL-4 and IL-13 secretion from CD4^{+} T-cells. When rRELMα was applied to Th2 polarised T-cells, IL-5 was inhibited in a dose and BTK dependent manner (Nair, Du *et al.* 2009). In both reports using the *Retnla*$^{-/-}$ mice, IL-5 and IL-13 were impacted more significantly than IL-4. This disparity in regulation of Th2 cytokines fits with a report linking IL-4 signalling to the RELMα induced PH model, in which it was shown that in the lung, rRELMα enhanced IL-4 production (Yamaji-Kegan, Su *et al.* 2010).

Retnla has not been found in the human genome. However, the expression pattern of human resistin is more similar to murine RELMα than murine resistin (Nair, Guild *et al.* 2006). Thus investigating RELMα in mice may provide useful information about human resistin, a protein involved in insulin regulation (Bastard, Maachi *et al.* 2006).

1.6.4 Mannose receptor

The inaugural AAM marker, the (macrophage) mannose receptor (*Mrc1*, MR, CD206) is a phagocytic receptor of the CLR family that mediates the binding of sulphated sugars (via a cysteine-rich domain), collagen (via a fibronectin type II domain), and mannose, fucose and N-acetyl-glucosamine containing sugars (via a C-type lectin domain) (Gazi and Martinez-Pomares 2009). The first report defining IL-4 treated macrophages as being 'alternatively' activated described the *in vitro* upregulation by IL-4 of the MR on peritoneal exudate macrophages (Stein, Keshav *et al.* 1992). The IL-4Rα dependence of MR expression was shown in macrophages recruited to granulomas in *S. mansoni* infection (Linehan, Coulson *et al.* 2003). The generation of anti-MR monoclonal antibodies aided the study of MR on macrophage populations (Martinez-Pomares, Reid *et al.* 2003). More recently, IL-10 has been shown to activate $MR^{+}Ym1^{+}$ macrophages in the absence of macrophage-specific IL-4Rα signalling (Dewals, Marillier *et al.* 2010) and also to enable the upregulation of MR on thioglycollate elicited macrophages (Martinez-Pomares, Reid *et al.* 2003). In the absence of MR, BMM are less able to phagocytose the excretory/secretory products of the invasive form of *S. mansoni* (Paveley, Aynsley *et al.* 2011), and DC uptake of the SEA component Omega-1 was recently shown to be MR dependent (Everts, Hussaarts *et al.* 2012), highlighting a role for macrophage expression of MR in the immune response against schistosomes.

1.6.5 Dectin-1

A second receptor associated with alternative activation is the dendritic cell c-type lectin (dectin-1; *Clec7a*), a *β*-glucan receptor re-discovered on macrophages in 2002 (Brown, Taylor *et al.* 2002) and associated with alternative macrophage activation in 2003 (Willment, Lin *et al.* 2003). Dectin-1 is a non-classical CLR as compared to classical CLRs as dectin-1 recognition of carbohydrate is metal-ion independent (Brown 2006; Gazi, Rosas *et al.* 2011). IL-4 and IL-13 were shown to enhance the expression of this receptor on peritoneal macrophages within 4 hours, and dectin-1 was shown to bind zymosan, a β-glucan rich cell wall mixture from the yeast *Saccharomyces cerevisiae* (Di Carlo and Fiore 1958; Willment, Lin *et al.* 2003). Following engagement of dectin-1, its intracellular ITAM is phosphorylated by Src family kinases, leading to activation of Syk kinase and downstream signalling events including activation of the MAPK, ERK (Slack, Robinson *et al.* 2007; Eberle and Dalpke 2012). Zymosan contains a mixture of antigens (55% β-glucan, 20%

mannan, 15% protein, 10% lipids, inorganic materials and chitin (Di Carlo and Fiore 1958) and as such is able to signal via a compelx of TLR2/TLR6 as well as dectin-1. However, zymosan depleted of contaminating TLR2 ligands can be used as a specific dectin-1 stimulus (dZym) (Eberle and Dalpke 2012). The binding of β-glucan component of zymosan to dectin-1 has since been shown to cause the shedding of MR into the supernatant as a soluble non-membrane bound form (sMR) (Gazi, Rosas *et al.* 2011). This shedding was shown to be dependent upon, MMP, Syk, and PI3K signalling, but did not require AKT or internalisation of the β-glucan particles (Gazi, Rosas *et al.* 2011).

The interaction between MR and dectin-1 has the potential to modulate responses to extracellular pathogens, particularly in the presence of Th2 cytokines and a fungal pathogen because, as explained above, IL-4 has been shown to induce expression of both MR and dectin-1 in macrophages (Stein, Keshav *et al.* 1992; Willment, Lin *et al.* 2003).

To detect the intracellular proteins arginase, RELMα and Ym1/2 via flow cytometry it is necessary to permeabilise a cells membrane, thus making functional assays following cell sorting an impossibility. As both dectin-1 and MR are surface receptors, they are useful for the identification of AAM populations for use in functional studies.

1.6.6. AAM associated chemokines

In addition to surface receptors, novel proteins, and well characterised enzymes, AAM are also associated with secretion of defined chemotactant proteins (chemokines). IL-4 activation of macrophages has been associated with enhanced secretion of C-C type chemokines including CCL17 (Thymus and activation regulated chemokine; TARC) and CCL24 (eotaxin-2) (Mantovani, Sica *et al.* 2004).

CCL17 is recognised by the chemokine receptor (CCR) 4 (Imai, Baba *et al.* 1997) which is highly expressed on Th2 cells, skin homing memory T-cells and NK cells (Andrew, Ruffing *et al.* 2001; Alferink, Lieberam *et al.* 2003; Mantovani, Sica *et al.* 2004). CCL17 expression by DCs has been shown to recruit activated and memory T-cells (Alferink, Lieberam *et al.* 2003) and, more recently, has been shown to limit regulatory T-cell expansion, resulting in enhanced atherogenesis (Weber,

Meiler *et al.* 2011). CCL17 is thought to be expressed only in the $CD11b^{+}CD8^{-}$ DC subset (Alferink, Lieberam *et al.* 2003).

CCL24 binds to CCR3, a receptor predominantly expressed by eosinophils (Forssmann, Uguccioni *et al.* 1997). CCL24 was shown to be differentially induced by LPS and IL-4 in human monocytes and macrophages, with LPS enhancing secretion of this chemokine in monocytes and IL-4 enhancing secretion of CCL24 by macrophages (Watanabe, Jose *et al.* 2002). LPS was unable to induce macrophage CCL24 and likewise IL-4 could not induce monocytes to secrete CCL24 (Watanabe, Jose *et al.* 2002). Murine CCL24 was identified as being a constitutively expressed eosinophil chemoattractant that could be induced in lungs following intra-tracheal instillation of IL-4 (Zimmermann, Hogan *et al.* 2000). CCL24 has been shown to be a potent recruiter of eosinophils in models of airway inflammation (Kurowska-Stolarska, Stolarski *et al.* 2009).

1.6.7 Metabolism and AAM

Obesity and the associated metabolic syndrome (insulin resistance, glucose intolerance, dyslipidemia and hypertension) is one of the major causes of morbidity and mortality in recent times; 300-400,000 deaths every year in the USA are directly attributable to obesity (Odegaard and Chawla 2011). Multiple studies have begun to link the activation status of macrophage populations with their intrinsic metabolic capacity and that of their host organism (Olefsky and Glass 2010; Odegaard and Chawla 2011). Obesity is characterised by an underlying inflammatory state, with adipose tissue of obese individuals expressing high levels of TNFα, IL-6 and iNOS (Olefsky and Glass 2010). A microarray study was undertaken to assess the transcriptional profile of adipose tissue taken from a spectrum of lean to severely obese mice; of the 100 genes most significantly correlated with increased body mass, 30% were transcripts characteristically expressed by macrophages (Weisberg, McCann *et al.* 2003). Weisberg and colleagues isolated fractions of perigonadal adipose tissue from obese mice (B6.V*Lep*$^{ob/ob}$) and showed that the $F4/80^{+}$ macrophage fraction expressed the highest levels of pro-inflammatory cytokines (Weisberg, McCann *et al.* 2003). Further work looking into adipose tissue macrophages (ATM) of obese versus lean animals showed that ATM of lean mice have AAM characteristics, expressing *Chi3l3* and *Arg1* (Lumeng, Bodzin *et al.* 2007). As mentioned previously, the initiating factors required for alternative

activation are the type-2 cytokines IL-4 and IL-13 (Varin and Gordon 2009) (Section 1.4). In 2006 it was shown that IL-4 treatment of macrophages results in a switch to oxidative metabolism via the upregulation of the transcriptional co-activator PGC1β (Vats, Mukundan *et al.* 2006). In contrast, CAM use a program of glycolysis (Rodriguez-Prados, Traves *et al.* 2010). Inhibition of fatty acid oxidation in macrophages resulted in a decrease in IL-4 driven arginase 1, dectin-1 and MR showing a dependence on oxidative metabolism for alternative activation (Vats, Mukundan *et al.* 2006). These studies highlight that the availability of metabolites within a tissue site can have a large impact on the activation state of immune cells within that tissue.

A requirement for the ligand-activated transcription factors, peroxisome proliferator-activated receptors (PPARs) in the maintenance of macrophage alternative activation downstream of IL-4 has been established (Odegaard, Ricardo-Gonzalez *et al.* 2007; Odegaard, Ricardo-Gonzalez *et al.* 2008). PPARs are members of the nuclear receptor superfamily and are vital for controlling fatty acid metabolism (Odegaard and Chawla 2011). Mice with a macrophage/neutrophil specific deletion ($LysM^{Cre}$) of PPARγ were unable to maintain an alternatively activated state (Odegaard, Ricardo-Gonzalez *et al.* 2007). Furthermore, macrophage deficiency in PPARγ resulted in enhanced obesity and increased insulin resistance when compared to wild type mice fed a high fat diet, serendipitously highlighting a beneficial function for AAM in protecting against the metabolic consequences of obesity (Odegaard, Ricardo-Gonzalez *et al.* 2007).

The most recent correlation between AAM and metabolism shows that upon exposure to cold environments, macrophages of the brown and white adipose tissue become alternatively activated (Nguyen, Qiu *et al.* 2011). In addition, these AAM are able to synthesise catecholamines that contribute to adaptive thermogenesis, a response that enables survival during cold stress (Nguyen, Qiu *et al.* 2011).

This section highlights an important biological role for AAM outwith the context of helminth infection and allergic disease, and show that even in the absence of strongly Th2 polarised environments, IL-4 signalling is essential for alternative activation. The availability of metabolites and changes in temperature are factors that will impact directly all cells of the immune system, it is going to be

interesting to see in future years how the results of these macrophage studies are applied to other cell types, for example the DCs.

1.6.8 Other cytokines implicated in the induction of AAM

Although most studies investigating AAM use the classical IL-4R dependence as defining this population, a few studies have investigated expression of AAM associated markers in response to other cytokines. One example of such is a study which investigated the role of IL-21 on account of the IL-21R having significant sequence and structural similarity to the IL-4R (Pesce, Kaviratne *et al.* 2006). Using IL-21R deficient mice, Pesce *et al* found a correlation between the level of AAMs present following schistosome infection and the ability to respond to IL-21 (Pesce, Kaviratne *et al.* 2006). Mice that could not receive an IL-21 signal had reduced IL-4, IL-13, RELMα, Ym1/2 and AMCase transcripts in granulomatous lung tissue 7 days after sensitisation (i.p.) and challenge (i.v.) with *S. mansoni* eggs (Pesce, Kaviratne *et al.* 2006). This work also found that IL-21 signalling to BMM enhanced expression of both the IL-4R and IL-13Rα (Pesce, Kaviratne *et al.* 2006).

Another cytokine that has recently been identified as being involved in Th2 settings is IL-33. IL-33 is an IL-1 family member purported to be an apoptotic signal (Schmitz, Owyang *et al.* 2005; Luthi, Cullen *et al.* 2009). IL-33 signals via a receptor composed of ST2L and the IL-1R (Schmitz, Owyang *et al.* 2005). IL-33 has been shown to enhance the alternative activation of macrophage populations in co-operation with IL-4 signalling (Kurowska-Stolarska, Stolarski *et al.* 2009). In particular IL-33 was shown to enhance IL-4Rα expression in the lung and promote CCL24 production from macrophages, which then recruited eosinophils and mediated allergic inflammation in the lung (Kurowska-Stolarska, Stolarski *et al.* 2009).

The TGFβ super family member Activin-A has been implicated in the regulation of both murine and human alternative activation. A 2006 study suggested that Activin-A (shown to be produced by Th2 cells) was able to induce the expression of Arg1 from peritoneal macrophages whilst simultaneously limiting iNOS expression (Ogawa, Funaba *et al.* 2006). Activin-A induces antigen-specific regulatory T-cells that are able to suppress Th2 effector cells in allergic disease (Semitekolou, Alissafi *et al.* 2009), so a role in the promotion of alternatively

activated macrophages may not be that likely. In agreement with this, human PBMC derived macrophages were found to have reduced M2 characteristics when cultured in the presence of Activin-A (Sierra-Filardi, Puig-Kroger *et al.* 2011).

These studies show that while other cytokines can support IL-4 dependent induction of macrophage alternative activation, expression of the IL-4R is required. In the next section we discuss the expression of alternative activation associated molecules in cells other than macrophages (1.6.9).

1.6.9 Alternative activation in non-macrophage populations

Reports of expression of macrophage associated alternative activation products by cells other than macrophages have been published. RELMα is produced at high levels by eosinophils (Nair, Du *et al.* 2009; Pesce, Ramalingam *et al.* 2009; Cook, Aynsley *et al.* 2011) and intestinal epithelial cells (Holcomb, Kabakoff *et al.* 2000; Munitz, Waddell *et al.* 2008). Ym1/2 can also be produced by mast cells (Lee, Yook *et al.* 2005) and neutrophils (Harbord, Novelli *et al.* 2002). IL-4 culture of BM with stem cell factor resulted in induction of BM-mast cells that expressed Ym1/2 however, no role for the presence of Ym1/2 in mast cells was described (Lee, Yook *et al.* 2005). Neutrophil derived Ym1 was shown to have functional β-N-acetylhexosaminidase activity, the authors also suggest that macrophage associated Ym1 is dependent in part due to their phagocytosis of short lived apoptotic Ym1 producing neutrophils (Harbord, Novelli *et al.* 2002). A confocal microscopy paper however disputed the presence of Ym1/2 in neutrophils in the skin (HogenEsch, Dunham *et al.* 2006).

A few reports have been published in which markers associated with alternative activation have been shown to be induced in DCs. Arginase activity was induced in BMDC by the Th2 cytokines IL-4, with a synergistic increase seen when IL-10 was also included in the 48h culture (Munder, Eichmann *et al.* 1999). BMDCs were also shown to upregulate transcript for Ym1/2 and RELMα following overnight culture with IL-4 (Nair, Gallagher *et al.* 2005). Additionally, the same authors showed upregulation of RELMα and Ym1/2 in DCs sorted from the draining lymph nodes of animals implanted with the filarial nematode *Brugia malayi* (Nair, Gallagher *et al.* 2005). As well as these reports on the impact of IL-4 and nematodes on DC expression of alternative activation markers, Ym1/2 was shown to be expressed by

BMDCs exposed to the lipid lowering drug simvastatin and to be involved in DC priming of Th2 responses *in vitro* (Arora, Chen *et al.* 2006). Simvastatin promoted Th2 responses, but addition of anti-Ym1 antibody to simvastatin treated DC and T-cell co-cultures abrogated the ability of these DCs to induce Th2 cytokine secretion from the T-cells (Arora, Chen *et al.* 2006). Arora *et al* showed that simvastatin induction of Ym1/2 was dependent upon STAT6 and IL-4Rα, but independent of IL-4 presence (confirmed using neutralising antibodies) suggesting that the statin is able to utilise the IL-4Rα/STAT6 axis in DCs to upregulate Ym1/2 (Arora, Chen *et al.* 2006). Ym1/2 was also confirmed to be expressed in mLN DCs from allergic mice, and addition of recombinant Ym1/2 *in vitro* to *Il13*$^{-/-}$ DC: WT T-cell co-cultures from OVA sensitised animals resulted in amplified IL-5 secretion by the T-cells (Cai, Kumar *et al.* 2009). The expression of these AAM associated molecules in DCs was shown in all previous reports to be dependent upon IL-4 signalling, a topic which is discussed in detail below (1.7).

1.7 IL-4R SIGNALLING

As mentioned above, a central component of alternative activation is the ability to respond to IL-4. IL-4 signals via binding to heterodimeric receptor complexes, including the 140-kDa IL-4Rα chain which pairs with either the common gamma chain (first identified as a component of the IL-2 receptor) to create the type-I IL-4R (Fig. 1.8), or the IL-13Rα1 to create the type-II IL-4R through which IL-13 can also signal (Lutz, Schnare *et al.* 2002; Varin and Gordon 2009; Junttila, Creusot *et al.* 2012). T-cells were at first thought not to express the type-II IL-4R and to be unable to respond to IL-13 but a 2009 study reported that Th17 cells express a functional IL-13R (Newcomb, Zhou *et al.* 2009). IL-4 can be recognised by the IL-4Rα in the absence of a secondary receptor chain, though receptor heterodimerisation is required for intracellular signalling (Nelms, Keegan *et al.* 1999). As neither the IL-4Rα or γc have endogenous kinase activity, receptor associated kinases are required to transduce the intracellular signal (Johnston, Kawamura *et al.* 1994; Witthuhn, Silvennoinen *et al.* 1994). Both the janus associated kinases 1 and 3 (JAK1/JAK3) (Johnston, Kawamura *et al.* 1994; Witthuhn, Silvennoinen *et al.* 1994) and Src kinase families (Izuhara, Yang *et al.* 1993) are activated downstream of IL-4 binding. JAK3 associates with the γc of the type-I IL-4R (Witthuhn, Silvennoinen *et al.* 1994). Five tyrosine residues become phosphorylated in the IL-4Rα tail, enabling the binding of signalling proteins via their

src homology domain 2 (SH2) regions (Varin and Gordon 2009). Signal transducer and activator of transcription (STAT) 6 becomes phosphorylated by the receptor associated kinases (Velazquez, Fellous *et al.* 1992; Darnell 1997), forms a dimer with another STAT6 molecule and enters the nucleus to change gene transcription. The 170-kDa phosphoprotein insulin receptor substrate (IRS2 also known as 4PS) was reported to be uniquely phosphorylated in response to IL-4 treatment of haematopoietic cell lines (Wang, Keegan *et al.* 1992). IRS1/2 becomes activated following binding to one of the phosphorylated tyrosine residues in the IL-4 bound IL-4Rα chain and activates downstream PI3K and MAPK pathways (Nelms, Keegan *et al.* 1999). The catalytic p110 subunit of PI3K is activated following interaction of the p85 subunit with IRS1/2. The lipid kinase activity of PI3K then triggers the transfer of phosphates from ATP to inositol in the cell membrane to produce phosphoinositides that act as second messengers to activate Protein Kinase C (PKC) and AKT (Nelms, Keegan *et al.* 1999) (Fig. 1.8).

AKT and PKC are important for the control of cell growth, survival and proliferation and PI3K driven AKT has recently been shown to be required for IL-4 driven macrophage proliferation *in vivo* (Ruckerl, Jenkins *et al.* 2012). Phosphorylated IRS1/2 also interacts with the adaptor protein Grb2 an adaptor protein that constitutively localises with the guanine nucleotide exchange factor son of sevenless (SOS); SOS functions to activate Ras the upstream activator of MAPK pathways (See Fig. 1.3 & Fig. 1.5) (Nelms, Keegan *et al.* 1999). The IL-4 driven activation of Ras/MAPK is proposed to be cell type specific and dependent upon the expression of other signalling molecules within the IL-4 treated cells (Nelms, Keegan *et al.* 1999). However, in T-cells IL-4R signalling has been shown to be controlled by Ras/MAPK signalling, and JNK signalling has been shown to promote Th1 and thus limit Th2 and also inhibit proliferation (Yamashita, Kimura *et al.* 1999; Jorritsma, Brogdon *et al.* 2003).

IL-4 signalling downstream of the IL-4R is thus relatively complex, with the interaction of multiple signalling pathways. In relation to macrophage alternative activation, dependence upon IL-4Rα and STAT6 are the most frequently investigated components, however roles for other components have been investigated, for example AKT (Ruckerl, Jenkins *et al.* 2012). To date, the majority of research investigating the impact of IL-4 on DCs has focused upon IL-4 induced

changes in the secretion of T-cell polarising cytokines, a topic discussed in more detail in the next section (1.7.1 IL-4 induction of DC IL-12).

1.7.1 IL-4 induction of DC IL-12

IL-4R signalling to DCs has been investigated previously in studies aiming to determine how IL-4 can alter the polarisation of DC and influence their ability to promote Th1 or Th2 responses (Hochrein, O'Keeffe *et al.* 2000; Kalinski, Smits *et al.* 2000; Biedermann, Zimmermann *et al.* 2001; Lutz, Schnare *et al.* 2002; Yao, Li *et al.* 2005; Guenova, Volz *et al.* 2008). Many of these studies were going on prior to or simultaneous with the work investigating IL-4 driven alternative activation in macrophages; as such the potential for molecules such as Ym1/2 and RELMα to influence the outcome of T-cell polarisation was not yet/only just being published. Thus this facet of IL-4 dependent immune changes was not investigated by the DC community.

In their role as professional APCs, DCs excel at efficiently activating naïve T-cells (Steinman 2012). DCs were shown in 1995 to be able to secrete IL-12 and preferentially polarise naïve T-cells toward a Th1 phenotye (Macatonia, Hosken *et al.* 1995). It was subsequently shown that the DC:T-cell interaction was not sufficient to induce IL-12 secretion by DCs and that exogenous factors such as IFNγ and CD40:CD40L engagement were required to promote optimal Th1 immunity (Cella, Scheidegger *et al.* 1996; Hilkens, Kalinski *et al.* 1997). Bioactive IL-12 is an heterodimeric cytokine of 70kD (IL-12p70), composed from the association of p35 and p40 subunits (Hochrein, O'Keeffe *et al.* 2000). IL-12p40 can be secreted in excess both *in vitro* and *in vivo* as a (p40) homodimer, which is able to antagonise the effects of bioactive IL-12p70 by binding, without signalling, to the IL-12R in mouse and human (Mattner, Fischer *et al.* 1993; Gillessen, Carvajal *et al.* 1995; Ling, Gately *et al.* 1995).

The role of IL-4 in altering the balance of IL-12 and IL-10 production by DCs has been investigated frequently *in vitro* (Hochrein, O'Keeffe *et al.* 2000; Kalinski, Smits *et al.* 2000; Lutz, Schnare *et al.* 2002; Yao, Li *et al.* 2005; Guenova, Volz *et al.* 2008). IL-4 (but not IL-13) (Lutz, Schnare *et al.* 2002) was shown to enhance IL-12p70 whilst inhibiting IL-12p40 secretion from PRR stimulated splenic DCs (Hochrein, O'Keeffe *et al.* 2000), CD40L stimulated human or monocyte derived

DCs (Kalinski, Smits *et al.* 2000), or IFNγ stimulated monocytes or GMDC (Bullens, Kasran *et al.* 2001; Lutz, Schnare *et al.* 2002). IL-4 was also shown to instruct resistance to *L. major*, and this was dependent upon induction of IL-12 in DCs (Biedermann, Zimmermann *et al.* 2001). The ability of IL-4 to enhance IL-12p70 production from LPS stimulated splenic or GMDC was subsequently shown to be dependent upon inhibition of IL-10 production (Yao, Li *et al.* 2005). High or low dose IL-4 treatment of human monocyte derived DCs has been shown to alter their ability to promote Th1 or Th2 during *in vitro* culture, with 5ng/ml IL-4 resulting in DCs that secrete low IL-12p70 and prime naïve T-cell toward Th2, and 50ng/ml IL-4 treatment of DCs resulting in high IL-12p70 and Th1 induction from naïve T-cells (Guenova, Volz *et al.* 2008). Although the ability of IL-4 to limit PRR stimulation induced DC IL-10 and promote DC IL-12p70 has been shown *in vitro*, the functional ability of IL-4 stimulated DCs to prime Th1 responses *in vivo* has not been addressed. As *in vitro* conditions cannot accurately represent the *in vivo* environment, for example in terms of the mixed antigenic, cytokine and cellular environment, it is important to address whether the impact of IL-4 on DC derived IL-12p70 also influences the outcome of DC T-cell polarisation *in vivo*.

1.8 VITAMIN A METABOLISM

In the second half of this thesis vitamin A metabolites are investigated in relation to their role in modulating DC priming of T cell responses in the context of an IL-4 rich environment. Vitamin A (retinol) is a fat-soluble nutrient (Hall, Grainger *et al.* 2011) that is converted to retinoic acid (RA) in a step-wise process via the intermediate product retinal (Fig.1.9). Retinol cannot be synthesised endogenously and as such it must be absorbed from the diet. Diet acquired retinol is converted by lecitihin retinol acyl transferases (LRAT) in small intestinal enterocytes into retinyl esters and is transported in chylomicrons through the lymph into the bloodstream (Blomhoff and Blomhoff 2006; Jaensson-Gyllenback, Kotarsky *et al.* 2011). The major site of retinol storage in the body is the liver (Blomhoff and Blomhoff 2006). To allow mobilisation of retinol around the body, retinol is bound by retinol binding protein (RBP) (Theodosiou, Laudet *et al.* 2010). Retinol can freely diffuse into cells prior to binding RBP, although retinol complexed to RBP (holoRBP) must be delivered via the novel surface receptor *stimulated by retinoic acid 6* (STRA6) (Bouillet, Sapin *et al.* 1997; Berry, Jin *et al.* 2011; Kawaguchi, Zhong *et al.* 2012). Following mobilisation from the liver, holo-RBP complexes with a second carrier

protein transthyretin (TTR), which increases the size of the complex protecting clearance by filtration in the kidney (Episkopou, Maeda *et al.* 1993). The RBP/retinol/TTR complex binds to the RBP-receptor STRA6 and retinol is delivered directly into the cell (Kawaguchi, Yu *et al.* 2007), where it is bound by either to cellular retinol binding protein-I (CRBP-I) (Kawaguchi, Zhong *et al.* 2012) or LRAT (Isken, Golczak *et al.* 2008; Amengual, Golczak *et al.* 2012). STRA6 sophisticatedly controls the balance of intracellular LRAT and CRBP-1 bound retinol to extracellular RBP-retinol (Kawaguchi, Yu *et al.* 2011; Berry, Croniger *et al.* 2012; Kawaguchi, Zhong *et al.* 2012).

It was recently shown that in addition to mediating the uptake of retinol into target cells, STRA6 is able to signal (Berry, Jin *et al.* 2011). Transfer of extracellular retinol from RBP to intracellular CRBP results in the phosphorylation of STRA6 and the recruitment and activation of the signalling molecules JAK2 and STAT5 (Berry, Jin *et al.* 2011; Berry, O'Byrne *et al.* 2012). Following cellular uptake, retinol is converted into retinal by short chain or alcohol dehydrogenase (SDR/ADH), then retinal is converted to RA via retinal dehydrogenase enzymes (RALDHs;*Aldh1a1, Aldh1a2, Aldh1a3*) (Blomhoff and Blomhoff 2006). RALDH1 (*Aldh1a1)* is expressed in the dorsal retina of embryos and in adult epithelia tissues (Blomhoff and Blomhoff 2006), and was found to be expressed in PP DCs, liver and weakly in peripheral LNs (Iwata, Hirakiyama *et al.* 2004). RALDH2 (*Aldh1a2)* is expressed in many cell types of both embryonic and adult tissue and is required for embryonic development (Niederreither, Subbarayan *et al.* 1999). RALDH2 is the most highly expressed RALDH in the mLN, specifically in mLN DCs (Iwata, Hirakiyama *et al.* 2004; Coombes, Siddiqui *et al.* 2007; Jaensson, Uronen-Hansson *et al.* 2008), and was initially not thought to be expressed in the spleen (Iwata, Hirakiyama *et al.* 2004), however upregulation in the spleen has since been reported, as discussed further in sections 1.8.4 and 1.8.5. RALDH3 (*Aldh1a3)* null animals die within 10h of birth due to nasal defects (Dupe, Matt *et al.* 2003). *Aldh1a3* was shown to be weakly expressed in the mLN, PP and peripheral LNs (Iwata, Hirakiyama *et al.* 2004) and has recently been shown to be expressed in liver leukocytes during *S. mansoni* infection (Broadhurst, Leung *et al.* 2012).

1.8.1 Retinoic acid signalling

RA signals via binding to heterodimeric nuclear hormone receptors of the retinoic acid receptor (RAR) or retinoid x receptor (RXR) families, which have 3 members in each family, α, β & γ (Chambon 2004; Blomhoff and Blomhoff 2006). All trans-RA has a high affinity for RARs but lower affinity for RXRs, whereas 9-cis-RA (9cRA), an isomer of atRA, is an high affinity RXR ligand (Kane 2012). Although studies had failed to detect 9cRA in tissues, leading to the suggestion that it did not have a functional *in vivo* role, it has since been detected in the pancreas (Kane, Folias *et al.* 2010).

RARα is the dominant RAR isoform in myeloid cells (Nagy, Szanto *et al.* 2012), though all RAR isoforms have been detected in murine splenic DCs (Manicassamy, Ravindran *et al.* 2009). Ligand-bound RAR/RXRs associate with retinoic acid response elements (RARE) in the promoter regions of target genes to mediate their downstream effects. RAREs are composed of repeats of a core hexameric motif PuG(G/T)TCA (Bastien and Rochette-Egly 2004). The classical RARE has direct repeats spaced by 5bp, and as such is referred to as DR5 (Bastien and Rochette-Egly 2004). DR5 elements are found in the promoters of RARβ (Bastien and Rochette-Egly 2004) and RARα (Leroy, Nakshatri *et al.* 1991) genes, suggesting that RA can modulate expression of its own receptors.

1.8.2 Intestinal homing

In order to explain how RA is generally perceived to be involved in immune system function, it is necessary to first explain how T-cells are recruited to intestinal sites (Fig. 1.10) (Iwata 2009). Pathogens generally enter the body at peripheral sites such as the skin, GI tract and lung, locations at which naïve T-cells are infrequently present/largely excluded (Ebert, Schaerli *et al.* 2005). In order for naïve T-cells to come into contact with APCs expressing antigen they continually circulate through secondary lymphatics in an attempt to locate an antigen loaded DC with whom they can form a cognate interaction (Randolph, Ochando *et al.* 2008). Naïve T-cell migration through the secondary lymphatics is dependent upon expression of CD62L (L-selectin) and CCR7: CCR7 expression allows naïve and central memory T-cells to enter the T-cell zones of lymphoid organs (LOs) by recognition of a gradient of CCL19/ELC (Ebert, Schaerli *et al.* 2005). Short lived effector T-cells result from this interaction and are released into the circulation such that they can

enter peripheral tissues to perform their effector function (Ebert, Schaerli *et al.* 2005). Circulating effector T-cells express cellular adhesion receptors that allow them entry into defined tissues expressing their cognate vascular endothelial ligands (Agace 2009). For entry into mucosal sites, T-cells are required to express CCR9, α4β7, and have low level expression of CD62L (Johansson-Lindbom, Svensson *et al.* 2003), a phenotype only thought to be generated in gut associated lymphoid tissue (GALT) such as the mLN (Johansson-Lindbom, Svensson *et al.* 2003). $CCR9^+$ T-cell are able to enter the SI mucosa via interaction with the ligand CCL25 (Svensson, Marsal *et al.* 2002). CCL25 is constitutively expressed by epithelial cells of the small intestine, and is present on the surface of intestinal vascular endothelial cells, where it is able to mediate the interaction of T-cells with the intestinal mucosa (Kunkel, Campbell *et al.* 2000). T-cell recognition of CCL25 induces clustering of α4β7 on the cell surface, resulting in enhanced affinity for its ligand MadCAM1, which is expressed constitutively on the vascular endothelium of the LP (Agace 2009). Following entry into the intestinal LP, intraepithelial lymphocytes (IELs) downregulate expression of α4β7 and upregulate the integrin αEβ7 (CD103), allowing interaction with E-cadherin expressed on epithelial cells (Ericsson, Svensson *et al.* 2004).

1.8.3 $CD103^+$ DCs, RA production and RA effects on T-cells

mLN DCs were found to be superior at inducing intestinal homing markers on circulating T-cells (Svensson, Johansson-Lindbom *et al.* 2008; Agace and Persson 2012). In particular, a DC subset that also expressed CD103 was also found to be able to induce CCR9 and α4β7 on $CD8^+$ T-cells *in vitro* (Johansson-Lindbom, Svensson *et al.* 2005). It was then determined using $CCR7^{-/-}$ mice that the $CD103^+$ DCs in the mLN were migratory DCs, arriving from the LP in a CCR7 dependent manner (Johansson-Lindbom, Svensson *et al.* 2005). Reports in other laboratories revealed that RA was also able to induce CCR9 and α4β7 expression by naïve αCD3/αCD28 stimulated $CD4^+$ T-cells *in vitro* (Iwata, Hirakiyama *et al.* 2004). mLN DCs were found to express *Aldh1a2* (RALDH2) and produce RA by irreversible conversion of retinal, and this was sufficient to induce CCR9, α4β7 expression by and gut homing in T-cells (Iwata, Hirakiyama *et al.* 2004). Further, the effect of RA was found to be dependent upon RARβ/RARα but not RXRs (Iwata, Hirakiyama *et al.* 2004).

CD103^{+} mLN and LP DCs have also been shown to promote spontaneous extra-thymic conversion of naïve T cells into Foxp3^{+} expressing regulatory T cells in a TGFβ and RA dependent manner (Coombes, Siddiqui *et al.* 2007; Sun, Hall *et al.* 2007). In addition, it was again shown that CD103^{+} mLN DCs produced RA via their expression of *Aldh1a2* (Coombes, Siddiqui *et al.* 2007). CD103^{+} mLN DCs were confirmed to be a tissue-derived population of migratory cells required for the presentation of orally derived antigens to both CD4^{+} and CD8^{+} T-cells, and an equivalent population (both functionally and phenotypically) was found in human mLN (Jaensson, Uronen-Hansson *et al.* 2008). Induction of T-cell Foxp3 by TGFβ and IL-2 was shown to be limited by DC provision of co-stimulation, and this could be overcome by using DCs deficient in CD80/86 or addition of exogenous RA to the cultures (Benson, Pino-Lagos *et al.* 2007). Interest in the role of RA in influencing Th differentiation was further enhanced by studies showing that RA was able to limit the conversion of Th cells toward a Th17 phenotype. RA and other agonists of RARα were shown to inhibit Th17 whilst promoting Foxp3 expression in collaboration with TGFβ (Mucida, Park *et al.* 2007; Elias, Laurence *et al.* 2008). These studies were important for defining how the tolerogenic intestinal environment was maintained in the steady state, and highlighted an important role for vitamin A metabolites acting at the site of vitamin A acquisition. Further studies have begun to investigate what factors drive RALDH2 expression in DCs, an area discussed in more detail in the next section (1.8.4).

1.8.4 Induction of RALDH2 in DCs

Although *in vitro* culture of mLN DCs had been reported to result in the expression of CCR9 and α4β7 on co-cultured T-cells (Johansson-Lindbom, Svensson *et al.* 2003), it was only shown recently that this ability was abolished if retinoids were depleted from complete medium via UV-irradiation of FCS prior to use (Jaensson-Gyllenback, Kotarsky *et al.* 2011). Addition of retinal to serum free medium (XVIVO10) could also rescue the ability of mLN DCs to induce gut homing markers on T-cells (Jaensson-Gyllenback, Kotarsky *et al.* 2011). In accordance with this recent finding, previous studies have reported a role for RA in modulating DC ability to induce gut homing molecules *in vitro* (Feng, Cong *et al.* 2010). Inclusion of RA during DC differentiation resulted in DC populations that expressed CCR9 and expressed *Aldh1a2* (Feng, Cong *et al.* 2010). The same study also reported BM expression of *Aldh1a2*, leading the authors to propose a model whereby RA

expression within BM results in the imprinting of 'mucosal DCs' that express CCR9 to enable migration to GALT (Feng, Cong *et al.* 2010). PPARγ activation has also been implicated in the induction of RALDH2, resulting in enhanced RA signalling to RARα and upregulation of CD1d expression and leading to altered presentation of lipid antigens (Szatmari, Pap *et al.* 2006). The authors suggest that at intestinal sites there would be high levels of lipid traffic, which would provide a source of PPARγ ligands to drive RALDH2 production. However, this work was all carried out *in vitro* (Szatmari, Pap *et al.* 2006).

Until recently, a functional role for RA in induction of small intestine DC RALDH2 activity *in vivo* had not been shown. However, several studies have since found that animals kept on a VAD diet display reduced expression of *Aldh1a2* and RALDH activity in their $CD11c^+$ mLN cells (Yokota, Takeuchi *et al.* 2009), and that this also holds true for the $CD103^+$ subset (Jaensson-Gyllenback, Kotarsky *et al.* 2011). The ability of $CD103^+$ DCs to metabolise retinal was shown to occur locally, as pre-DCs had no aldehdye dehydrogenase activity, in direct contrast to the report by Feng *et al* (Feng, Cong *et al.* 2010; Jaensson-Gyllenback, Kotarsky *et al.* 2011). DR5-luciferase reporter mice were used to show that $CD103^+$ SI-LP DCs received an RA signal *in vivo,* and that the signal was higher in SI DCs than colonic $CD103^+$ DCs (Jaensson-Gyllenback, Kotarsky *et al.* 2011). Contrary to expectation, the local source of retinol required to imprint RALDH2 activity in SI DCs was found to be bile retinoids released from the liver, and not a dietary retinol source (Jaensson-Gyllenback, Kotarsky *et al.* 2011).

We will now discuss work detailing a role for DC RA production at sites other than the gut. Splenic DCs were initially reported not to express *Aldh1a2* (Johansson-Lindbom, Svensson *et al.* 2003; Iwata, Hirakiyama *et al.* 2004). However, a consensus is emerging that in response to certain stimuli, or on removal of inhibition, RALDH activity can be upregulated in splenic DCs both *in vitro* (Manicassamy, Ravindran *et al.* 2009; Yokota, Takeuchi *et al.* 2009; Stock, Booth *et al.* 2011; Wang, Villablanca *et al.* 2011) and *in vivo* (Manicassamy, Ravindran *et al.* 2009; Stock, Booth *et al.* 2011; Wang, Villablanca *et al.* 2011).

One of the few stimuli that have been reported to induce *Aldh1a2* in splenic DCs is signalling via TLR2. The *S. cerevisiae* fungal cell wall preparation, zymosan

was shown to induce *Aldh1a2* production by splenic CD11c$^+$ DCs (Manicassamy, Ravindran *et al.* 2009). Zymosan enhancement of DC *Aldh1a2 in vitro* was shown to be partially dependent upon TLR2, independent of dectin-1 but dependent upon ERK signalling (Manicassamy, Ravindran *et al.* 2009). Injection of zymosan was found to induce total splenic CD11c$^+$ cell populations to increase production of *Aldh1a2* mRNA and RALDH2 protein (assessed by western blot) (Manicassamy, Ravindran *et al.* 2009). The TLR4 and TLR9 agonists LPS and CpG were unable to induce splenic DC expression of *Aldh1a2* (Manicassamy, Ravindran *et al.* 2009), highlighting that *Aldh1a2* induction is linked to TLR2 signalling rather than generic pathogen recognition. In a more recent study, *Aldh1a2* expression and RALDH activity was shown to be impaired in mLN but not the splenic DCs of MyD88$^{-/-}$ mice (Wang, Villablanca *et al.* 2011), and TLR2 was shown to be expressed by mLN, PP and spleen DCs (Wang, Villablanca *et al.* 2011). Furthermore, the TLR2 specific agonist P3C was shown to induce *Aldh1a2* in splenic DCs, repeating the findings of Manicassamy *et al* in highlighting a role for TLR2 signalling in increasing RALDH in extra-intestinal DCs (Wang, Villablanca *et al.* 2011). Additionally, CD103$^+$ mLN DCs were found to have reduced RALDH activity in TLR2$^{-/-}$ mice compared to WT animals (Wang, Villablanca *et al.* 2011). These data highlight a role for TLR2 signalling in inducing DC expression of RALDH2, and suggest that DC expression of this enzyme may function in the immune response to both bacterial and fungal pathogens which signal via TLR2.

1.8.5 Immune mediators implicated in induction and regulation of DC RALDH

Interestingly, IL-4 has been shown to cause the induction of *Aldh1a2* mRNA in mLN DCs resulting in the upregulation of CCR9, but not α4β7 on T-cells (Elgueta, Sepulveda *et al.* 2008). IL-4 and IL-13 were both shown to induce RALDH activity in BM cells following culture with FLT3L and either Th2 cytokine (Stock, Booth *et al.* 2011). However, an additional study reported that intestinal DCs from *Il4ra*$^{-/-}$ mice had normal or enhanced levels of RALDH activity (Yokota, Takeuchi *et al.* 2009). Given the reports that RA could directly drive *Aldh1a2* in DCs, the literature available on the interaction of these two immune mediators is limited. A topic also underrepresented in the RA literature is evidence for macrophage expression of retinal converting enzymes, however this topic is beginning to be addressed (Broadhurst, Leung *et al.* 2012). Due to the sparse references available on DC alternative activation (Modolell, Corraliza *et al.* 1995; Nair, Gallagher *et al.* 2005;

Arora, Chen *et al.* 2006; Cai, Kumar *et al.* 2009), and the limited interest (so far) in macrophage vitamin A metabolism, the cross talk between IL-4 induction of alternative activation and retinoic acid production has not yet been addressed. We will begin to address the interaction between DC alternative activation and vitamin A metabolites/metabolism within chapter 5 of this thesis.

GM-CSF has also been implicated in the acquisition of RA producing capacity in intestinal DCs, in combination with IL-4 (Yokota, Takeuchi *et al.* 2009), and alone in $CD11c^+$ cells of the mLN and spleen. Interestingly RALDH activity was enhanced in GM-CSF treated DCs in the presence of LPS (Stock, Booth *et al.* 2011). GM-CSF binds to the GM-CSFR, and deficiency in this receptor (*Csf2r*$^{-/-}$) was shown to reduce $CD103^+CD11b^+$ LP DCs, the population reported to have the highest level of endogenous RALDH activity (Bogunovic, Ginhoux *et al.* 2009).

Prostaglandin E2 (PGE_2) has been purported to be the factor responsible for the low levels of RALDH activity in extra-intestinal DCs (Stock, Booth *et al.* 2011). PGE_2 was shown to repress the GM-CSF dependent transcription of RALDH in mouse and human moDCs, *in vitro* via the EP-2 and EP-4 receptors respectively (Stock, Booth *et al.* 2011). Reducing PGE_2 levels at steady state *in vivo,* via administration of a cyclooxygenase inhibitor indomethacin, resulted in a four to five fold increase in RALDH expressing splenic DCs (Stock, Booth *et al.* 2011), with stromal cells identified as being the source of the RALDH inhibitory PGE_2.

1.8.6 Vitamin A and Th1/Th2

Work on Treg/Th17 induction was carried out in the steady state conditions and did not assess the role that RA plays in regulating Th1/Th2 balance. Prior to the studies investigating the role for vitamin A metabolism in DCs and Treg/Th17 induction, animals kept on a VAD diet had been shown to have enhanced production of IFNγ by T-cell cultures *in vitro* (Carman and Hayes 1991) and in response to *Trichinella spiralis* infection *in vivo* (Carman, Pond *et al.* 1992). In addition, RA was found to influence the outcome of T-cell polarisation *in vitro* when added to cells under Th2 differentiation conditions, promoting the secretion of IL-4 (Hoag, Nashold *et al.* 2002). A study comparing the *in vitro* polarising ability of RA and 9cRA in a co-culture of APCs with DO11.10 TCR transgenic T-cells reported a role for 9cRA and RXR agonists in promoting Th2 responses in primary stimulations

of $CD4^+$ T-cells (Stephensen, Rasooly *et al.* 2002). This report provides useful information on the capacity of RXRs to alter T-cell responses to RA, but may not be representative of an *in vivo* situation due to the relatively low level presence of 9cRA (Kane 2012). Interestingly, the study by Stephensen *et al* found no role for primary exposure to RA in promoting Th2 polarisation in either secondary or tertiary restimulation (Stephensen, Rasooly *et al.* 2002), but did identify a role for RA in reducing IFNγ secretion by already polarised Th1 cells.

RA acting via RARs was shown to directly suppress Th1 development and enhance Th2 development in a report from a different laboratory (Iwata, Eshima *et al.* 2003). In this work, RA was added to *in vitro* cultures of isolated T-cells in the absence of APCs. RA also enhanced expression of the IL-4Rα by thymocytes cultured under Th2 conditions (Iwata, Eshima *et al.* 2003), suggesting that T-cells exposed to RA would be more receptive to IL-4 and thus to becoming Th2. Additionally, RA has been shown to promote human T-cell Th2 cytokine production and GATA3 expression and to limit expression of T-bet and IFNγ (Dawson, Collins *et al.* 2006). A study from 2011 showed that RA signalling occurs concurrent with immunisation (CFA, LPS) and inflammation following allogeneic skin grafting (Pino-Lagos, Guo *et al.* 2011). Furthermore, investigations into RA signalling in the inflammatory settings of *Toxoplasma gondii* infection and vaccination also revealed a broader role for RA in mediating T-cell activation via cell intrinsic RARα signalling (Hall, Cannons *et al.* 2011). Hall *et al* have suggested that the contrasting data available in the literature regarding the role for RA in mediating Treg/Th1/Th2/Th17 responses is due to RA acting in a biphasic manner, driving T-cell activation and differentiation early during an immune response but regulating the response at later stages (Hall, Cannons *et al.* 2011). In this context, interpretation of the previous studies mentioned above may need re-evaluation taking timing into consideration.

1.8.7 Vitamin A and Schistosomiasis

Deficiency in dietary vitamin A, and thus reduced systemic vitamin A availability, is associated with increased morbidity and mortality in response to infectious disease (Semba 1999), and dietary supplementation with vitamin A has been shown to significantly reduce all-cause mortality in children (Sommer 2008). Serum retinol levels in school-age children have been inversely correlated with *S. mansoni* infection (Friis, Mwaniki *et al.* 1997). A recent study comparing the impact

of murine vitamin A deficiency during infection with *S. mansoni* or LCMV found enhanced numbers of Foxp3$^+$ Tregs in the spleen and colons in control, helminth, or virally infected animals (Broadhurst, Leung *et al.* 2012), a result also seen in the SI LP of naïve animals in a separate previous study (Hall, Cannons *et al.* 2011). Hepatic leukocytes of *S. mansoni* infected VAD animals displayed significantly reduced intracellular CD4$^+$ T cell IL-4, reduced secretion of IL-4 and IL-10 following restimulation with *S. mansoni* egg antigens, and decreased *Il4*, *Il5* and *Il13* mRNA, compared to vitamin A sufficient infected controls (Broadhurst, Leung *et al.* 2012). This study was the first to reveal that AAM present within liver granulomas express RALDH, and can be an important source of RA following schistosome infection. It also showed that IL-4 was able to promote *Aldh1a2* induction in macrophages (Broadhurst, Leung *et al.* 2012). The role for DC RALDH2 expression in the context of *S. mansoni* is a topic that has not yet been investigated. The lack of investigation into DC RALDH outwith intestinal settings is perhaps due to work suggesting that RALDHs are not expressed in extraintestinal tissues such as the spleen, this finding has since been clarified by the detection of the inhibitory role of stromal cell derived prostaglandins (Stock, Booth *et al.* 2011).

1.9 THESIS AIMS

That IL-4 can alter the activation status of macrophages is well documented. However, whether IL-4 has the ability to alter the activation and function of DCs in a similar manner is much less clear.

Chapter 3 of this thesis compares the impact that IL-4 has on DCs and macrophages, both *in vitro* and *in vivo*, in response to exogenous IL-4 treatment and schistosome infection. Expression of molecules usually associated with AAM (arginase, RELMα, Ym1/2, dectin-1, CCL17, CCL24, MR) is systematically investigated in IL-4 exposed DC populations. In addition, the APC ability of BMDC and BMM are directly compared *in vivo* in response to Th1/17 (Pa, St) or Th2 (SEA) polarising antigens.

Chapter 4 extends this theme to determine whether the capacity of DCs to alternatively activate (comparing WT to *Il4ra*$^{-/-}$ cells), alters their ability to prime the immune system in response to Th1 and Th2 antigens. Furthermore, the functional impact of DC expression of one specific AAM-associated molecule, RELMα, is investigated. An *in vivo* DC transfer system and *in vitro assays* are used to assess the role of RELMα deficient DCs in T-cell polarisation. A microarray study is used to determine the global transcriptional changes that occur in WT, IL-4Rα and RELMα deficient DCs. A new theme introduced in chapter 4 is the role of IL-4 in modulating the response of DCs to TLR stimulation, in particular the impact that IL-4 has on DC secretion of T-cell polarising cytokines is investigated.

Chapter 5 builds on work from other laboratories and chapter 4 of this thesis, in which it was found that DC expression of *Aldh1a2* (the retinal dehydrogenase enzyme) is modulated by IL-4. With the use of a panRAR inhibitor (LE540) and RARα deficient DCs, the relationship between RA synthesis, RA signalling and IL-4 driven alternative activation in DCs is investigated for the first time.

In chapter 6, themes taken from the previous 3 chapters are integrated with experiments investigating the interrelationship between TLR2 agonists (P3C, zymosan) and IL-4 signalling in the modulation of DC RA production and alternative activation.

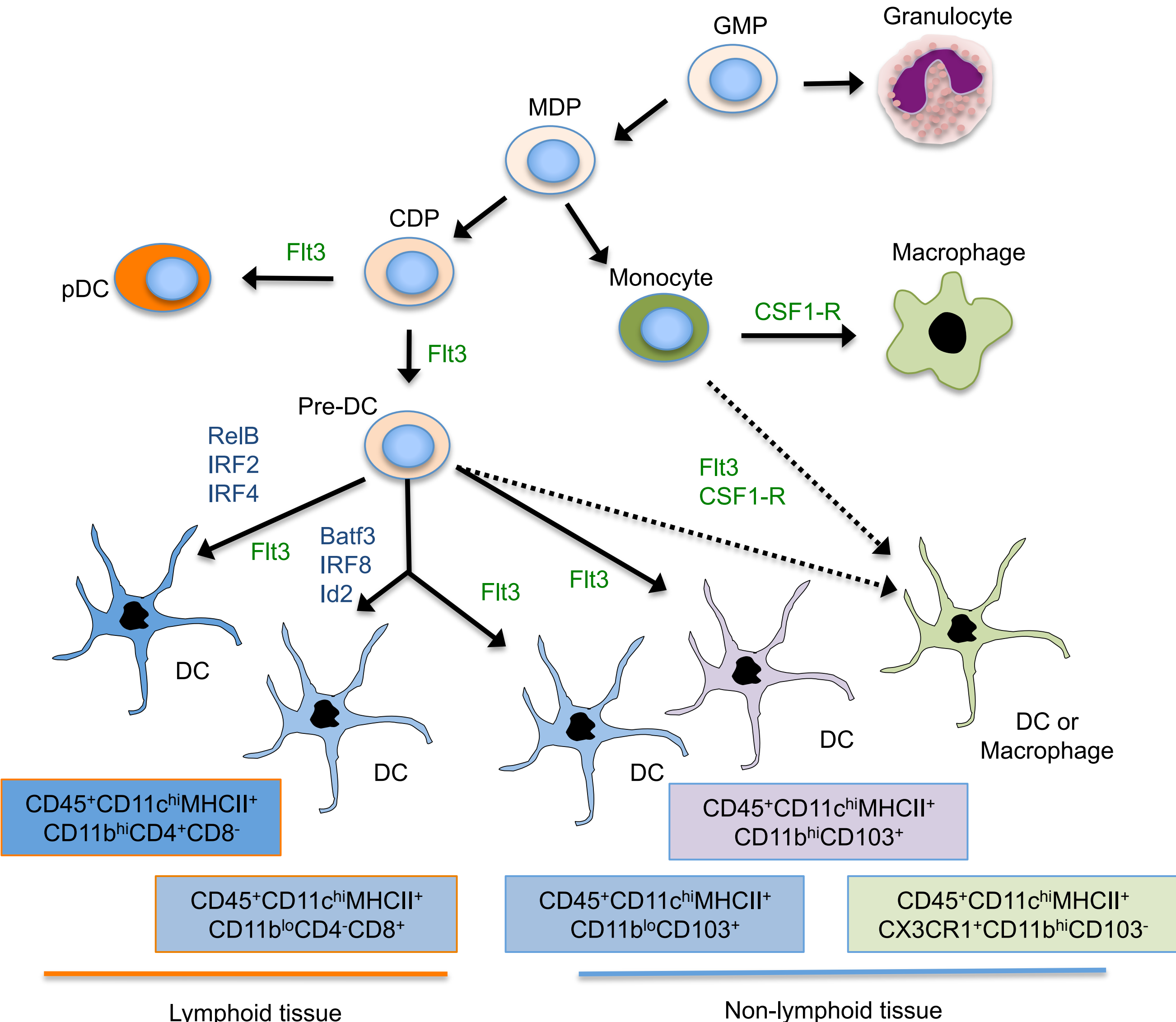


Figure 1.1. Murine DC and macrophage ontogeny. This figure highlights the precursor cells, transcription factors (blue) and cytokines (green) known to be required for development of DC and macrophage populations. The granulocyte/macrophage progenitor (GMP) gives rise to macrophage-dendritic cell progenitor MDP, which gives rise to monocytes and the common DC precursors (CDP). CDPs have lost monocyte-macrophage differentiation potential and give rise exclusively to plasmacytoid DCs (pDCs) and pre-DCs. Pre-DCs migrate to lymphoid tissues where they differentiate into lymphoid tissue $CD4^-CD8^+$ and $CD4^+CD8^-$ DCs and to non-lymphoid tissue where they differentiate into $CD103^+CD11b^-$ DCs and $CD103^+CD11b^+$ DCs. Flt3 controls myeloid precursor commitment to the DC lineage as well as the differentiation of mature DCs in tissue. The transcription factors Batf3, Id2 and IRF8 control the differentiation of lymphoid tissue $CD8^+$ DC and non-lymphoid tissues CD103+$CD11b^-$ DC. The transcription factors RelB, IRF2 and IRF4 control the development of $CD4^+$ DC. Macrophage development from monocytes is dependent upon expression of the M-CSF/Csf1 receptor CSF1-R. Figure is adapted from Merad and Manz 2009; Helft, Ginhoux *et al* 2010 and Hashimoto, Miller *et al* 2011.

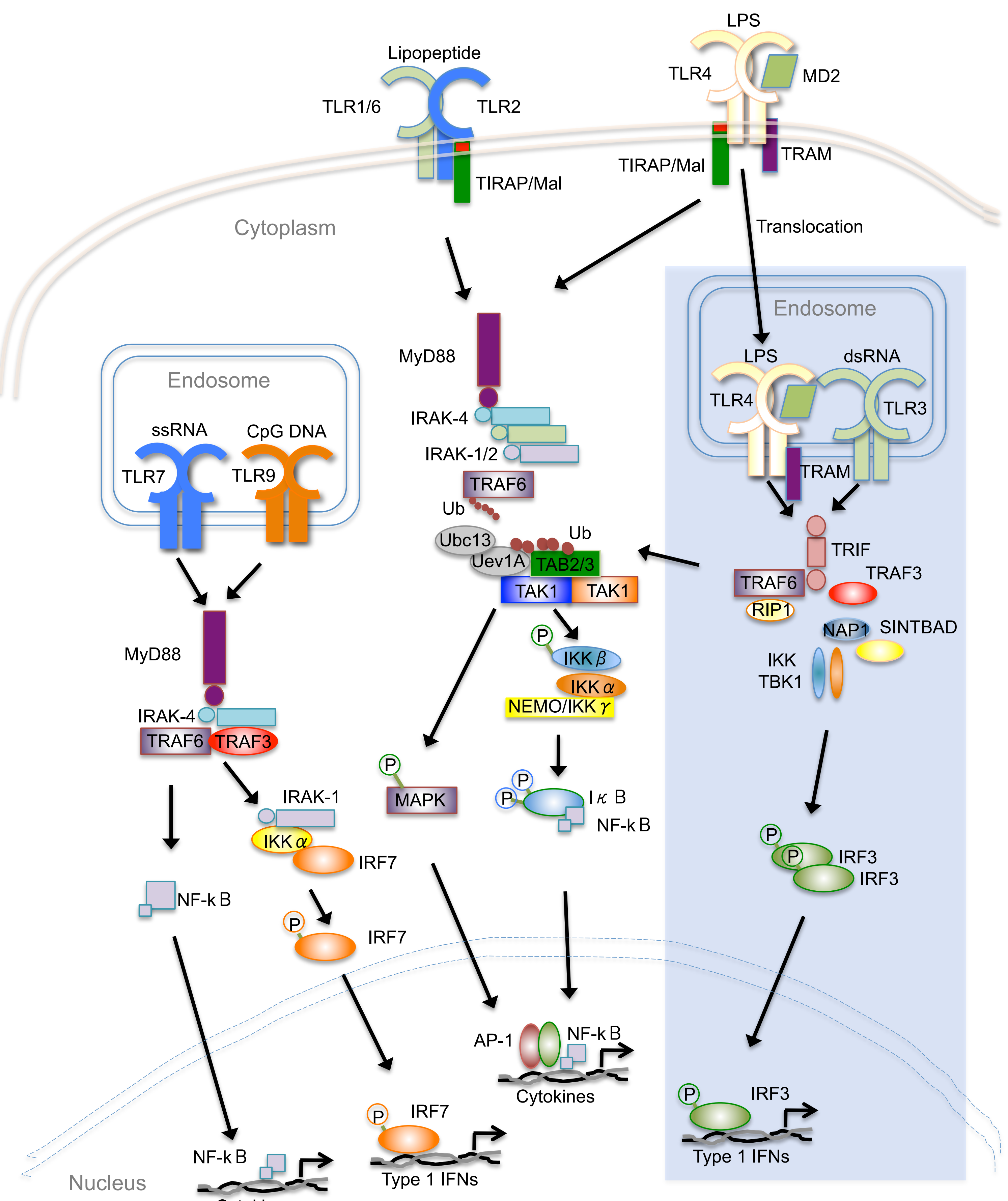


Figure 1.2 TLR signalling. This figure highlights the signalling pathways downstream of the main TLRs. Shaded=TRIF dependent/MyD88 independent pathway. Figure is adapted from Takeuchi and Akira 2010.

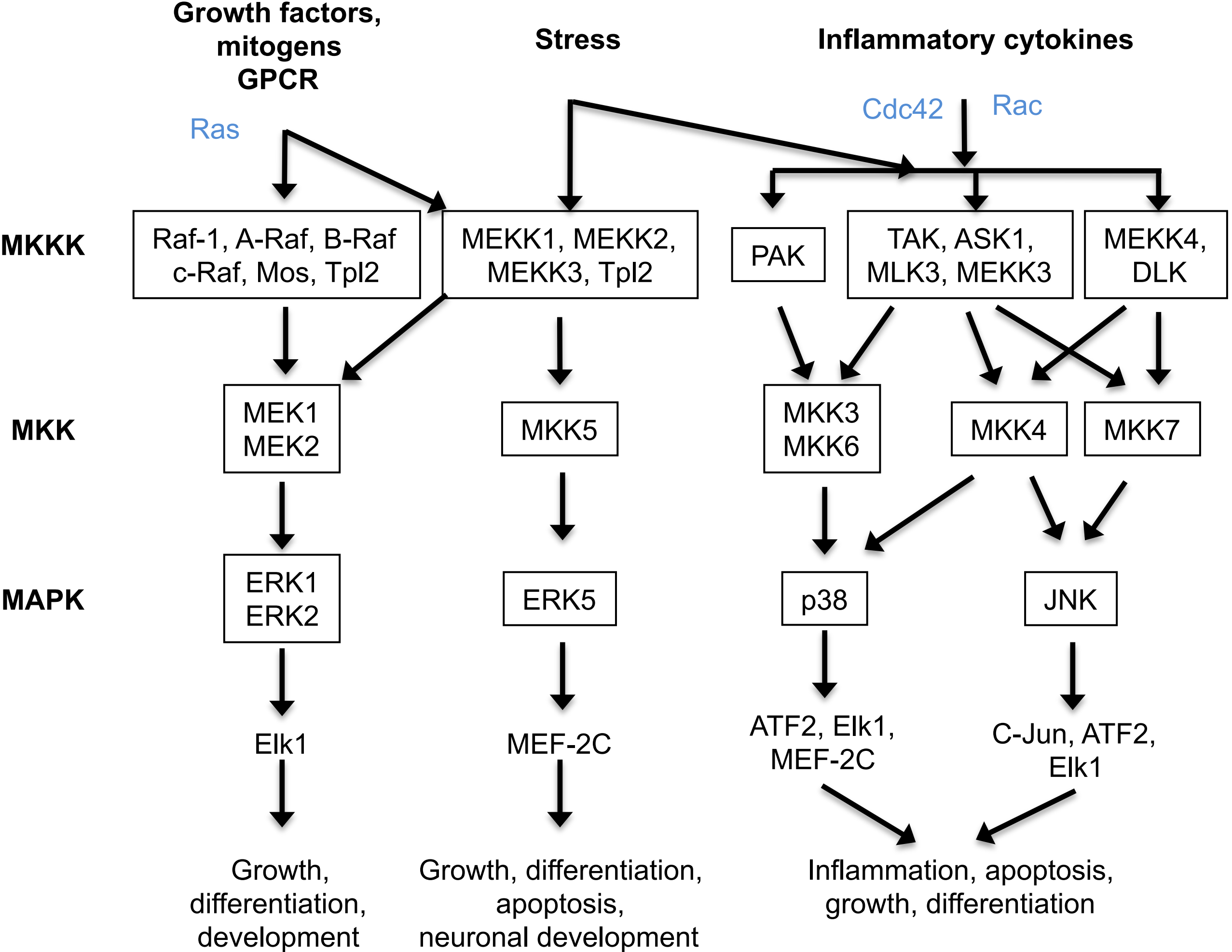


Figure 1.3. MAPK signalling. This figure highlights the hierarchy of MAPK signalling, which functions as a triple kinase cascade. The major MAPKs families (ERK1/2, ERK5, p38 and JNK) are activated by dual phosphorylation on Threonine and Tyrosine residues by specific MKKs, which have previously been activated by MKKKs. Small GTPase proteins are responsible for initiating the phosphorylation cascade upstream of MKKKs (blue). Figure is adapted from Zhang and Dong 2005.

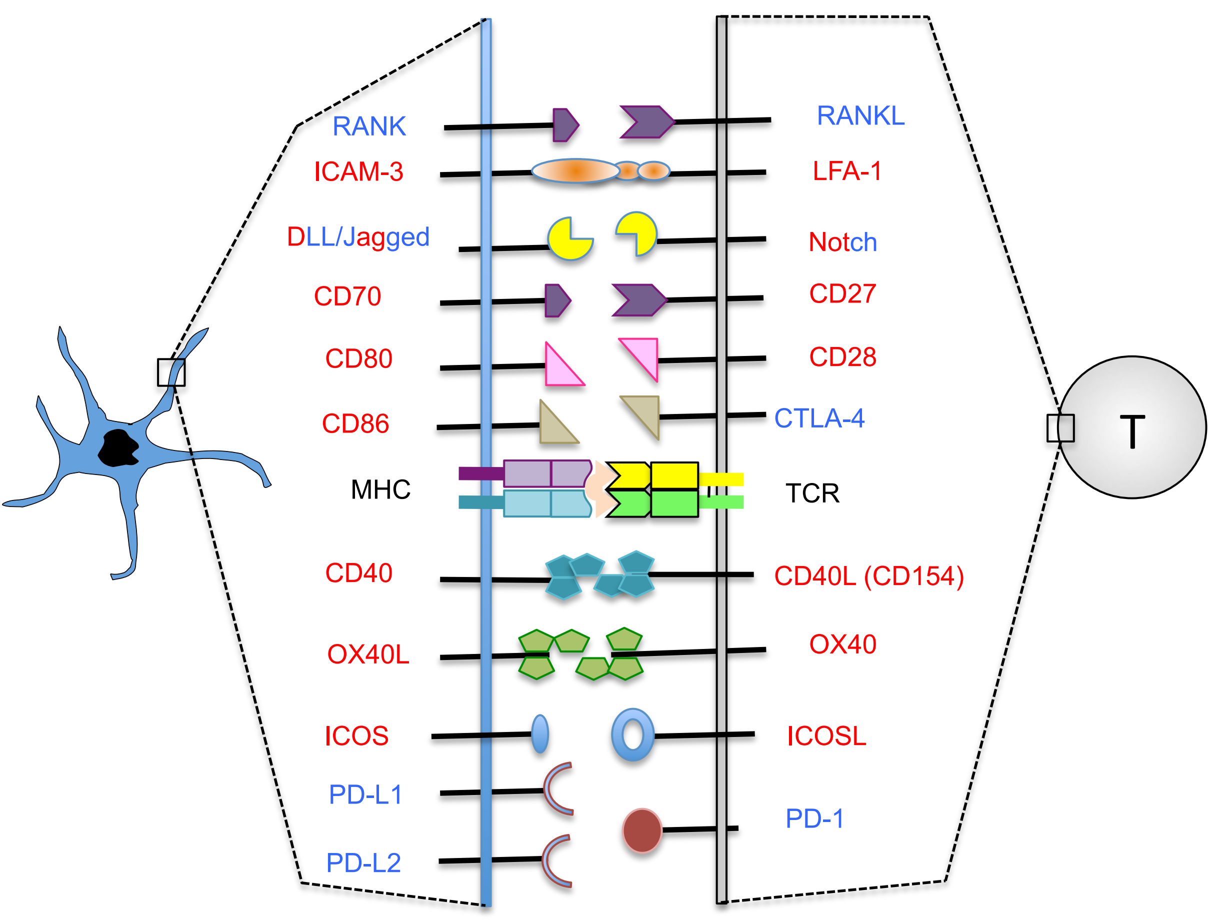


Figure 1.4. Co-stimulatory/-inhibitory molecules. This diagram displays ligands and receptors expressed on DCs and T-cells that form the immune synapse required for the activation of naïve T-cells. The proteins can be broadly grouped as Ig-superfamily or TNF/TNFR family members. Co-stimulatory (red) Co-inhibitory (blue). This figure is adapted from Nair, Amsen *et al* 2011.

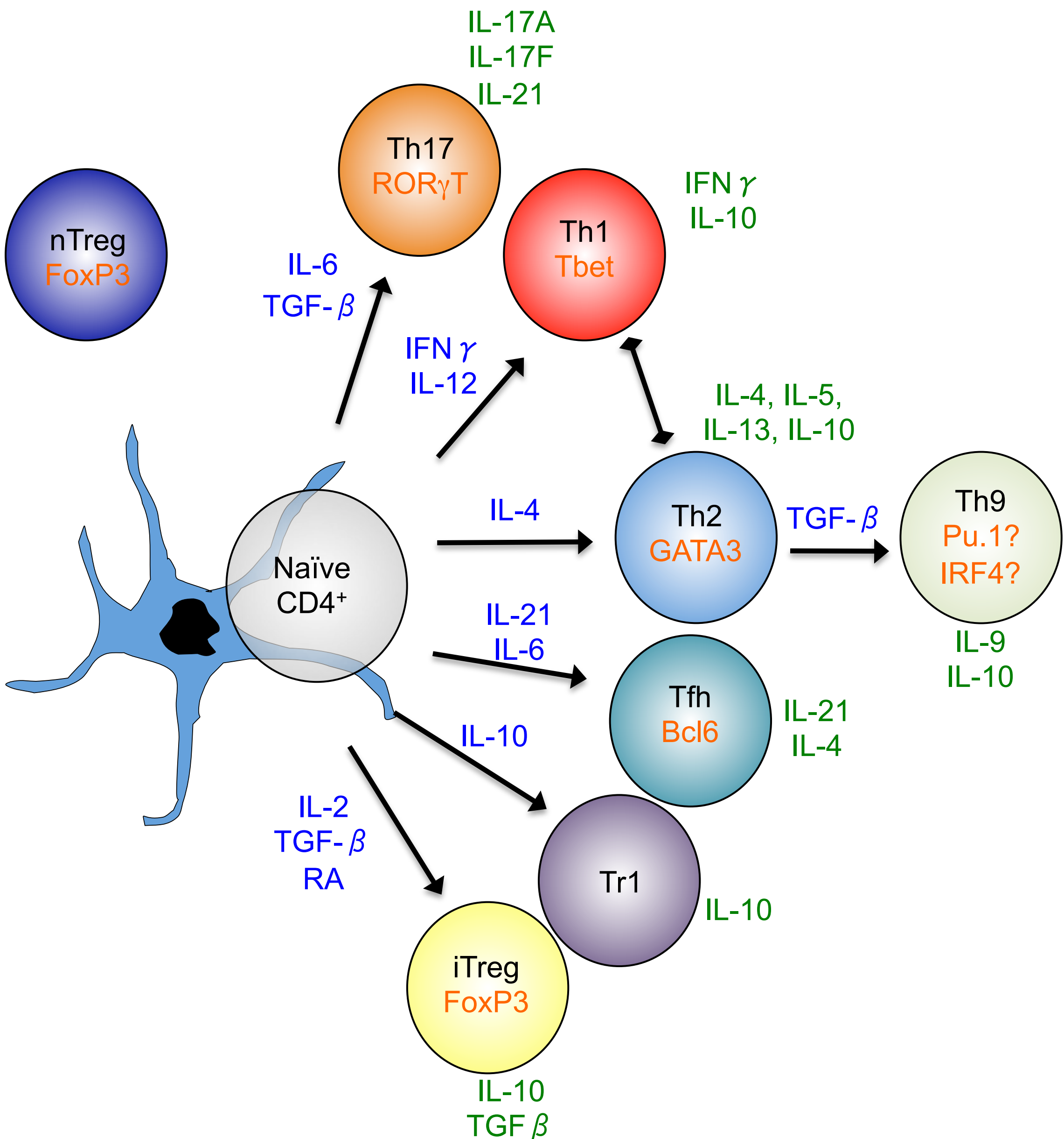
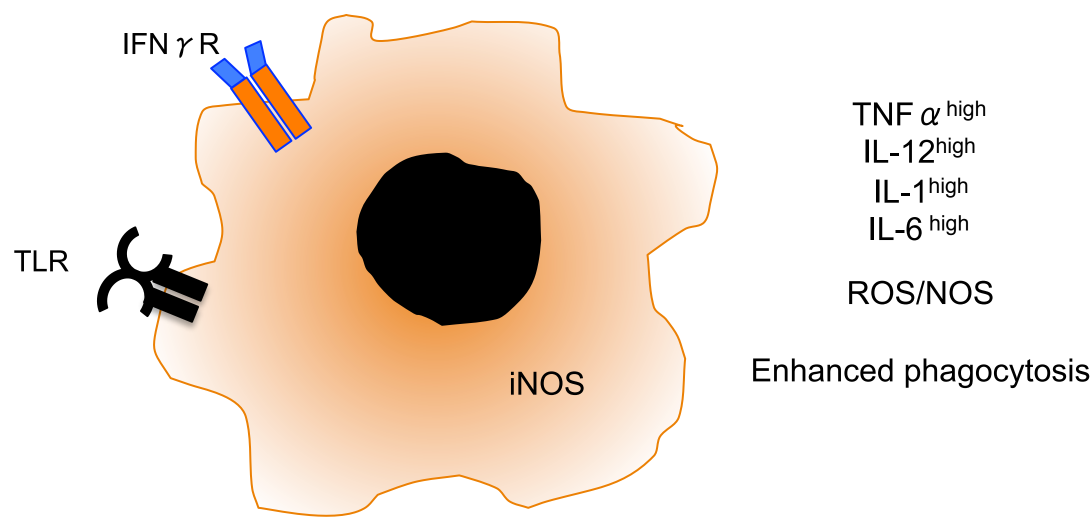


Figure 1.5. T-helper cell subsets. This figure highlights the cytokines (blue) and transcription factors (orange) required for the differentiation and specification of defined CD4+ T-helper cell subsets. Figure is adapted from Zhou, Chong *et al* 2009 and Pulendran and Artis 2012.

IFN γ
Microbial stimulus



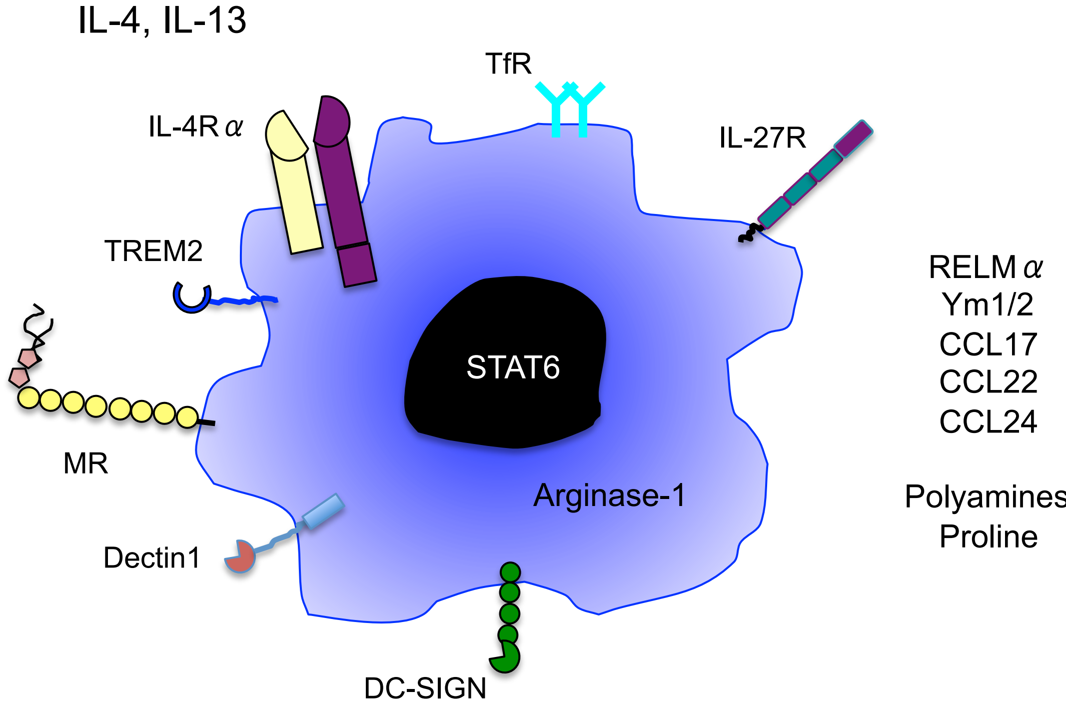


Figure 1.6. Diversity in molecules expressed by CAM and AAM. Figure is adapted from Varin and Gordon 2009 and Martinez, Helming *et al* 2009

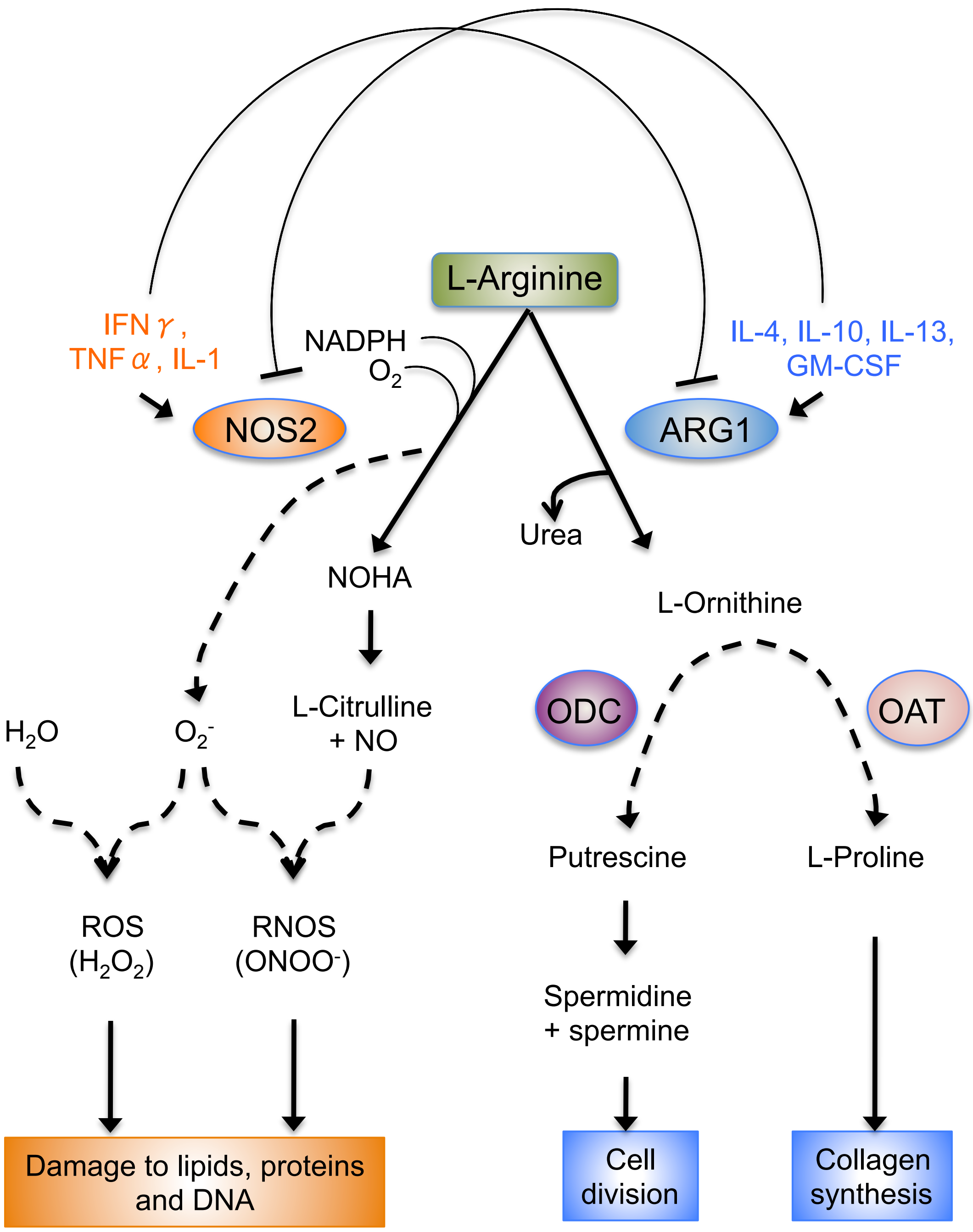


Figure 1.7. Arginine metabolism. This figure details the impact that Th1 and Th2 cytokines have on cellular metabolism of arginine, via the activation of inducible nitric oxide synthase (*Nos2*) and arginase 1 (*Arg1*). Th1 cyokines activate NOS2 an enzyme that converts arginine into reactive (nitrogen)-oxide species (ROS/RNOS), which damage cells. Th2 cytokines drive Arg1 activity leading to the conversion of L-arginine into L-ornithine. Ornithine is converted by ornithine decarboxylase (ODC) into polyamines that fuel cell division and by ornithine aminotransferase (OAT) into L-Proline which is important for collagen synthesis. Figure is adapted from Hesse, Modolell *et al* 2001 and Bronte and Zanovello 2005.

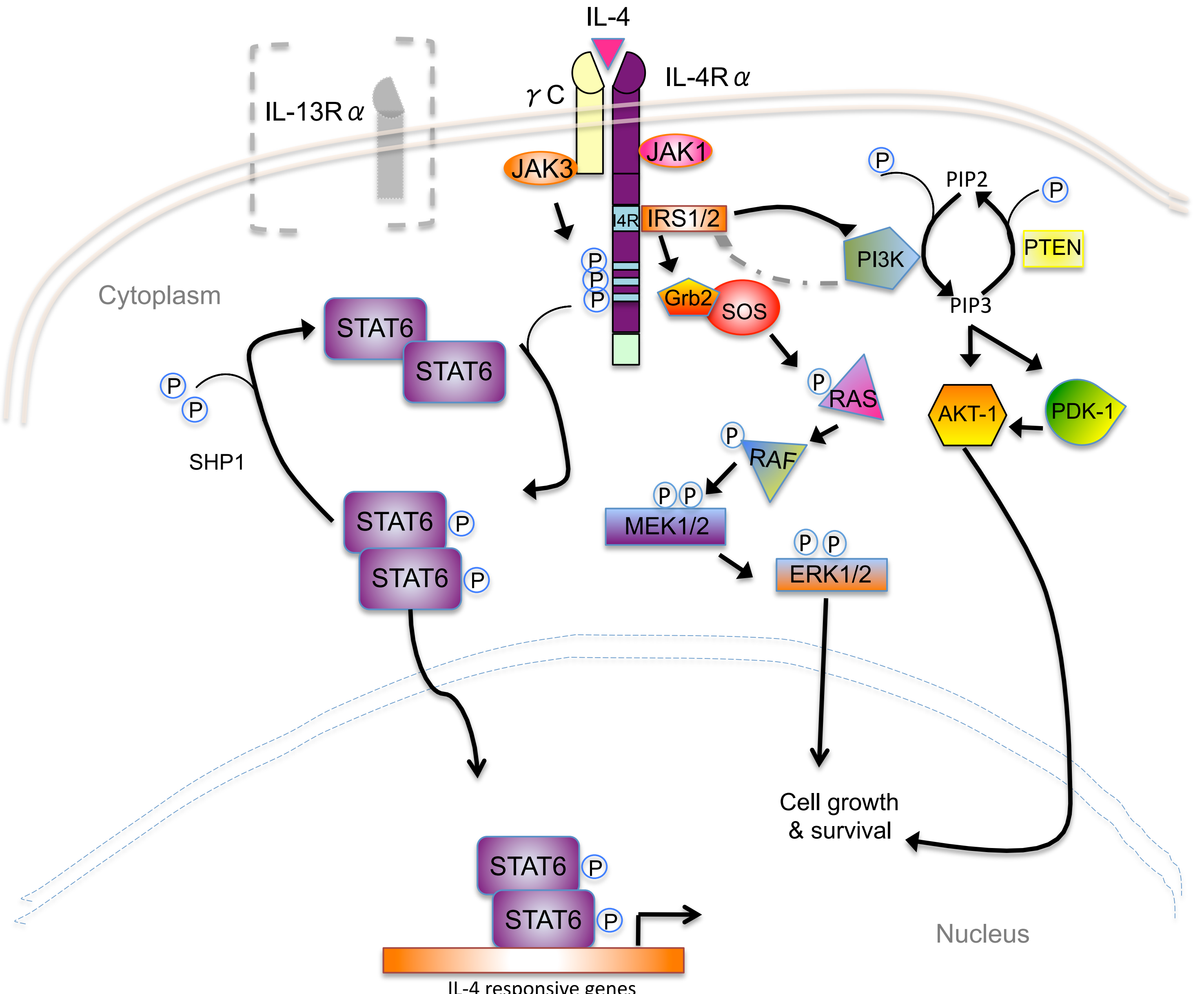


Figure 1.8. IL-4Rα signalling. IL-4 binds extracellularly to the IL-4Rα causing heterodimerisation of this receptor with either the common gamma chain (γC) or the IL-13Rα (grey brackets). JAK and Src kinases are activated leading to phosphorylation of the intracellular tail of IL-4Rα, enabling binding of SH2 domain proteins which themselves become activated. STAT6 becomes phosphorylated and forms a homodimer prior to nuclear entry and activation of IL-4 responsive genes. IRS1/2 is able to dock at an I4R site and activate the downstream signaling molecules PI3K and Grb2.
Figure is adapted from Nelms, Keegan *et al* 1999.

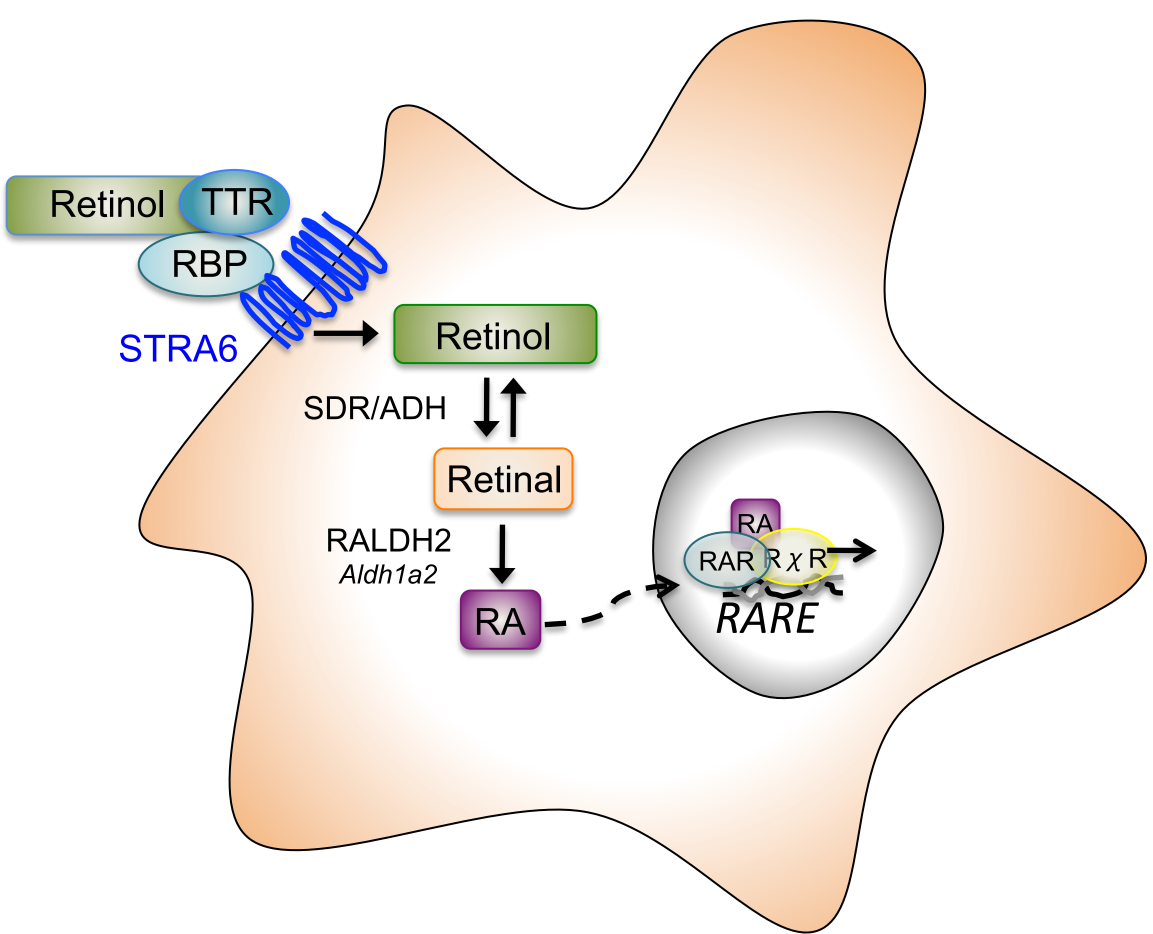


Figure 1.9. Retinol metabolism. This figure highlights the transport proteins, receptors and enzymes involved in the cellular conversion of retinol (vitamin A) into bioactive retinoic acid (RA). Retinol is transported bound to transthyretin (TTR) and the retinol binding protein (RBP), binding of RBP to the receptor stimulated by retinoic acid 6 (STRA6) facilitates delivery of retinol into the target cell. Retinol is reversibly converted into retinal by short chain/alcohol dehydrogenase (SDR/ADH). Retinal is irreversibly converted to retinoic acid (RA) by retinal dehydrogenases (RALDH). RA binds to hetero or homo-dimer nuclear hormone receptor pairings of retinoic acid receptor (RAR) $\alpha/\beta/\gamma$ and retinoid X receptor (RXR) $\alpha/\beta/\gamma$ that recognise retinoic acid response elements (RARE) in RA responsive genes. Figure is adapted from Theodosiou, Laudet *et al* 2010.

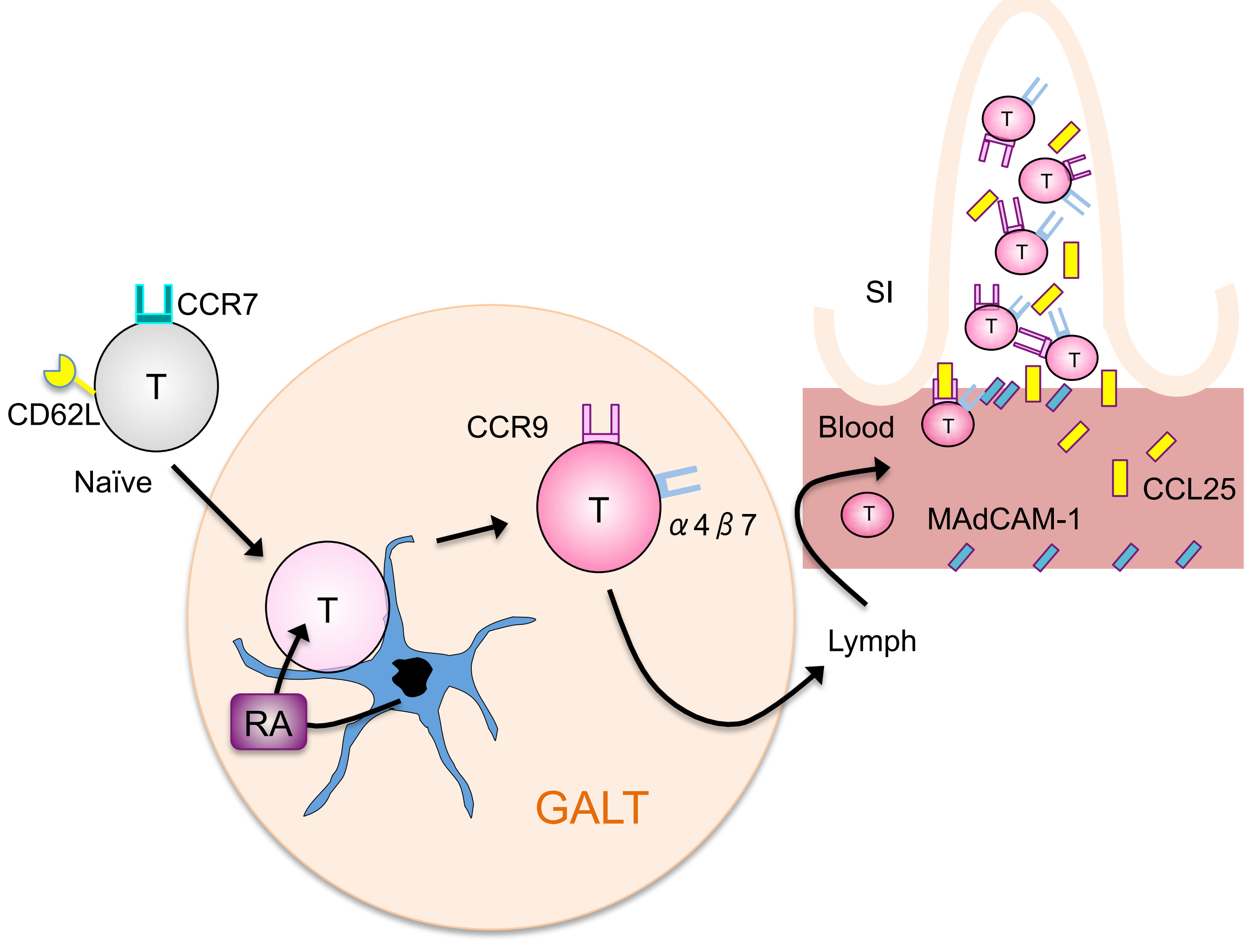


Figure 1.10. DC imprinting of gut homing function in T-cells. Naïve T-cells enter the GALT from the circulation, interact with antigen presenting DCs in the context of RA, upregulate expression of gut homing receptors CCR9 and $\alpha 4 \beta 7$, leave the GALT and enter the small intestinal tissue via interaction with MAdCAM1 and CCL25. Figure adapted from Iwata, 2009.

Chapter 2. Materials and Methods

2.1 MICE, INFECTIONS AND *IN VIVO* EXPERIMENTS

2.1.1 Mice

C57BL/6, BALB/c, *Il4ra*$^{-/-}$ (BALB/c & C57BL/6), IL-10GFP TIGR (C57BL/6), KN2 (C57BL/6), IL-10GFP x KN2 (C57BL/6), Ly5.1xOTII (C57BL/6), 4Get (BALB/c), *Tlr2*$^{-/-}$ (C57BL/6) and *Retnla*$^{-/-}$ (C57BL/6) mice were maintained under specific pathogen free conditions at the University of Edinburgh Animal Facilities. CD11c^{cre}RARα$^{fl/fl}$ and littermate control bone marrow was a generous donation from Dr. Yasmine Belkaid, NIAID, NIH, Bethesda. All experiments were approved under a Project License granted by the Home Office (U.K.) and conducted in accordance with local guidelines.

2.1.2 *S. mansoni* infection

Cercariae were shed from patent *S. mansoni* infected *B. glabrata* snails by placing the snails in pond water under a heat lamp for 50 minutes. A sample of Cercariae were counted in a 1:1 dilution of Lugol's iodine solution under a dissection microscope. Female C57BL/6 mice were anesthetised with 0.01ml/g of 0.25ml domitor and 0.19ml vetalar mixed in 2.6ml PBS (Sigma), hair was removed from abdomen with clippers, and animals taped to a pre-warmed heat pad to help maintain body temperature during the procedure. A stainless steel ring was taped over the shaved part of the abdomen, 80 cercariae were placed into the ring in 200μl of pond water, with an additional 200μl of pond water added on top. Cercariae were allowed to penetrate for 30 minutes, following this time, steel ring and remaining cercariae were removed from the animals, and animals were revived by a sub cutaneous injection of 0.1ml of 0.04ml antisedan diluted in 0.96ml PBS (Sigma). Following infection animals were monitored with weights measured 3 times a week starting at week 5. At week 8 organs were harvested following CO_2 asphyxiation.

2.1.3 Intra-peritoneal injections

Long acting IL-4 (IL-4c): IL-4/anti-IL-4 mAb complexes (IL-4c) were prepared as described previously (Herbert, Yang *et al.* 2009; Jenkins, Ruckerl *et al.* 2011). Recombinant murine IL-4 (Peprotech) was combined with rat IgG1 anti-IL-4 mAb 11B11 (BioXcell) at a 1:5 weight ratio (Urban, Maliszewski *et al.* 1995). Mice were injected intra-peritoneally with 50μl PBS or 0.625μg, 4μg, or 2 injections of 5μg IL-4 complexed to 11B11 in a volume of 50μl, and peritoneal exudate cells (PEC) and

spleen were harvested 4 days later.

2.1.4 Thioglycollate elicited peritoneal macrophages

300-500µl of 4% BBL Brewers modified thioglycollate medium (BD Europe) was injected i.p. to recruit inflammatory cells to the peritoneal cavity. 4 days later mice were killed using CO_2 asphyxiation, PEC were harvested, washed and plated onto 6cm petri dishes and left to adhere for 4 hours in a humidified environment, 37^oC, 5% CO_2. Non-adherent cells were aspirated, 3mM EDTA (Ambion) and 10mM Glucose (Sigma) was added to the dishes and cells were returned to 37^oC for 15 mins, following this incubation, adherent cells were detached using mechanical perturbation with the centre of a 1ml syringe (BD), cells were pooled, washed to remove EDTA/Glucose and re-suspended in complete RPMI-1640 medium without additional growth factors.

2.1.5 Sub-cutaneous injections into the feet

BMDC transfer: Wild type and *Il4ra*$^{-/-}$ BALB/c BMDC or Wild type and *Retnla*$^{-/-}$ C57BL/6 BMDC were cultured as below (section 2.3 and 2.4), with SEA, Pa or medium alone. 5 x 10^5 BMDC were injected subcutaneously into the feet of recipient WT BALB/c or C57BL/6 mice (2.5 x 10^5 per foot). 5-7 days following transfer the draining popliteal LNs were harvested. Single cell suspensions of LN cells (1 x 10^6 cell/ml) were cultured in X-vivo 15 medium (BioWhittaker) containing 2mM L-Glutamine and 50 mM 2-ME (Invitrogen) in 96 well plates at 37°C in a humidified atmosphere of 5% CO_2 with or without 15 µg/ml SEA, or 1 µg/ml Pa. Supernatants were harvested from the cultures after 72h and cytokine production assessed by ELISA.

2.2 CELL ISOLATIONS

2.2.1 Innate cell isolation

Single cell suspensions were prepared following protocols previously published by the MacDonald Laboratory (Phythian-Adams, Cook *et al.* 2010). Spleens were diced and digested at 37°C for 20 min with 1.75 Wunsch Units/ml Liberase TI (Roche Diagnostics) and 80 Kunitz Units/ml DNase I type VI (Sigma) in HBSS (Sigma) containing 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen). 100µl 0.1M pH 7.3 EDTA (Ambion) stop solution per ml was then added and the tube topped up with DMEM containing 50 U/ml penicillin and 50µg/ml streptomycin.

The resulting suspension was then passed through a 70μm cell strainer to obtain a single cell suspension, RBCs were lysed using RBC lysis buffer (Sigma) 3ml, 5 minutes and cells counted and resuspended for use. For restimulation assays, LNs were disrupted without enzymatic digestion. Single cell suspensions were prepared from the peritoneal cavity by flushing with 3 x 5ml PBS + 10% FCS (Hyclone/Sigma). RBC were lysed (as above), cells counted and resuspended for flow cytometric analysis.

2.2.2 Splenic DC purification

Spleens from 5-6 naïve C57BL/6 mice were mechanically disrupted, DC enriched using NycoDenz density gradient separation and CD11c^{hi}B220^{-} cDC were sorted from B220^{+}CD11c^{mid} pDC using a BD FACs Aria II. Post sort, 50,000 DCs were cultured overnight with 20ng/ml IL-4, prior to RNA extraction, reverse transcription and quantification of transcript levels using qPCR.

2.2.3 T-cell isolation for DC:T-cell co-culture

Spleen, mesenteric, brachial and inguinal lymph nodes were pooled, crushed through gauze to release cells, centrifuged at 1200g for 5 minutes at room temperature, RBC were lysed using RBC lysis buffer (Sigma), cells were washed and stained with CD4-APC, PE or AlexaFluor e780 (Table 2.1), prior to sorting using a BD Aria-II.

2.3 *IN VITRO* BONE MARROW DIFFERENTIATION

2.3.1 Bone marrow preparation

Tibias and fibulas of the hind legs of mice were collected into ice cold PBS (Sigma), bones were then sterilised in 70% Ethanol (Sigma) for 3 minutes, before washing with PBS (Sigma), to remove all ethanol. Individual bones were selected, the ends of the bone were removed using a scalpel and 5ml of PBS (Sigma) was injected through the bone using a 5ml syringe (BD) and a 21G needle (BD), flushing the bone marrow into a 50 ml falcon tube (BD). Flushed bone marrow samples pooled from all bones collected were then disrupted by repeatedly passing through a 23G needle (BD). A sample of BM was mixed in a 1:1 dilution with trypan blue and the numbers of HSCs present were counted based on estimated size.

2.3.2 Heat inactivation of fetal calf serum

The foetal calf serum serum (FCS) used in BMDC medium was placed at 58ºC for 50 minutes to heat inactivate it prior to use. Heat inactivation was used to denature the components of the complement cascade in the serum that could damage the cells being cultured or prepared.

2.3.3 rGM-CSF BMDC culture

Bone marrow (BM)-derived dendritic cells (DC) were prepared following protocols previously published from the MacDonald laboratory (MacDonald, Straw *et al.* 2001). $2x10^6$ bone marrow cells were seeded in 10ml complete medium (RPMI-1640 (Sigma) containing 20ng/ml rGM-CSF (Peprotech), 10% Fetal Calf Serum (FCS, Hyclone/Sigma), 2mM L-Glutamine (Gibco), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen)). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. On day 3, 10ml of complete medium was added, on days 6 and 8, 9ml of media was gently (to avoid undesired activation of cells) aspirated and replaced with 10ml of fresh complete medium, cells were harvested gently on day 10. Immature day 10 BMDCs were re-suspended in fresh medium containing 5ng/ml GM-CSF for *in vitro* stimulations, see below (section 2.4).

Due to the nature of the feeding process and the sensitivity of GMDCs to movement and temperature there is variability in the basal activation status of the cells used in all GMDC experiments. The method to count HSCs in BM preparations is not precise and down to personal assessment of cell size, and as such there is also variability in the yield of cells after the 10 day differentiation period. Another confounding factor in the activation status of the DCs used is that FCS is not a uniform preparation due to its biological nature. To minimise the variation in GMDC on account of FCS (and GM-CSF), we spend a considerable amount of time batch testing from various companies to ensure we get comparable growth, and then bulk buy the FCS and GM-CSF batch that provides the highest yield, lowest activation status, at the lowest price, in the hope that this will last for the duration of a project. Unfortunately the FCS stock had to be renewed toward the end of my PhD, and as such the activation status in some experiments may be slightly different to the basal activation in others. In addition I have found over the course of my PhD that the more people sharing an incubator in which DCs are grown, the lower the yield of cells achieved, which suggests that the increased frequency that the doors are

being opened is creating vibrations within the incubator and fluctuations in temperature.

2.3.4 Fms tyrosine like kinase-3 ligand BMDC culture

FL-DCs were cultured essentially as in (Naik, Proietto *et al.* 2005), flushed BM was RBC lysed and 1.5 x 10^5 /ml bone marrow cells were re-suspended in complete FLT3 medium (as above with the addition of 50mM 2-Mercapto ethanol (Invitrogen), omission of GM-CSF and addition of 200ng/ml rFLT3L (Peprotech)). Flasks were cultured at 37°C in a humidified atmosphere of 5% CO_2 for 8 days without feeding. On day 8 cells were harvested, and re-suspended in fresh medium containing 50ng/ml FLT3L for *in vitro* stimulations, see below. For analysis, FL-DC were gated based on expression of B220 and CD11c (pDC=B220$^+$CD11c^{int}, cDC=B220$^-$CD11c$^+$), representative gating strategy is shown in Fig. 2.1.

2.3.5 rMCSF BMM culture

Optimisation was carried out to increase macrophage (F4/80^{hi}CD11b^{hi}CD11c$^-$) yield from culture wells, cells were harvested on days 4, 5, 6, 7, and 8. Feeding on days 4 and 6, and unfed wells were compared (Fig. 2.2). Following optimisation the following protocol was used routinely: 1 x 10^6 bone marrow cells were seeded in 6 well tissue culture plates in 4ml of complete macrophage medium (as for GM-CSF DCs but with 20ng/ml rM-CSF (Peprotech) replacing GM-CSF) at 37°C in a humidified environment of 5% CO_2. On days 4 and 6, 2ml of medium was aspirated and replaced with 2ml of pre-warmed complete macrophage medium. On day 7 all medium was aspirated, 2ml of pre-warmed PBS (Sigma) containing 3mM EDTA (Ambion) and 10mM Glucose (Sigma) was added to the wells and cells were returned to 37°C for 15 mins, following this incubation, adherent cells were detached using mechanical perturbation with the plunger from a 1ml syringe (BD) cells were pooled, washed to remove EDTA/Glucose and re-suspended in complete medium without M-CSF.

2.4 *IN VITRO* STIMULATIONS

2.4.1 Preparation of antigens

Propionibacterium acnes (Pa) is an anaerobic bacterium and was grown by the lab of Professor Ian R Poxton (Microbial Pathogenicity Research Laboratory, University of Edinburgh) before being heat-killed and stored under sterile conditions

at 4ºC. The heat-killed *P. acnes* was aliquoted and the concentration of protein per ml was determined for each aliquot using a Bradford (coomassie) protein assay and a known BSA standard.

Attenuated *Salmonella typhimurium* strain SL3261 was supplied by Dr. Maurice Gallagher (University of Edinburgh) was heat-killed and stored under sterile conditions at 4ºC prior to use. The heat-killed *S. typhimurium* was aliquoted and the concentration of protein per ml was determined for each aliquot using a Bradford (coomassie) protein assay and a known BSA standard.

Endotoxin-free soluble egg antigen (SEA) from *S. mansoni* was prepared in-house as previously described (MacDonald et al., 2001). The stored eggs were thawed and transferred to a Tenbroeck 7 ml tissue grinder to which 5 ml sterile PBS was added.The eggs were then carefully homogenised using a twisting and grinding motion. After ~20 rotations, egg disruption was checked. This was repeated ~15 times on ice until 95% of the eggs were disrupted. The homogenate was transferred to a 15 ml tube and spun at 2800 xg for 15 minutes at 4ºC. The supernatants were transferred to 1 ml micro-centrifuge tubes and spun at 16,000 xg for 10 minutes. The pooled supernatants were filter sterilised through a 0.45 µm filter. The SEA protein concentration was determined using a Bradford (coomassie) protein assay and a known BSA standard. The SEA was aliquoted and stored at at -80ºC.

2.4.2 Prolonged stimulus

Cells were stimulated with or without rIL-4 (2ng/ml, 20 ng/ml, 200ng/ml; Peprotech) to optimise alternative activation (Fig. 2.3). Following optimisation, 20ng/ml rIL-4 was used for all other experiments. A 6h and 18h IL-4 stimulation time was assessed (Fig. 2.4). The 18h incubation was used for most experiments as RELMα and Ym1/2 proteins were only detected in the culture supernatant at the 18hr time point (Fig. 2.4A) and intracellular RELMα and Ym1/2 staining was similar or enhanced at 18hr (Fig. 2.4B+C).

In selected experiments, recombinant murine Interleukin 13 (rIL-13, 20ng/ml Peprotech), recombinant resistin like molecule alpha (rRELMα, 20ng/ml, Peprotech), endotoxin-free soluble egg antigen (SEA, 25µg/ml, prepared in-house), heat killed *P. acnes* (Pa, 10µg/ml), heat killed *S. typhimurium,* (St, 5µg/ml), Lipopolysaccharide

(LPS, 250ng/ml, Sigma). CpG oligodeoxynucleotide 1826 (ODN 1826, 5µg/ml, InvivoGen) or N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine x 3 HCl (Pam3CSK4 (P3C), 250ng/ml, InvivoGen), Zymosan ((Zym)100µg/ml, Sigma). U0126 (10µM, Sigma), Phorbol 12, 13-dibutyrate (PdBU; 100nM, Sigma), all-trans retinoic acid (RA, 20nM, 10µM, reconstituted in DMSO, Sigma), LE540 (10µM Wako) or the arginase inhibitor (S-(2-boronoethyl)-l-cysteine) (BEC, 1µM, 10µM, 100µM; Cayman Chemical) were included in the post differentiation cultures. For initial RA experiments, control wells were stimulated with media containing DMSO. The TLR agonists chosen and concentrations used were based on previous studies (Hochrein, O'Keeffe *et al.* 2000; Kane, Cervi *et al.* 2004; Guenova, Volz *et al.* 2008; Manicassamy, Ravindran *et al.* 2009).

2.4.3 Short term stimulus

In order to assess phosphorylation of the kinase ERK, cells were surface stained with antibodies against CD11c and MHC-II (eBioscience) cultured for 15 minutes at 37°C in the presence of media or the ERK inhibitor U0126, prior to addition of antigens (as listed above) for 15 minutes.

2.4.4 αCD40 stimulation

To assess any further activation triggered by the ligation of CD40, BMDC were harvested after stimulation, washed, and re-plated at 4 x 10^{6} DC/ml in a volume of 200µl in a 96 well tissue culture plate, for a further 24 h in the presence or absence of 20µg/ml of an agonistic anti-CD40 Ab(Perona-Wright, Jenkins *et al.* 2009) (clone FGK.45) or an isotype control (clone Mac-1) both produced in house.

2.4.5 DC:T-cell co-culture

50,000 IL10-eGFP^{-}CD4^{+}, IL-4eGFP^{-}CD4^{+}, KN2 CD4^{+} or IL-10eGFP^{-}xKN2 CD4^{+} T-cells were cultured in 96 well plates for 3-4d with 2,500 WT, *Il4ra*$^{-/-}$ or *Retnla*$^{-/-}$ GMDCs, 1µg/ml anti-CD3 (produced in house) and with or without IL-4 (20 ng/ml, Peprotech) and Retinoic acid (10µm, Sigma).

2.4.6 OT-II: DC cultures

For CFSE dilution assays CD4^{+} OT-II TCR Transgenic T-cells were purified from spleen and LN using CD4^{+} Dynabeads (Invitrogen) following the

manufacturer's protocol. T-cells were labelled with 5µM CFSE (Invitrogen) for 15 mins at 37°C, excess CFSE was allowed to leach from the cells prior to culture with 5 x 10^4 WT, *Retnla*$^{-/-}$ or *Il4ra*$^{-/-}$ BMDCs in the presence of 0.01µg/ml $OVA_{323-339}$ or 5µg/ml OVA protein (Sigma) which had been endotoxin depleted in-house. Cultures were incubated at 37°C for 4 days prior to assessment of CFSE dilution by flow cytometry.

2.5 ANALYSIS TECHNIQUES

2.5.1 Flow cytometry

For initial intracellular staining of BMDC, GolgiStop (1µl/ml, BD) was added to cells for 6 hours prior to RELMα and Ym1/2 staining to increase the intracellular protein levels. For subsequent RELMα and Ym1/2 staining, golgistop was not used as it was found to reduce rather than enhance RELMα detection (Fig. 2.5). For innate cell staining panels, cells isolated from the spleen and peritoneal cavity were stained directly *ex vivo.* Following FcR-Block using αCD16/CD32 (2.4G2, produced in house), cells were surface stained using combinations of the following mAb conjugations listed in Table 2.1.

For intracellular staining, cells were fixed in 1% formaldehyde, permeabilised using Cytoperm (BD PharMingen) and innate and BM derived cells were stained with anti-RELMα, and anti-Ym1/2-biotin followed by incubation with Alexa 488–conjugated Zenon anti–rabbit antibody and Streptavidin-APC or –PerCP/Cy5.5 (See Table 2.1).

To assess antigen uptake, 2 x 10^5 BMDCs were incubated with 200µg of FITC conjugated dextran (Sigma) for 30 minutes at 37°C or on ice prior to acquisition.

For analysis of phospho-ERK, pre surface stained samples were fixed immediately following activation using 1% formaldehye, cells were transferred to a 96 well FACs plate, washed and permeabilised with 90% Methanol (Sigma) for 30 minutes on ice up to 12h at -20°C, following the pERK antibody manufacturers protocol. Permeabilised cells were washed in PBS +1%BSA (Sigma), prior to staining for 1h on ice with anti Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) Rabbit mAb (Cell Signalling Technology); bound Rabbit antibody was then detected using Anti-Rabbit IgG (H+L)$F(ab')_2$- Alex Fluor 488 (Invitrogen).

Samples were acquired using a FACS LSR II or FACS Canto II flow cytometer using BD FACS Diva software and analyzed with FlowJo v.9 software (Tree Star, Inc.).

2.5.2 The aldefluor assay

Following surface staining, cells were washed, resuspended in aldefluor assay buffer (Stemcell Technologies) containing (0.25μl of 300μM stock, final concentration/well =3μM) of activated aldefluor substrate, and incubated at 37°C for 30 minutes. Control samples had 3μl of the aldehyde dehydrogenase inhibitor diethyl aminobenzaldehyde (DEAB, 1.5mM stock = 90μM/well) added prior to the 30 minute incubation. The activated aldefluor substrate contains a bodipyaminoacetaldehyde subtrate (BAAA) that diffuses into cells, and is converted by active aldehyde dehydrogenases to bodipyaminoacetate (BAA) which fluoresces, BAA cannot diffuse from the cells. Samples were washed following incubation in ice cold Aldefluor assay buffer (which contains ABC transporter inhibitors to block BAA active transport from the cells) 3 x, prior to sample acquisition using a a FACS LSR II or FACS Canto II flow cytometer, with analysis of aldefluor activity in the FITC channel, using BD FACS Diva software and analysis with FlowJo v.9 software (Tree Star, Inc.).

2.5.3 Enzyme linked immunosorbent assay (ELISA)

Cytokine ELISAs were performed on culture supernatants using paired mAb (See Table 2.2) purified in house, purchased from eBioscience or BD Pharmingen, and recombinant cytokine standards purchased from PeproTech. TNFα, CCL24, CCL17 and Ym1 ELISAs were perfomed using DuoSet ELISA development kits (R&D systems). RELMα ELISAs were performed using products purchased from PeproTech. As Ym1/2 is the predominant protein in lavage fluid from litomosoides sigmodontis infected animals, this was used as a standard for ECL-F (Ym1/2) ELISA. Primary/capture antibodies were coated onto 96 well plates (NUNC) in a volume of 50μl in PBS overnight at room temperature (TNFα, Ym1/ECLF/CCL24) or 4°C (RELMα, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-5, IL-4, IL-17, CCL17, CCL24) or for 3 hours at 37°C (all). Plates were blocked using 10% NCS or FCS/PBS for >1 hour. Supernatants and doubling dilutions of recombinant protein standards were added to 96 well plates in a volume of 50μl, in duplicate where possible, single wells were used in situations where supernatant or plate space was

limiting. Secondary/detection antibodies were added in a volume of 50µl in 10% NCS/PBS, and allowed to bind for 1h at 37°C (IL-10), 1h at room temperature (IL-12p40, IL-12p70, IL-6) or 2h at room temperature (CCL17, CCL24, Ym1/2, RELMa, TNFα). Streptavidin-peroxidase was added to plates in a volume of 50µl and incubated at 37°C for 30 minutes. 100µl of the colorimetric substrate of peroxidase, TMB (Sigma), was added to each well, following development of blue colour, reaction was stopped by addition of 0.18M H_2SO_4 acid (Sigma). Between steps, plates were washed using PBS 0.05% Tween-20 (Sigma).

2.5.4 Griess reaction for NO_2

NO_2 levels within supernatants following *in vitro* cell culture were assessed using the Griess reaction (Tsikas 2007). Solution A: Sulfanilamide (1%)(Sigma S9251) in 2.5% H_3PO_4 (Phosphoric acid, Sigma) and Solution B: Napthylethylenediamine dihydrochloride (0.1%)(Sigma N9125) in 2.5% H_3PO_4 (Phosphoric acid, Sigma) were prepared and mixed 1:1 immediately before use in the assay. 50µl of supernatant was placed in duplicate (where possible) into well of a 96 well plate, a standard curve of doubling dilutions of $NaNO_2$ was made in media with a top concentration of 1mM, 50µl of the mixed A:B solutions was added to each sample and standard well, absorbance was read at 570nm using a microplate spectrophotometer.

2.5.5 Arginase activity assay

Arginase activity was measured following a protocol previously published by the Allen Laboratory (Mylonas, Nair *et al.* 2009). Briefly, 1.5 x 10^5 BMDC/BMM were lysed with 100µl of 0.1% Triton X-100, following a 30 min incubation at room temperature, 100µl of 25mM Tris-HCL (pH 7.2) and 20µl of 10mM $MnCl_2$ were added and the enzyme was activated by heating to 56°C for 10 min. $_L$-Arginine hydrolysis was conducted by incubating 10µl of this lysate with 10µl of 0.5M $_L$-arginine (pH 9.7) at 37 ^{o}C for 30-120 min. The reaction was stopped using 80µl H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7 v/v/v). 4µl of 9% isonitrosopropiophenone (Sigma) was then added followed by heating to 95°C for 30 min. Reactions were conducted in 200 µl PCR tubes, and incubations carried out using a PCR block. 50 µl of each sample was transferred to a 96 well plate for measurement at 570nm using a microplate spectrophotometer. A standard curve of urea was used to determine sample concentration. One unit of arginase enzyme activity is defined as

the amount of enzyme that catalyzed the formation of 1μmol of urea per minute at 37 ^{o}C.

2.5.6 Microscopy

50,000 day 10 GM-CSF DCs were cultured overnight with or without 20ng/ml IL-4 in 24 well plates (Corning Incorporated) containing 22mm coverslips. Media was aspirated, adherent cells were washed with PBS (Sigma) and then fixed at 4°C in 4% formaldehyde (Sigma) for 30 minutes. Formaldehyde was aspirated, cells were washed with PBS, and incubated at room temperature in 50mM Glycine to quench the formaldehyde. Glycine was aspirated, cells were washed with PBS and permeabilised at room temperature in 0.1% TritonX100 (Sigma) for 6 minutes. Cells were washed in PBS and then blocked with PBS containing 10%FCS (Sigma) and 2% Bovine Serum Albumin (BSA, Sigma) for 2 hours. Block was aspirated and cells were incubated at 4°C overnight with BODIPY-FL Phallacidin (Invitrogen) to label F-actin and enable visualization of DC architecture, rabbit anti-murine RELMα (Peprotech) or polyconal rabbit IgG (produced in house). Primary stain was aspirated, cells were washed with PBS and counterstained with Zenon anti-rabbit alexa-fluor 546 (Invitrogen) for 45 minutes at room temperature. Secondary antibodies were aspirated, cells were washed with PBS, coverslips were dried and mounted onto 4 well slides using Prolong gold anti-fade mounting media (Invitrogen) containing 1μg/ml 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were acquired as bidirectional Z-stacks (0.25μM series) using an inverted Leica confocal laser scanning microscope SP5C, using a 63x oil objective, 700Hz, 1024 x 1024 image, 3 frame average. Images were analysed using Volocity (Perkin Elmer).

2.5.7 RNA isolation and qPCR

RNA was recovered from cells following suspension in the phenol-based product TRIzol (Invitrogen). Phase separation of DNA/protein and RNA was performed using Chloroform. RNA was precipitated using 2-propanol, visualisation of RNA pellet was enhanced by addition of glycogen (Ambion) during precipitation. 75% ethanol was used to wash RNA, prior to air drying and resuspension in RNAse free DEPC water (Ambion). 0.1-0.25μg RNA was used for the synthesis of cDNA using Superscript-III and oligo-dT (Invitrogen). Relative quantification was performed by qPCR analysis using the Roche Light Cycler 480, with LightCycler SYBR Green I master mix (Roche). Five serial 1:4 dilutions of a positive control sample of cDNA

were used to create standard curves. Expression was normalised to the housekeeping genes (genes expressed constitutively for the maintenance of cellular function) hypoxanthine-guanine phosphoribosyltransferase (*Hprt)* or glyceraldehyde 3-phosphate dehydrogenase *Gapdh.* For primers used see Table 2.3.

2.6 MICROARRAY

2.6.1 Microarray sample preparation

3 biological replicates of GM-CSF BMDCs were set up for each strain (C57BL/6, *Retnla*$^{-/-}$ and *Il4ra*$^{-/-}$). RNA was isolated using Purelink RNA mini extraction kit (Ambion), following manufacturers protocol with on-column DNAse treatment. Samples were then checked for RNA quantity and quality (260/230 ratio) on a nanodrop spectrophotemeter (ThermoScientific) before biotin end labeling and amplification was performed using illumina total prep RNA amplification kit (Ambion). End-labelled cRNA was shipped to the Wellcome trust clinical research facility genetics core, Western General Hospital, Edinburgh. cRNA quality was assessed using an Agilent 2100 bioanalyser, samples were condensed where needed and hybridized to Illumina Mouse WG6_V2_0_R3_11278593_A arrays.

2.6.2 Microarray post hybridisation analysis

Subsequent analysis of microarray data was carried out by Al Ivens (CIIE bioinformatics core support). All 18 arrays were quality control analysed using the arrayQualityMetrics package in Bioconductor (Kauffmann and Huber 2010). Data were transformed using a variance stabilisation transformation prior to normalisation across all arrays using robust spline normalisation. Linear modeling was used to compare 6 data sets (1. IL-4 WT relative to medium WT 2. IL-4 *Retnla*$^{-/-}$ relative to medium *Retnla*$^{-/-}$ 3. IL-4 *Il4ra*$^{-/-}$ relative to medium *Il4ra*$^{-/-}$ 4. Medium *Il4ra*$^{-/-}$ relative to medium *Retnla*$^{-/-}$ 5. Medium *Il4ra*$^{-/-}$ relative to medium WT 6. Medium *Retnla*$^{-/-}$ relative to Medium WT). p= Adj. p significance values were assessed using Bayesian analysis, corrected using Benjamini & Hochberg method for multiple testing (Al Ivens, CIIE Bioinformatics support).

2.7 STATISTICAL ANALYSIS

Statistical analyses were carried out using GraphPad Prism 5 software. Student's T tests and one-way analysis of variance were employed to determine significant differences between sample groups. In situations where only two

replicate wells or individual animals were used, statistical tests are not valid and as such have not been used.

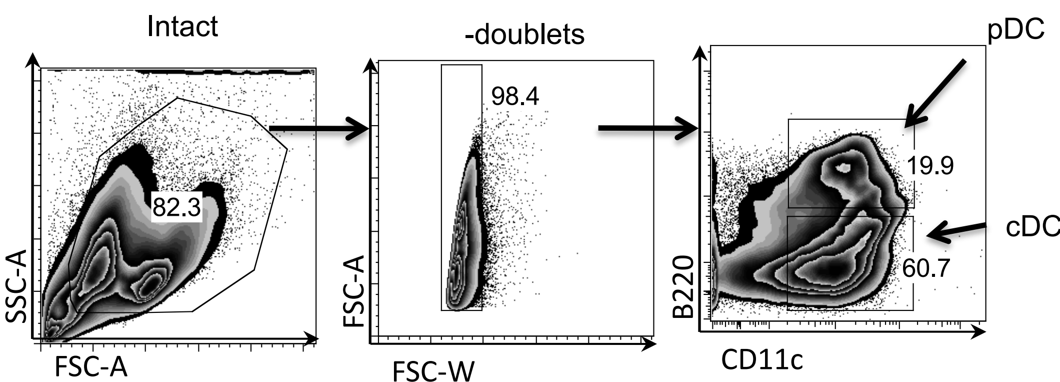


Figure 2.1 Gating strategy for FLT3L cultures. Bone marrow was cultured for 8 days with FLT3L prior to staining for B220 and CD11c, pDC or cDC were defined based on their expression of these markers.

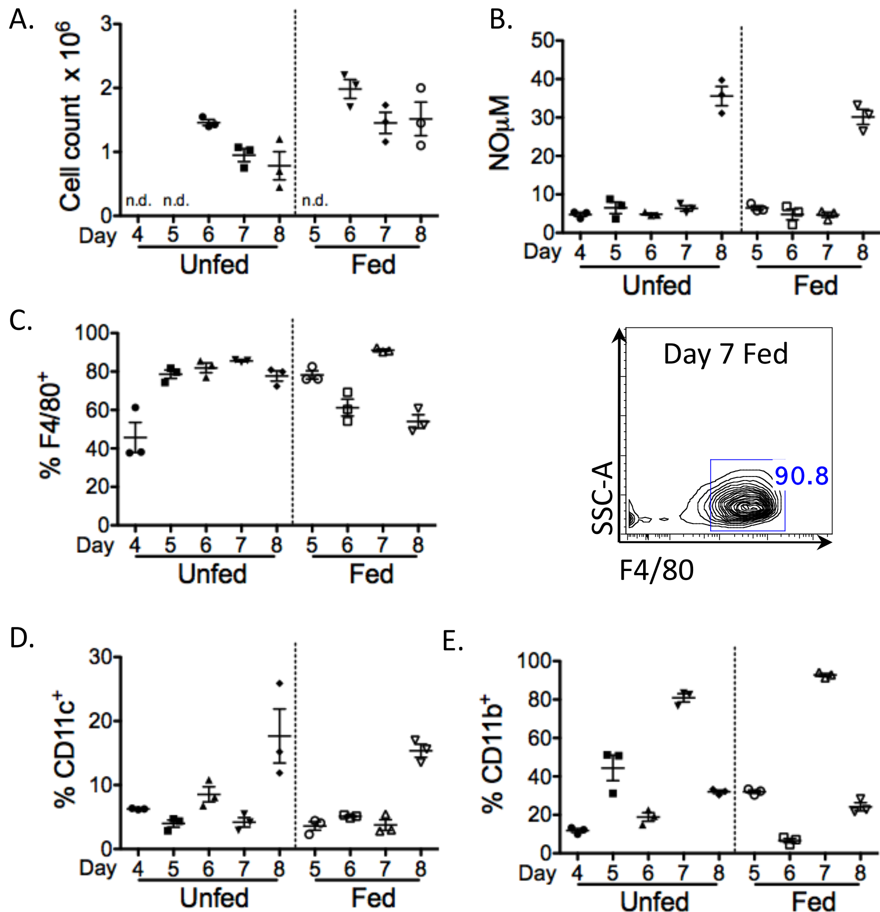


Figure 2.2 Optimisation of M-CSF BMM culture. Bone marrow was cultured in the presence of 20ng/ml rMCSF for the number of days indicated, either with (fed) or without (unfed) media replacement. Cultures were assessed for cell number (A), NO production (B), F4/80 (C), CD11c (D) and CD11b (E) expression. Optimisation was carried out once.

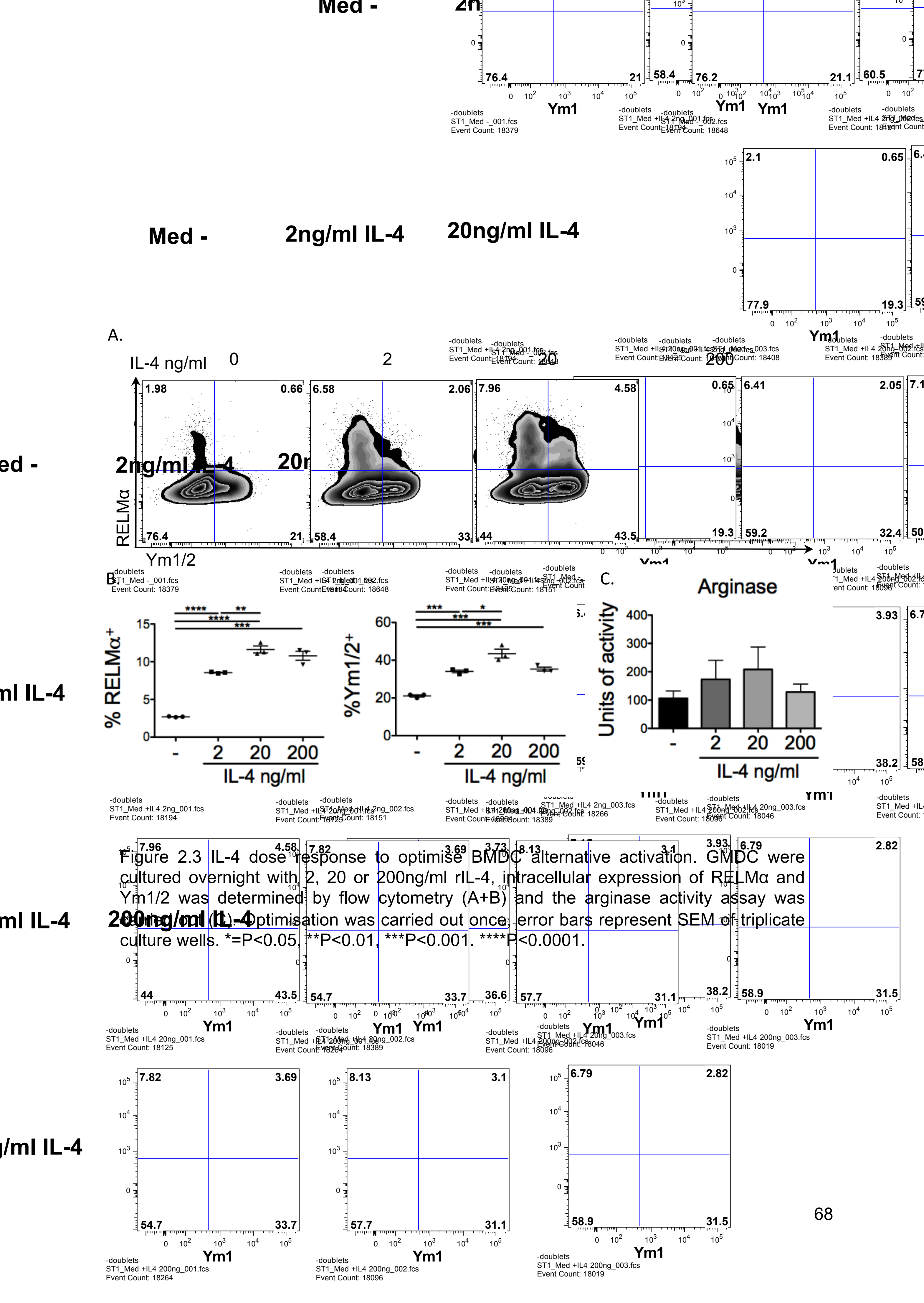


Figure 2.3 IL-4 dose response to optimise BMDC alternative activation. GMDC were cultured overnight with 2, 20 or 200ng/ml rIL-4, intracellular expression of RELMα and Ym1/2 was determined by flow cytometry (A+B) and the arginase activity assay was carried out (C). Optimisation was carried out once, error bars represent SEM of triplicate culture wells. *=P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

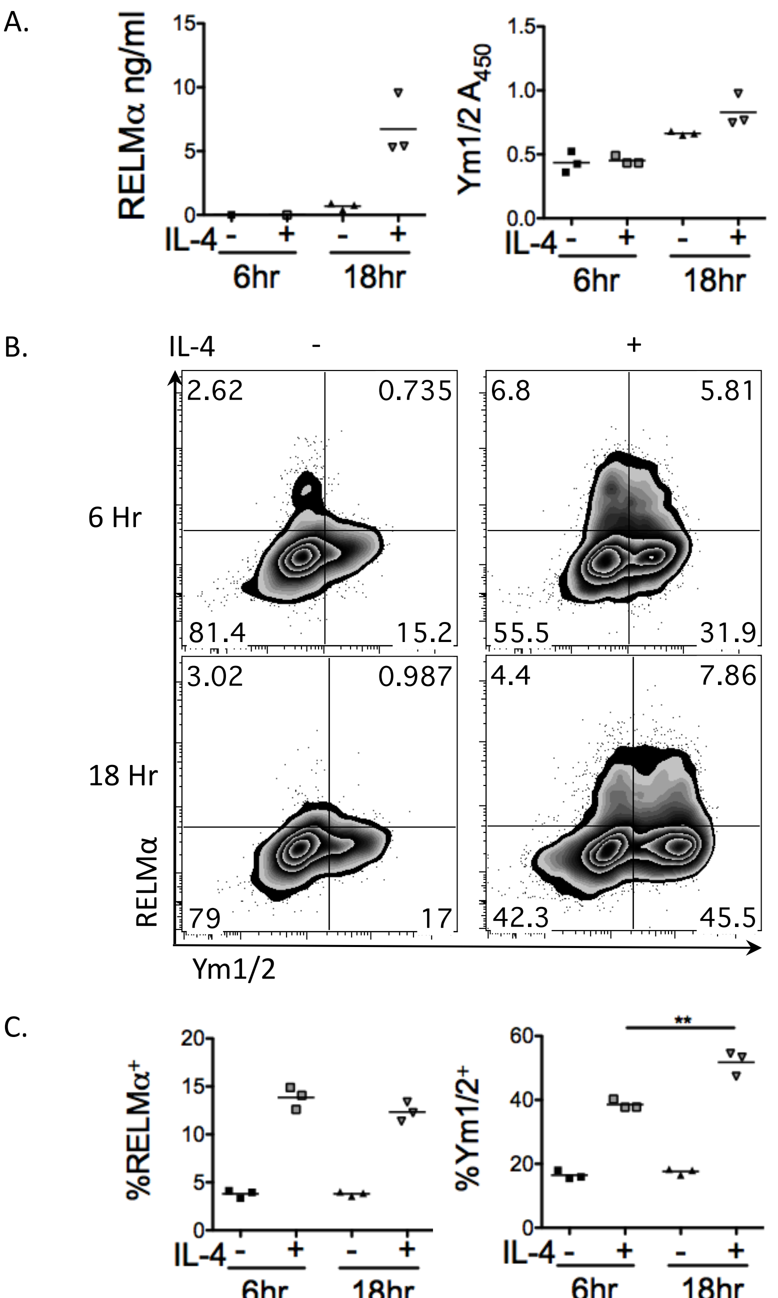


Figure 2.4 Optimisation of the timing of IL-4 treatment. GMDC were cultured with 20ng/ml IL-4 for 6h or 18h, prior to analysis of RELMα and Ym1/2 secretion (A) and intracellular expression (B+C). Optimisation was carried out once, data points represent triplicate culture wells **P<0.01.

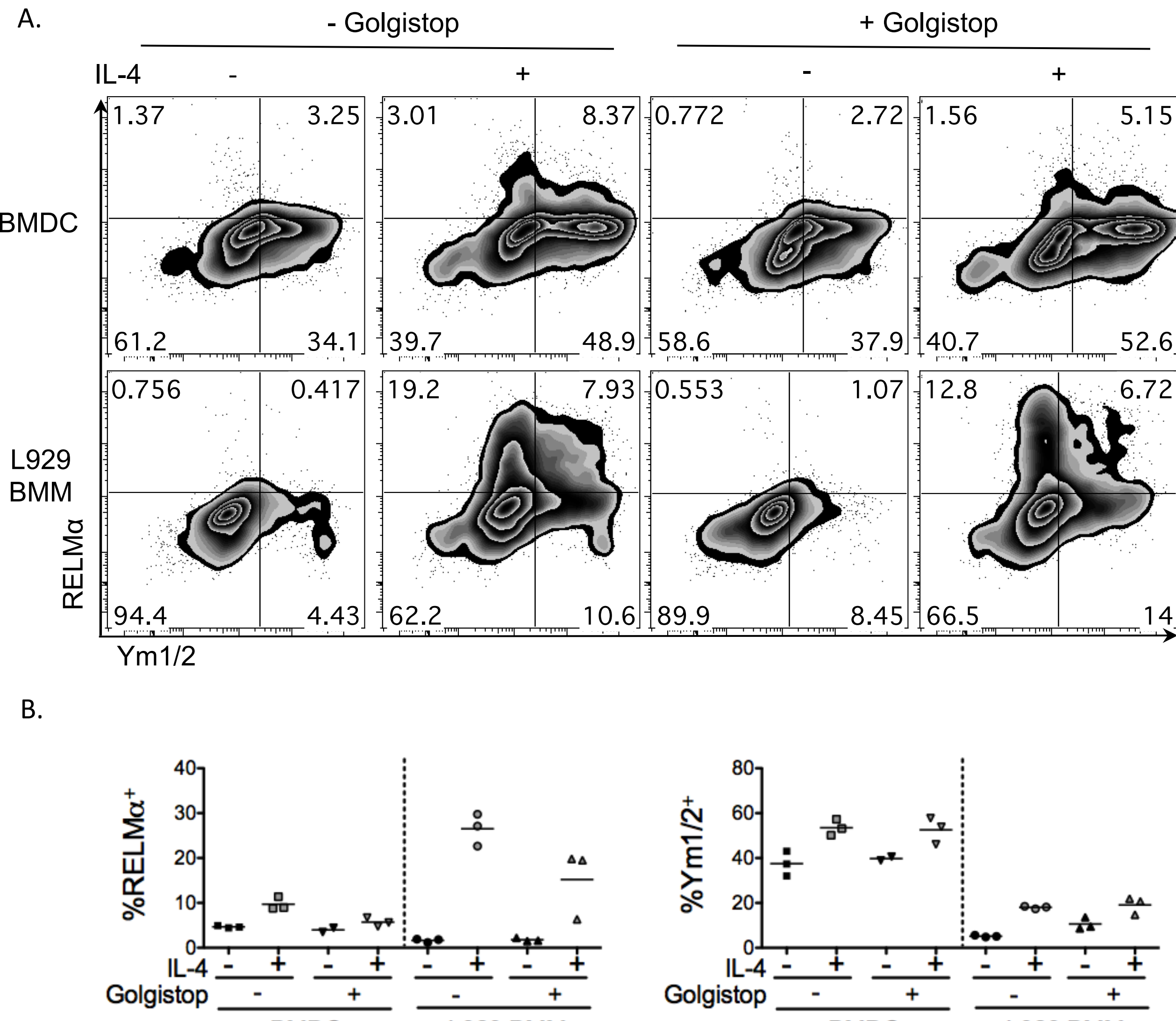


Figure 2.5. Golgistop does not enhance intracellular levels of RELMα or Ym1/2. GMDC or BMM (differentiated with L929 supernatant) were cultured for 12h with medium or IL-4 prior to addition or not of 1µl/ml of Golgistop (BD) for the final six hours. RELMα and Ym1/2 expression was determined by flow cytometry (A+B). Bars represent mean of triplicate culture wells.

Table 2.1 Flow antibody clones, manufacturer and titrations						
Antigen Name	**Clone**	**Isotype**	**Host**	**Conjugate**	**Titration**	**Manufacturer**
Live/Dead Aqua				Aqua		Invitrogen
huCD2	S5.5	IgG2a	Mouse	PE	1/200	Invitrogen
CD4	RM4-5	IgG2a k	Rat	APC/eFluor780 or PE or AlexaFluor647	1/800	eBioscience
CD8	53-6.7	IgG2a k	Rat	PE/Cy7	1/800	Biolegend
CD11b	M1/70	IgG2b k	Rat	PE/APC/eFluor780	1/600	Biolegend
CD11c	N418	IgG	Ar Ham	Alexa-647/PE/APC/ eFluor780	1/200	eBioscience
CD19	1D3	IgG2a, k	Rat	Alexa-700/PE	1/200	Biolegend
CD40	3/23	IgG2a k	Rat	PE	1/200	BD Pharmingen
CD45R (B220)	RA3-6B2	IgG2a, k	Rat	APC/eFluor780	1/400	eBioscience
CD80	16-10A1	IgG2 k	Ar Ham	PerCP/Cy5.5	1/200	BD Pharmingen
CD86	GL-1	IgG2a, k	Rat	Alexa-700/AlexaFluor488	1/200	BD Pharmingen
CD124 (IL-4Rα)	mIL4R-m1	IgG2a k	Rat	Bio	1/50	BD Pharmingen
Chi3l3/ECF-L	Polyclonal	IgG	Goat	Bio	1/50	R&D
Dectin-1	2A11	IgG2b, k	Rat	AlexaFluor647	1/50	AbD Serotech
F4/80	BM8	IgG2a	Rat	PeCy7	1/400	Biolegend
Gr1 (Ly6G+6C)	RB6-8C5	IgG2b k	Rat	PerCP/Cy5.5	1/200	BD Pharmingen
MHC class II (I-A/I-E)	M5114	IgG2b, k	Rat	PerCP/Cy5.5	1/1600	eBioscience
MHC class II (I-A/I-E)	M5115	IgG2b, k	Rat	Alexa-450	1/800	eBioscience
MR	MR5D3	IgG2a, k	Rat	PE	1/25	AbD Serotech
NK1.1	PK136	IgG2a k	Mouse	APC_BV570	1/200	BD Pharmingen
RELMα	Polyclonal	IgG	Rabbit		1/100	Peprotech
Siglec F	E50-2440	IgG2a, k	Rat	PE	1/200	BD Pharmingen
Streptavidin				APC/PerCP/Cy5.5	1/2500	eBioscience

Table 2.2 ELISA Antibodies					
Specificity	**Conjugation**	**Clone**	**Host**	**Usage Conc.**	**Manufacturer**
Primary Antibody (Purified)					
IL-4	Purified	11B11	Rat	2ug/ml	Homegrown
IL-5	Purified	TRFK5	Rat	2ug/ml	Homegrown
IL-6	Purified	MP5-20F3	Rat	2ug/ml	Homegrown
IL-10	Purified	JES16-E3	Rat	1ug/ml	eBioscience
IL-12p40	Purified	C15.6	Rat	2ug/ml	eBioscience
IL-12p70	Purified	9A5	Rat	2ug/ml	BD
IL-13	Purified	ebio13A	Rat	2ug/ml	eBioscience
IL-17	Purified	TC11-18H10	Rat	0.5ug/ml	BD
IFN-gamma	Purified	R4-6A2	Rat	2ug/ml	Homegrown
TNF-alpha	Purified	Duoset DY410		0.8ug/ml	R&D
Ym1/2/Chi3l3/ECF-L	Purified	Duoset DY2446		1ug/ml	R&D
RELMα	Purified	500-P214	Goat	0.5ug/ml	Peprotech
CCL24/Eotaxin-2	Purified	Duoset DY528		2ug/ml	R&D
CCL17/TARC	Purified	Duoset DY529		2ug/ml	R&D
Recombinant Standard					
IL-4	Recombinant			20ng/ml	Peprotech
IL-5	Recombinant			20ng/ml	BD
IL-6	Recombinant			20ng/ml	Peprotech
IL-10	Recombinant			50ng/ml	BD Pharmingen
IL-12	Recombinant			20ng/ml	Peprotech
IL-13	Recombinant			50ng/ml	Peprotech
IL-17	Recombinant			20ng/ml	eBioscience
IFN-gamma	Recombinant			50ng/ml	Peprotech
TNF-alpha	Recombinant			4000pg/ml	R&D
Ym1/2	Pleural lavage			50ng/ml	Homegrown (Allen)
RELMα	Recombinant			400ng/ml	Peprotech
CCL24	Recombinant			60ng/ml	R&D
CCL17	Recombinant			50ng/ml	R&D
Secondary Antibody (Biotinylated)					
IL-4	Biotin	BVD6-24G2	Rat	0.25ug/ml	BD
IL-5	Biotin	TRFK4	Rat	0.3ug/ml	eBioscience
IL-6	Biotin	MP5-32C11	Rat	0.5ug/ml	eBioscience
IL-10	Biotin	SXC-1	Rat	0.2ug/ml	BD
IL-12/IL-23p40	Biotin	C17.8	Rat	0.2ug/ml	eBioscience
IL-13	Biotin	Polyclonal Sera	Rabbit	0.1ug/ml	Peprotech
IL-17	Biotin	TC11-8H4.1	Rat	0.25ug/ml	BD
IFN-gamma	Biotin	XMG1.2	Rat	0.2ug/ml	BD
TNF-alpha	Biotin	Duoset DY410		0.15ug/ml	R&D
Ym1/2/Chi3l3/ECL-F	Biotin	Duoset		0.2ug/ml	R&D
RELMα	Biotin	500-P214-Bt	Rabbit	0.125ug/ml	Peprotech
CCL24/Eotaxin-2	Biotin	Duoset DY528		0.05ug/ml	R&D
CCL17/TARC	Biotin	Duoset DY529		0.2ug/ml	R&D

Table 2.3 qPCR primers used			
Gene name	**Fwd/Rev**	**Sequence**	**TM °C**
Arg1	Fwd	GTCTGTGGGGAAAGCCAAT	59
	Rev	GCTTCCAACTGCCAGACTGT	60
Retnla	Fwd	TATGAACAGATGGGCCTCCT	59
	Rev	GGCAGTTGCAAGTATCTCCAC	59
Il4ra	Fwd	GAGTGGAGTCCTAGCATCACG	59
	Rev	GGATGCAGAGGCAGGAGAT	60
Mrc1	Fwd	TCATTGGAAGATCCACTCTGG	60
	Rev	CAGCGCTTGTGATCTTCATTATAG	61
Chi3l3	Fwd	GAACACTGAGCTAAAAACTCTCCTG	60
	Rev	GACCATGGCACTGAACGAG	60
Il27ra	Fwd	AGTTCCGGTACAAGGAATGC	59
	Rev	ACAGGAGTCAGCCCATCTGT	59
Clec7a	Fwd	ATGGTTCTGGGAGGATGGAT	60
	Rev	GCTTTCCTGGGGAGCTGTAT	60
Stat6	Fwd	TCTCCACGAGCTTCACATTG	59
	Rev	GACCACCAAGGGCAGAGAC	60
Nos2	Fwd	CTTTGCCACGGACGAGAC	60
	Rev	TCATTGTACTCTGAGGGCTGAC	59
Ccl17	Fwd	TGCTTCTGGGGACTTTTCTG	60
	Rev	GAATGGCCCCTTTGAAGTAA	59
Ccl22	Fwd	TCTTGCTGTGGCAATTCAGA	60
	Rev	GAGGGTGACGGATGTAGTCC	59
Ccl24	Fwd	GCAGCATCTGTCCCAAGG	60
	Rev	GCAGCTTGGGGTCAGTACA	59
Pparg	Fwd	GGGGGTGATATGTTTGAACTTG	60
	Rev	GAAAGACAACGGACAAATCACC	60
Aldh1a2	Fwd	CATGGTATCCTCCGCAATG	59
	Rev	GCGCATTTAAGGCATTGTAAC	59
Gmcsf	Fwd	GCATGTAGAGGCCATCAAAGA	60
	Rev	CGGGTCTGCACACATGTTA	59
Mcsf	Fwd	CAACAGCTTTGCTAAGTGCTCTA	59
	Rev	CACTGCTAGGGGTGGCTTTA	60
Flt3l	Fwd	CCTAGGATGCGAGCCTTGT	58
	Rev	TGTTTTGGTTCCCAACTCG	47
Tlr2	Fwd	GGGGCTTCACTTCTCTGCTT	60
	Rev	AGCATCCTCTGAGATTTGACG	59
Hprt	Fwd	TCCTCCTCAGACCGCTTTT	60
	Rev	CCTGGTTCATCATCGCTAATC	60
Gapdh	Fwd	AATGTGTCCGTCGTGGATCT	60
	Rev	CCCAGCTCTCCCCATACATA	59

Chapter 3. Can Dendritic Cells be Alternatively Activated?

3.1 INTRODUCTION

DCs bridge the innate and adaptive immune systems, and are responsible for the activation of T-cells via presentation of antigen on their surface MHC molecules (Kapsenberg 2003; Steinman 2012). DCs are often defined by their surface expression of the integrin CD11c (Phythian-Adams, Cook *et al.* 2010; Murray and Wynn 2011), although a functional role for this marker is rarely detailed. DC and macrophages arise from a common bone marrow resident precursor (Naik 2008; Hashimoto, Miller *et al.* 2011), and share expression of many phenotypic markers (Murray and Wynn 2011) leading to contention within the field as to the role of the DC compared to the macrophage (Hume 2008; Geissmann, Gordon *et al.* 2010). The recent discovery of DC specific transcription factors such as *Batf3* (Hildner, Edelson *et al.* 2008) and *Zbtb46* (Meredith, Liu *et al.* 2012; Satpathy, Wumesh *et al.* 2012) has begun to facilitate accurate naming of DC versus macrophage subsets.

Alternatively activated macrophages (AAM) are a well characterised cell population whose 'alternative' activation status is induced following signalling through the IL-4Rα (Gordon 2003; Gordon and Martinez 2010). In contrast to their pro-inflammatory counterparts, the classically activated macrophage (CAM), AAM are a more regulatory cell population (Taylor, Harris *et al.* 2006). AAM are involved in the 'fast and dirty' repair of wounds (Allen and Wynn 2011), for example following infection with parasitic worms. AAM both cause disease pathology and mediate survival during infection with Th2 inducing pathogens such as the trematode *S. mansoni* (Herbert, Holscher *et al.* 2004). AAM are induced in non-infectious disease states during cancer, asthma and allergy, and in non-disease settings in the adipose tissue of lean animals (Lumeng, Bodzin *et al.* 2007). The mRNA expression profile of an AAM is much different to that of a CAM, for example, AAM express high levels Ym1/2 (*Chi3l3)* and RELMα (*Retnla*) transcripts that are not associated with IFNγ stimulated CAM (Loke, Nair *et al.* 2002; Nair, Gallagher *et al.* 2005; Mantovani, Sica *et al.* 2007; Gordon and Martinez 2010; Thomas, Ruckerl *et al.* 2012).

Although the impact of IL-4 on DC has been investigated previously (Hochrein, O'Keeffe *et al.* 2000) (Kalinski, Smits *et al.* 2000) (King, Mueller Hoenger

et al. 2001; Lutz, Schnare *et al.* 2002; Webb, Cai *et al.* 2007; Liao, Schones *et al.* 2008), the ability of IL-4 treated DCs to express many of the molecules associated with AAM has not been addressed fully (Nair, Gallagher *et al.* 2005; Cai, Kumar *et al.* 2009). Given the dramatically different functional outcome of alternative vs. classical activation in macrophages (Varin and Gordon 2009; Gordon and Martinez 2010; Murray and Wynn 2011), we reasoned that it is important to understand the extent to which DCs can also be alternatively activated.

In the work detailed in this chapter, we have carefully characterised the ability of DCs to become 'alternatively activated' in response to the canonical Th2 cytokine IL-4. The relevance of these experiments has been addressed by investigating the presence of alternatively activated DCs during murine infection with the parasitic helminth *S. mansoni*. Finally, we have compared the similarities and differences in how DCs and macrophages become alternatively activated, to provide information to guide subsequent work (chapter 4) addressing the function of some key alternative activation products on DC function.

3.2 SPECIFIC AIMS

1. To characterise the impact of IL-4 on activation of DCs *in vitro* and *in vivo*
2. To determine whether DCs express molecules associated with AAM in response to infection with *S. mansoni*.
3. To compare the influence of IL-4 on activation of DCs and macrophages, both *in vitro* and *in vivo.*

3.3 RESULTS

3.3.1. DCs express IL-4Rα, which is downregulated following exposure to IL-4 *in vitro*

Initially we decided to investigate the impact of IL-4 on a defined population of *in vitro* generated cells, GMDC. To determine what impact IL-4 has on the basal activation status of bone marrow derived DC, changes in expression of well characterised co-stimulatory molecules and the IL-4Rα were assessed by flow cytometry. GM-CSF derived DC expressed the IL-4Rα (Fig. 3.1B), in addition we revealed the novel finding that DC expression of IL-4Rα was reduced following culture with IL-4 (Fig. 3.1B). Signalling via IL-4Rα resulted in a slight but significant reduction in fluorescence intensity of CD80 and MHC-II in the $CD11c^+$ population, however no significant difference was found upon addition of IL-4 to surface expression of CD86 or CD40 (Fig. 3.1C). We next assessed whether IL-4 altered the basal secretion of cytokine following overnight culture, IL-4 significantly reduced the secretion of both IL-12p40 and IL-6 (Fig. 3.1D). We also assessed TNFα, IL-10 and IL-12p70 secretion all of which were most consistently not detected in the absence of stimulation. Having confirmed that in our hands GMDC are able to respond to IL-4, we next assessed whether IL-4 was able to induce expression of AAM markers within GMDCs.

3.3.2. IL-4 upregulates DC expression of RELMα and Ym1/2

In order to assess the impact that IL-4 has on DC activation, we investigated whether two of the major molecules associated with alternative activation of macrophage populations (RELMα and Ym1/2) were upregulated in GMDC populations following culture with IL-4 (Fig. 3.2A). Intracellular staining for both RELMα and Ym1/2 was carried out simultaneously using a protocol developed in the Allen Laboratory, University of Edinburgh. Addition of IL-4 strongly enhanced intracellular expression of both RELMα and Ym1/2 single and double positive cells

(Fig 3.2B&C). Unstimulated GMDCs secreted high levels of RELMα and Ym1/2 into the culture supernatant and this was increased upon addition of IL-4 (Fig. 3.2D). Growing DCs from bone marrow deficient in expression of the IL-4Rα confirmed that increased expression of these alternative activation markers was dependent upon signalling through this receptor (Fig. 3.2B-D). Interestingly, IL-4Rα$^{-/-}$ DCs had a reduced basal level of Ym1/2 production (p=0.0001; ICS and secretion) compared to WT cells. However, RELMα expression in IL-4Rα$^{-/-}$ was comparable to WT (Fig. 3.2B-D) suggesting a possible disparity in the requirement for IL-4 signalling in the induction of these alternative activation products. As IL-4 has now been confirmed to induce intracellular expression and secretion of AAM associated molecules, we were interested to determine the localisation of these molecules within individual cells.

3.3.3. IL-4 driven RELMα is located throughout the cytoplasm of BMDCs

We decided to use fluorescence and confocal microscopy to determine the expression pattern of the resistin like molecule RELMα within IL-4 treated GMDCs (Fig. 3.3A). A phalloidin toxin based stain for filamentous actin enabled the visualisation of the DC cytoskeleton. RELMα was detectable within medium GMDCs, however expression was increased upon addition of IL-4 to the overnight cultures. RELMα protein was detected throughout the cytoplasm of the IL-4 treated GMDCs, spreading out along the extended dendrites, whereas it was less prominent within the dendrites of medium DCs (Fig. 3.3B). Areas with the appearance of vacuoles were detected within the IL-4 treated samples, and this was most easily visualised when contrasted to the strongly positive RELMα staining.

3.3.4 IL-4 upregulates multiple transcripts associated with AAM, but not *Arg1*

Having found that both RELMα and Ym1/2 proteins were increased in GMDC on addition of IL-4 we wanted to confirm that this was due to increased mRNA transcript being produced within the cells and not due to a change in post-transcriptional regulation. IL-4 upregulated mRNA for both RELMα and Ym1/2 in GMDC (Fig. 3.4B). Following on from this finding, it seemed reasonable that other markers associated with AAM may also be transcriptionally regulated by IL-4 in DCs. Interestingly, the transcript for arginase-1, an enzyme whose expression is often used as a defining marker of an AAM (Gordon and Martinez 2010), was not significantly increased by IL-4 (Fig. 3.4C). Correspondingly, there was no significant

difference in the expression of inducible nitric oxide synthase (*Nos2*) at the mRNA level between medium and IL-4 treated GMDC (Fig. 3.4C). iNOS and arginase are known to counter regulate one another (Fig. 1.7) and in AAM arginase expression is enhanced by IL-4 (Modolell, Corraliza *et al.* 1995; Munder, Eichmann *et al.* 1999). Both *Arg1* and *Nos2* transcripts were at a high basal level in the medium samples (Fig. 3.4C). The chemokines CCL17 and CCL24 are both expressed by AAM (Mantovani, Sica *et al.* 2004), and it was found that both mRNA and secreted protein was enhanced in DC populations on addition of IL-4 (Fig. 3.4D&E). CCL24 was highly significantly upregulated by IL-4 perhaps due to it being expressed at a much lower level in the medium GMDCs than many of the other markers tested (Fig. 3.4E). Various scavenger receptors and C-type Lectin family members have been associated with AAM. The macrophage mannose receptor (*Mrc1*, CD206/MR) was significantly upregulated by IL-4 at the level of transcript, but no shift in surface protein expression was detected (Fig. 3.4F). Dendritic cell C-type lectin-1 (*Clec7a,* Dectin-1) was significantly enhanced in DC upon addition of IL-4 at the level of both mRNA and protein (Fig. 3.4G). Other markers (*Il27ra, Pparg, Stat6, Ccl22*) that have been linked to AAM were not found to be consistently upregulated to a significant level in GMDC (Fig. 3.4H).

3.3.5. Arginase-1 is not a marker of an alternatively activated DC (AADC)

To further probe the *Arg1* qPCR result an arginase assay was performed to assess whether IL-4 treatment of GMDCs resulted in an enhanced conversion of arginine to urea. There was no significant difference in arginase activity between medium and IL-4 treated DCs (Fig. 3.5B). To confirm the lack of a role for arginase-1, GMDCs were treated with an arginase inhibitor (BEC). No impact upon GMDC arginase activity was seen on addition of varying doses (1, 10, 100μM) of BEC to the overnight culture, with or without IL-4. The lack of impact found upon addition of BEC could suggest that the inhibitor was non-functional. Having confirmed that with the exception of arginase upregulation, GMDCs can alternatively activate in response to IL-4 we decided to confirm whether IL-4 could also alternatively activate FLDCs.

3.3.6 FLT3L derived DCs do not become alternatively activated in response to IL-4

There are two growth factors used for the *in vitro* differentiation of murine DCs from bone marrow: GM-CSF and FLT3L. The majority of BMDC work carried out previously within the MacDonald Laboratory used GM-CSF, which enables the differentiation of high numbers of a homogenous population of conventional CD11c^{+} DCs. FLT3L differentiates BM into a heterogenous population of conventional CD11c^{+}B220^{-} and CD11c^{mid}B220^{+} plasmacytoid DC (cDC/pDC). FLT3L generated DCs (FLDC) have been reported to better represent *in vivo* steady state DCs, compared to the more inflammatory GMDC (Xu, Zhan *et al.* 2007; Naik 2008). In order to determine whether FL-DC could be alternatively activated in a comparable manner to GMDC, the two types of BMDC were generated using either GM-CSF or FLT3L. Following differentiation, cells were cultured overnight with IL-4 or IL-13 and intracellular RELMα and Ym1/2 staining was performed (Fig. 3.6A). The IL-4 related cytokine IL-13 was capable of inducing comparable percentages of RELMα and Ym1/2 in GMDC as IL-4 (Fig. 3.6B&C). It was very clear that both IL-4 and IL-13 failed to upregulate comparable levels of RELMα or Ym1/2 in either pDC or cDC from FLT3L cultures to those seen in GMDC (Fig. 3.6B&D). This failure was unlikely to be due to an inability to detect IL-4, as both cDC and pDC expressed IL-4Rα (Fig. 3.6E), with expression being lower on pDC than cDC.

3.3.7 *Ex vivo* splenic DC can be alternatively activated by IL-4

To determine whether splenic cDCs respond to IL-4 similarly to GMDCs or FLT3L DCs, we enriched DC from naïve wild type mice using density gradient centrifugation and magnetic beads, then sorted cDCs (CD11c^{+}B220^{-}) from pDCs (CD11c^{int}B220^{hi}) using FACS (Fig. 3.7A). The enriched DC population was sorted based on size, width and surface expression of CD11c and B220 (Fig. 3.7B&C). Quantitative real time PCR (qPCR) was used to assess the level of expression of alternative activation markers following overnight culture of the sorted splenic cDCs with IL-4. Reassuringly, cDC expressed the IL-4Rα and presence of IL-4 caused down regulation of this transcript, as was shown previously following IL-4 treatment of GMDC (Fig. 3.7D). Due to low cell numbers achieved following FACS, alternative activation status following IL-4 treatment was only determined by qPCR and not by flow cytometry. A strong upregulation of RELMα mRNA expression was detected upon addition of IL-4 to splenic cDC, whereas there was no increase in *Chi3l3* transcript (Fig. 3.7E). *Clec7a* and *Ccl24*, markers of alternative activation that we

found to be enhanced by IL-4 in GMDC populations, were also enhanced by IL-4 addition to splenic cDC (Fig. 3.7F).

3.3.8 DCs can be alternatively activated *in vivo*.

Having confirmed that GMDCs could be alternatively activated *in vitro* and splenic DCs *ex vivo* following culture with IL-4 (Fig. 3.7), we next sought to determine whether we could directly isolate alternatively activated DCs from mice exposed to IL-4 *in vivo*. Although recombinant cytokines have a short half-life when injected *in vivo*, it has been shown previously that mixing rIL-4 with anti-IL-4 mAb (IL-4 complex; IL-4c) prior to injection enables the slow release of cytokine, increasing its half life of activity for 2-3 days (Finkelman, Madden *et al.* 1993). Based on previous studies (Finkelman, Madden *et al.* 1993) we chose to inject mice i.p. with either PBS vehicle control or a 1:5 weight to weight mixture of IL-4 and 11B11 antibody. Animals were given either one (Fig. 3.8A-C) or two (Fig. 3.8D-F) doses of IL-4c. Four days following injection of IL4c or vehicle, peritoneal exudate cells (PEC) (Fig. 3.8A) and splenocytes (Fig. 3.8D) were isolated. Flow cytometry was then used to stringently define DC (F4/80^{-}FSC^{lo}CD11c^{hi}MHC-II^{hi}) and macrophage (F4/80^{+}FSC$^{int/hi}$MHC-II$^{int/lo}$) populations following prior gating to exclude granulocytes, monocytes and B cells based on cell size, Gr1 and CD19 expression (Fig. 3.8B). We have recently shown that IL-4c induces macrophage proliferation in the peritoneal cavity and at distant tissue sites (Jenkins, Ruckerl *et al.* 2011). In contrast to the expected enhanced macrophage numbers, IL-4c did not significantly increase the numbers of DCs within the cavity (Fig. 3.8B). In order to determine the alternative activation status of the cavity DC and macrophage populations, PEC were intracellularly stained for RELMα and Ym1/2. As expected, IL-4c significantly upregulated both RELMα and Ym1/2 within the peritoneal macrophage population, in a dose dependent manner (Fig. 3.8C). Interestingly, the peritoneal DC population displayed strong IL-4 induced upregulation of RELMα within a large percentage of the population, whereas Ym1/2 expression was restricted to a smaller proportion of cells and only in animals that had been injected with a higher concentration of IL-4c. To address whether the impact of IL-4 on alternative activation of DCs was a localised effect, we also isolated splenocytes from IL-4c injected animals and assessed the levels of RELMα and Ym1/2 by intracellular staining and flow cytometry (Fig. 3.8D). IL-4c injection i.p. clearly alternatively activated splenic DCs, indicating that this effect was not restricted to the site of injection alone (Fig.

3.8E&F). IL-4c elicited splenic AADC expressed significantly enhanced levels of both RELMα and Ym1/2 compared to PBS elicited control cells and, similar to PEC, the percentage of Ym1/2^{+} cells was considerably lower than that of RELMα^{+} (Fig. 3.8F).

3.3.9 Markers of alternative activation are upregulated following *S mansoni* infection.

Having confirmed the ability of recombinant IL-4 to alternatively activate DCs in the peritoneal cavity and the spleen, we wanted to assess whether DC alternative activation was also a feature of relevant infection settings wherein IL-4 is the predominant cytokine. We chose to assess alternative activation status of DCs from *Schistosoma mansoni* infected animals that display a robust Th2 response following release of the egg stage of the parasite life cyle (Pearce and MacDonald 2002; Phythian-Adams, Cook *et al.* 2010). In order to assess global changes in levels of alternative activation following *S. mansoni* infection, cDNA samples were used from a time course infection that had previously been carried out in the MacDonald laboratory (Rachel Lundie). Tissue samples from sites of immune priming, egg recruitment and transit (spleen, liver and gut) were collected at time points relevant for *S. mansoni* infection. Samples were collected 4 weeks following infection prior to egg release, 6 weeks following infection as egg release begins, 8 weeks following infection as the Th2 response peaks and 12 weeks post infection as the Th2 response begins to decline (Pearce and MacDonald 2002). qPCR for markers of alternative activation *Arg1* (Fig. 3.9B), *Retnla* (Fig. 3.9C) and *Chi3l3* (Fig. 3.9D) were carried out. The liver was the tissue that had the most significant change in levels of alternative activation markers during infection, with significantly reduced *Arg1*, and significantly upregulated *Retnla* and *Chi3l3* (Fig. 3.9B-D). Highest levels of expression of both *Retnla* and *Chi3l3* were detected at week 8 in the liver, corresponding to the peak of Th2 activity in this model of infection (Pearce and MacDonald 2002). Interestingly, *Chi3l3* was down regulated in the liver between weeks 8 and 12 of infection (Fig. 3.9D). The inhibition of *Arg1* in the liver upon infection could be due to an alteration in the cellular architecture of the liver as arginase activity is known to be highly abundant within the liver (Cai, Kumar *et al.* 2009). The global expression of alternative activation markers within the spleen was much more varied than the liver; the only significant upregulation of any of the markers assessed in the spleen was *Chi3l3* at week 6 of infection (Fig.3.9B-D).

Retnla was significantly upregulated in gut tissue from week 6 of infection and *Arg1* from week 8, and a trend for infection-induced expression at this tissue site was apparent at later time points, though this did not reach significance, *Chi3l3* expression was not assessed in the gut (Fig. 3.9B&C). During the course of *S. mansoni* infection, the damage induced by the granulomatous response in the liver increases due to the increased egg burden, as such increased expression of AAM related markers in this tissue makes sense due to their proposed role in wound healing (Allen and Wynn 2011). Likewise, intestinal damage will occur later in infection as the eggs traffic out of the intestines, and infection induced levels of *Arg1* and *Retnla* were highest in the gut following 8 weeks of infection (Fig. 3.9B&C). Infection induced changes in AAM markers in the spleen were most apparent at the earlier time points assessed as the switch from a mixed Th1/Th2 to more pronounced Th2 response is occuring (Pearce and MacDonald 2002). It is important to keep in mind when assessing this data set that there are multiple cell types able to express *Arg1, Retnla* and *Chi3l3* in the liver, spleen and gut. Infection induced changes in individual cell types can still occur but be masked by the unchanged or reduced levels of expression within the other cells within the mixed population used to create the cDNA of interest.

3.3.10 Parasitic helminth infection induces alternative activation of splenic DCs.

As mentioned above, the lack of splenic global transcript increase seen for alternative activation markers upon infection with *S. mansoni* does not rule out the possibility that DCs present in this immune organ are alternatively activated. Furthermore, the ability of recombinant IL-4 delivered intra-peritoneally to alternatively activate DCs in the spleen (Fig. 3.8E&F) shows that these cells can be alternatively activated. In order to determine if *S. mansoni* can induce AADCs, we chose to focus on splenic DCs isolated from animals at 8 weeks post infection with *S. mansoni*, the time around which the peak of the Th2 response occurs (Pearce and MacDonald 2002). Spleens were harvested from mice at this 8 week timepoint, and intracellular staining was used to determine RELMα and Ym1/2 expression (Fig. 3.10A) within the MHC-II^{+}CD11c^{hi} DC population of individual spleens (Fig. 3.10B). RELMα was significantly upregulated in DCs by *S. mansoni* infection, whereas Ym1/2 was actually significantly decreased compared to naïve controls (Fig. 3.10B&C). This finding was not totally unexpected given that addition of IL-4 to

splenic DCs *ex vivo* did not enhance *Chi3l3* expression (Fig. 3.7E), and that upregulation of Ym1/2 in splenic DCs was much lower than RELMα when IL-4c was injected into the peritoneal cavity (Fig. 3.8E&F).

3.3.11 Defining a population of AAMs for direct comparison to AADCs.

Having identified that AADC can be induced by IL-4 *in vitro* and *in vivo*, and found during helminth infection, we then wanted to compare some of the aspects of their alternative activation to AAM. DCs and macrophages isolated from the spleens of infected animals would be the ideal populations to carry out competence assays to compare the functional capacity of AAM and AADC side by side. However, isolation of DC populations is technically difficult and extremely expensive to achieve due to their rarity. As a compromise, we chose to compare macrophages to GMDC differentiated from bone marrow, a method which provides a readily available supply of defined cells in the quantities required for our assays (Lutz, Kukutsch *et al.* 1999). In order to allow a direct comparison between AADC and AAM it was necessary to first define a population of macrophages to compare GMDCs with. Two commonly used types of experimental macrophage are 1) those generated *in vitro* from bone marrow using macrophage-colony stimulating factor (M-CSF) or 2) isolated *ex vivo* following intra-peritoneal injection of an sterile inflammation inducing medium (thioglycollate). BMM and ThioM both expressed F4/80, CD11b and CD11c on their surface (Fig. 3.11B). ThioM displayed higher expression of F4/80 and CD11b than BMM, and addition of IL-4 during overnight culture resulted in upregulation of CD11c on both BMM and ThioM (Fig. 3.11C). IL-4 induced production of RELMα and Ym1/2 was then assessed by ICS and ELISA and, as expected, the two proteins were significantly upregulated by IL-4 in both macrophage populations (Fig. 3.11D&E). Interestingly, there was a disparity in the expression of these hallmark proteins of alternative activation between the macrophage types, with BMM producing more RELMα than the ThioM and ThioM producing more Ym1/2 (Fig. 3.11D&E). Since both BMM and ThioM could be alternatively activated in our hands in response to IL-4, and BMM can be generated in large numbers from few mice, we decided to focus on use of BMM in our DC comparison studies.

3.3.12 BMDC and BMM are distinct cells types.

In general, there is broad agreement in the field that most DC and macrophages represent quite distinct myeloid cell types (Hashimoto, Miller *et al.* 2011). However, in some tissues or inflammatory conditions, it can sometimes be difficult to distinguish DCs and macrophages based on surface marker expression alone, allowing a small but vocal minority to continue to raise discussion as to the delineation of DC and macrophage populations in general (Hume 2008) (Geissmann, Gordon *et al.* 2010). In experiments carried out prior to the identifiaction of the DC specific transcription factor zDC (Meredith, Liu *et al.* 2012) (Satpathy, Wumesh *et al.* 2012) we decided to compare the surface phenotypes of BMM and GMDC differentiated *in vitro* (Fig. 3.12A). GMDC expressed very little F4/80 and high levels of CD11c (Fig. 3.12B). BMM expressed significantly higher levels of F4/80, comparable levels of CD11b and significantly reduced CD11c compared with GMDC (Fig. 3.12B). Thus, the levels of expression of the expected defining markers CD11c and F4/80 clearly distinguish BMMs from GMDCs. In the experiment shown, the GMDC expressed comparable levels of MHC-II to the BMM, however this was found to vary between experiments (Fig. 3.12B) and as no antigen was included in the culture such variability is dependent upon how activated the cells have become during the differentiation process. As AAM are known to express enhanced arginase activity in the presence of IL-4, we direclty compared their phenotype with IL-4 treated GMDC (Fig. 3.12C), which we have shown do not upregulate *Arg1* transcript (Fig. 3.4C) or arginase activity (Fig. 3.5) in response to IL-4. The basal arginase activity of GMDC was significantly higher than that of BMM and although addition of IL-4 enhanced arginase activity in the BMM this did not reach the basal level of activity present within GMDC (Fig. 3.12D). This result lends credence to the theory that GMDC express such a high level of arginase that the amount of BEC inhibitor added was not sufficient to inhibit the activity (Fig. 3.5C).

3.3.13 GMDC express higher levels of co-stimulatory molecules and secrete more cytokine than BMM.

A defining feature of DCs that helps to distinguish them from macrophages is their ability to migrate to the secondary lymphatics and efficiently activate naïve T cells (Geissmann, Gordon *et al.* 2010). Adoptive transfer of GMDCs stimulated with either heat killed bacteria (gram positive *P. acnes/ gram negative S. typhimurium*) or soluble egg antigens (SEA) from *S. mansoni* efficiently primes Th1 or Th2 immune responses in lymph nodes draining the site of injection (MacDonald, Straw *et al.*

2001; Perona-Wright, Jenkins *et al.* 2009). We differentiated GMDC and BMM from C57BL/6 bone marrow and stimulated them overnight with media, *P. acnes, S. typhimurium* or SEA*,* prior to quality control assessment by ELISA and flow cytometry (Fig. 3.13A) Surface phenotyping by flow cytometry showed that pre-transfer GMDC expressed higher levels of the co-stimulatory molecules MHC-II, CD80 and CD86 than BMM (Fig. 3.13B). Bacterial antigens elicited the highest levels of expression of all three markers of APC activation by GMDC, whereas the only evident shift in BMM surface expression was in CD80 following stimulation with *S. typhimurium* (Fig. 3.13B). Correspondingly, the only cytokines secreted by BMM pre-transfer at levels comparable to GMDC was also in response to stimulation by *S. typhimurium* (Fig. 3.13.C; IL-10 & TNFα). The Th1 & Th17 polarising cytokines IL-12p70, IL-12p40 and IL-6 were secreted in much larger amounts by GMDC than BMM in response to bacterial stimuli (Fig. 3.13C). BMM all produced comparable levels of the anti-microbial molecule nitric oxide in response to *S. typhimurium* as GMDC (Fig. 3.13D).

3.3.14 GMDC are significantly better than BMM at priming both Th1 and Th2 responses *in vivo.*

Following overnight culture with either SEA, *P. acnes* (Pa) or *S. typhimurium* (St) GMDCs were phenotyped (Fig. 3.13) and equal numbers of GMDC or BMM were injected subcutaneously into the top of the feet of naïve WT recipients. 7 days later the draining popliteal lymph nodes (pLN) were harvested, cells isolated and restimulated with the priming antigens for 72 hours in order to elicit a recall cytokine response from any T-cells present (Fig. 3.14A). Following transfer, SEA pulsed GMDC primed significantly higher levels of SEA specific IL-4, IL-5, IL-13, IL-10 and IFNγ than SEA pulsed BMM that elicited very little SEA specific Th2 cytokine (Fig. 3.14B). BMM were slightly more capable at priming Th1 responses than Th2, with detectable levels of *P. acnes* specific IFNγ and *S. typhimurium* specific IL-10 being induced (Fig. 3.14C) however these levels were significantly reduced in comparison to the GMDC response. The inability of bacterially stimulated BMM to polarise Th1 and Th17 responses with the same magnitude as bacterially stimulated GMDCs is potentially due to the inability of these cells to secrete comparable quantities of the Th1 polarising cytokine IL-12 and the Th17 polarising cytokine IL-6 pre-transfer (Fig. 3.13C & see Fig. 1.5).

3.3.15 BMM are less competent than GMDC at polarising CD4$^+$ T-cells towards Th2 *in vitro.*

The adoptive transfer experiments show that BMM are much less capable than GMDCs at induction of adaptive responses *in vivo*, and it is possible that the impaired ability is largely due to the well-documented poor migratory ability of macrophages (Niess, Brand *et al.* 2005; Bogunovic, Ginhoux *et al.* 2009; Geissmann, Gordon *et al.* 2010). To be able to compare the basic APC capacity of the GMDCs and the BMM, we used an *in vitro* culture system to remove migratory capacity from the equation. We sorted CD4$^+$IL-10GFP$^-$ T-cells from the spleen and LNs of HuCD2xIL-10GFP mice. Upon transcription of *Il10* mRNA, cells from this strain of mouse express green fluorescent protein. Additionally, when IL-4 protein is translated, human-CD2 is expressed on their cell surface (Mohrs, Wakil *et al.* 2005), enabling the detection and identification of cells that are either singly or doubly positive for these cytokines. We stimulated the sorted T-cells for 4 days using anti-CD3 (αCD3) (a requirement of this polyclonal T cell system to enable cytokine secretion) and determined if there was induction of IL-4 protein or IL-10 mRNA on addition of IL-4 when GMDC or BMM were included in the culture (Fig. 3.15A). IL-4 was added to this system to determine whether the impact of IL-4 on DCs was instructive in their ability to modulate T-cell cytokine secretion. In the absence of αCD3 or an antigen presenting cell, the T-cells did not proliferate. GMDC supported enhanced blasting when compared to BMM, as assessed by cell pellet size at day 4 and time taken to acquire similar numbers of T-cells by flow cytometry. Addition of IL-4 resulted in significant increases in percentage expression of huCD2 (IL-4 protein) and GFP (IL-10 mRNA) within the CD4 population by day 4, in the presence of either GMDC or BMM (Fig. 3.15B&C). However, the percentage induction within the GMDC culture wells was significantly higher than in the BMM wells (Fig. 3.15C). ELISA data confirmed that BMM were less capable at inducing T-cell cytokine secretion in response to IL-4 (Fig. 3.15D). It is not possible to assess accurately using this experimental design to what extent the IL-4 is acting directly on the T-cell or via the DC/BMM. However, this shows that, as expected given their limited surface activation phenotype, BMM have a basic APC ability and are much less capable than GMDCs in this regard. Further, these *in vitro* data suggest that the more dramatically imparied APC ability of BMM in the adoptive transfer experiments (Fig. 3.14) may be largely down to their poor migratory capacity.

3.4 SUMMARY

- IL-4 can trigger a profile of 'alternative activation' in DCs similar, but not identical, to that of macrophages, both *in vitro* (Fig. 3.2), *ex vivo* (Fig. 3.7) and *in vivo* (Fig. 3.8). For example, DC arginase activity was not enhanced by IL-4 treatment (Fig. 3.5).
- Global expression levels of *Retnla* and *Chi3l3* in whole tissue following *S. mansoni* infection do not correlate with DC alternative activation (Fig. 3.9).
- Alternative activation of splenic DCs is a feature of infection with the parastiic helminth *S. mansoni* (Fig. 3.10)
- BMDCs, BMM and ThioM are phenotypically different cell populations (Fig. 3.11-3.15)
- BMDCs are more efficient at priming Th1, Th2 and Th17 immune responses than BMM (Fig. 3.13-3.15).

3.5 DISCUSSION

3.5.1 IL-4 and GM-CSF dose

A previous study had found that overnight culture of splenic DCs with a higher concentration of IL-4 (100ng/ml) resulted in moderate downregulation of CD80 (B7.1 in this study) and significant upregulation of CD86 (B7.2 in this study), and that these changes were reversed by addition of anti-IL-4 antibody (11B11) to the culture well (King, Mueller Hoenger *et al.* 2001). A second study that included IL-4 in the culture during differentiation of GMDCs found no impact of IL-4 presence on DC expression of MHC-II or B7.2 (Lutz, Schnare *et al.* 2002) when the cells were differentiated using high dose GM-CSF (200U/ml). It is possible that the dose of IL-4 used impacts the ability of IL-4 to alter the activation status of GMDCs. As shown in the materials and methods (Fig. 2.3), changes in the concentration of IL-4 that DCs are exposed to can impact DC phenotype. In particular, we focussed on a concentration of IL-4 at which alternative activation was most evident. The Lutz paper investigated the changes in maturation status of GMDCs cultured in the presence of different concentrations of both IL-4 and GM-CSF (Lutz, Schnare *et al.* 2002). It would be interesting to assess the alternative activation status of DCs differentiated in the presence of IL-4. We would hypothesise that this would result in a much increased basal level of alternative activation, such that addition of exogenous IL-4 following differentiation may no longer result in significant enhancement of the alternatively activated state.

3.5.2 IL-4 down modulation of the IL-4Rα

We confirmed, as had been reported previously (Lutz, Schnare *et al.* 2002) that GMDCs express the IL-4Rα (Fig. 3.1). In addition, we have reported the novel finding that IL-4 presence results in reduced detection of this receptor on the surface of GMDCs (Fig. 3.1). It is possible that IL-4 binding to the IL-4Rα may be blocking the binding of the IL-4Rα antibody to its epitope. To address this possibility, a different IL-4Rα antibody clone could have been used that targets an epitope outwith the IL-4 recognition domain. An alternative and more interesting possibility is that IL-4 binding may initiate a negative feedback loop to increase internalisation of it's receptor via phagocytosis. However, some of our data actually suggests that IL-4 may mediate a reduction in the expression of the IL-4Rα at the level of transcription, as splenic DCs cultured overnight with IL-4 had a trend for reduced *Il4ra* transcript when compared to cells cultured overnight with medium alone (Fig. 3.7D). This

observation was not statistically significant, so to clarify this issue, repeat experiments would be required.

3.5.3 Differential expression of RELMα and Ym1/2

We found that overnight culture with 20ng/ml IL-4 was able to upregulate the high level expression of both RELMα and Ym1/2 in GMDCs (Fig. 3.2). Interestingly, although the intracellular expression of Ym1/2 was ablated in IL-4Rα deficient GMDCs a low level of Ym1/2 was detectable in the supernatant (Fig. 3.2D). The same basal level of RELMα expression was maintained in IL-4Rα deficient cells prior to the addition of IL-4, as was detected in WT cells. This intracellular protein data suggests that RELMα expression can be maintained in GMDCs in the absence of a basal signal through the IL-4Rα. As GM-CSF is a potent stimulus to DCs, and 5ng/ml is included in the overnight cultures with IL-4, it is possible that this may be resulting in IL-4R independent RELMα production. A useful experiment to assess this would be to directly compare GMDCs stimulated overnight with IL-4 with and without GM-CSF. Further, exposure of the GMDCs to a dose curve of both would likely be the most informative, given the findings of Lutz *et al.,* as discussed in section 3.5.2.

In addition to characterising the response of GMDCs to IL-4 *in vitro*, we also looked at DC populations *ex vivo* and their ability to alternatively activate. *Ex vivo* culture of FACS sorted $CD11c^+$ splenic DCs with IL-4 resulted in enhanced expression of *Retnla,* but not *Chi3l3* (Fig. 3.7E). DCs isolated from the peritoneal cavity following IL-4c treatment only expressed detectable intracellular Ym1/2 when a higher concentration (4µg) of IL-4 was injected (Fig. 3.8C). Although splenic DCs were capable of expressing intracellular Ym1/2 in response to IL-4, the population of cells expressing Ym1/2 was clearly more minor than those expressing RELMα (Fig. 3.8E). This apparently poor ability of DCs to express Ym1/2 in response to IL-4c *in vivo*, at least relative to GMDCs *in vitro*, may be due to dose, as mice received two injections of IL-4c prior to spleen harvest. Macrophages isolated from PEC following injection of IL-4c were able to express both RELMα and Ym1/2 at high levels even at the lower concentrations of IL-4 (Fig. 3.8C), suggesting that these cells *in vivo* are more sensitive to IL-4 than the $F4/80^-$ DC population. In accord with the qPCR data following overnight incubation of splenic DCs with IL-4 (Fig. 3.7), splenic DCs isolated following 8 weeks of *S. mansoni* infection also failed to dramatically

upregulate Ym1/2 in response to this Th2 cytokine dominated infection. It is possible that the level of IL-4 present in the spleen following infection is not sufficient to induce Ym1/2 expression in DCs. For example previous studies from the MacDonald laboratory have shown that, upon restimulation with SEA, 2 million splenocytes produce in the region of 100pg/ml IL-4 over 72h (Jenkins, Perona-Wright *et al.* 2008). An additional possibility is that the IL-4 level is sufficient to induce Ym1/2, but that *S. mansoni* worm or egg secretions may actively interfere with Ym1/2 upregulation in DCs.

A less pleasing thought for a DC laboratory such as ours, is that GMDCs may be more closely related to *in vivo* macrophage populations, and that high level expression of Ym1/2 as well as RELMα in GMDCs in response to IL-4 proves to highlight this fact. However, as shown in later experiments, GMDCs have enhanced APC activity as compared to BMM and are able to migrate to sites of naïve T-cell priming (as assessed by antigen-specific restimulation assays following DC transfer Fig. 3.14). Both migration and activation of naïve T-cell are characteristics of DCs (Geissmann, Gordon *et al.* 2010; Steinman 2012). Furthermore, the recent papers identifying the DC specific transcription factor zDC have shown that DC differentiated with GM-CSF *in vitro* express this gene (Satpathy, Wumesh *et al.* 2012), whereas BMM do not.

Although GMDCs may not be identical to a defined *in vivo* DC subset, they do possess defining characteristics of DCs, and provide a useful source of DCs for use in assays that would otherwise be too costly both in terms of mouse usage and reagents for isolating DCs *ex vivo*.

3.5.4 What are the vacuoles in IL-4 treated GMDCs?

In Figure 3.3, we showed the intracellular expression of RELMα within IL-4 treated GMDCs. It is possible that the regions with a vacuolar appearance are lipid bodies, as lipid mediators such as the PPARs are known to be involved in mediating nematode elicited macrophages that have an M2 like phenotype (Thomas, Ruckerl *et al.* 2012). However, an accumulation of lipid in DCs in response to IL-4 has never been reported previously. Further staining for markers known to induce lipid body formation such as adipocyte differentiation related protein (ADRP) may prove informative (Maya-Monteiro and Bozza 2008). An accumulation of lipid within DCs

may provide a fuel source for fatty acid oxidation by the DCs; as in macrophages, IL-4 has previously been shown drive a switch from glycolysis to fatty acid oxidation (Vats, Mukundan *et al.* 2006).

3.5.5 DC arginase expression and activity

In contrast to what has been reported previously for macrophages, which we have confirmed in Fig. 3.12D, IL-4 failed to consistently induce increased expression and activity of arginase in GMDCs (Fig. 3.4, 3.5, 3.12). In our experiments, the inability of the arginine analogue BEC to influence arginase activity (Fig. 3.5), is likely due to a failure of the BEC to inhibit arginase activity at the range of concentrations used. Repeating this experiment using higher doses of BEC would address this suspicion. Alternatively, it may be the case that GMDCs produce such a high basal level of arginase that the inhibitor is unable to effectively reduce activity. To address if this might be the case, it would be important to first ensure that the inhibitor was functional, for example through use of this inhibitor with BMM, as BMM display a lower level of arginase activity than GMDCs (Fig. 3.12).

Addition of 0.2% BEC in the drinking water of mice infected with *S. mansoni* (Herbert, Orekov *et al.* 2010) has been shown to replicate the disease phenotype of animals either deficient in arginase (Pesce, Ramalingam *et al.* 2009) or deficient in *Arg1* in their BM compartment (via use of a chimera)(Herbert, Orekov *et al.* 2010). Inhibition of arginase activity by various inhibitors has been reported to have off target effects, and as such use of an inhibitor may not be the best system to block DC expression of this enzyme for further studies(Bronte and Zanovello 2005). It would be interesting to determine whether the absence of GMDC expression of arginase alters the ability of IL-4 to enhance RELMα and Ym1/2 expression, for example via generation of a DC specific arginase deficient animal (eg.CD11c^{Cre}Arg-1$^{fl/fl}$). Interestingly, chimeric mice with a deficiency in *Arg1* in their BM compartment have enhanced levels of pro-inflammatory cytokines within their intestinal compartment following 8 weeks of *S. mansoni* infection compared to WT BM chimeras, but no difference in expression of *Retnla (Herbert, Orekov et al. 2010).*

As mentioned above, GMDCs expressed a higher basal level of arginase activity than BMM in the absence of IL-4 treatment (Fig. 3.12). However, GM-CSF has been reported to itself be able to induce arginase activity. Incubation of BMM

with GM-CSF results in a dose dependent increase in arginase activity (Jost, Ninci *et al.* 2003; Martin, Comalada *et al.* 2006), and increases the upregulation of an arginine transporter, CAT2 (Martin, Comalada *et al.* 2006). Assessment of GMDC arginase activity immediately following differentiation, or following overnight culture in media not supplemented with 5ng/ml GM-CSF, may abrogate the inability of IL-4 to enhance DC arginase activity in our studies. Interestingly, a role for the SH2 domain containing inositol-5' phosphatase (SHIP) in blocking IL-4 induced *Arg1* has been reported in AAM (Weisser, McLarren *et al.* 2011). Assessment of the levels of this phosphatase within GMDC cultures could provide a potential avenue to understand why IL-4 is unable to enhance *Arg1* expression and activity in these cells.

The lack of enhanced IL-4 driven arginase in AADCs, highlights that these cells have a different function to AAM. AAM are evolutionarily very important for the repair of tissue damage, and as such an IL-4 driven increase in production of polyamines and proline, (which promote collagen deposition and tissue repair) by these cells is necessary for their function (Allen and Wynn 2011). DCs on the other hand are the prime initiators of T-cell activation (Kapsenberg 2003; Steinman 2012), and enhanced production of arginase by DCs may deplete this important amino acid from the T-cells which they are in the process of activating (Bronte and Zanovello 2005).

3.5.6 AADC secretion of chemokines

In vivo, DC expression of chemokines and chemokine receptors is important not only for mediating their migration between bone marrow, sites of immune priming and effector function, but also for controlling DC interactions with other cells of the immune system (Mantovani, Sica *et al.* 2004). We confirmed that, like AAM, AADCs can secrete high levels of the eosinophil chemoattractant CCL24/eotaxin-2 (Fig. 3.4E). It is not yet clear what the *in vivo* role for DC expression of this chemokine may be. However, eosinophils have been shown to be a potential source of IL-4 for the maintenance of alternative activation of macrophages in adipose tissue during helminth infection (Wu, Molofsky *et al.* 2011). So it is possible that DC expression of CCL24, may result in the propagation of a Th2 response via the recruitment of IL-4 secreting cells. IL-4 also significantly increased DC expression of CCL17 (Fig. 3.4D). A recent report suggests that DC derived CCL17 is able to limit

T-regulatory cell induction in atherosclerotic lesions (Weber, Meiler *et al.* 2011). In this study, transwell migration assays were also carried out that showed reduced CD4$^+$ T-cell migration towards BMDCs deficient in CCL17, suggesting that CCL17 may function to increase CD4$^+$ T-cell accumulation at sites of an ongoing immune response (Weber, Meiler *et al.* 2011).

3.5.7 IL-4 Vs. IL-13 in DC alternative activation

IL-4 can signal via the type-I IL-4R, whereas IL-13 can signal via the type-II IL-4Rα (a combination of IL-4Rα and IL-13Rα1) (Nelms, Keegan *et al.* 1999). Genetic ablation of IL-4Rα thus blocks signalling by both IL-4 and IL-13. We found that IL-13 induced comparable levels of alternative activation in GMDCs to IL-4 (Fig. 3.6C). IL-13 has also been shown to induce comparable alternative activation in macrophage populations (Doyle, Herbein *et al.* 1994; Van den Bossche, Bogaert *et al.* 2009). As IL-13 was found to induce comparable levels of alternative activation within DCs, we decided to focus only on IL-4, to remove the complexity of dual IL-4 and IL-13 signalling. A separate study on IL-13 induction of AADCs would be interesting and would most likely highlight functional differences between the phenotype of DCs induced by these different Th2 cytokines.

A recent study in human monocytes/macrophages has shown that different Jaks and STATs are activated downstream of IL-4 and IL-13 signalling (Bhattacharjee, Shukla *et al.* 2012). IL-4 was found to preferentially recruit Jak1 to regulate STAT3 and STAT6, and IL-13 both Jak2 and Tyk2, which then activated STAT3, and STAT1 and STAT6 respectively (Bhattacharjee, Shukla *et al.* 2012). A thorough characterisation of Jak/STAT usage within IL-4 and IL-13 treated DCs would likely provide useful information about the mechanism(s) by which these two cytokines mediate programmes of alternative activation. Such signalling studies would also be a good starting point to investigate the differential expression of RELMα and Ym1/2 within different DC populations, for example to determine if FLDCs activate specific JAK/STAT combinations downstream of IL-4 binding, or if GMDCs deficient in components of the IL-4R signalling machinery alternatively activate in a different way. Studies into Jak/STAT usage may also provide information as to why RELMα and Ym1/2 are upregulated with different magnitudes in response to the same concentration of IL-4 both *in vitro* and *in vivo* and in different cells types (ie DCs vs. macrophages) (Fig. 3.2, 3.6, 3.7, 3.8, 3.10, 3.11). As

discussed in the introduction, other Th2 related cytokines IL-21 (Pesce, Kaviratne *et al.* 2006), IL-33 (Kurowska-Stolarska, Stolarski *et al.* 2009) and Activin A (Ogawa, Funaba *et al.* 2006) have been implicated in modulating AAM activation, similar investigations into the impact of these cytokine on DC activation status should also be carried out.

3.5.8 Why aren't FLDCs alternatively activated by IL-4?

FLDCs have been described as being the equivalent of 'steady state' splenic DCs (Naik, Proietto *et al.* 2005). It was somewhat surprising, given their expression of IL-4Rα (Fig. 3.6), that they were unable to express RELMα or Ym1/2 in response to IL-4 (Fig. 3.6D), when overnight culture of splenic DCs from naïve mice (also described as 'steady state' DCs) with IL-4 did induce RELMα expression.

It is possible that FLDCs simply require an additional signal to allow their alternative activation. This signal is not IL-13, as addition of this cytokine also failed to induce RELMα or Ym1/2 in either the pDCs or cDCs from the FLDC culture (Fig. 3.6B&D). A possibility is that the absence of GM-CSF within this system is the reason that FLDCs are unable to alternatively activate, a simple experiment to assess this would be the addition of exogenous GM-CSF to the overnight culture of FLDC +IL-4. Alternatively, the pDCs present within the mixed FLDC cultures could inhibit the alternative activation of the cDCs. It may be informative to sort pDC and cDC populations prior to culture with IL-4 and phenotyping for alternative activation.

A further possible explanation for the ability of splenic DCs to alternatively activate, when FLDCs cannot, could be that isolation of splenic DCs *ex vivo* results in their low level activation, which may be sufficient to license them for alternative activation.

It is also interesting to note that permeabilisation of FLDCs substantially altered their size and granularity as detected by flow cytometry (Fig. 3.6). It is possible that use of a different method to permeabilise these cells may enable better detection of intracellular proteins.

3.5.9 Comparing the alternative activation of BMM and ThioM

Thioglycollate medium is a mixture of proteins, sugars and sodium thioglycollate (Li, Baviello *et al.* 1997) which induces 'sterile' inflammation and recruitment of monocytes to the peritoneal cavity (Gallily and Feldman 1967). IL-4 was able to induce higher levels of RELMα protein from M-CSF stimulated BMM than from ThioM, which had a preponderance of Ym1/2 expression (Fig. 3.11). It is possible that such thioglycollate recruitment results in macrophages with a more inflammatory phenotype, and Ym1 has been reported previously to promote rather than limit T-cell cytokine secretion (Cai, Kumar *et al.* 2009). It is also possible that the ThioM population contains cells with a more neutrophilic phenotype, as injection with thioglycollate initially recruits an influx of neutrophils to the peritoneal cavity (Kuhn, Godshall *et al.* 2001) and the ThioM expressed higher levels of CD11b than BMM (Fig. 3.11B). Additionally neutrophils have been reported to express high levels of Ym1/2 (Harbord, Novelli *et al.* 2002). These findings prove to highlight that BMM and ThioM are not necessarily comparable cell populations.

3.5.10 APC function of BMM vs. BMDC

BMDCs were more efficient at priming of Th1, Th2 and Th17 immune responses following transfer into WT recipients than BMM (Fig. 3.14). Assessment of cytokine production prior to transfer highlighted that bacterially stimulated BMM secreted much lower levels of IL-12p70 and IL-6 than BMDCs stimulated with the same amounts of the same antigens (Fig. 3.13C). Upon transfer, Pa or St stimulated BMM induced pLN T-cells to secrete low level IFNγ and very low level IL-17, as may have been predicted by their failure to secrete significant amounts of the Th1 and Th17 polarising cytokines IL-12p70 and IL-6 (Fig. 3.13). BMM did secrete IL-10 in response to overnight culture with St (Fig. 3.13C) and, following transfer, St BMM induced higher levels of T-cell IL-10 than Pa BMM, which did not secrete detectable IL-10 pre transfer (Fig. 3.14C). Furthermore BMM also failed to upregulate high level expression of MHC-II and co-stimulatory molecules in response to antigen challenge prior to transfer (Fig. 3.13B), suggesting that these cells would be less competent at providing signal 2 to activate naïve T-cells.

SEA pulsed BMM failed to induce any detectable recall response in the dLN following transfer into WT recipients (Fig. 3.14B). It is likely that SEA activated BMM fail to migrate to the LN following transfer. In order to assess whether this may the cause of the reduced priming ability of BMM, a cell tracking experiment could be

undertaken using congenic BM or fluorescently labelled cells, such as those from dsRed animals (Vintersten, Monetti *et al.* 2004).

The results we gained from *in vitro* co-culture experiments suggest that failure to migrate to the sites of immune priming are not the only reason why BMM are unable to effectively prime Th2 responses. BMM still induced lower levels of IL-4, IL-13 and IL-10 from T-cells than equivalent numbers of DCs, in the presence of the Th2 polarising cytokine IL-4 (Fig. 3.15). This result supports the idea that BMDCs are able to supply factor(s) that BMM cannot to support T-cell activation and polarisation. Co-stimulation may be one factor that is missing, or cytokine production, and further work to address what these factors may be would be interesting. IL-2 has been implicated in supporting DC induction of T-cell IL-4 secretion (Cote-Sierra, Foucras *et al.* 2004) (Mandron, Aries *et al.* 2006) and IL-2 secretion by DCs has been reported (Granucci, Vizzardelli *et al.* 2001). So, it is possible that DCs provide IL-2 to support T-cell induction of IL-4, and that macrophages do not. However, preliminary experiments in our laboratory have so far failed to detect IL-2 secretion by GMDCs, following 2 hours of culture with IL-4.

Chapter 3 of this thesis has compared the impact that IL-4 has on DCs and macrophages, both *in vitro* and *in vivo*, in response to exogenous IL-4 treatment and schistosome infection. Expression of molecules usually associated with AAM RELMα, Ym1/2, dectin-1, CCL17, CCL24 and MR were found to be upregulated in IL-4 exposed DC populations, whereas arginase was not. In addition, the APC ability of BMDC and BMM were directly compared *in vivo* with BMM being found to be much less capable of activating naïve T-cells than BMDC.

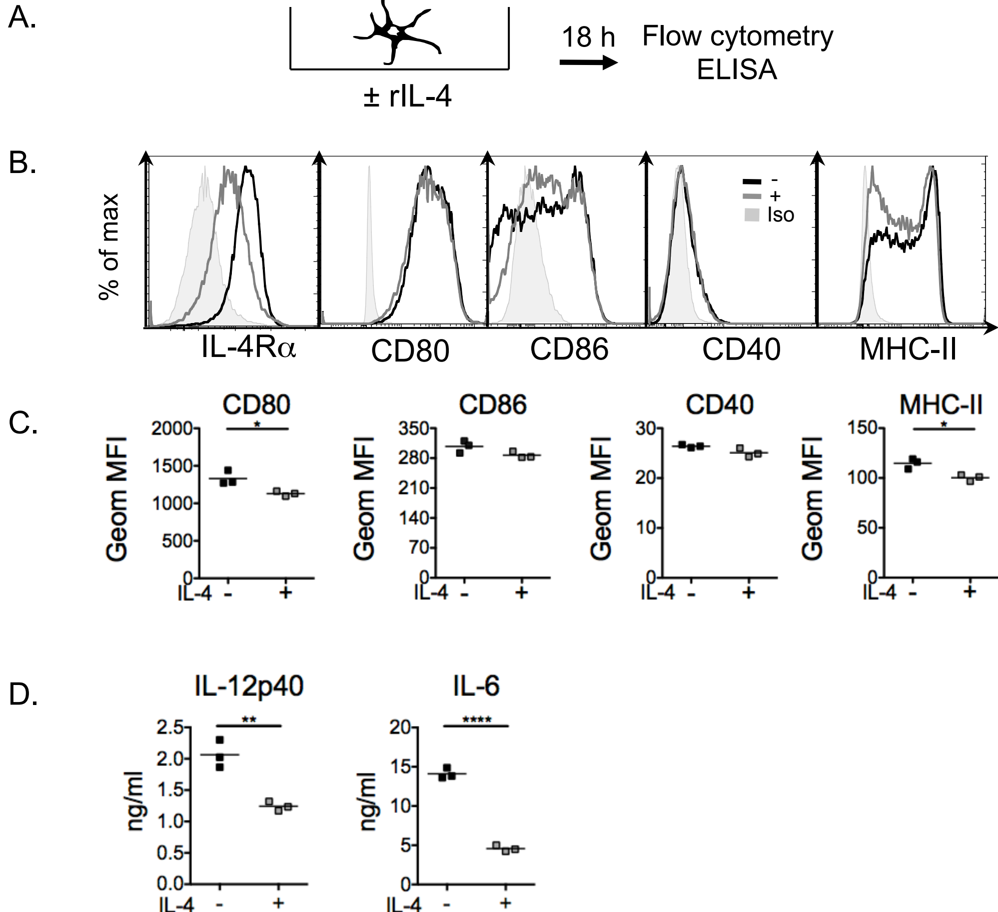


Figure 3.1. IL-4 stimulated BMDCs do not dramatically alter their activation phenotype. WT BMDCs were stimulated overnight with 20ng/ml IL-4 and their expression of the IL-4Rα, CD80, CD86, CD40 and MHC-II was assessed by flow cytometry (A-B). Shaded=isotype control, black line=media, grey line=+IL-4. Geometric mean fluorescence intensity (MFI) of marker expression by CD11c^{+} cells (C) and cytokine secretion was assessed by ELISA (D). Data representative of >5 experiments, carried out in triplicate. *=P<0.05, **P<0.01, ****P<0.0001.

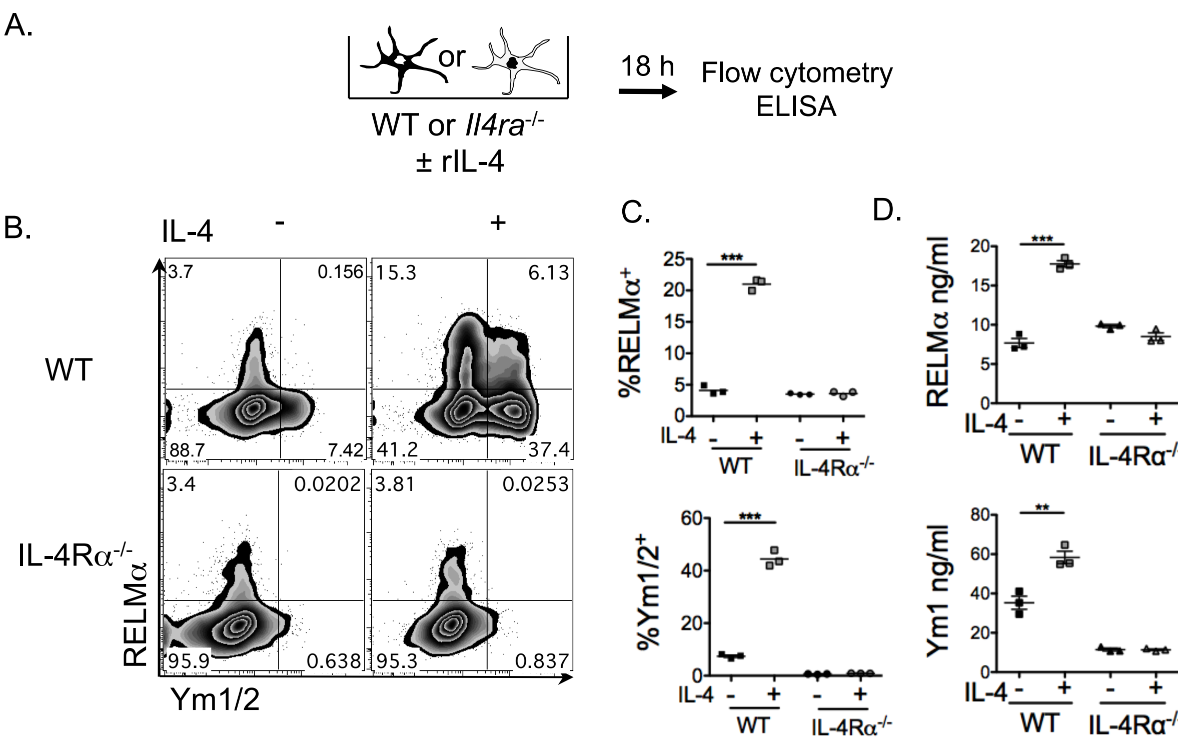


Figure 3.2. IL-4 upregulates RELMα and Ym1/2 in BMDCs via the IL-4Rα. Overnight culture of BMDCs with IL-4 induces increased intracellular RELMα and Ym1/2 (A&B) and secretion (C), that depends upon IL-4Rα expression (D). Data shown are representative of 3-5 independent experiments, carried out in triplicate **P<0.01, ***P<0.001.

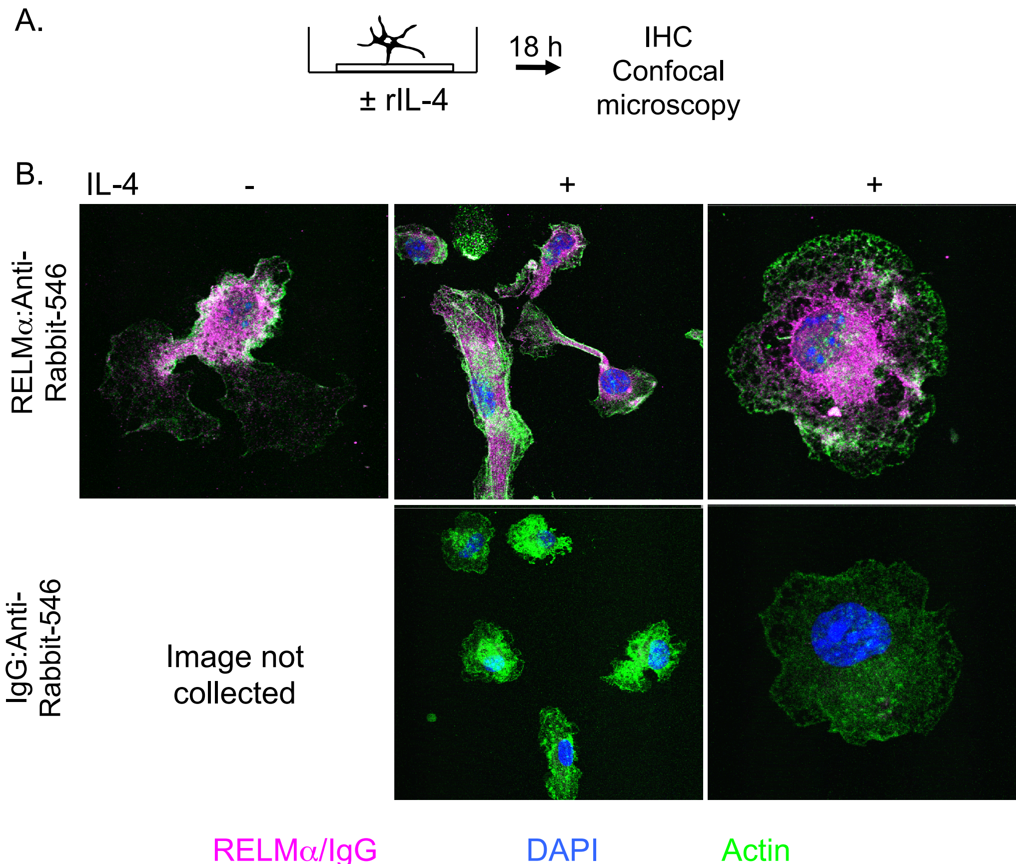


Figure 3.3. IL-4 treated DCs produce RELMα protein throughout their cytoplasm. GMDC were cultured overnight with or without 20ng/ml IL-4 in wells containing coverslips (A). Expression of the alternative activation associated molecule RELMα (pink) was assessed by confocal microscopy (B). The nucleus of the cells are stained using DAPI (blue) and DC structure is visualized using phallacidin staining for filamentous actin (green). An isotype control antibody is used as a control in the lower panels.

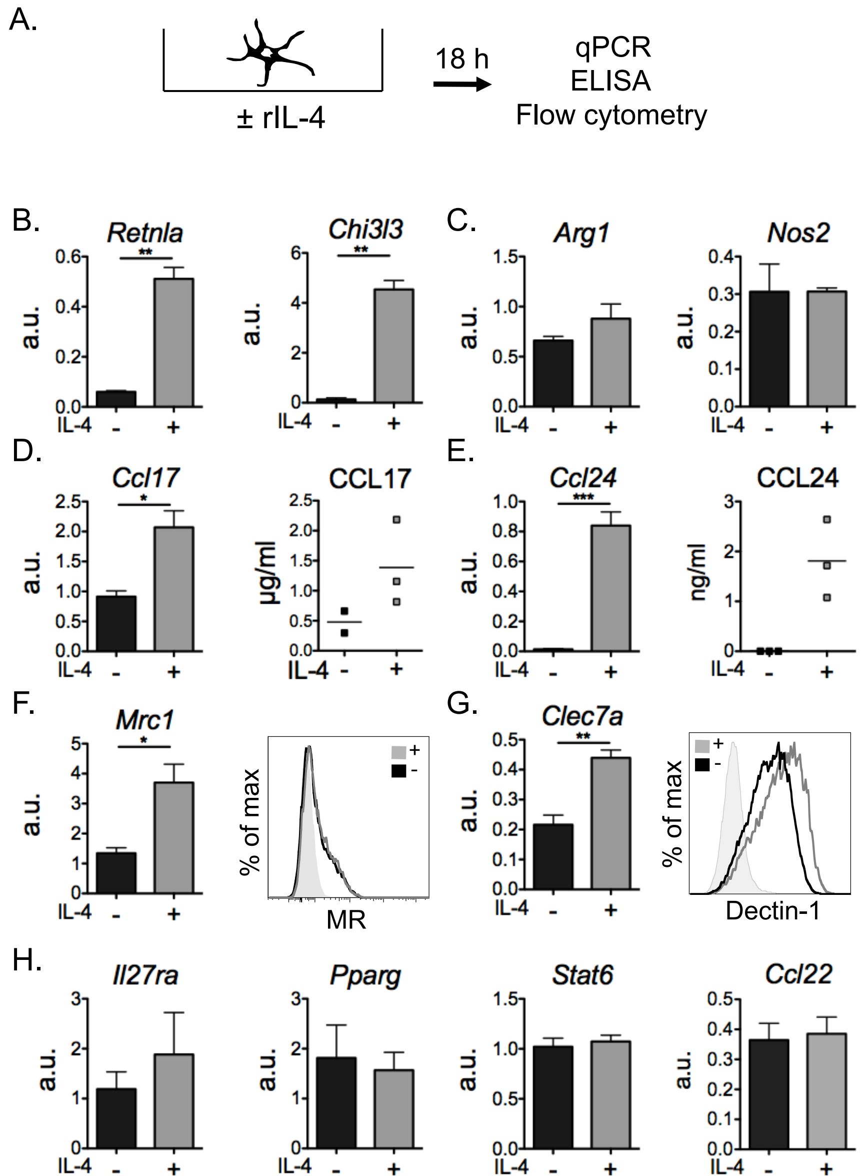


Figure 3.4. IL-4 upregulates multiple markers associated with AAM, but not *Arg1*. Overnight culture of GMDCs with IL-4 (A) upregulated Retnla and Chi3l3 (B), but did not enhance Arg1 or Nos2 (C) Chemokines (D+E) and C-type lectins (F+G) were upregulated by IL-4 at both transcript and protein level. Other markers associated with macrophage alternative activation were not upregulated by IL-4 in GMDCs (H). Black= media, Grey = +IL-4, shaded =isotype. Data shown are representative of 3-5 independent experiments, carried out in triplicate *=P<0.05, **P<0.01, ***P<0.001.

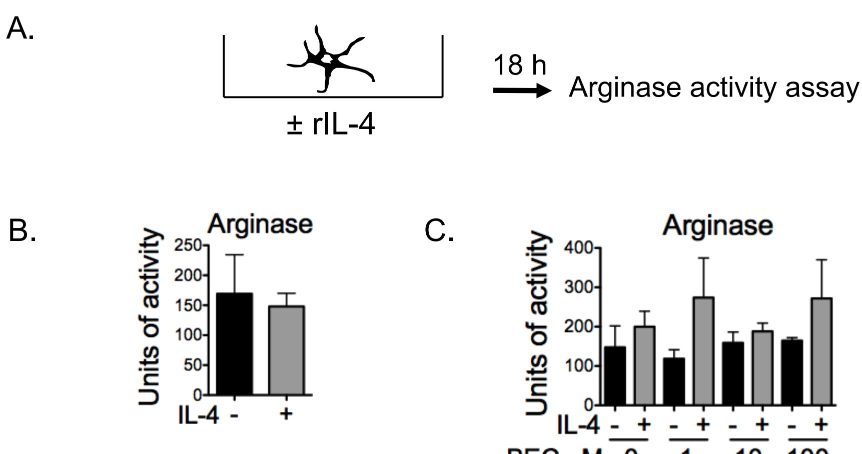


Figure 3.5 Arginase-1 is not a marker of an AADC. GMDC were treated overnight with IL-4 and units of Arginase activity were quantified (A+B) The Arginase inhibitor BEC was unable to limit DC Arginase activity at a range of doses (0-100 μM) (C). Data is representative of >5 experiments (B) and 1 experiment (C) carried out in triplicate.

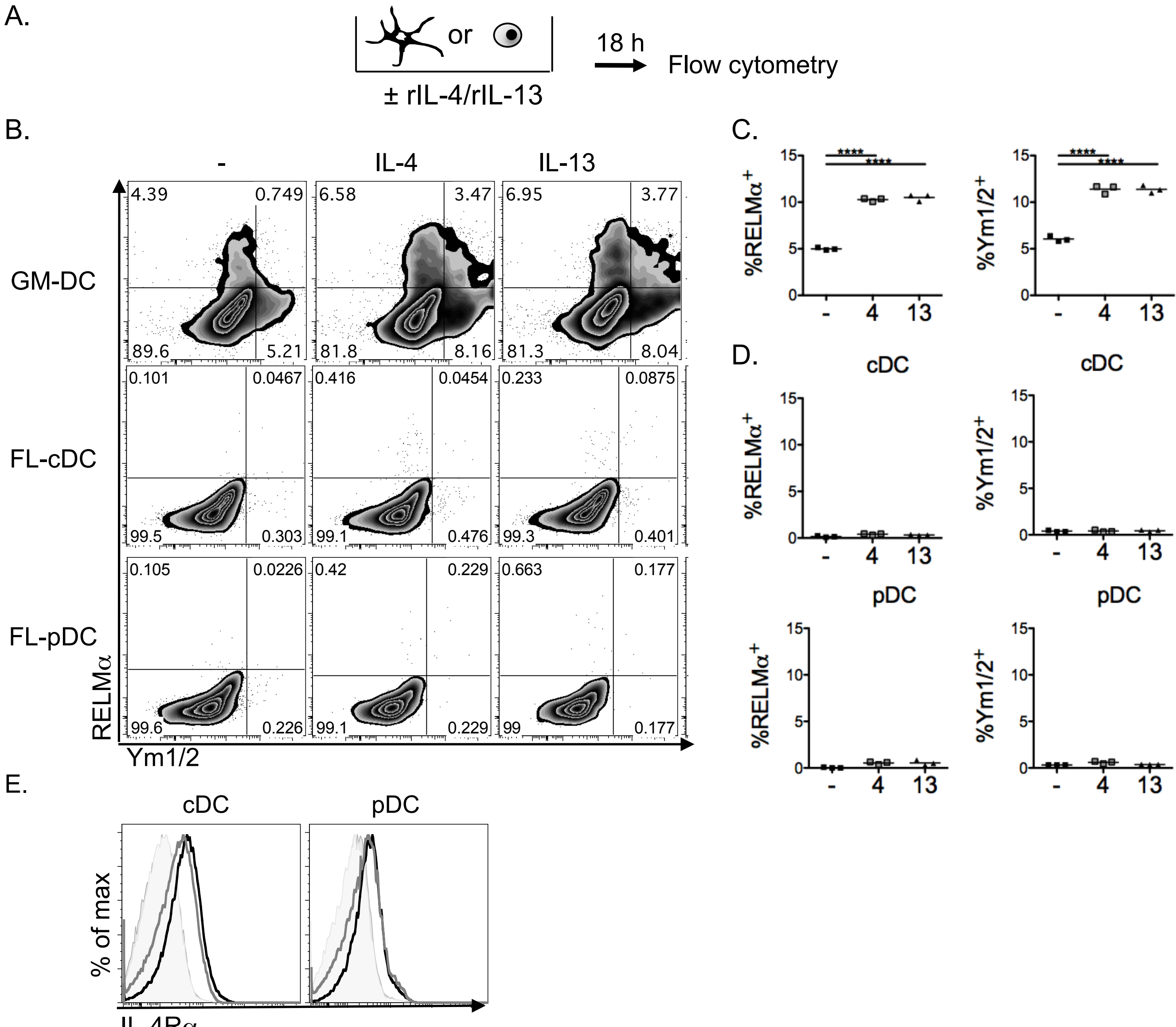


Figure 3.6 FLDCs do not become alternatively activated in response to IL-4. Bone marrow derived DCs were differentiated in the presence of GM-CSF (GMDC) or FLT3L (FLDC) prior to overnight culture with IL-4 or IL-13 (A-D) RELMα and Ym1/2 were significantly up-regulated by IL-4 and IL-13 in GMDCs (B+C), neither IL-4 or IL-13 upregulated pDC or cDC expression of RELMα or Ym1/2 in FLDC cultures (D). Both cDC and pDC from FLDC cultures express the IL-4Rα (E) shaded=isotype control, black line=media, grey line=+IL-4. Data shown are representative of 3 independent experiments carried out in triplicate *=P<0.05, **P<0.01, ***P<0.001.

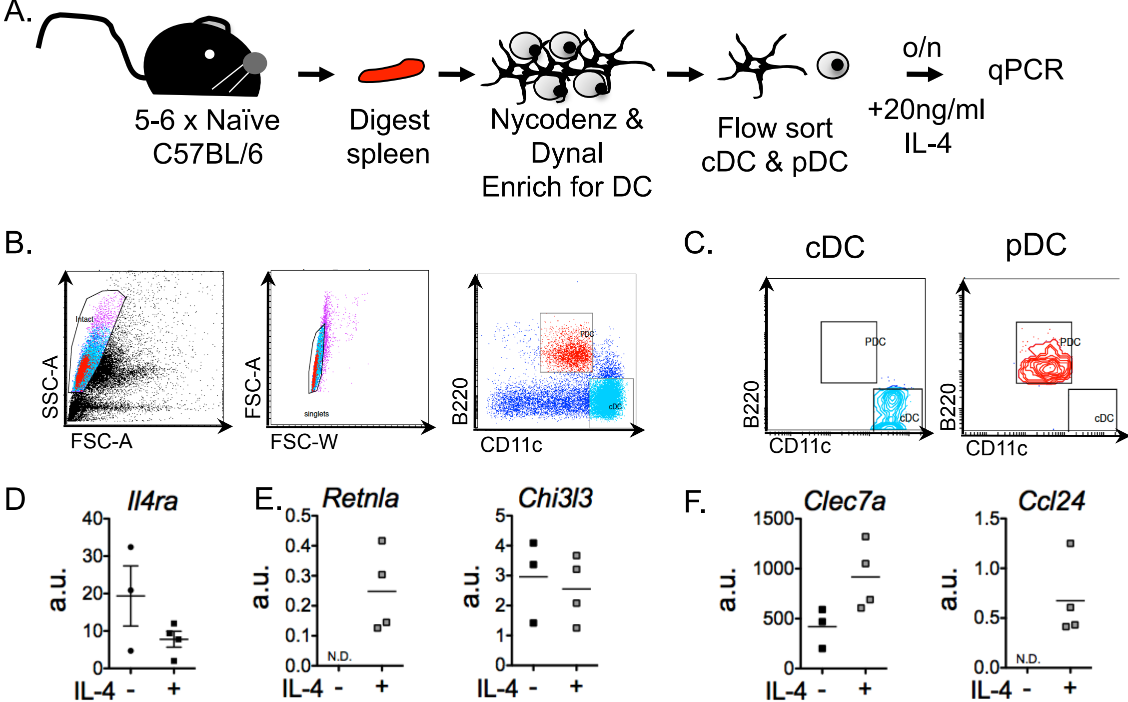


Figure 3.7. IL-4 alternatively activates splenic *ex vivo* DCs *in vitro*. Following enrichment for DC populations, splenic cDCs ($CD11c^{hi}B220^{-}$) from naïve mice were separated from pDCs ($B220^{+}CD11c^{mid}$) (B-C) and cultured overnight in the presence of 20ng/ml IL-4. IL-4 increased mRNA expression of alternative activation markers (D-F). Data representative of 2 experiments, 3-4 replicate wells per group, a.u.=arbitrary units, N.D.= not detected.

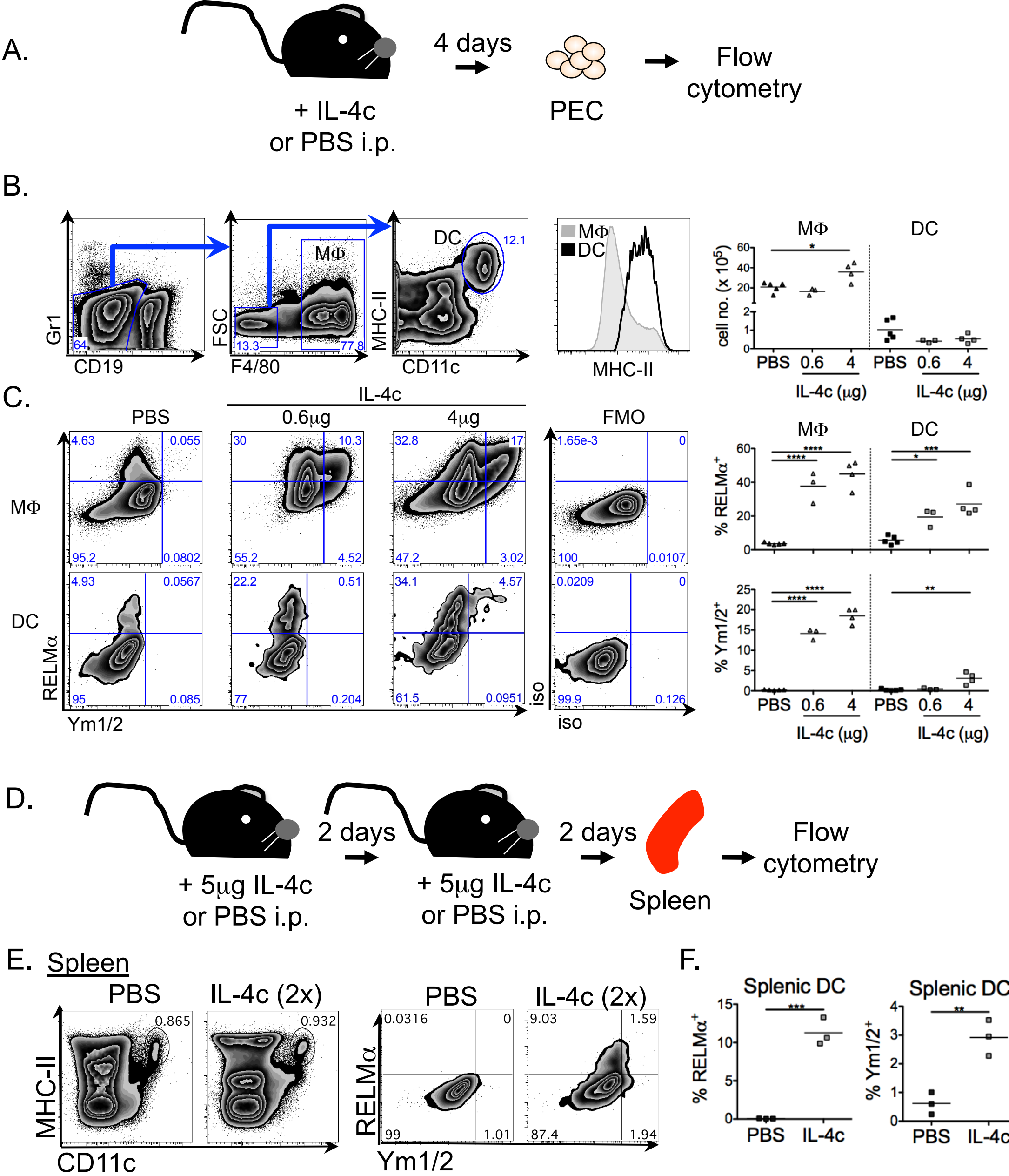


Figure 3.8 IL-4 alternatively activates DCs *in vivo*. C57BL/6 animals were injected i.p. with 50 μl PBS or IL-4c (0.6 or 4 μg), peritoneal exudate cells (PEC) were harvested 4 days later (A). Relative abundance of Macrophage (MΦ) and DC populations in peritoneal exudate cells (PEC) from PBS or IL-4c treated mice was assessed by flow cytometry (B). MΦ and DC populations as defined in (B) stained for intracellular expression of RELMα and Ym1/2 (C). Splenic DCs (MHC-II^{+} CD11c^{hi}) from PBS or IL-4c treated mice (2 x 5 μg, d0 and d2) were harvested at d4 and stained for intracellular expression of RELMα and Ym1/2 (D-F). Data are representative of 5 (B-C) or 3 (D-F) independent experiments, graphs show % expression for individual mice. FMO = Fluorescence minus one control. Error bars indicate SEM of 3-5 mice per group. *=P<0.05 **=P<0.01 ***= P<0.001 ****P<0.0001.

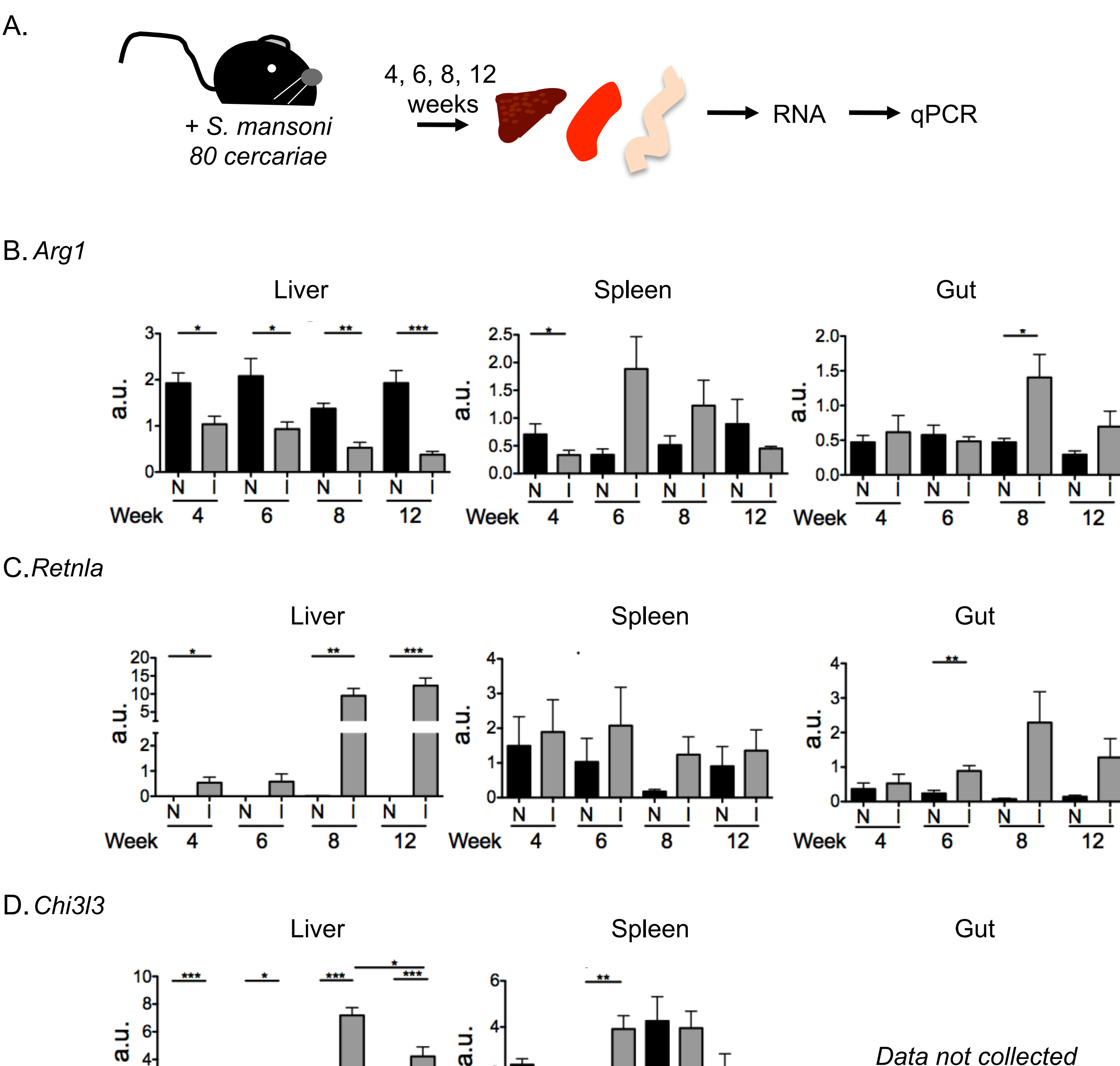
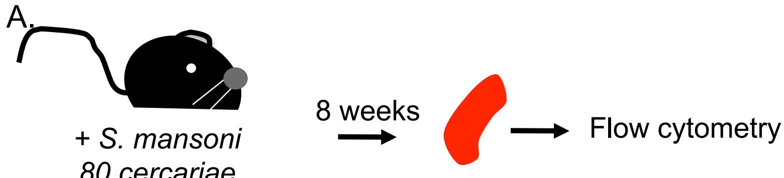


Figure 3.9 Markers of alternative activation are upregulated following *S .mansoni* infection. Whole tissue sections from Liver, Spleen, and Gut of *S. mansoni* infected mice were harvested at 4, 6, 8 and 12 weeks post infection, RNA was extracted, reverse transcribed and qPCR was carried out (A) to detect *Arg1* (B), *Retnla* (C) and *Chi3l3* (D). Data are representative of 2 experiments, error bars represent SEM of 5-8 mice per group. *=P<0.05, **P<0.01, ***P<0.001.



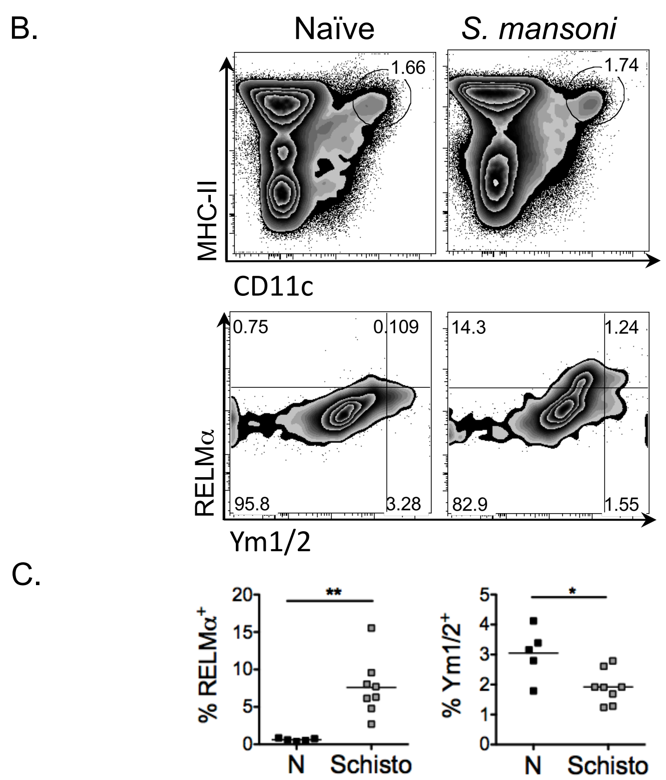


Figure 3.10. Parasitic helminth infection induces alternative activation of Splenic DCs. Splenic DCs (MHC-II^{+}CD11c^{hi}) from naïve and *S. mansoni* (Schisto) infected mice were stained for intracellular expression of RELMα and Ym1/2 (A-C). Data is representative of 3-6 independent experiments, graphs show % expression for individual mice, 5-8 per group. *=P<0.05 **= P<0.01.

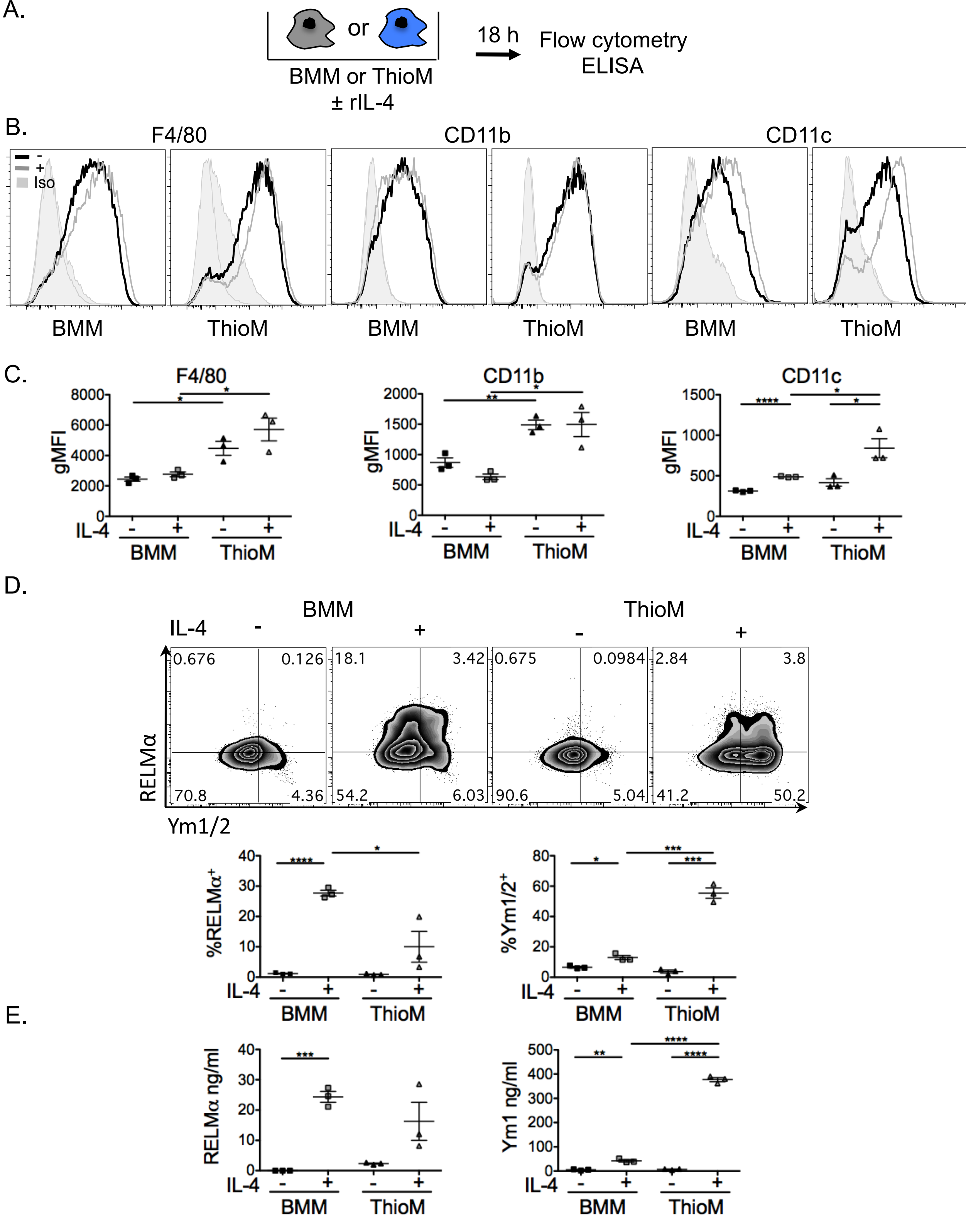


Figure 3.11 BMM produce more RELMα than Ym1/2, the opposite is true of ThioM. BM derived macrophages were differentiated *in vitro* with M-CSF (BMM) or elicited from the peritoneal cavity following injection of Thioglycollate (ThioM), cells were cultured overnight with IL-4 or media (A). Surface expression of F4/80, CD11b and CD11c was quantified by flow cytometry (B+C) and alternative activation by intracelluar RELMα and Ym1/2 staining and ELISA (D+E). Data is representative of 2 independent experiments carried out in triplicate. *=P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

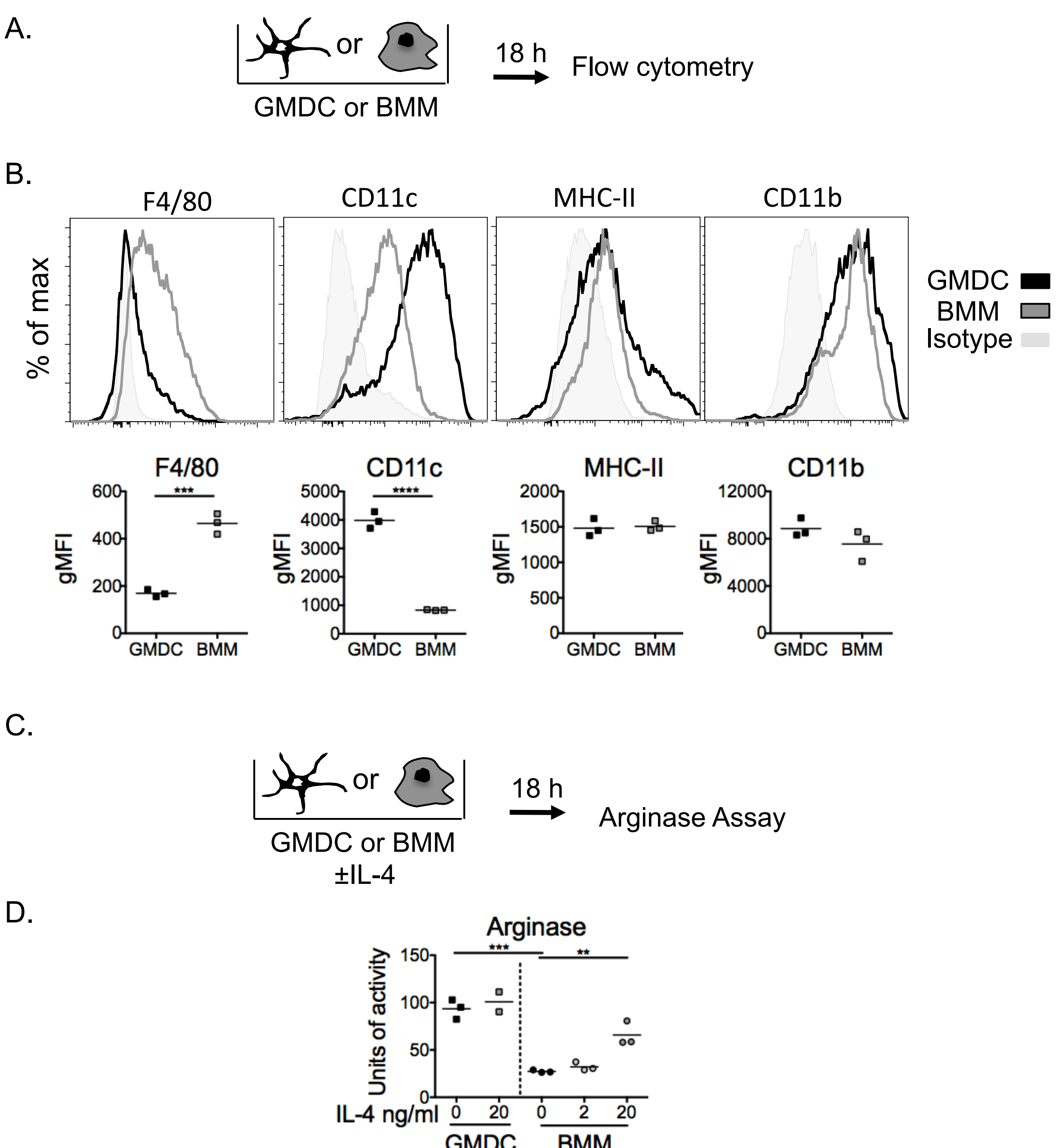


Figure 3.12 BMDC and BMM are different cells types. BM was differentiated with either GM-CSF (BMDC) or M-CSF (BMM) and expression of surface markers was assessed via flow cytometry (A&B) Following overnight culture with IL-4 an Arginase activity assay was carried out (C&D). Data is representative of 3 (B) or 1 (C&D) independent experiments, carried out in triplicate **P<0.01.

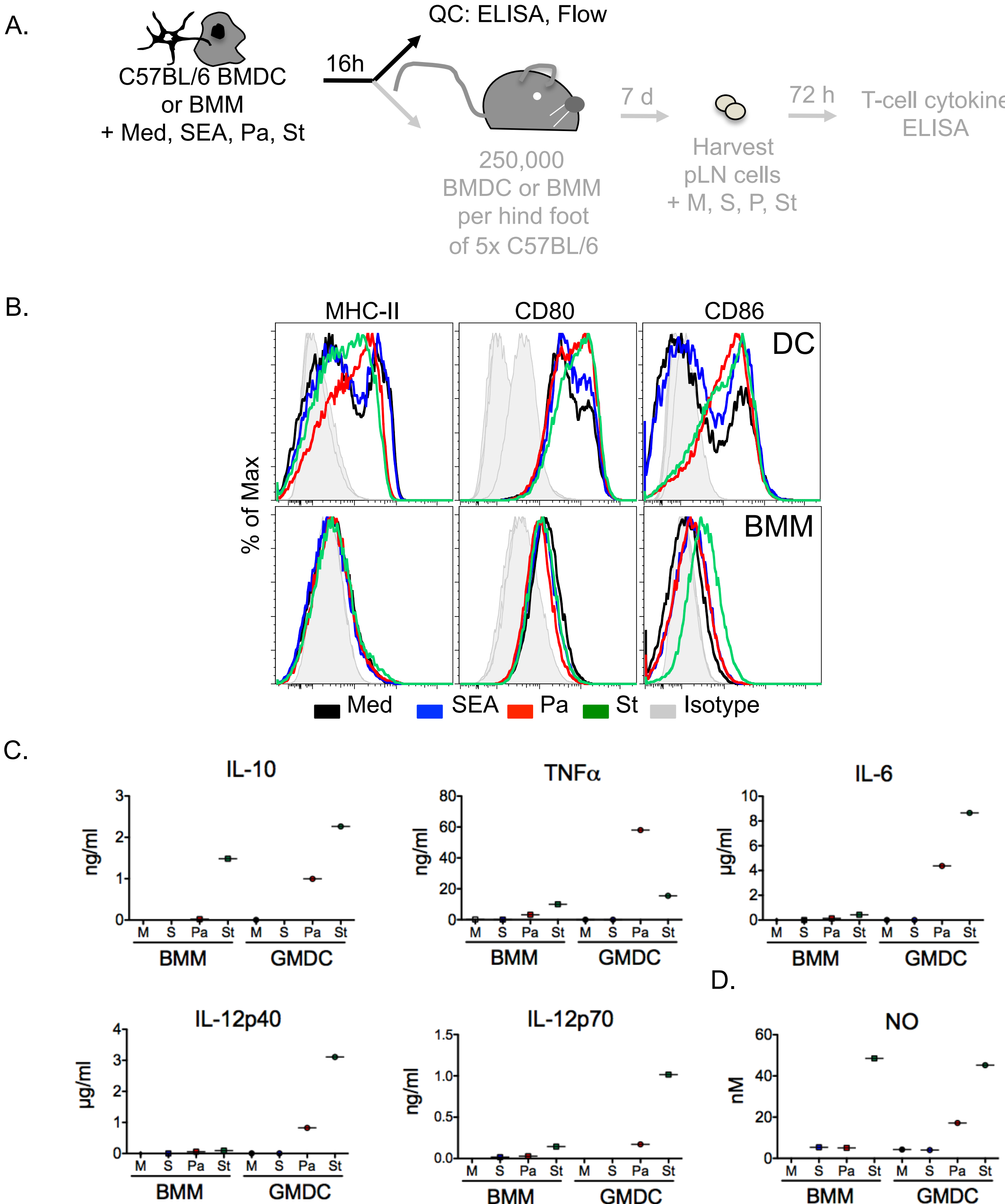


Figure 3.13 GMDC express higher levels of co-stimulatory molecules and secrete more cytokine than BMM. WT BMM or BMDCs were cultured overnight in medium alone (M), SEA (S), *P. acnes* (Pa) or *S. typhimurium* (St), prior to transfer into *naïve* recipients (A) Co-stimulatory molecule expression (B), cytokine secretion (C) and nitric oxide production (D) was assessed pre transfer. Data are representative of 2 independent experiments, with single culture wells.

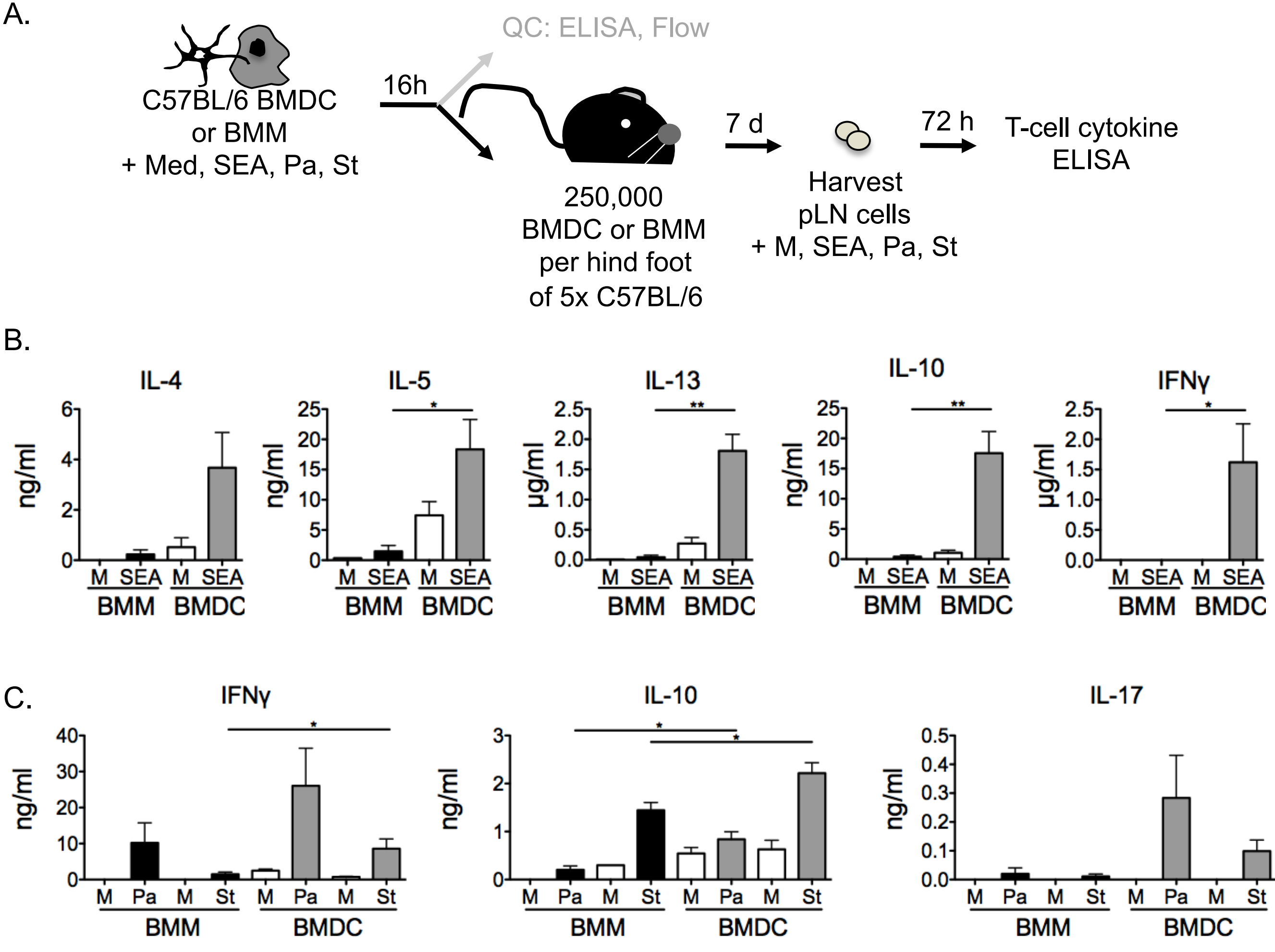


Figure 3.14 GMDC are significantly better than BMM at priming both Th1 and Th2 responses *in vivo* . WT BMM or BMDCs were cultured overnight in medium alone (M), SEA, P. acnes (Pa) or S. typhimurium (St), and injected subcutaneously into WT mice, 7d later pLN were harvested, cells restimulated for 72h with M, SEA, Pa or St and cytokine secretion was assessed by ELISA (A). Cytokines produced following restimulation with S (B), Pa or St (C) was assessed by ELISA. Data is representative of one independent experiment, error bars = SEM of 3-5 mice per group, *=P<0.05 **= P<0.01.

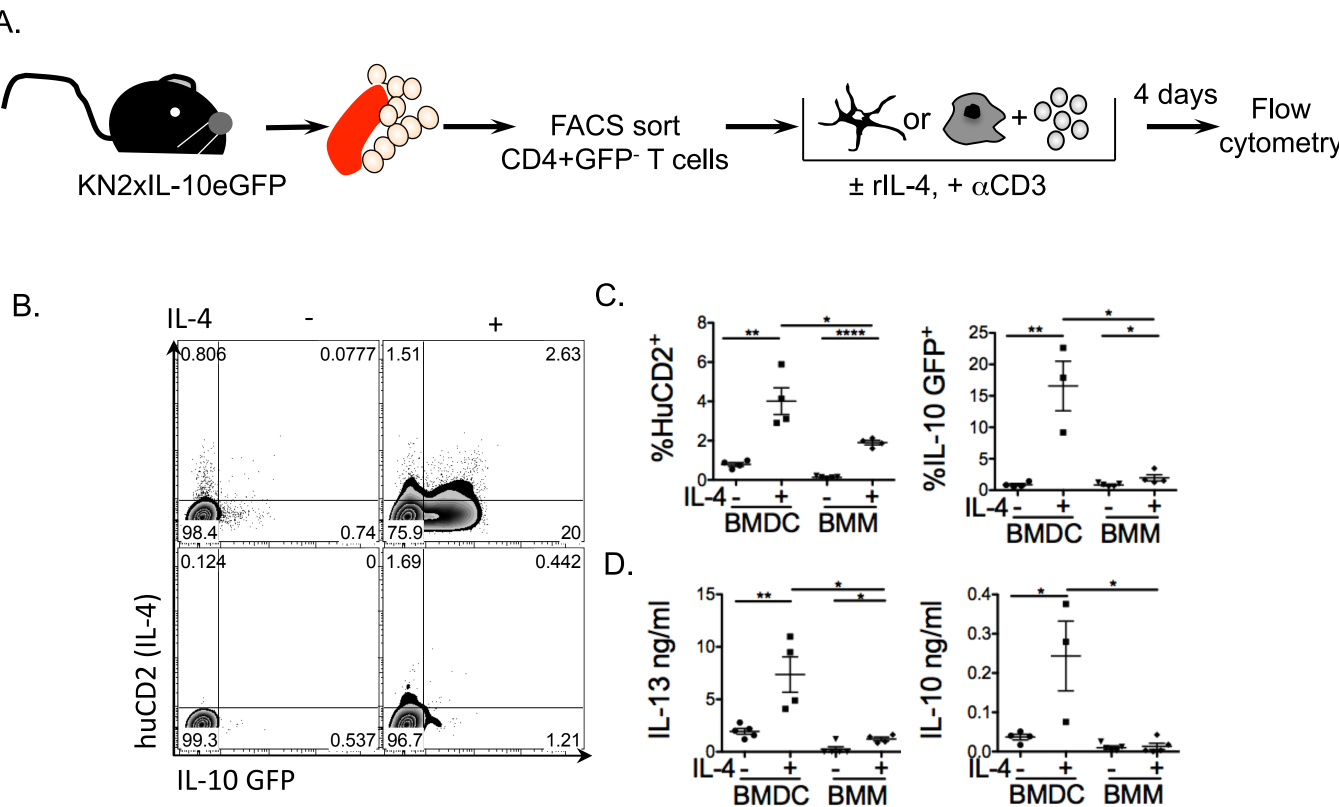


Figure 3.15 BMM are less competent than GMDC at polarising CD4⁺ T-cells towards Th2 *in vitro.* CD4⁺ cells sorted from KN2xIL-10eGFP mice (IL-4/IL-10 dual reporters) were cultured for 4 days with GMDCs or BMM and agonistic anti-CD3 antibody with or without IL-4 (A). Cells were assessed for IL-4 protein production and IL-10 mRNA production by flow cytometry (B+C) and cytokine secretion by ELISA (D). Data are representative of 2 experiments, error bars represent SEM of 3- 5 replicate wells per group, *=P<0.05 **= P<0.01 ****P<0.0001.

Chapter 4. What is the Impact of IL-4 and Alternative Activation on DC Function?

4.1 INTRODUCTION

Having confirmed that DCs can be alternatively activated both *in vitro, ex vivo* and *in vivo* we wanted to determine how such alternative activation might alter the ability of DCs to respond to antigenic stimuli, and to function as APCs.

DCs are professional antigen presenting cells, able to migrate and activate naïve T-cells (Geissmann, Gordon *et al.* 2010; Steinman 2012), as shown in the previous chapter, GMDC are able to do this efficiently, whereas BMM were less capable (Fig. 3.14&3.15). In their role as APCs, DC expression of cell polarising factors such as cytokines and chemokines is very important in determining not only the outcome of T-cell priming but also in defining the amplitude and duration of an ongoing immune response. We showed in chapter 3 of this thesis that in response to the Th2 cytokine IL-4, DC isolated from the spleen or peritoneal cavity and differentiated from bone marrow are able to produce high levels of RELMα, and in situations of high dose IL-4, the chitinase like molecule Ym1/2. RELMα production by macrophages has previously been suggested to function to limit Th2 cytokine production (Nair, Du *et al.* 2009; Pesce, Ramalingam *et al.* 2009), as animals deficient in *Retnla* had enhanced T-cell production of IL-4, IL-5 and IL-13 in *S. mansoni* egg induced models of pulmonary granuloma. However other studies have shown that rRELMα can increase IL-4 expression in the mouse lung and the authors propose that RELMα can act as an amplifier of Th2 cytokines (Yamaji-Kegan, Su *et al.* 2010). DC production of Ym1/2 has been reported to enhance Th2 cytokine secretion (Arora, Chen *et al.* 2006; Cai, Kumar *et al.* 2009).

The contrasting and potentially confusing roles for these alternative activation products in modulating the immune response warrant further investigation. The reports investigating RELMα do not investigate expression of this protein in DCs, we feel that this is an area that needs to be addressed more fully, on account of the high level of RELMα that was detected in all IL-4 exposed DC populations assessed in chapter 3, and given the importance of DCs in directing the immune response (Steinman 2012).

In a normal *in vivo* situation, where a DC is continually sampling the environment, it is likely to come into contact with a mixed antigenic and cytokine milieu. Unless an animal has come upon their very first pathogenic antigen, the activation of DCs in response to antigen is going to occur in the context of an already established immune environment. For example, in places in which helminth infection is endemic, such as schistosomiasis in sub-saharan Africa (Wilson, Mentink-Kane *et al.* 2007), young children (who have not yet developed immunity to this parasite) are likely to have an ongoing Th2 polarised immune response, should they come into contact with a Th1 driving antigen, such as a bacterial or viral infection, they need to be able to mount an appropriate response (Kamal and El Sayed Khalifa 2006). Thus, it is important to investigate how DCs deal with simultaneous exposure to Th1 polarising antigen in the context of Th2 cytokines such as IL-4.

In the work detailed in this chapter, we aimed to build upon some older reports in the literature that had identified some aspects of the impact of IL-4 on DC cytokine production *in vitro*. Although one theme emerged from these studies – somewhat surprisingly, IL-4 enhancement of DC IL-12p70 production – the authors of this earlier work only speculated on what this might mean functionally (Hochrein, O'Keeffe *et al.* 2000; Kalinski, Smits *et al.* 2000; Guenova, Volz *et al.* 2008). The intention of the work presented in this chapter was to more deeply interrogate the role for DC IL-4 responsiveness in the activation of both Th2 and Th1 immune responses, a facet of DC function that had not been exhaustively investigated *in vivo* (Hochrein, O'Keeffe *et al.* 2000; Biedermann, Zimmermann *et al.* 2001; King, Mueller Hoenger *et al.* 2001). To complement such studies, experiments were also carried out to identify the impact of IL-4 on the response of DC to antigen exposure, as well as investigate the involvement of specific products of AADCs, produced downstream of IL-4R engagement, in naïve T-cell priming.

As AADC have not previously been carefully characterised, the functional significance of DC alternative activation with regard to immune system priming has not been investigated fully. Two previous publications have investigated the role of DC expression of Ym1/2 in the promotion of Th2 responses in the context of allergy (Cai, Kumar *et al.* 2009) and following treatment with a statin (Arora, Chen *et al.* 2006). However a functional role for RELMα in DCs has not previously been addressed.

4.2 SPECIFIC AIMS

1. To determine to what extent IL-4 alters the response of DC to polarisng antigenic stimuli.
2. To investigate the broad requirements for DC IL-4 responsiveness in their ability to prime both Th1 and Th2 responses.
3. To identify the specific role for DC expression of the alternative activation associated molecule RELMα in their ability to polarise $CD4^+$ T cells.

4.3 RESULTS

4.3.1 TLR ligation upregulates DC co-stimulatory molecule expression

We wanted to further investigate the impact of IL-4 on DC function, to assess whether the presence of IL-4 is able to modulate the ability of DCs to perform their function as APCs, and whether IL-4 is able to modulate the response of DCs to antigen preparations from pathogens. To do this, we initially chose to assess the changes in DC phenotype upon exposure to well defined TLR ligands (CpG/TLR9 or LPS/TLR4) in combination with IL-4. In their role as professional APC, the expression of co-stimulatory molecules and secretion of T-cell polarising cytokines are major functional requirements of a DC, at least in the case of Th1 and Th17 polarisation (Ouyang, Ranganath *et al.* 1998; Kapsenberg 2003; Harrington, Hatton *et al.* 2005; Zhou, Chong *et al.* 2009). DCs were cultured overnight in the presence of the bacterial TLR ligands CpG and LPS with and without addition of IL-4 and co-stimulatory molecule expression was assessed by flow cytometry (Fig. 4.1A). TLR9 or TLR4 ligation did not significantly enhance expression of MHC-II above the medium control samples (Fig. 4.1B&C) but did significantly increase expression of CD80, CD86 and CD40 (Fig. 4.1B&D-F). As shown in Fig. 3.1, IL-4 significantly decreased CD80 expression on control DCs (Fig. 4.1D), but the impact of IL-4 on MHC-II was not significant in this experiment (Fig. 4.1C), unlike in Fig. 3.1. The only influence that IL-4 had on the ability of TLR ligation to upregulate CD80, CD86 and CD40, was a subtle but significant enhancement of the ability of LPS to upregulate CD86 (Fig. 4.1E).

4.3.2 Concurrent exposure to IL-4 at the time of TLR ligation boosts IL-12 and reduces IL-10 secretion by DCs

It has been previously reported that concurrent IL-4 and LPS or CpG treatment of murine splenic DCs *ex vivo* results in enhanced production of bioactive

IL-12 heterodimers (IL-12p70) and reduced antagonistic IL-12p40 homodimers (Mattner, Fischer *et al.* 1993) suggesting that the Th2 polarising cytokine IL-4 is involved in a negative feedback loop whereby its presence increases the levels of the Th1 promoting cytokine IL-12p70 (Hochrein, O'Keeffe *et al.* 2000). Such a feedback mechanism would make sense in a co-infection setting, as would often occur in nature, in which a host is exposed to a Th1 polarising infection such as a bacterium in the context of an ongoing Th2 response, for example to a helminth. To ascertain whether, in our hands, GMDCs also respond to LPS/CpG and IL-4 in a similar manner to that reported for splenic DCs (Hochrein, O'Keeffe *et al.* 2000), we cultured GMDCs overnight in the presence of IL-4 and either LPS or CpG and collected the supernatants for ELISA analysis (Fig. 4.2A). The addition of IL-4 to the cultures significantly enhanced IL-12p70 secretion in response to CpG and LPS above the levels elicited by either antigen in the absence of IL-4 (Fig. 4.2B). As was previously reported for splenic DCs, IL-4 addition significantly impaired DC secretion of IL-12p40 in response to the TLR9 agonist CpG, whereas only a trend towards decreased secretion was apparent in the case of LPS and IL-4 (Fig. 4.2C). The LPS and CpG induced secretion of the regulatory cytokine IL-10 was decreased by the addition of IL-4 (Fig. 4.2D), lending further credence to the idea that, when in the presence of a strong bacterial stimulus such as LPS or CpG, IL-4 functions to promote a Th1 polarising environment (Kalinski, Smits *et al.* 2000). The pro-inflammatory cytokines TNFα and IL-6 were not significantly affected by the presence of IL-4 at the time of either TLR9 or TLR4 ligation (Fig. 4.2E).

4.3.3 TLR agonists limit IL-4 induced alternative activation

Having confirmed in chapter 3 that IL-4 was able to alternatively activate DCs, and in Fig. 4.2 that TLR ligation concurrent with IL-4 treatment modulated conventional DC cytokine production, we thought it would be prudent to investigate the impact that TLR ligation has on IL-4 induction of AADC, given the immune modulatory functions previously reported for RELMα (Yamaji-Kegan, Su *et al.* 2006; Nair, Du *et al.* 2009; Pesce, Ramalingam *et al.* 2009; Yamaji-Kegan, Su *et al.* 2010) and Ym1/2 (Arora, Chen *et al.* 2006; Cai, Kumar *et al.* 2009). In order to determine the impact of TLR9 or TLR4 ligation on IL-4 driven GMDC alternative activation, we detected RELMα and Ym1/2 protein by intracellular staining and ELISA following overnight culture with the defined ligands CpG and LPS, alone or with the addition of IL-4 (Fig. 4.3A). TLR9 ligation with CpG DNA was able to significantly reduce IL-4

driven RELMα and Ym1/2 protein, however, this inhibition was not as complete as that seen with LPS, as substantial amounts of intracellular protein were still upregulated by IL-4 even in the presence of CpG (Fig. 4.3B-D). The presence of LPS dramatically abrogated IL-4 induced RELMα both within the DCs (Fig. 4.3B&C) and secreted by the DCs over the 18 hour time point (Fig. 4.3D). However, LPS did not completely impede basal secretion of RELMα as ~0.5ng/ml was detectable within the supernatant in both the media and LPS treatment groups (Fig. 4.3D). LPS also abrogated intracellular IL-4 driven Ym1/2 protein (Fig. 4.3B&C), although ~0.1μg/ml of IL-4 driven Ym1/2 was detectable by ELISA (Fig. 4.3D).

To assess more broadly the activation status of DCs following either LPS+IL-4 or CpG+IL-4 treatment, qPCR was carried out on a panel of alternative activation associated markers (Fig. 4.3E&F). Shown as arbitrary units relative to the housekeeping gene *Hprt*, inclusion of all genes on the same axis allows a global view of the activation state of the DCs. The addition of CpG (E; striped bars) or LPS (F; striped bars) reduced transcript levels of *Retnla* below the basal level for media only. IL-4 driven *Chi3l3* transcripts were decreased by the presence of both agonists. However, in a similar pattern to protein production, an IL-4 driven response was still apparent. As shown in chapter 3 (Fig. 3.4) *Arg1* was not enhanced by IL-4 and neither CpG nor LPS significantly affected the transcript for this arginine converting enzyme (Fig. 4.3E). *Nos2*, which encodes the enzyme iNOS, competes with arginase for the shared substrate arginine (Modolell, Corraliza *et al.* 1995). As expected, *Nos2* mRNA expression was stimulated by either TLR agonist (Fig. 4.3E&F), and this was not significantly altered by the addition of IL-4. Expression of the CLR *Mrc1* (MR) was almost completely abrogated by the presence of LPS or CpG even in the presence of IL-4, whereas *Clec7a* (Dectin-1), though reduced by the presence of CpG, was still significantly upregulated by addition of IL-4 (Fig. 4.3E), however IL-4 was unable to overcome the LPS inhibition of *Clec7a* (Fig. 4.3F). Neither LPS nor CpG significantly altered IL-4 induced upregulation of the eosinophil attractant *Ccl24* (Eotaxin2). Interestingly, expression of both *Ccl17* and *Ccl22* (MDC) was highest when CpG and IL-4 were simultaneously provided to the DCs; in macrophages a similar outcome has previously been shown in a microarray analysis of BMM or ThioM primed by IL-4 for 18h and then stimulated with LPS (Major, Fletcher *et al.* 2002; El Chartouni and Rehli 2010) however in this work, addition of LPS and IL-4 simultaneously did not

significantly upregulate *Ccl17* or *Ccl22* over the level induced by either stimulus alone (Fig. 4.3E). The data in this figure prove to highlight that similar to the ability of IL-4 to modulate TLR driven cytokine production in Fig. 4.2, TLR ligation can modulate IL-4 driven responses in DCs.

4.3.4 Complex bacterial, but not helminth antigens modulate DC co-stimulatory molecule expression and cytokine production in a manner similar to defined TLR ligands

In order to determine whether the response of GMDCs to defined TLR ligands could be mirrored with complex antigens more akin to what the innate immune system encounters in reality, we compared the effects of culturing DCs with both IL-4 and the Th1 polarising gram positive bacterium *P. acnes* (Pa) or the Th2 polarising soluble egg antigen (SEA) from *S. mansoni* (Fig. 4.3A). Following overnight culture with Pa, DCs significantly upregulated expression of MHC-II, CD86 and CD40, and IL-4 did not alter such upregulation (Fig. 4.4B&C). As previously reported (MacDonald, Straw *et al.* 2001), SEA did not significantly increase DC expression of MHC-II, CD80, CD86 or CD40 and inclusion of IL-4 in the culture did not change this outcome (Fig. 4.4B&C). Pa strongly induced GMDC secretion of IL-12p70 and IL-10 following overnight culture (Fig. 4.4D) and addition of IL-4 to the culture significantly enhanced secretion of IL-12p70 while reducing secretion of IL-10. As previously reported SEA did not stimulate GMDC to secrete the T-cell polarising cytokines IL-12 and IL-10 (MacDonald, Straw *et al.* 2001), and the inclusion of IL-4 did not alter this (Fig. 4.4D).

4.3.5 Complex bacterial and helminth antigens modulate DC alternative activation in a manner similar to defined TLR ligands

The secretion (Fig. 4.5B), intracellular production (Fig. 4.5C) and transcription (Fig. 4.5D) of alternative activation products by IL-4 and antigen exposed DCs was also assessed. Pa was found to abrogate secretion of both IL-4 induced RELMα and Ym1/2 whereas SEA displayed little inhibitory impact in this regard upon Ym1/2, but significantly inhibited IL-4 driven RELMα (Fig. 4.5B-D). The differential inhibition of SEA on RELMα production compared to Ym1/2 suggests that a component of this antigenic mix is able to modulate the IL-4R signalling pathway after the point at which induction of RELMα and Ym1/2 diverges.

4.3.6 IL-4 alters the response of DCs to CD40 ligation

Having determined that IL-4 alters the production of both alternative activation markers and T-cell polarising cytokines, we decided to investigate how such changes may alter the interaction of DCs with T-cells, both in terms of changes to the DC themselves and to the polarisation of the T-cells. To initially assess this, we chose to ligate the co-stimulatory molecule CD40 on the DC surface using agonistic monoclonal antibodies (Jenkins, Perona-Wright *et al.* 2007). DCs express CD40 and interact with CD154 on the surface of T-cells. This interaction enhances DC cytokine production, an important mechanism in the induction of Th1, Th2 and Th17 responses (Elgueta, Benson *et al.* 2009; Perona-Wright, Jenkins *et al.* 2009). GMDCs were cultured overnight with medium, Pa or SEA in the presence or absence of IL-4, then washed and re-cultured with anti-CD40 antibody for a further 24 hours. Anti-CD40 ligation of DC following treatment with Pa enhanced secretion of IL-12, IL-10 and IL-6 compared to cells treated with an isotype control antibody that did not ligate CD40 (Fig. 4.6B). Pre-culture with Pa and IL-4 resulted in enhanced IL-12p40, IL-12p70 and IL-6 secretion following αCD40 treatment when compared to Pa treatment alone (Fig. 4.6B). There was also a trend for reduced IL-10, however this did not reach significance. Neither RELMα or Ym1/2 secretion by the DCs was altered by αCD40 ligation (Fig. 4.6C). Interestingly, the DCs continued to secrete both RELMα and Ym1/2 following removal of IL-4 from the culture supernatant (Fig. 4.6C). Prolonged secretion of alternative activation products in the absence of IL-4 stimulus suggests that DCs *in vivo* would be able to migrate away from the proximal site of IL-4 exposure to alter the immune response at distal tissues or LNs.

4.3.7 IL-4Rα$^{-/-}$ DCs are less able to induce IFNγ and IL-17 responses

Having determined that DC exposure to IL-4 triggers production or expression of a wide range of molecules, and alters their response following CD40 ligation, we next wanted to ascertain what impact IL-4 signalling to DCs had on their ability to polarise either Th1 or Th2 immune responses. As presented in chapter 3, Pa and SEA pulsed DCs efficiently prime Th1/Th17 and Th2 responses, respectively, following adoptive transfer *in vivo* (Fig. 3.14). We initially hoped to determine whether culture with IL-4 prior to transfer would alter the resultant immune response in the dLN of recipient mice. However, there was a lot of variation in the immune response following transfer of IL-4 treated DCs, suggesting

that the impact of IL-4 *in vitro* may be too transient to detect a consistent impact of this exposure on DC function *in vivo.* As an alternative approach to the question of how IL-4 alters DC APC function, we instead chose to assess the polarising ability of DCs unable to receive an IL-4 signal following transfer *in vivo*. IL-4 signals via the IL-4Rα, so we used *Il4ra*$^{-/-}$ bone marrow to generate GMDCs and compared their ability to prime T-cell responses with DCs grown from WT bone marrow. WT or IL-4Rα deficient GMDCs were cultured overnight with media, Pa or SEA. DCs were then transferred into the feet of WT naïve recipients, the pre-transfer supernatants were assessed by ELISA; following transfer cells were left for 7 days, the draining popliteal LN were harvested, and the cells re-stimulated for 72h (Fig. 4.7A). Prior to transfer, Pa stimulated IL-4Rα deficient DCs had significantly increased IL-12p40 and significantly reduced IL-10 secretion but had no difference in the levels of Pa induced pro-inflammatory molecules IL-12p70, NO, TNFα or IL-6 (Fig. 4.7B). Interestingly, upon transfer, the Pa stimulated IL-4Rα deficient DCs were less capable of priming IL-17 and IFNγ T-cell responses than their WT counterparts (Fig. 4.7C), suggesting that in a WT situation, receipt of an IL-4 signal in the context of a bacterial pathogen drives a feedback loop to enhance Th1/Th17 cytokine production, even in the absence of high levels of Th2 cytokine. It was interesting to note that Pa pulsed DCs also induced IL-13 production upon restimulation, however no significant difference in IL-13 was found between WT and IL-4Rα deficient cells (Fig. 4.7C). IL-4Rα deficient DCs were also significantly less competent at inducing SEA driven IL-10 and IFNγ than their WT counterparts but had no defect in induction of the Th2 cytokines IL-4 or IL-13. This data shows that IL-4Rα expression by DCs is not an absolute requirement for Th2 induction in response to helminth antigen. Moreover, it reveals that IL-4 responsiveness is necessary for optimal DC polarisation of T cells in Th2 or Th1/17 settings via fine-tuning of IL-10, IL-17 and IFNγ production (Fig. 4.7C&D).

4.3.8 DCs unable to respond to IL-4 are less able to support early Th2 polarisation *in vitro*

The DC transfer model provides an important readout of *in vivo* priming ability. However, following transfer it is hard to determine which precise cell types are involved. As a more reductionist and controllable complementary approach, we set up *in vitro* DC:T-cell co-culture assays incorporating huCD2 (IL-4) reporter T-cells and DCs either sufficient or deficient in IL-4Rα expression, to determine if the

DC response to IL-4 changed their capacity to polarise T-cells. Anti-CD3 was used to stimulate T-cells in the presence of DCs, with or without addition of exogenous recombinant IL-4 (Fig. 4.8A), this method was modified from a study in which TGFβ was used (in place of IL-4) to convert αCD3 stimulated $CD4^{+}FoxP3^{-}$ T-cells (in place of huCD2 reporters) to a Treg phenotype in the presence of DCs (Travis, Reizis *et al.* 2007). Following 3 days of culture, IL-4 induced increased expression of huCD2 by reporter T-cells cultured in the presence of WT DCs (Fig. 4.8B&C). By day 3 of culture, there was a significantly reduced induction of huCD2 (IL-4 protein) in T cells cultured with DCs lacking IL-4Rα expression (Fig. 4.8B&C). This reduced Th2 polarisation was also evidenced by reduced IL-10 and IL-13 protein secretion (Fig. 4.8D). There was no significant difference in the levels of IFNγ secreted by any of the groups (Fig. 4.8E), however, the level of IFNγ was very low as there was no antigenic stimulus to promote IFNγ cytokine secretion, unlike in the SEA/Pa stimulated *Il4ra*$^{-/-}$ transfer (Fig. 4.7). This system allows conclusions to be drawn as to the direct versus indirect effects of IL-4 on T-cell activation. In the absence of DC IL-4Rα expression, recombinant IL-4 is able to induce IL-4 protein expression by T-cells, though at a reduced level. In conclusion, early IL-4 driven Th2 polarisation is partially dependent upon provision of an unknown signal provided by IL-4 responsive DCs. IL-4 signalling to the IL-4Rα changes the gene expression profile of DCs in multiple ways, including (but not limited to) the initiation of a programme of alternative activation, we were interested to determine what signal was being produced by IL-4 responsive DCs to induce Th2 polarisation and whether alternative activation products played a role in such DC dependent T-cell polarisation.

4.3.9 DC expression of RELMα during Th2 priming regulates IFNγ and promotes IL-10 and IL-13 production

Using IL-4Rα deficient cells enabled us to determine the global changes that occur in the absence of complete IL-4 signalling, one facet of which is alternative activation. As we were predominantly interested in the functional impact of DC alternative activation rather than the large scale changes that occur following IL-4 treatment, we decided to use cells deficient in one dominant aspect of the alternative activation profile, RELMα (Fig. 3.2-4&3.7-10). We adoptively transferred WT or *Retnla*$^{-/-}$ GMDCs into WT recipients, and assesed their ability to prime Th1/Th17 (Pa) or Th2 (SEA) immune responses (Fig. 4.9A). In contrast to the result found with IL-4Rα deficient DCs, there was no defect in Th1/Th17 induction

following transfer of Pa pulsed *Retnla*$^{-/-}$ DCs (Fig. 4.9B). There was, however, a significant role for DC derived RELMα in the induction of IL-10 and IL-13 and in the regulation of IFNγ in the context of the Th2 polarising antigen SEA (Fig. 4.9C). IL-10 and IL-13 were both significantly reduced when the priming DCs could not produce RELMα, whereas IFNγ was enhanced. We saw a different outcome in terms of immune response induction and polarisation when the priming DC could not become alternatively activated on account of having no IL-4Rα (decreased Pa specific IL-17 and IFNγ, decreased SEA specific IL-10 and IFNγ) than when the DCs were deficient only in RELMα (decreased SEA specific IL-10, IL-13 and enhanced IFNγ). These experiments suggest that IL-4 causes greater changes to the functional antigen presenting and T-cell polarising capability of a DC than induction of RELMα expression.

4.3.10 DC derived RELMα promotes IL-4 driven IL-10, and inhibits T-cell IL-4

In order to investigate specifically whether DC alternative activation was involved in supporting Th2 polarisation, αCD3 stimulated T-cell co-cultures were set up using DCs deficient in expression of the alternative activation related molecule RELMα (Fig. 4.10A). When DCs were unable to produce RELMα, a significantly increased level of $CD4^+$ T-cell huCD2 (IL-4) expression was detected (Fig. 4.10B&C). This result suggests that RELMα usually functions to limit the induction of T-cell IL-4. Due to the addition of exogenous rIL-4 to the culture wells it was not possible to assess IL-4 secretion. However, the production of IL-13 was measured in the culture supernatants by ELISA (Fig. 4.10D). In contrast to increased IL-4 protein production (huCD2), IL-13 secretion was reduced in the presence of RELMα deficient DCs (Fig. 4.10D). IL-10 was initially reported as being a Th2 cytokine, was later associated with Tregs and most recently has been shown to have broad associations with many cells of the immune system (Saraiva and O'Garra 2010). We wondered whether the reduction in IL-4 driven IL-13 was mediated via altered induction of IL-10. A surprisingly clear reduction in the levels of IL-4 driven IL-10 (GFP Fig. 4.10E&F) and protein (Fig. 4.10G) was found when DCs were unable to produce RELMα, confirming a role for RELMα in promoting T-cell IL-10. This data recapitulates the findings from the *Retnla*$^{-/-}$ GMDC transfer (Fig. 4.9) where it was found that DCs deficient in RELMα failed to promote optimal Th2 responses, with reduced IL-10 and IL-13, and enhanced IFNγ. In this system, in the absence of

antigen or Th1 polarising cytokine, there was no difference in the secretion of IFNγ by T-cells exposed to WT or *Retnla*$^{-/-}$ DCs (Fig. 4.10H).

4.3.11 rRELMα does not activate GMDCs

We next aimed to ascertain whether RELMα could be acting in an autocrine manner to change DC function within the context of a WT DC transfer or T-cell co-culture. In order to assess this we cultured GMDC with recombinant RELMα in the presence or absence of IL-4 overnight and phenotyped the cells to determine if there were changes in IL-4Rα expression (Fig. 4.11B), co-stimulatory marker expression (Fig. 4.11C), RELMα or Ym1/2 production (Fig. 4.11D), or cytokine secretion (Fig. 4.11E). There were no significant differences detected between the rRELMα treated and control wells for any of the parameters tested. No IL-12p70 or IL-10 were secreted by the cells in response to rRELMα, and no significant difference was apparent in those cytokines which were detected (CCL24, TNFα, IL-12p40, NO). This lack of impact of recombinant RELMα on DC activation phenotype suggests that RELMα may directly influence the T-cells. Alternatively, RELMα may affect a parameter of DC activation that we did not measure in these experiments.

4.3.12 WT, *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs are similarly capable at antigen uptake

To determine whether altered function of the IL-4Rα and RELMα deficient DCs was the cause of their impaired ability to prime T-cell response *in vitro* and *in vivo,* we first assessed whether the cells were equally competent at antigen uptake as WT DCs. The rationale behind this approach was that, if DCs are less capable at taking up antigen, then they would be less able to process and present components of that antigen to T-cells. We undertook a simple assay to assess endocytic/pinocytic activity (Steinman, Mellman *et al.* 1983), whereby WT, IL-4Rα deficient or RELMα deficient GMDCs were incubated with fluorescently labelled dextran at 37°C for 30 minutes, followed by analysis of fluorescence by flow cytometry to determine uptake as compared to control samples incubated on ice to stop biological processes (Fig. 4.12A). There was no major difference in the ability of either type of genetically deficient DC to uptake FITC conjugated Dextran when compared to WT cells (Fig. 4.12B&C), however IL-4Rα deficient cells had a significantly increased % of FITC positive cells and gMFI than did WT cells, and although a similar percentage of RELMα deficient cells had taken up FITC-dextran,

the gMFI data would suggest that each cell had pinocytosed less dextran than the WT cells (Fig. 4.12C).

4.3.13 Antigen processing and presentation is unimpaired in *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs

Although antigen uptake was intact in the IL-4Rα and RELMα deficient DCs, we next wanted to determine whether altered antigen processing or presenting function might be the cause of their impaired ability to prime T-cell response *in vitro* and *in vivo*. We assessed whether the IL-4Rα and RELMα deficient DCs were equally competent at processing and presentation of antigen (Fig. 4.13) to ovalbumin specifc (OT-II) TCR transgenic CD4^{+} T cells (Barnden, Allison *et al.* 1998) *in vitro*. To assess antigen presentation ability, OT-II T-cells labelled with the intracellular dye CFSE were first cultured with WT, *Il4ra*$^{-/-}$ or *Retnla*$^{-/-}$ DCs, in the presence of ovalbumin peptide (Fig. 4.13A). CFSE can not diffuse out of cells, and as such gets diluted at each round of cell division (Hawkins, Hommel *et al.* 2007). CFSE dilution was assessed by flow cytometry following 4d of culture; using peptide rather than whole ovalbumin protein allowed determination of the ability of a cell to present the ovalbumin peptide to T-cells outwith the requirements to first process the antigen. There was no deficit in the ability of the DCs to present pOVA resulting in T-cell proliferation. In fact, *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ deficient DCs induced enhanced T-cell proliferation compared to the control WT cells (Fig. 4.13B). Next, in order to assess whether there was a defect in the ability of the DCs to process antigen prior to presentation to T-cells, whole OVA protein was used instead of pOVA in an otherwise identical assay (Fig. 4.13C). There was no deficit in ability to drive T-cell proliferation. Indeed, similar to what was observed with the addition of peptide (Fig. 4.13B), *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs also induced significantly enhanced T-cell proliferation compared to WT cells when cultured with whole OVA protein (Fig. 4.13C). This enhanced proliferation was consistent with expectation, given the reduced IL-10 levels evident following transfer of *Il4ra*$^{-/-}$ (Fig. 4. 7) or *Retnla*$^{-/-}$ (Fig. 4.9) DCs *in vivo*, and in T-cell co-culture assays *in vitro* (Fig. 4.8 & Fig. 4.10).

4.3.14 IL-4 treatment of RELMα deficient DCs significantly alters transcription of more genes than IL-4 treatment of WT DC

In an attempt to determine the gene(s) responsible for the changes in T-cell polarising ability of *Retnla*$^{-/-}$ DC compared to WT DC, Illumina whole genome

expression profiling was undertaken. This system allows determination of transcription changes within cell populations by biotion labelling of total cellular cRNA prior to hybridisation to standard mouse reference genome chips which (in this study) contained probes for 45,281 transcripts. In this microarray, we chose to assess the impact of IL-4 treatment on WT and *Retnla*$^{-/-}$ GMDC, with *Il4ra*$^{-/-}$ GMDC serving as a control population. In order to enhance the statistical power of the data produced by gene expression profiling, we used 3 biological rather than technical replicates per genotype tested. GM-CSF was used to differentiate DCs from bone marrow harvested from 3 individual mice per genotype (9 mice total). GMDCs were stimulated with IL-4 for 6 hours, then counted, and equal numbers per biological replicate were used to produce pooled RNA, which was converted to biotin-labelled complementary RNA. Quality control (flow cytometry, ELISA) was carried out on aliquots of cells not used for RNA extraction (Fig. 4.14B-E). The biotin labelled cRNA samples were transferred to the Clinical Research Facility at the Western General Hospital, Edinburgh, for chip hybridisation and sample normalisation (Fig. 4.12A). *Il4ra*$^{-/-}$ DCs did not express the IL-4Rα (Fig. 4.14B&C), *Retnla*$^{-/-}$ DCs expressed slightly reduced levels of IL-4Rα compared to WT cells and 6 hours of IL-4 treatment downregulated the IL-4Rα (Fig. 4.14B&C) to a similar extent to 18h of IL-4 treatment (as shown previously in Fig. 3.1). With regard to co-stimulatory marker expression, all three genotypes of DC were fairly comparable, with the only significant difference being enhanced CD40 expression on *Retnla*$^{-/-}$ DCs and a trend for reduced CD80 when DCs were unable to produce RELMα or respond to IL-4 (Fig. 4.14B&C). *Retnla*$^{-/-}$ DCs were unable to produce RELMα but produced comparable levels of intracellular Ym1/2 in response to IL-4 (Fig. 4.14D&E). IL-4 failed to upregulate RELMα or Ym1/2 in *Il4ra*$^{-/-}$ DCs (Fig. 4.14D&E), unfortunately the on plate standard failed for the Ym1/2 ELISA, and as all of the supernatant had been used, this ELISA could not be repeated, as such OD values have been plotted. Following hybridisation, QC analysis was carried out by Al Ivens (CIIE, bioinformatics support) using the arrayQualityMetrics package in BioConductor (Kauffmann and Huber 2010). One of the IL-4 treated *Il4ra*$^{-/-}$ samples failed the QC analysis, this sample had a low concentration of RNA following initial extraction, and had to be concentrated prior to chip hybridisation, and unfortunately failed to hybridise correctly. Due to this failure, the IL-4Ra data set was excluded from further analysis. Although statistical significance could not be assessed in this group

due to the low sample number (n=2), no genes were changed by IL-4 treatment of the IL-4Rα deficient DCs, as expected.

Following IL-4 treatment of WT DCs, 109 genes were significantly changed (up or down regulated) ($p<0.05$). Interestingly, 308 genes were significantly altered by IL-4 treatment of *Retnla*$^{-/-}$ DCs, 199 more genes than were changed by IL-4 treatment of WT DCs; 79 of the IL-4 induced gene changes were shared by WT and RELMα deficient cells (Fig. 4.14F). In order to reduce the size of the gene lists, stringent fold change cut offs of ≤0.5 and ≥2 were chosen, such that IL-4 treatment must reduce the expression of a gene of interest by at least 50% or conversely enhance gene expression by 100% or more. Using these cutoffs reduced the number of significant genes of interest to 53, genes are listed in tables in order of significance with most significant first.

Only *Thbs1* (Thrombospondin-1, a glycoprotein involved in cell adhesion) was down regulated by IL-4 in both DC strains (Table 4.3.1, Fig. 4.14G). IL-4 treatment of *Retnla*$^{-/-}$ DCs (Table 4.3.2) significantly downregulated an additional 14 genes. The most significantly down regulated gene was *Il1rn* (interleukin 1 receptor antagonist $p=0.0025$). Of the 14 downregulated genes, the two with the lowest fold change (and thus most reduced by IL-4 treatment) were *Tlr2* (Toll like receptor 2) and *Clec4e* (C-type lectin domain family 4, member e; also known as Mincle).

21 genes were significantly increased by 2 fold or greater following IL-4 treatment of WT DCs. Of these, 18 were also upregulated in *Retnla*$^{-/-}$ DCs (Table 4.3.3), and an additional 17 genes were upregulated by IL-4 only in *Retnla*$^{-/-}$ DCs (Table 4.3.4) (Fig. 4.14H).

Only 3 genes were upregulated by IL-4 in WT DCs that were not upregulated in *Retnla*$^{-/-}$ DCs, indicating that these genes potentially require RELMα to be present for their expression. *Retnla* itself was one of these genes, the other 2 being *Hfe* (Hemochromatosis) and *Apol7c* (Apolipoprotein 7c) (Table 4.3.5).

Of the 18 genes upregulated by IL-4 in both WT and *Retnla*$^{-/-}$ DCs (Table 4.3.3) the eosinophil chemoattractnat *Ccl24* (also known as Eotaxin-2) was both the most significantly upregulated gene and also had the highest fold change (16),

Chi3l3 (Ym1) and *Chi3l4* (Ym2) also had a high fold change (≥4), these changes had already been identified by qPCR, ELISA and flow cytometry (chapter 3).

The most significantly upregulated gene >2FC in *Retnla*$^{-/-}$ DCs was *Edn1* (Endothelin 1)(Table 4.3.4), although this gene was also significantly upregulated in IL-4 treated WT DCs, but did not reach the 2FC cut off. As IL-4 treatment significantly enhanced more genes in *Retnla*$^{-/-}$ deficient cells than in WT cells, this suggests that RELMα may normally act to inhibit, either directly or indirectly, the upregulation of those genes.

4.4 SUMMARY

- IL-4 enhanced IL-12p70 and reduced IL-10 secretion from DCs stimulated with bacteria or bacterial products (LPS, CpG, heat killed *P. acnes*) (Fig. 4.2, Fig. 4.4).
- Markers of IL-4 driven alternative activation were not uniformly down regulated by TLR ligation (Fig. 4.3 & Fig. 4.5).
- Helminth antigens did not modulate IL-4 driven Ym1/2, but did modulate IL-4 induced RELMα (Fig. 4.5).
- Pre-culture with IL-4 enhanced DC cytokine production following CD40 ligation (Fig. 4.6).
- DC RELMα and Ym1/2 secretion was maintained for at least 24h following removal of IL-4 (Fig. 4.6).
- *Il4ra*$^{-/-}$ DCs were less capable of priming mixed Th1/Th17 responses against *P. acnes,* and induced reduced SEA specific IL-10 and IFNγ (Fig. 4.7 & Fig. 4.8). This was not due to a defect in antigen uptake (Fig. 4.12), processing or presentation (Fig. 4.13).
- *Retnla*$^{-/-}$ DCs were less capable of priming some aspects of the Th2 response: SEA specific IL-10 and IL-13 were reduced, but IFNγ was enhanced (Fig. 4.9 & Fig. 4.10). This was not due to a defect in antigen uptake (Fig. 4.12), processing or presentation (Fig. 4.13).
- IL-4 significantly increased expression of more genes in *Retnla*$^{-/-}$ DCs than in WT DCs (Fig. 4.14): hence RELMα may act to some extent as a feedback regulator of DC alternative activation.

4.5 DISCUSSION

4.5.1.TLR and bacterial modulation of DC co-stimulatory molecule expression

We showed in Fig. 4.1 that TLR9 or TLR4 ligation with the specific agonists CpG or LPS significantly enhanced expression DC expression of the co-stimulatory molecules CD80, CD86 and CD40, as has been reported previously (Dearman, Cumberbatch *et al.* 2009). Also as reported previously for LPS, there was no significant increase in the level of MHC-II expression following overnight culture (Dearman, Cumberbatch *et al.* 2009), though this is in contrast with an earlier report (MacDonald, Straw *et al.* 2001). The ability of exogenous PRR stimuli to activate GMDCs is dependent upon the basal level of expression of the marker of interest. For example, if DCs have become activated during differentiation, the ability to enhance MHC-II expression further may be compromised, which might explain the differences between the studies. IL-4 is able to downregulate the basal level of DC CD80 expression (Fig. 3.1, 4.1D). Interestingly, in the presence of strong TLR ligation, IL-4 no longer limits CD80 expression (Fig. 4.1D). However, IL-4 does instead promote CD86 (Fig. 4.1E). The IL-4 mediated reduction in any of the conditions, though significant, is only a minor alteration in the absolute level of expression, so may not make any biological difference to the ability of the DCs to activate T-cells. In contrast to the LPS and CpG enhancement of CD80, CD86, and CD40 but not MHC-II, Pa enhanced MHC-II, CD86 and CD40 expression but not CD80 (Fig. 4.4B&C). Indeed, the more subtle induction of CD80 by Pa than LPS was published previously (MacDonald, Straw *et al.* 2001). IL-4 did not cause any alteration in the amount of MHC-II, CD80, CD86 or CD40 upregulated on DCs in response to stimulation with Pa.

4.5.2 IL-4 regulation of bacterially induced DC function

We also confirmed results from previous studies (Hochrein, O'Keeffe *et al.* 2000; MacDonald, Straw *et al.* 2001) showing that LPS, CpG and Pa enhanced DC secretion of T-cell polarising cytokines IL-12p70, IL-12p40, IL-10, TNFα and IL-6 (Fig. 4.2 & 4.4D). Furthermore, we confirmed in Fig. 4.2, that IL-4 enhances the secretion of IL-12p70, and decreases secretion of IL-10 (Yao, Li *et al.* 2005) and IL-12p40 (Hochrein, O'Keeffe *et al.* 2000) during TLR ligation with LPS or CpG. We add to the findings of these previous studies by showing that IL-4 mediates the same cytokine changes in IL-12p70 and IL-10 in cells stimulated with the more complex antigen preparation of *P. acnes* (Fig. 4.4).

If a DC is exposed to Th1 inducing pathogen and Th2 cytokine at the same time, it makes sense that the cytokine (in our study, IL-4) should help to enable a Th1 response to develop (e.g. enhancing IL-12p70) as Th1 pathogens are likely to be more immediately life threatening (Allen and Wynn 2011). This raises interesting questions about flexibility and stability of the impact of IL-4 on DC function. One theory is that later, once the immediate Th1 threat has been dealt with, the DC would be competent to drive a Th2 response. To test this theory, following culture of a DC with LPS or Pa and IL-4, we could wash the cells then re-expose the DC to IL-4 only and assess the cytokines that are secreted, and the ability of those DCs to support naïve Th differentiation.

Prior to *in vivo* transfer it was found that Pa stimulated *Il4ra*$^{-/-}$ DC secreted significantly enhanced levels of IL-12p40 and reduced levels of IL-10, but had no increase in IL-12p70, NO, TNFα or IL-6 (Fig. 4.7B). IL-4 has previously been shown to inhibit the secretion of (p40)2 homodimers from TLR stimulated DCs (Hochrein, O'Keeffe *et al.* 2000). (p40)2 has been shown to function as an antagonist of both mouse and human IL-12p70 signalling (Mattner, Fischer *et al.* 1993; Gillessen, Carvajal *et al.* 1995; Ling, Gately *et al.* 1995). The ability of IL-4 to mediate enhanced IL-12p70, (via relieving the antagonism of (p40)2), was suggested to result in a feedback mechanism whereby IL-4 was able to promote a Th1 response (Hochrein, O'Keeffe *et al.* 2000). Although we did not determine whether the IL-12p40 that we detected prior to DC transfer was of a (p40)2 conformation, it is possible that, as it was produced from *Il4ra*$^{-/-}$ DC at such high levels, it was in the antagonistic form. Further evidence that the DC derived IL-12p40 was functioning in an antagonistic role *in vivo* is highlighted by the significantly reduced level of IFNγ that was primed upon transfer of Pa stimulated *Il4ra*$^{-/-}$ DCs (Fig. 4.7C).

Interestingly, IL-4 has been reported to inhibit IL-10 secretion from LPS/CpG stimulated DCs, which results in an enhancement of IL-12p70, an outcome which enables the polarisation of a Th1 type response *in vitro* (Yao, Li *et al.* 2005; Guenova, Volz *et al.* 2008). As such, the finding that Pa pulsed IL-4Rα deficient cells produced less IL-10 than WT DCs (Fig. 4.7B) is at first surprising. However, it has to be remembered that there is no detectable or exogenous IL-4 within this system pre-transfer. As Pa stimulated IL-4Rα deficient DCs were less capable of

priming IL-17 and IFNγ T-cell responses than their WT counterparts (Fig. 4.7C), this suggests that in a WT situation, receipt of an IL-4 signal in the context of a bacterial pathogen drives a feedback loop to enhance Th1/Th17 cytokine production (Fig. 4.15).

A question that should be addressed in relation to the transfer of Pa stimulated DCs *in vivo* is: what is the source of IL-4 (or IL-13) in this setting? No IL-4 was detected upon restimulation of LNs following Pa DC transfer, though a small amount of IL-13 was measured (Fig. 4.7C). Thus, IL-13 may be the factor that is influencing the outcome of Th1/Th17 polarisation by bacterially stimulated WT or *Il4ra*$^{-/-}$ DCs.

4.5.3 IL-4 and SEA regulation of DC function

As shown in previous studies, SEA caused no obvious maturation of DCs (MacDonald, Straw *et al.* 2001) in terms of MHC-II, co-stimulatory molecule or IL-10 and IL-12p70 expression/secretion, and this was the case irrespective of IL-4 presence (Fig. 4.4).

The ability of SEA pulsed DCs to induce potent Th2 responses following transfer *in vivo* has been reported previously (MacDonald, Straw *et al.* 2001; Perona-Wright, Jenkins *et al.* 2006; MacDonald and Maizels 2008). We extended the use of this transfer system to address how the ability of the polarising DC to respond to IL-4 alters the ensuing immune response. DCs that could not receive an IL-4 signal (*Il4ra*$^{-/-}$), primed less IL-10 and IFNγ, following restimulation of pLN cells with SEA (Fig. 4.7D). In this system, SEA delivery via the transferred DCs induces an IL-4 rich environment. *S. mansoni* eggs and the soluble antigens released from them activate mast cells and induce IL-4 production from eosinophils (Sabin, Kopf *et al.* 1996), and SEA exposure results in purified human basophil secretion of IL-4 (Falcone, Dahinden *et al.* 1996; Haisch, Schramm *et al.* 2001). However, in the absence of mast cells or eosinophils, *S. mansoni* eggs can induce normal Th2 responses, and it has been suggested that the main source of IL-4 is in fact the CD4^{+} T-cells (Pearce, C *et al.* 2004). An alternative source of IL-4 in Th2 settings are the innate lymphoid cells (ILCs) (Mjosberg, Bernink *et al.* 2012), a cell populations whose importance in the DC dependent induction of Th2 responses has yet to be investigated comprehensively.

Based on our data (Fig. 4.7D), we propose that the IL-4 induced following DC presentation of components of SEA *in vivo* then feeds back to signal to the priming DC, altering the magnitude of the resultant effector response. The concept that IL-4 can drive enhanced DC secretion of IL-12p70 and reduced IL-12p40 and IL-10 (as discussed above), provides a possible explanation for the reduced induction of SEA-specific IFNγ by IL-4Rα$^{-/-}$ DCs (Fig. 4.7C). No cytokines were induced by SEA prior to DC transfer (Fig. 4.7B), but this does not preclude the possibility that *in vivo* derived signals will promote SEA-DC cytokine secretion following transfer.

A study has addressed a related question, of how DCs that produce IL-4 may modulate priming of Th cell responses (Kaneko, Wang *et al.* 2003). Since DCs do not themselves produce IL-4 (MacDonald and Maizels 2008). This study used an adenoviral transduction system to create IL-4 secreting DCs, which they then trasferred *in vivo*, and restimulated draining LN cells 7 days later with γ-irradiated splenocytes (Kaneko, Wang *et al.* 2003). IL-4 secreting DCs promoted enhanced levels IL-4, IL-10 and IFNγ than control DCs (Kaneko, Wang *et al.* 2003). This data indicates that DC IL-4 acting in an autocrine manner is able to promote IFNγ and IL-10 expression by T-cells, confirming our finding of reduced IFNγ and IL-10 in the absence of DC IL-4R signalling. Kaneko *et al* confirmed that in response to CD40 ligation or LPS treatment, the IL-4 producing DCs also secreted more IL-12p70 (Kaneko, Wang *et al.* 2003) than control cells, highlighting a role for the secreted IL-4 in promoting Th1 responses (Hochrein, O'Keeffe *et al.* 2000; Kalinski, Smits *et al.* 2000; Yao, Li *et al.* 2005). Indeed, the *in vivo* signal that mediates altered SEA-DC cytokine production may be partly due to CD40 ligation, as this has previously been shown to be required for Th2 induction by SEA pulsed DCs transferred *in vivo* (MacDonald, Straw *et al.* 2002). DC CD40 expression was shown not to be required for induction of Th1 responses by Pa stimulated DCs (MacDonald, Straw *et al.* 2002), and *in vitro* ligation of CD40 did not alter DC cytokine secretion in response to SEA (Fig. 4.6). Nevertheless, *in vivo* CD40 stimulation may be required for the induction of Th1 cytokines (enhanced IFNγ) in the context of the Th2 polarising antigen SEA.

The ability of SEA to partially inhibit IL-4 dependent RELMα production by DCs (Fig. 4.5) was a little surprising given previous reports showing that SEA does very little to modulate detectable changes in DC activation (Perona-Wright, Jenkins *et al.* 2006). DC derived RELMα promotes SEA (and IL-4) dependent T-cell IL-10 and IL-13, but limits IFNγ *in vivo* (Fig. 4.9) as well as IL-4 *in vitro* (Fig. 4.10). SEA interference with RELMα production (Fig. 4.5) may be a factor contributing to the lack of significant difference seen in IL-4 or IL-13 priming by WT and IL-4Rα$^{-/-}$ DCs following their *in vivo* transfer (Fig. 4.7D). The differences observed in the *in vitro* DC:T-cell co-cultures, in which significant enhancement of IL-4 (Fig. 4.8B&C) and reduction of IL-13 was found when DCs lacked IL-4Rα (Fig. 4.8D) (See Fig. 4.16), could in part be due to the absence of SEA in the *in vitro* assay.

Given this complexity, we have attempted to put together working models of how IL-4 responsiveness and RELMα production may alter the ability of DCs to polarise CD4^{+} T cell cytokine production *in vitro* and *in vivo*, in the presence and absence of helminth antigen (Fig. 4.16). Based on our data, we would propose that, in the absence of helminth antigen, a DC IL-4Rα dependent factor (labelled 'A') may be primarily responsible for the promotion of T-cell IL-4 *in vitro* (Fig. 4.16A). Similarly, as illustrated in Fig. 4.16B, an SEA dependent factor 'B' may be responsible for the stringent promotion of T-cell IL-4 *in vivo*.

Although IL-10 and IFNγ were significantly decreased following transfer of SEA pulsed *Il4ra*$^{-/-}$ DCs, as compared to WT SEA DCs, IL-4 was not significantly reduced, suggesting that an IL-4R independent signal is promoting T-cell IL-4 in this setting (Fig. 4.7D). Furthermore, in contrast to what was seen *in vitro* (Fig. 4.10B), *Retnla*$^{-/-}$ DCs were not found to promote elevated IL-4 *in vivo* following transfer (Fig. 4.9C), suggesting that the SEA driven factor A may override or bypass the ability of RELMα to control DC IL-4 induction.

Factors A and B may be the same molecule, induced via two separate pathways (IL-4/SEA), or separate molecules, indeed, molecules A and B may also counter-regulate one another or synergise with each other. To try and identify Th2 polarising molecules expressed by DCs, a microarray, deep sequencing or proteomic screen comparing WT DCs +IL-4, WT DCs +SEA, and *Il4ra*$^{-/-}$ DC +SEA could be undertaken. We would hypothesis that, if a single DC molecule is

responsible for promoting T-cell IL-4 downstream of IL-4R and SEA receptor signalling, then it would be enhanced by both IL-4 and SEA in WT DCs, and by SEA in the absence of IL-4R signalling.

This leads into the question of which receptor is signalling downstream of SEA recognition? The lacto-N-fucopentaose III (LNFPIII) moiety of SEA has been shown to bind to DCs via DC-SIGN, a molecule that has been associated with IL-4 driven AAM (Relloso, Puig-Kroger *et al.* 2002; Martinez, Helming *et al.* 2009). Thus IL-4 may upregulate expression of CLRs such as DC-SIGN on DCs, enabling a more efficient interaction with SEA components, leading to an altered induction of RELMα. Although DC-SIGN was not addressed in this work, we did investigate the impact of IL-4 on DC expression of a different CLR, MR. MR is another molecule associated with both the uptake of the SEA component omega-1 and *in vivo* priming of Th2 by DCs (Everts, Hussaarts *et al.* 2012). MR is also enhanced by IL-4 in AAM (Stein, Keshav *et al.* 1992), and found to be significantly increased at the transcript level in AADCs (Fig. 3.4F). An investigation into the ability of SEA to limit IL-4 driven RELMα in *Mrc1*$^{-/-}$ DCs would be a good starting point to investigate whether SEA inhibition of RELMα is dependent upon this uptake receptor. Alternatively, anti-MR antibodies could be used to block this receptor in WT DCs.

Signalling molecules downstream of TLRs should also be considered, as TLR recognition of SEA components has been reported previously (van der Kleij, Latz *et al.* 2002; Aksoy, Zouain *et al.* 2005; Gao, Zhang *et al.* 2012). Another layer of complexity that could be particularly relevant here may also be provided by the recent identification of cross-talk between TLR and CLR signalling, an aspect of DC activation that has not yet been investigated in the context of Th2 induction and development (Geijtenbeek and Gringhuis 2009).

4.5.4 RELMα function

Previous studies using global RELMα deficient animals have suggested that, globally, RELMα functions as a negative regulator of Th2 responses (Nair, Du *et al.* 2009; Pesce, Ramalingam *et al.* 2009). In our study investigating the function of RELMα specifically in DCs, we found that RELMα limited T-cell IL-4 *in vitro* (Fig. 4.10) but promoted T-cell IL-10 and IL-13 both *in vitro* and *in vivo* (Figs. 4.9 & 4.10). These findings suggests that the role of RELMα in the immune system varies in a

context and timing dependent manner; DC secretion during T-cell priming influencing responses differently to RELMα produced more generally by other cell types at other timepoints of immune development. Using an *S. mansoni* lung granuloma model, Pesce *et al* found that there was enhanced expression of IL-4, IL-5 and IL-13 mRNA in global *Retnla*$^{-/-}$ mice, and T-cells secreted elevated IL-4 and IL-5. This contrasts our results in which we show that DC RELMα differentially modulates IL-4 and IL-13 (Fig. 4.10). In the liver during *S. mansoni* infection eosinophils were found to be the main source of *Retnla*, and animals globally deficient in *Retnla* had enhanced liver granuloma volume by 12 weeks post infection (Pesce, Ramalingam *et al.* 2009).

Neither of the published reports of RELMα deficiency in settings of helminth infection have described a role for RELMα in the modulation of IL-10, which is a novel aspect of our work (Figs. 4.9 & 4.10) (Nair, Du *et al.* 2009; Pesce, Ramalingam *et al.* 2009). Addition of rRELMα during LPS stimulation of BMM was found to enhance the production of the pro-inflammatory cytokines IL-6 and TNFα, whilst inhibiting LPS induced IL-10 (Munitz, Waddell *et al.* 2008). Thus the ability of RELMα to modulate IL-10 also appears to be cell type and context dependent, with rRELMα being able to limit IL-10 in BMM in the context of a Th1 stimulus, but absence of RELMα in GMDCs reducing their ability to promote T-cell IL-10 secretion in the context of Th2 polarising antigen (Fig. 4.9) or cytokine (Fig. 4.10). In agreement with Fig. 4.11, in which it was shown that treatment of GMDCs with rRELMα alone did not alter their co-stimulatory molecule or cytokine secretion, Munitz *et al*, also found rRELMα was unable to modulate IL-10, IL-6 and TNFα production from BMM in the absence of LPS (Munitz, Waddell *et al.* 2008). The modulation of BMM by rRELMα (Munitz, Waddell *et al.* 2008) underscores the ability of AAM/AADC derived RELMα to function in an autocrine manner. In future work to help better delineate the different impact of RELMα on macrophages vs. DCs, the addition of exogenous rRELMα to GMDCs should be repeated in the presence of LPS or other defined TLR ligands, as was carried out in the BMM study (Munitz, Waddell *et al.* 2008).

The 2008 study from Munitz *et al* (Munitz, Waddell *et al.* 2008) also reported that DSS colitis was reduced in *Retnla*$^{-/-}$ animals, so it is perhaps surprising that no similar role for RELMα in modulation of gut inflammation and pathology during

schistosomiasis was identified in the Pesce study (Pesce, Ramalingam *et al.* 2009). Another study has previously shown that LysM^{Cre}IL-4Rα$^{fl/-}$ animals that lack AAM display reduced survival following *S. mansoni* infection, linked to enhanced gut pathology, breakdown of barrier function and sepsis (Herbert, Holscher *et al.* 2004). In this study, in the absence of AAM, there was impaired intestinal wound healing, which was suggested to be reason for increased death. However, the LysM^{Cre}IL-4Rα$^{fl/-}$ animals would likely also have reduced RELMα at the site of intestinal damage, and thus less amplification of LPS driven IL-6 and TNFα, as shown in the DSS colitis model (Munitz, Waddell *et al.* 2008). Thus, the reduction or absence of RELMα production by the IL-4Rα deficient macrophage populations simultaneously exposed to gut bacteria and their products could have been beneficial in the Herbert schistosome infection study. To assess more precisely the role of macrophage derived RELMα in gut pathology during *S. mansoni* infection the use of LysM^{Cre}*Retnla*$^{-/fl}$ animals would be most informative. In the absence of such mice, providing exogenous rRELMα intestinally during *S. mansoni* infection of LysM^{Cre}IL-4Rα$^{fl/-}$ animals, could determine the contribution of RELMα in mediating intestinal inflammation.

The clear role for DC expression of RELMα in limiting T-cell IL-10 shown in Figs. 4.9 & 4.10 suggests that, in response to IL-4, RELMα deficient DCs are either producing a factor that directly inhibits T-cell IL-10, or not producing a factor that IL-4 treated WT cells produce to support T-cell IL-10 production. Alternatively, the factor could be acting indirectly on T cells by modulating other aspects of DC APC function. It is possible that RELMα itself is the factor, and/or *Retnla*$^{-/-}$ DCs may differentially express other T-cell/DC polarising factors. The identity of the RELMα receptor has not yet been determined, although BTK has been proposed as a binding partner (Su, Zhou *et al.* 2007; Nair, Du *et al.* 2009). Identification of a presumably, surface bound receptor for RELMα and generation of a mouse strain deficient in expression of the receptor would enable future experiments to determine the relative importance of DC Vs T-cell responsiveness to RELMα. In the absence of such information, we undertook a microarray study to investigate which molecules may be differentially transcribed in WT vs. *Retnla*$^{-/-}$ DCs in response to IL-4 treatment (Fig. 4.12), as discussed below in section 4.5.6. “Microarray targets”.

One fascinating feature of alternative activation of the GMDCs was that they continued to secrete RELMα and Ym1/2 up to 36 hours after removal of IL-4 from *in vitro* culture (Fig. 4.6C), clearly showing that the impact of IL-4 on DC alternative activation is not transient. The maintained expression of these AA markers suggests that, in the context of an immune response, a DC could become alternatively activated in the peripheral tissues and influence effector responses there, but also still be able to secrete RELMα and Ym1/2 following migration to the secondary lymphatics and so during T cell priming.

4.5.5 Antigen processing and presentation by WT, *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs

In order to control for the possibility that IL-4Rα and/or RELMα deficient DCs had an altered ability to acquire and present antigen to T-cells, compared to WT DCs, we undertook antigen uptake and presentation assays (Figs. 4.12 & 4.13). A similar percentage of the GMDC population was able to pinocytose FITC conjugated dextran in the absence of RELMα, though the per cell uptake may have been slightly lower than WT cells. In contrast IL-4Rα deficient DCs had significantly enhanced uptake at both the population and per cell level. The functional outcome of this finding is unclear, however increased uptake of antigen may result in enhanced processing and presentation of antigen which may in turn provide a stronger signal for T-cell proliferation. Importantly, the altered ability of *Il4ra*$^{-/-}$ or *Retnla*$^{-/-}$ DCs to polarise T-cell responses following *in vivo* transfer was not due to a fundamental inability to take up antigen.

Additionally, using the model antigen ovalbumin, we showed that both IL-4Rα and RELMα deficient DCs are better at driving the proliferation of OVA specific TCR transgenic T-cells (Fig. 4.13), highlighting that the altered cytokine induction following DC transfer (Figs. 4.7 & 4.9) is not due to a fundamental inability of these DCs to efficiently process and present antigen. The enhanced proliferation induced by IL-4Rα deficient DCs is in keeping with the increased signal strength idea proposed in the previous paragraph, as *Il4ra*$^{-/-}$ GMDCs pinocytosed higher levels of FITC-Dextran (Fig. 4.12).

4.5.6 Microarray targets

Having realised the complexity of the impact of IL-4 on DC activation, we took a more broad approach to attempt to assess the major mRNA changes within

AADCs. This approach has revealed a number of genes regulated by IL-4 that could have an important functional role in DCs, and with potential to be the focus of new studies out-with the scope of this thesis. As is the caveat with all assays measuring transcript levels, the results of this microarray analysis do not necessarily correlate with changes in protein production. However, many of the targets found to be upregulated by IL-4 at the transcript level (Table 4.3.3) had already been previously validated at the level of protein in WT DCs in chapter 3 of this thesis, for example RELMα, Ym1/2, and CCL24 (Figs. 3.2 & 3.4).

The only gene to be reduced by more than 50% by IL-4 treatment of both WT and RELMα deficient DCs was *Thbs1*, a gene that encodes Thrombospondin-1/TSP1, a protein known to be involved in cell adhesion (Lynch, Maillet *et al.* 2012) and previously reported to negatively regulate human monocyte derived DC cytokine production (Doyen, Rubio *et al.* 2003). IL-10, TGFβ and PGE_2 were previously shown to enhance human moDC secretion of TSP1, furthermore, TSP1 was proposed to mediate DC exhaustion (Doyen, Rubio *et al.* 2003). Recombinant TSP1 could be used in our studies to investigate whether in the context of TLR ligation and IL-4 treatment, this molecule is involved in the modulation of T-cell polarising cytokine secretion by DCs. In relation to the role of *Thbs1* in cell adhesion, it would be interesting to investigate whether reduction in *Thbs1* by IL-4 has any impact upon the duration or strength of interaction between DCs and T-cells. An assay to determine the length of DC:T-cell interactions with and without IL-4 presence could be carried out with collaborators (Fagerholm Laboratory) in Dundee, who use shear flow cell adhesion assays to assess such interactions (MacPherson, Lek *et al.* 2011)

As described in the results (Table 4.3.2), the gene most significantly down regulated by IL-4 treatment of *Retnla*$^{-/-}$ DCs was *Il1rn* (interleukin 1 receptor antagonist, IL-1RA, p=0.0025), a gene whose down-regulation by IL-4 in macrophages has previously been reported to be dependent upon the transcription factor IRF4 (El Chartouni, Schwarzfischer *et al.* 2010). *Il1rn* was also significantly down regulated following IL-4 treatment of WT DCs, but did not reach the 50% reduction in expression that was used as a cut off during this analysis (Adj p value=0.026, FC=0.54). IL-1RA potently suppresses the effects of the proinflammatory cytokine IL-1. Interestingly, *Ilrn*$^{-/-}$ animals have been shown to have

exacerbated Th2 cytokine production, which was associated with decreased DC IL-12 production in a non-helminth model of liver granuloma (Iizasa, Yoneyama *et al.* 2005). Furthermore, reduced DC IL-12p40 was also seen during cutaneous leishmaniasis in IL-1RA deficient animals (Kautz-Neu, Kostka *et al.* 2011). Decreased *Il1rn* expression in *Retnla*$^{-/-}$ DCs following IL-4 treatment may indicate a mechanism via which these cells are more capable at supporting T-cell IL-4 production than their WT counterparts, which maintain higher levels of expression of *Il1rn* (Fig. 4.8B&C).

Several PRRs were also identified as being differentially expressed in AADCs. The importance of TLR2 in the regulation of IL-4 treated DCs is the focus of Chapter 6 and will not be discussed in detail here. Mincle (*Clec4e)* was identified as a C-type lectin induced in macrophages by proinflammatory stimuli (LPS, IFNγ, TNFα, IL-6) (Matsumoto, Tanaka *et al.* 1999) and so its downregulation by IL-4 is not that surprising. Mincle functions as a danger receptor, recognising dead cells, damaged self and pathogen products from mycobacteria and fungus (Miyake, Ishikawa *et al.* 2010). IL-4 dependent downregulation of Mincle in DCs has not been reported previously.

Of the three genes upregulated greater than 100% by IL-4 treatment of WT but not *Retnla*$^{-/-}$ DCs (Table 4.3.5), one was *Retnla,* one *(Apol7c)* has an unknown function and the third was *Hfe. Hfe* encodes haemochromatosis, a protein involved in the regulation of iron transport (Evstatiev and Gasche 2011). Specifically, it interacts with and controls the activity of transferrin receptors. Classical versus alternative activation has previoulsy been reported to impact the iron handling ability of MCSF derived BMM (Corna, Campana *et al.* 2010). IFNγ exposed M1 cells were shown to have a sealed intracellular pool of iron, whereas IL-4 exposed M2 cells were poised for iron release and uptake allowing recycling of any available iron. The same study also found that in conditions of iron limitation, M2 cells had reduced expession of CD86 and MHC-II, whereas M1 cells maintained the same levels of costimulatory molecule expression. *Hfe* was significantly upregulated by IL-4 in RELMα deficient cells but the fold change did not meet the 2 fold cut off. Altered iron metabolism in DCs in the context of IL-4 and a pathogen (such as a bacteria) could influence the outcome of immune activation and host survival, as iron retention can be used as a mechanism to deplete iron from invading pathogens (Gaetano,

Massimo *et al.* 2010). Upregulation of the transferrin receptor c (*Tfrc*) by IL-4 in both WT and *Retnla*$^{-/-}$ DCs (Table 4.3.3) confirmed a role for IL-4 in altering DC iron metabolism.

Another gene dramatically altered in DCs by IL-4 was *Aldh1a2*, which encodes the retinal dehydrogenase enzyme RALDH2, and is involved in the conversion of retinal to RA (Blomhoff and Blomhoff 2006). There is already a substantial literature on the possible role of RA in DC activation and function, which prompted us to more fully address this observation, and *Aldh1a2* is the focus of Chapter 5 of this thesis.

Other upegulated genes with relevance to this thesis include *Irf4*, the interferon response factor originally shown to be required for homeostasis in B and T cells (Mittrucker, Matsuyama *et al.* 1997) and more recently shown to be the transcription factor required for GMDC differentiation (Lehtonen, Veckman *et al.* 2005). *Irf4* is a component of the T regulatory cell transcriptional machinery (Zheng, Chaudhry *et al.* 2009) and is thought to be required for alternative macrophage activation (El Chartouni, Schwarzfischer *et al.* 2010; Satoh, Takeuchi *et al.* 2010).. As *Il1rn* is known to be regulated by expression of *Irf4* (El Chartouni, Schwarzfischer *et al.* 2010), it is possible that the significant downregulation of *Il1rn* detected following IL-4 treatment of GMDCs, was due to the upregulation of this trasncription factor. A time course approach could be used to determine the dynamics of *Il1rn* and *Irf4* transcription following IL-4 treatment.

In terms of chemokine receptors, there was also a very significant upregulation by IL-4 of *Cxcr2* (also known as IL8RB) (Murphy and Tiffany 1991), a receptor known to be involved in neutrophil egress (Day and Link 2012). Only one previous report confirms Th2 cytokine induction of CXCR2 in human monocyte derived DCs (Bonecchi, Facchetti *et al.* 2000). Induction of CXCR2 expression by IL-4 or IL-13 in the study by Bonecchi *et al* was found to enable cells to respond to IL-8 as assessed by chemotaxis and release of oxygen radicals (Bonecchi, Facchetti *et al.* 2000). The authors speculate that IL-4 and IL-13 *in vivo* can reorient the action of IL-8 from neutrophils to monocyte derived cells via the upregulation of CXCR2 (Bonecchi, Facchetti *et al.* 2000).

Flt1 (FMS- related tyrosine kinase 1; Also known as VEGFR1) a receptor for the angiogenic factor vascular endothelial growth factor (VEGF) was upregulated by IL-4 with a fold change >3 (Table 4.3.3). Flt1 has previously been shown to be expressed by monocyte derived DCs differentiated in the presence of GM-CSF and IL-4 (Fernandez Pujol, Lucibello *et al.* 2001). Soluble Flt1 can also act as a decoy receptor, binding VEGF without signalling (Xie, Fan *et al.* 2009). Conceivably, this could alter the ability of DCs to respond to VEGF in a Th2 cytokine setting. It was interesting that Flt1 was also upregulated in *Retnla*$^{-/-}$ DCs, as RELMα has been described to enhance VEGF expression in the lung (Teng, Li *et al.* 2003; Tong, Zheng *et al.* 2006; Yamaji-Kegan, Su *et al.* 2006).

Edn1 is significantly upregulated by IL-4 in WT and *Retnla*$^{-/-}$ DCs (Tables 4.3.3 & 4.3.4), although in WT cells the upregulation did not reach the 2FC cut off (FC=1.88, P=0.00063). *Edn1* is a vasoconstrictive molecule, and the expression of endothelin receptors by human DCs is associated with maturation (Guruli, Pflug *et al.* 2004). Further, blockade of certain aspects of endothelin-1 signalling (ET_A) results in reduced IL-12 production by DCs, whereas blockade of ET_B results in enhanced DC survival (Guruli, Pflug *et al.* 2004). Interestingly, *Edn1* secretion by DCs has been linked to TLR2 and TLR4 agonists (Spirig, Potapova *et al.* 2009). RELMα acting under it's pseudonym HIMF, has also been shown to have vasoconstrictive action in the lung (Teng, Li *et al.* 2003). The potential for regulation of angiogenesis and vasodilation/vasoconstriction previously reported for various genes upregulated in GMDCs in response to IL-4, this should prove an exciting area for continued study.

Aqp3 (Aquaporin 3) was significantly upregulated within WT and *Retnla*$^{-/-}$ DCs in the presence of IL-4. Aquaporin 3 is a membrane water transport protein, that is permeable to water, urea, glycerol and was more recently shown to mediate uptake of H_2O_2 (Miller, Dickinson *et al.* 2010). Aquaporins play a role in maintaining the fluid balance of a cell, and are particularly important in immature DCs which constitutively take up fluid via macropinocytosis (de Baey and Lanzavecchia 2000). *Aqp3* has previously been shown to be expressed by immature DC and become downregulated following maturation; DCs in the De Baey study were differentiated from monocytes using GMCSF and IL-4. However, the authors noted that IL-4 was not required for the moDC expression of Aqp3 and that macrophages did not

express this protein (de Baey and Lanzavecchia 2000). A report from 2012 has further highlighted the importance of Aqp3 in the immune system by showing a requirement for aqauporin 3 mediated uptake of H_2O_2 in chemokine mediated migration of T-cells (Hara-Chikuma, Chikuma *et al.* 2012).

Finally, the transcription factor *Batf3* was – somewhat surprisingly – significantly induced by IL-4 in *Retnla*$^{-/-}$ DCs (but not induced at all in WT DCs), a result that has not previously been reported. *Batf3* is responsible for the development of CD8α^{+} DCs *in vivo*, a subset required for the cross presentation of antigens (Hildner, Edelson *et al.* 2008). It is not apparent as to why *Batf3* would be upregulated by IL-4 or why this molecule may be reguated by RELMα. *Batf3* has potential to be the factor that alters the priming capacity of RELMα deficient DCs, and analysis of the impact of IL-4 on *Batf3*$^{-/-}$ DCs, or the impact of rRELMα on WT DC *Batf3* expression could shed light on this matter.

As is inherent to transcriptomic approaches used to characterise cell populations, a large numer of possibly interesting candidate genes has been generated, in this instance with regard to AADC induction and function. In depth analysis of the available literature on each potential target gene will enable the most relevant target genes to be chosen for further interrogation. In this thesis, based upon the large body of literature available on DC vitamin A metabolism (section 1.8), we chose to interrogate further the role of *Aldh1a2* in IL-4 exposed DCs.

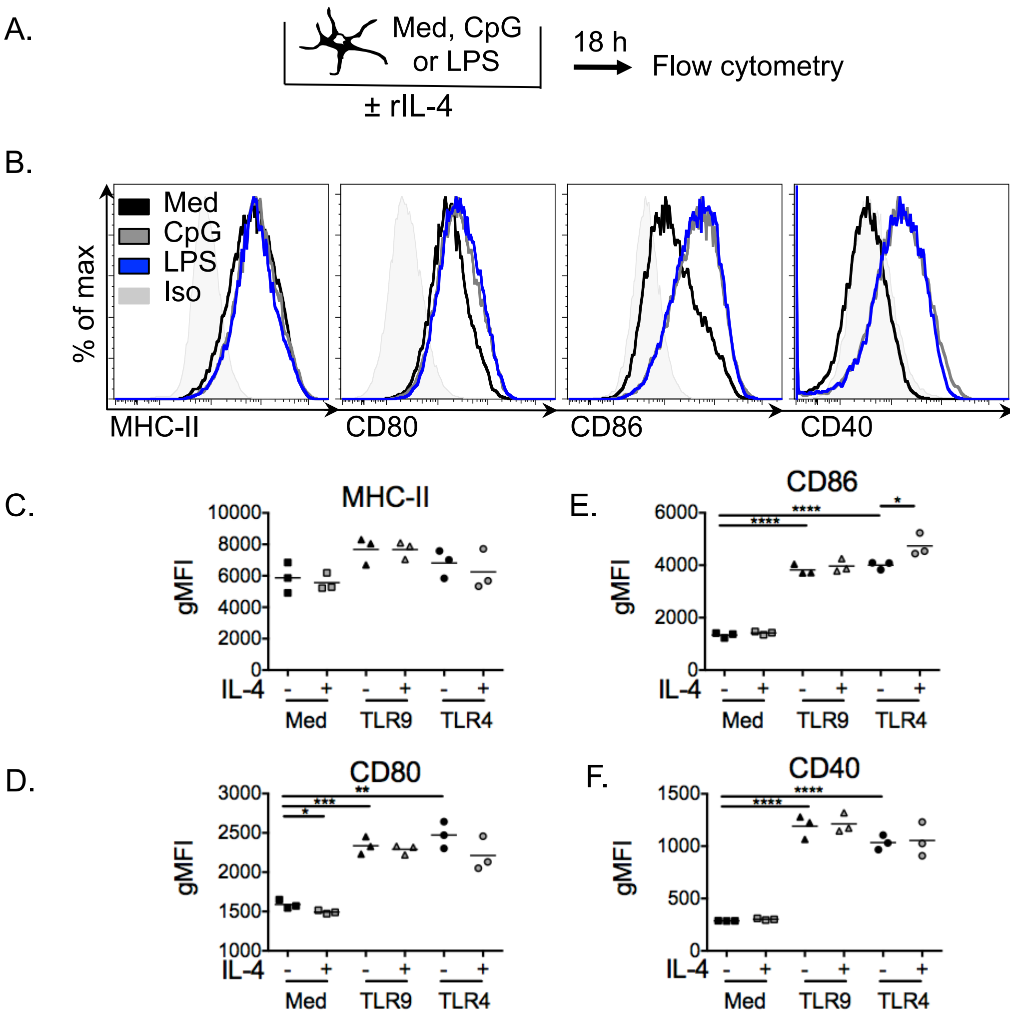


Figure 4.1 TLR stimulated GMDCs upregulate co-stimulatory molecules. WT GMDCs were stimulated overnight with 5 μg/ml CpG or 250ng/ml LPS and their expression of MHC-II, CD80, CD86 and CD40 was assessed by flow cytometry (B). Shaded=isotype control, black line=media, grey line=CpG, blue line = LPS. Geometric mean fluorescence intensity (MFI) of MHC-II (C), CD80 (D), CD86 (E), and CD40 (F) by CD11c^{+} cells following overnight culture with or without CpG, LPS and IL-4. Data is representative of 3 experiments (MHC-II), or 1 experiment (CD80, CD86, CD40), carried out in triplicate. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001.

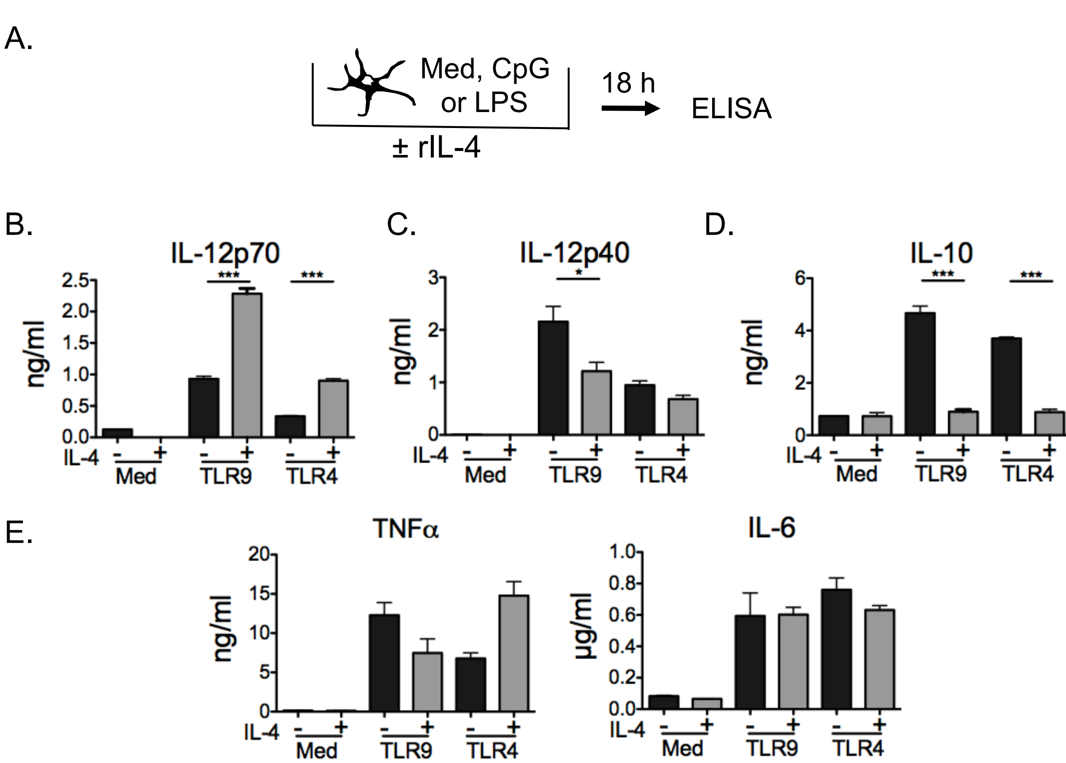


Figure 4.2 IL-4 alters the cytokine profile of DCs activated by TLR ligation. GMDCs were cultured overnight in the presence of 5 μg/ml CpG 1826 or 250ng/ml LPS with or without 20ng/ml IL-4 (A), their secretion of T-cell polarising cytokines was assessed by ELISA (B-E). Error bars represent SEM of triplicate wells, data is representative of 4 independent experiments *=P<0.05 **= P<0.01 ***P<0.001.

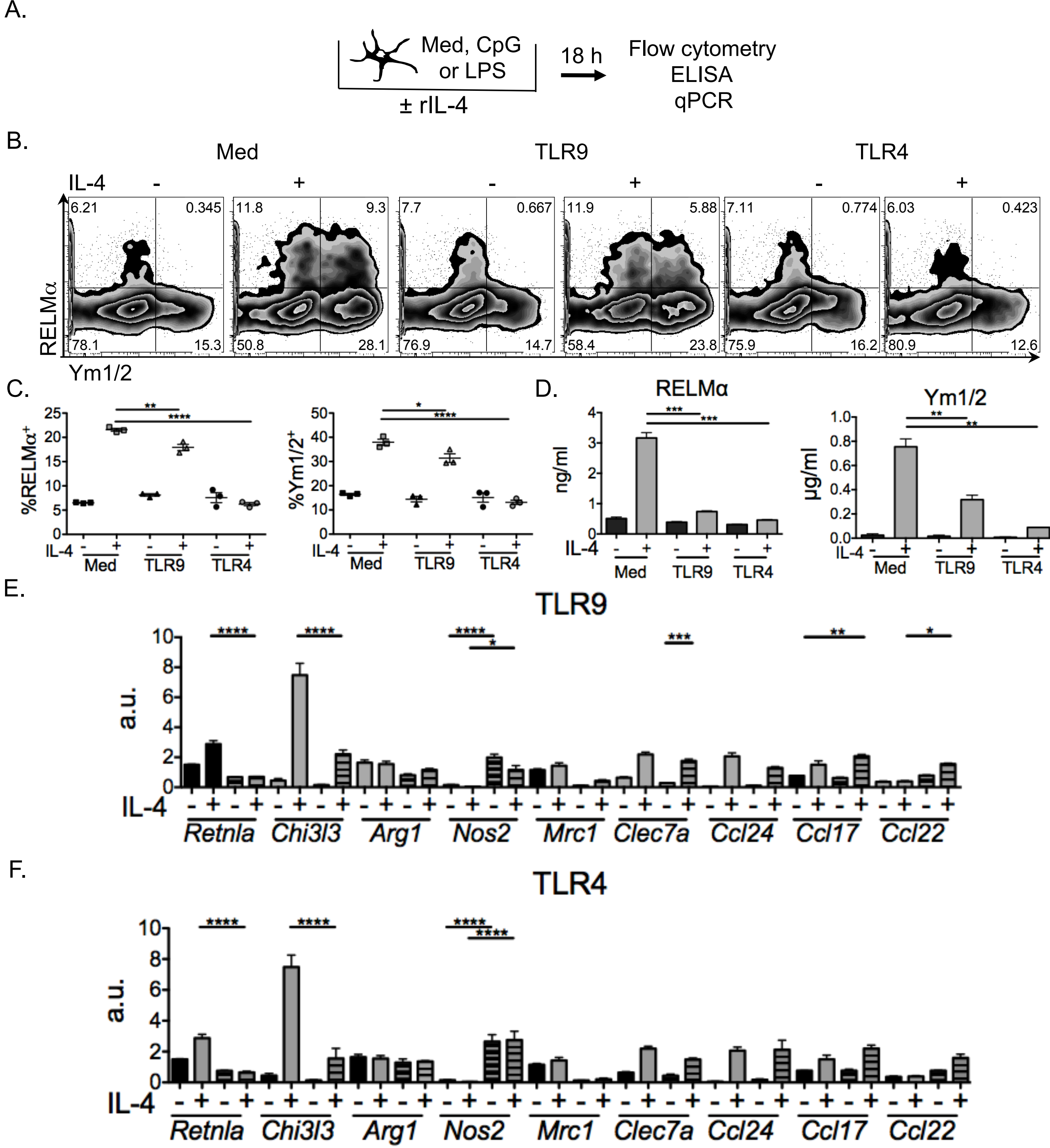


Figure 4.3 TLR agonists limit IL-4 driven alternative activation. GMDCs were cultured overnight in the presence of 5μg/ml CpG or 250ng/ml LPS with or without 20ng/ml IL-4 (A), alternative activation status was assessed by intracellular RELMα & Ym1/2 staining (B+C), ELISA (D) or qPCR analysis of alternative activation associated genes (E+F). Graphs in C show percentage positive populations, error bars represent SEM of triplicate wells. The medium control presented in part E are the same as those presented in part F, CpG (E) and LPS (F) stimulations were carried out within the same experiment but have been separated to improve clarity. Bars in parts E+F Black= Medium, Grey= +IL-4, Striped=+TLR ligand. Data is representative of 4 (A-C) or 2 (E+F) independent experiments *=P<0.05 **= P<0.01, ***=P<0.001, ****=P<0.0001.

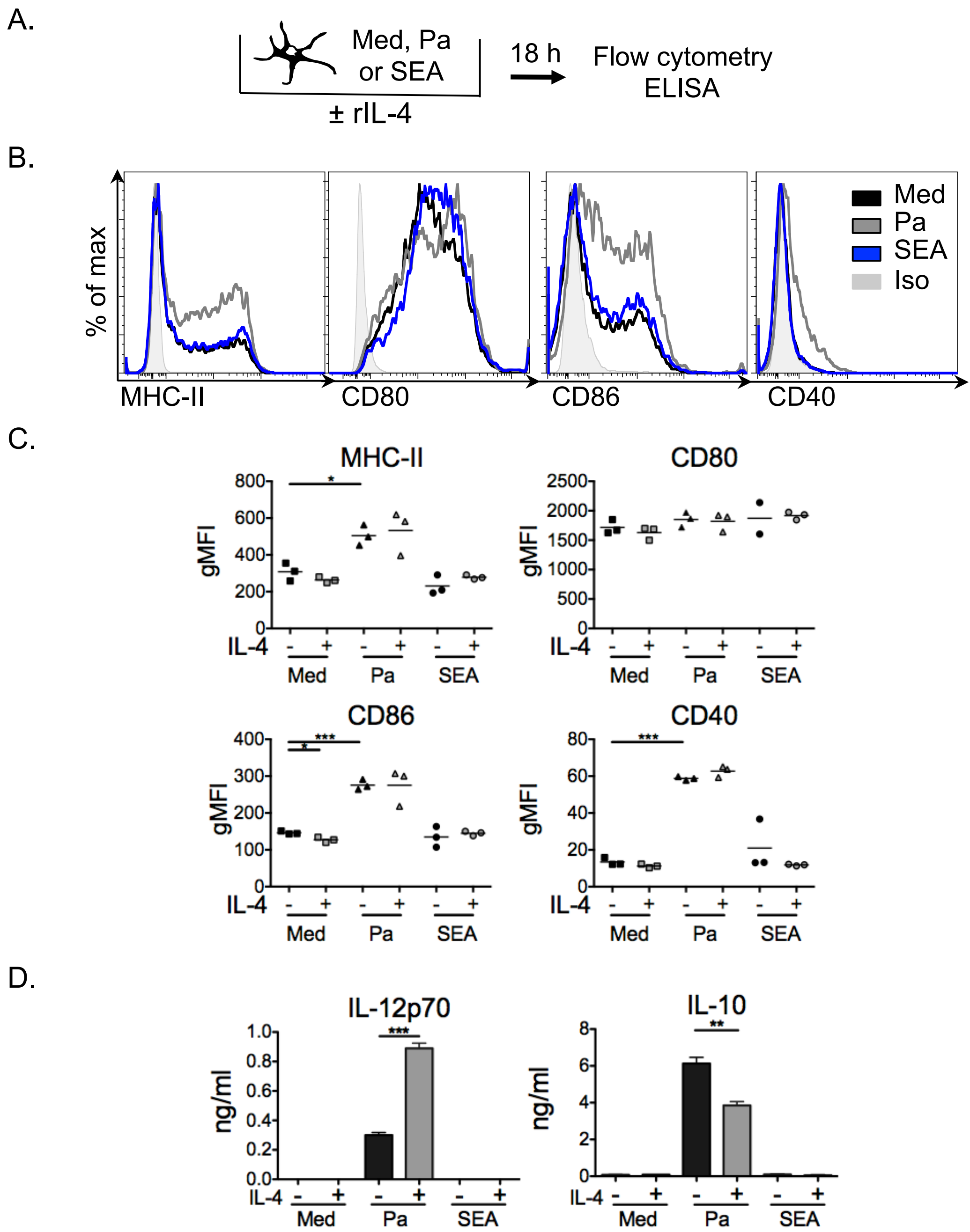


Figure 4.4 Complex bacterial but not helminth antigens modulate DC co-stimulatory molecule expression and cytokine production. GMDCs were cultured overnight in the presence of 10 μ g/ml *P. acnes* or 25 μ g/ml SEA with or without 20ng/ml IL-4 (A), co-stimulatory molecule expression (B+C) and secretion of IL-12p70 and IL-10 was assessed by ELISA (D). Graphs in C show mean fluorescence intensity. Error bars represent SEM of triplicate wells, data is representative of >3 independent experiments *=P<0.05 **= P<0.01 ***=P<0.001.

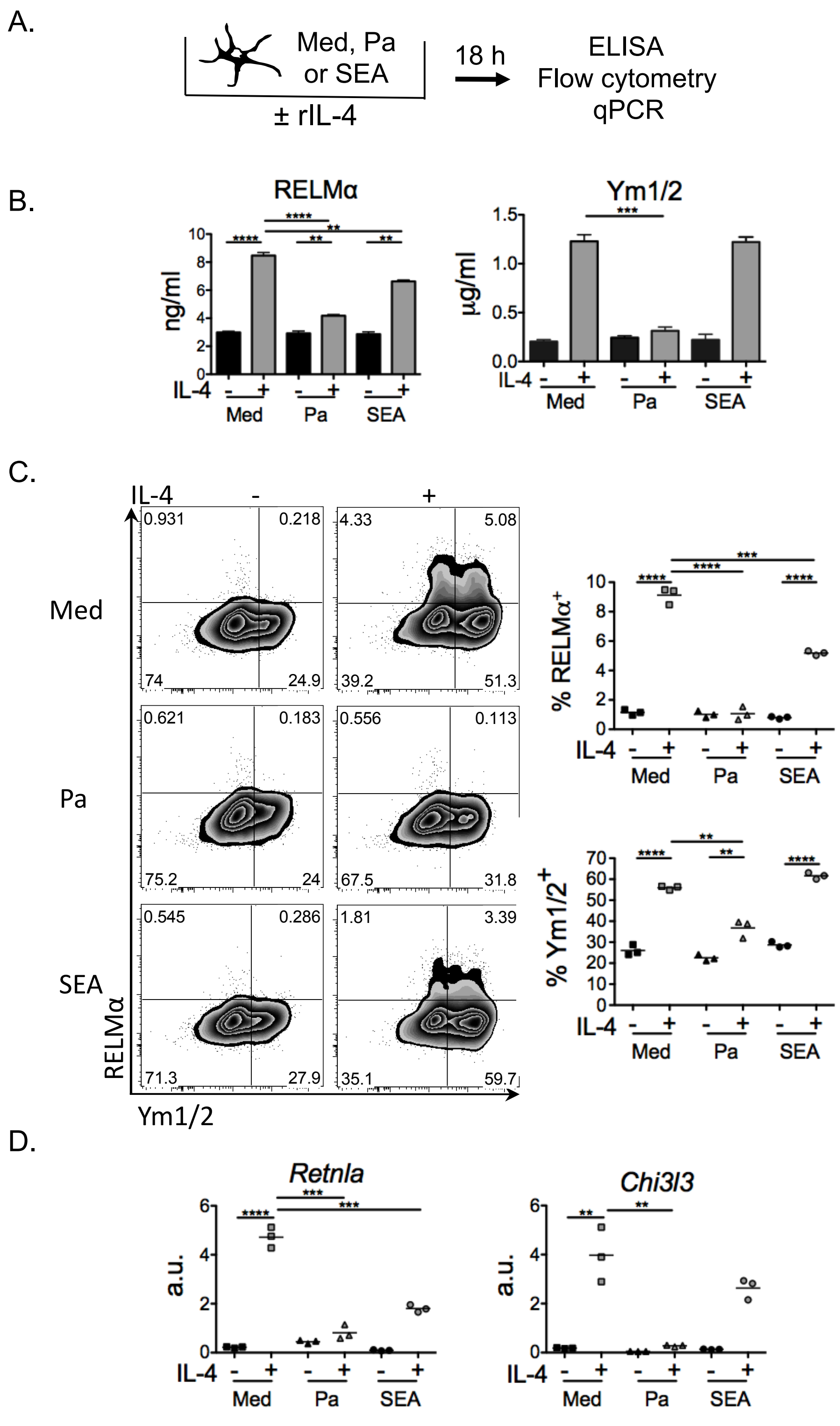


Figure 4.5 Complex bacterial and helminth antigens modulate DC alternative activation. GMDCs were cultured overnight in the presence of 10 μ g/ml *P. acnes* or 25 μ g/ml SEA with or without 20ng/ ml IL-4 (A), alternative activation status was assessed by ELISA (B), intracellular RELM α & Ym1/2 staining (C) and qPCR (D). Graphs in C show percentage positive populations, error bars represent SEM of triplicate wells, data is representative of >3 independent experiments, a.u.=arbitrary units relative to *Hprt*. *=P<0.05, **= P<0.01, ***=P<0.001, ****=P<0.0001.

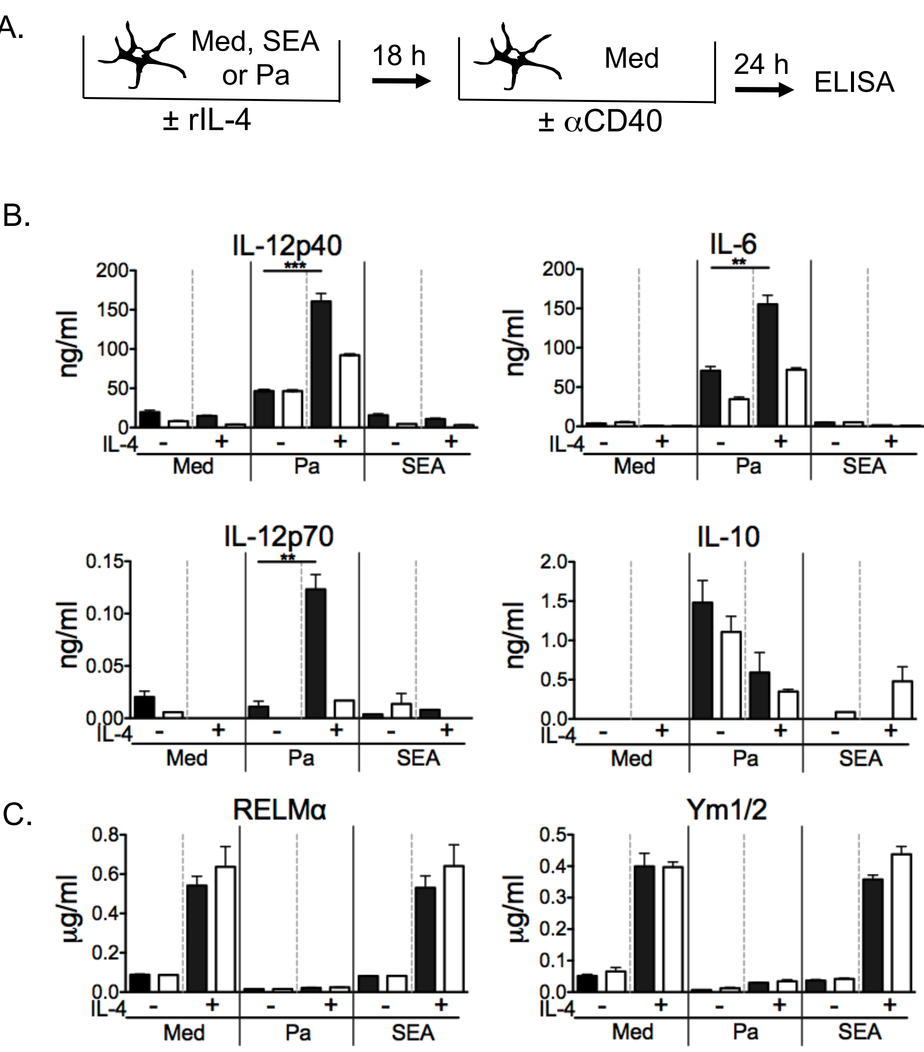


Figure 4.6 IL-4 alters the response of DCs following CD40 ligation. GMDCs cultured overnight with IL-4 and Pa or SEA were washed and re-cultured with agonistic anti-CD40 (black bars) or control antibody (white bars) for a further 24h (A). Cytokine (B) and RELMα and Ym1/2 (C) secretion was assessed by ELISA. Data representative of 3 experiments, error bars = SEM of triplicate culture wells **= P<0.01 ***=P<0.001.

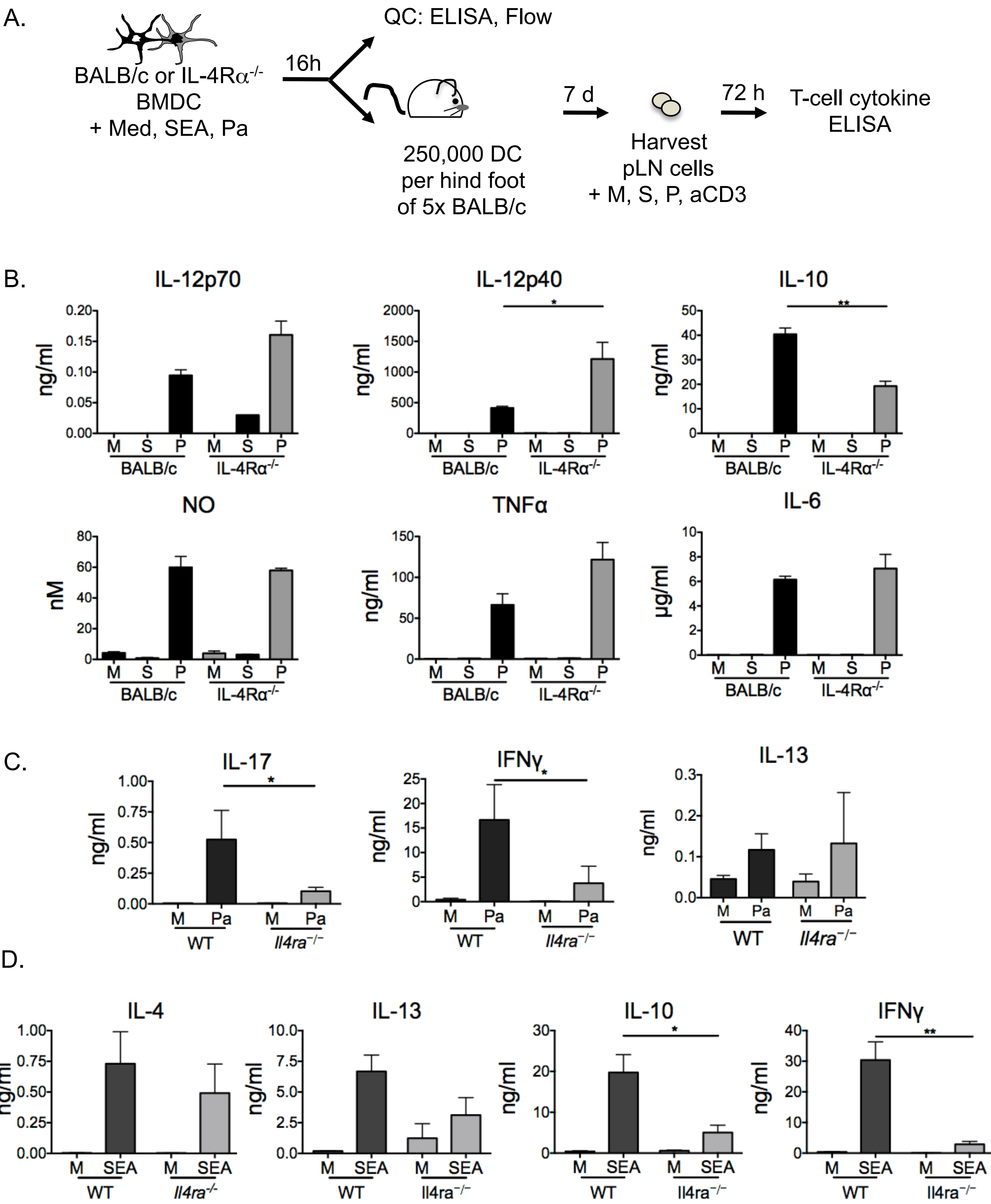


Figure 4.7 *Il4ra*$^{-/-}$ DCs are less able to induce IFNγ and IL-17 responses. WT or *Il4ra*$^{-/-}$ BMDCs were cultured overnight in medium alone (M), with Pa or SEA, harvested and injected subcutaneously into WT mice, 7 days later the draining pLN were harvested, cells restimulated for 72h with Pa or SEA and cytokine secretion was assessed by ELISA (A). Cytokine secretion was assessed pre transfer (B). Cytokines produced following restimulation with Pa (C) or SEA (D) was assessed by ELISA. Data are representative of 5 independent experiments, error bars = SEM of triplicate culture wells (B) or 3-5 mice per group (C&D), *=P<0.05 **= P<0.01.

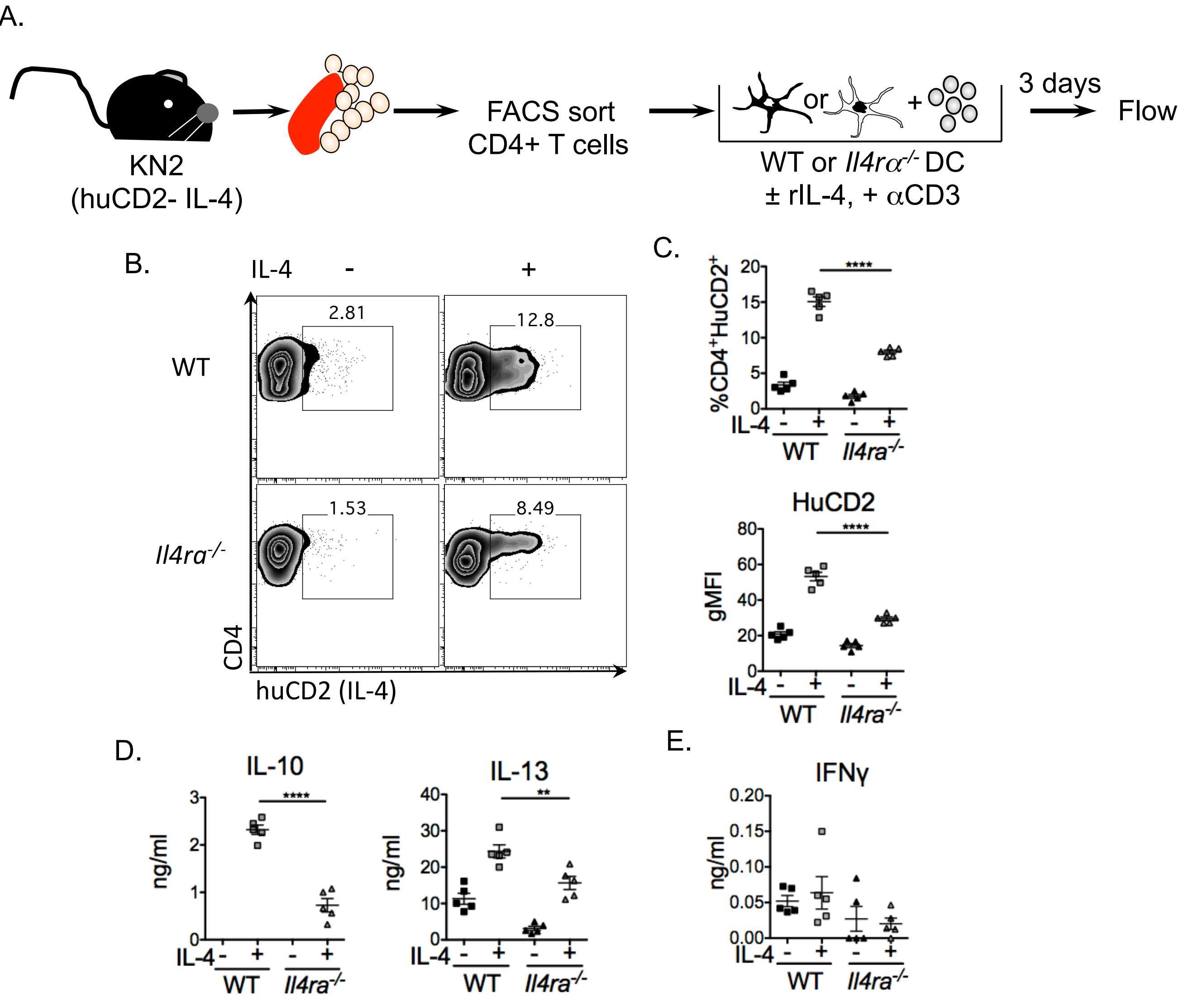


Figure 4.8 DC expression of IL-4Rα promotes early IL-4 driven T-cell IL-4, IL-10 and IL-13. $CD4^{+}$ cells sorted from KN2 IL-4 reporter mice were cultured for 3 days with WT or *Il4ra*$^{-/-}$ GMDCs and agonistic anti-CD3 antibody with or without IL-4 (A). Cells were assessed for IL-4 protein production by flow cytometry (B+C) and cytokine secretion by ELISA (D+E). Data are representative of 3 experiments, error bars = SEM of 5 replicate wells per group, **= P<0.01 ****=P<0.0001.

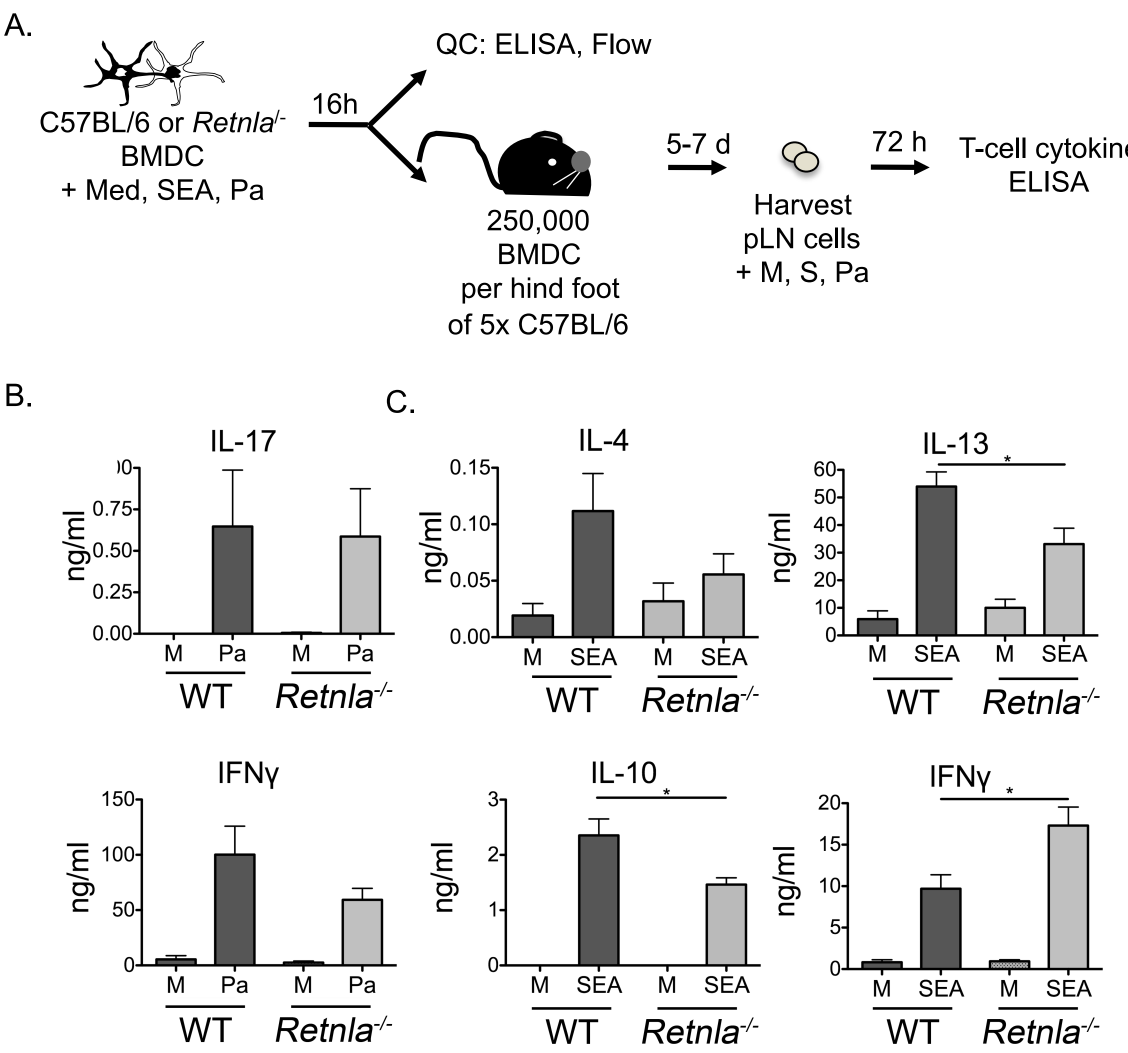


Figure 4.9 DC expression of RELMα during Th2 priming regulates IFNγ and promotes IL-10 and IL-13 production. WT or *Retnla*$^{-/-}$ BMDCs were cultured overnight in medium alone (M), Pa or SEA and injected subcutaneously into WT mice, 5-7d later pLN were harvested, cells restimulated for 72h (A) with Pa (B) or SEA (C) and cytokine secretion assessed by ELISA.. Data representative of 2 (B) or 4 (C) experiments, error bars = SEM of 3-5 mice per group, *=P<0.05.

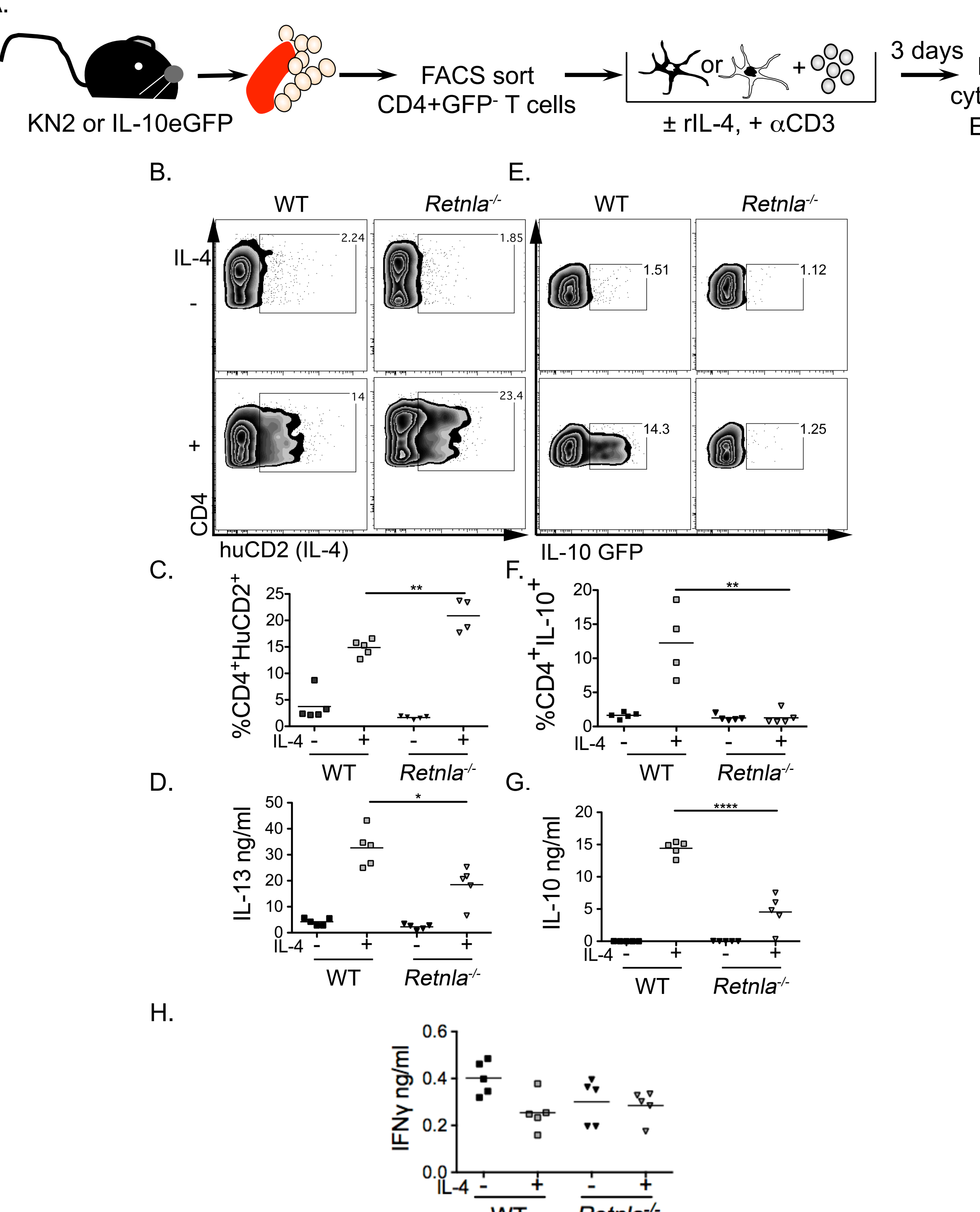


Figure 4.10 DC derived RELMα promotes IL-4 driven IL-10 but limits T-cell IL-4. CD4$^+$ cells sorted from KN2 IL-4 reporter mice (B+C), or IL-10eGFP$^-$-CD4$^+$ cells (E+F), were cultured for 4 days with WT or *Retnla*$^{-/-}$ BMDCs and agonistic anti-CD3 antibody with or without IL-4 (A) and assessed for IL-4 protein production or IL-10 mRNA expression by flow cytometry (B+C,E+F) and cytokine secretion by ELISA (D,G-H) Data is representative of 2 (B+C, E+F) or 4 (D,G-H) experiments, error bars = SEM of 4-5 replicate wells per group, *=P<0.05 **= P<0.01 ****=P<0.0001.

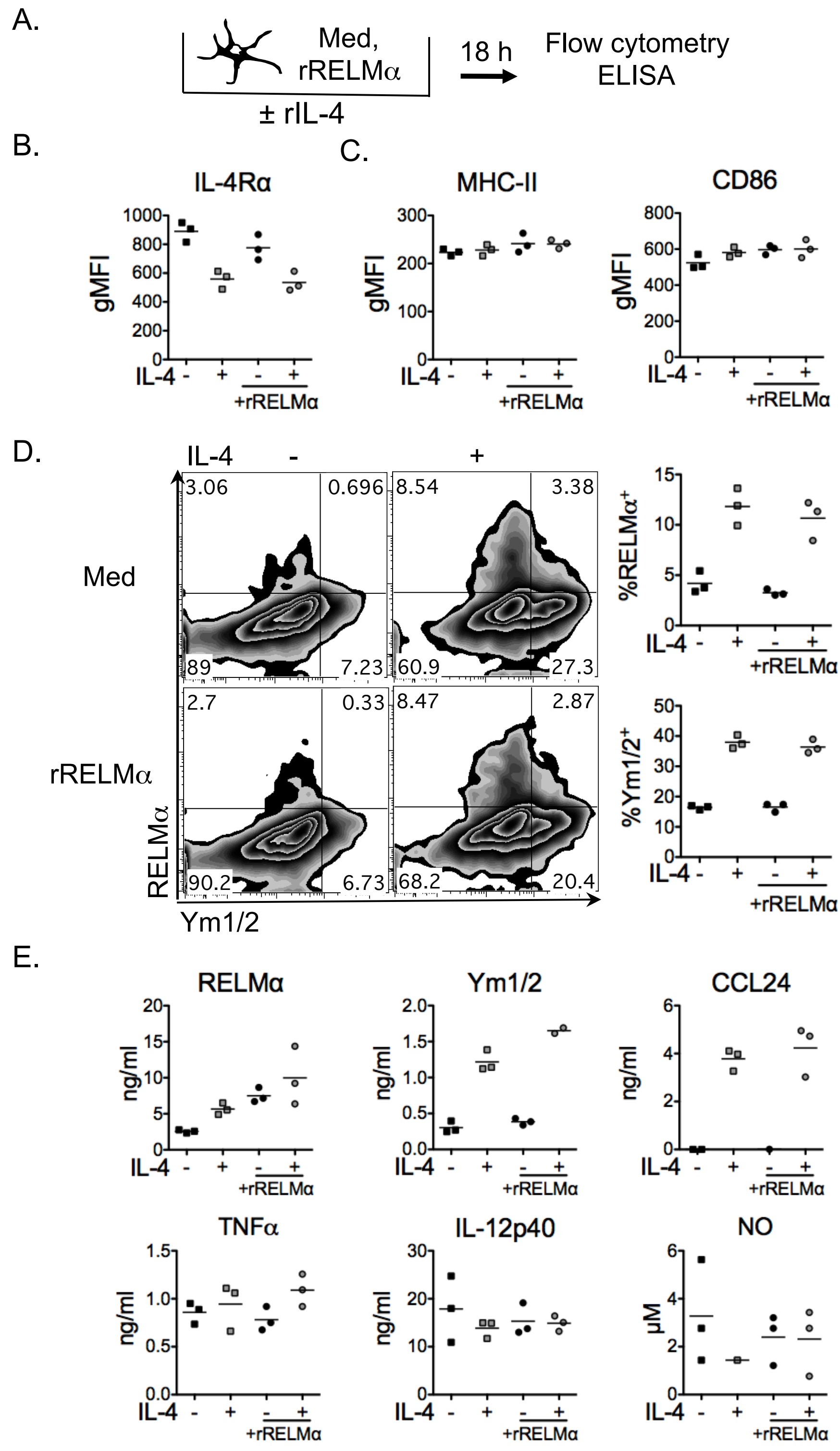


Figure 4.11 rRELMα does not activate GMDCs. GMDCs were cultured overnight in the presence of 20ng/ml rRELMα with or without 20ng/ml IL-4 (A). Expression of IL-4Rα was confirmed by surface staining (B), changes in levels of expression of MHC-II and CD86 were determined by flow cytometry (C). The alternative activation status of the cells was assessed by intracellular RELMα & Ym1/2 staining (D), and cytokine secretion was assessed by ELISA (E). Graphs in D show percentage positive populations, bars in D represent mean of triplicate wells, data is representative of 2 (E) or 3(B-D) independent experiments, gMFI=Mean fluorescence intensity.

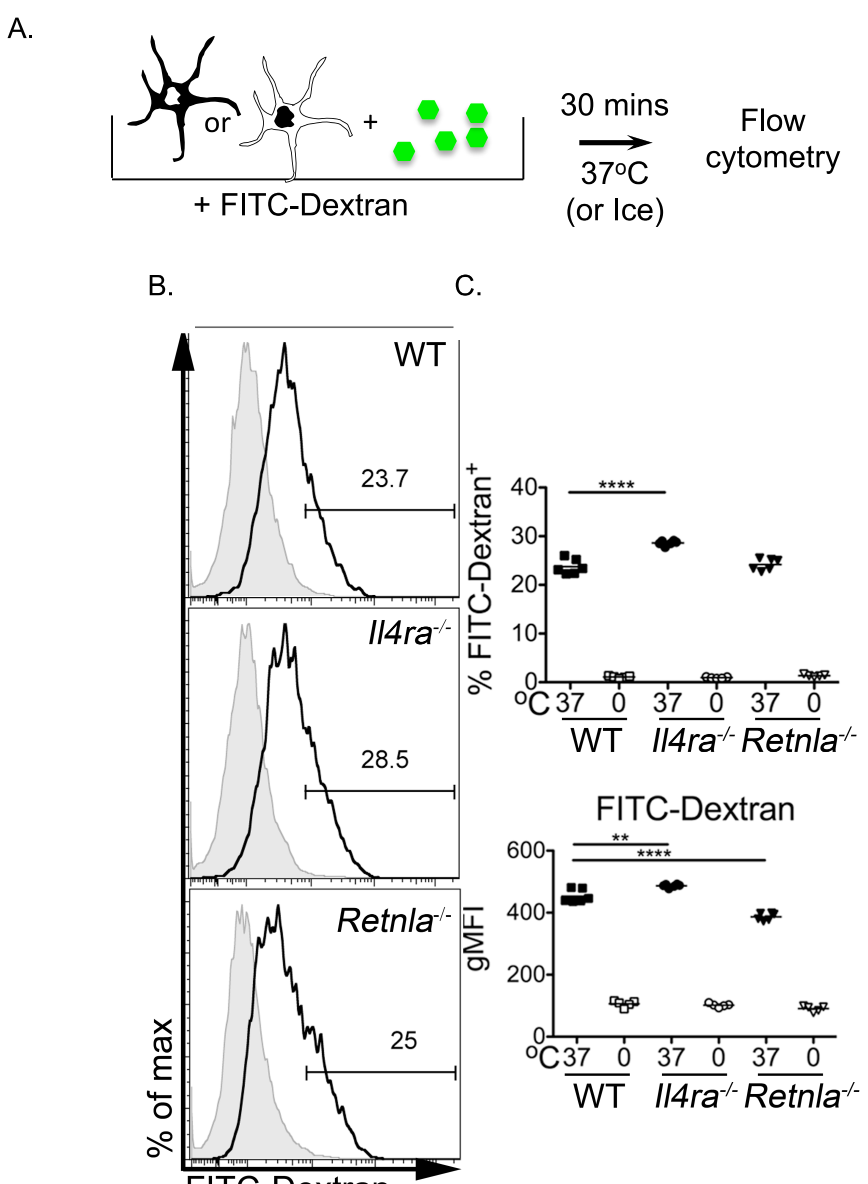


Figure 4.12. *WT, Il4ra$^{-/-}$ and Retnla$^{-/-}$* DCs are equally capable at antigen uptake. WT, *Il4ra$^{-/-}$* or *Retnla$^{-/-}$* BMDCs were incubated with FITC conjugated dextran at 37°C and antigen uptake assessed by flow cytometry (A-C). Shaded=0°C, Black line=37°C. Data is representative of 2 experiments, 5-6 replicate wells per group. **= P<0.01 ****P<0.0001.

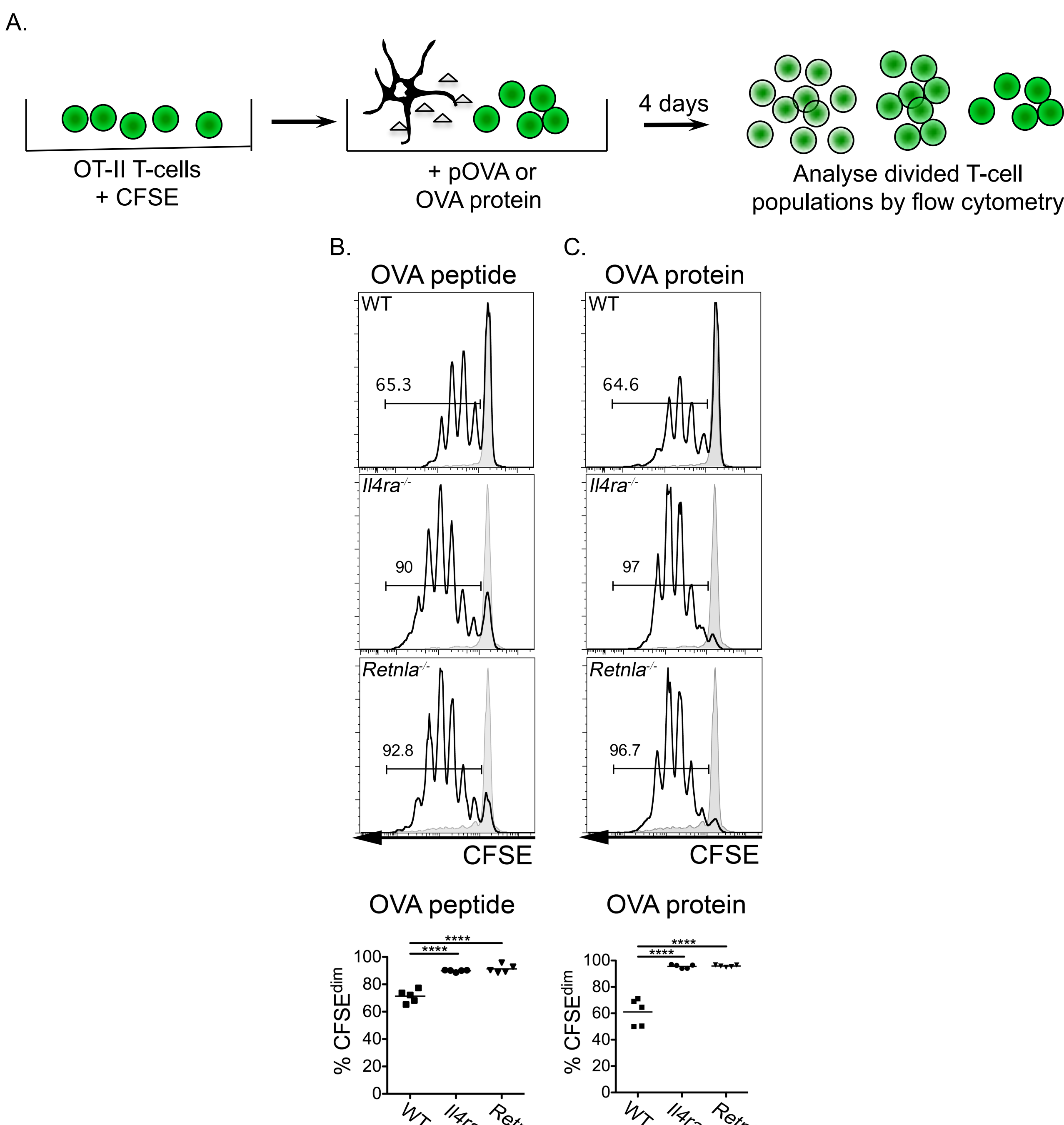


Figure 4.13. Antigen processing and presentation is unimpaired in *Il4ra-/-* and *Retnla-/-* DCs. WT, *Il4ra-/-* or *Retnla-/-* GMDCs were cultured for 4d with CFSE labelled OT-II Tg T-cells in the presence of OVA peptide (B) or protein (C) and the ability to stimulate proliferation was assessed by flow cytometric analysis of CFSE dilution (A). Shaded=no peptide/protein, Black line=0.01 μ g/ml OVA peptide (A) or 5 μ g/ml OVA protein (B). Data representative of 2 experiments, 5 replicate wells per group. ****P<0.0001.

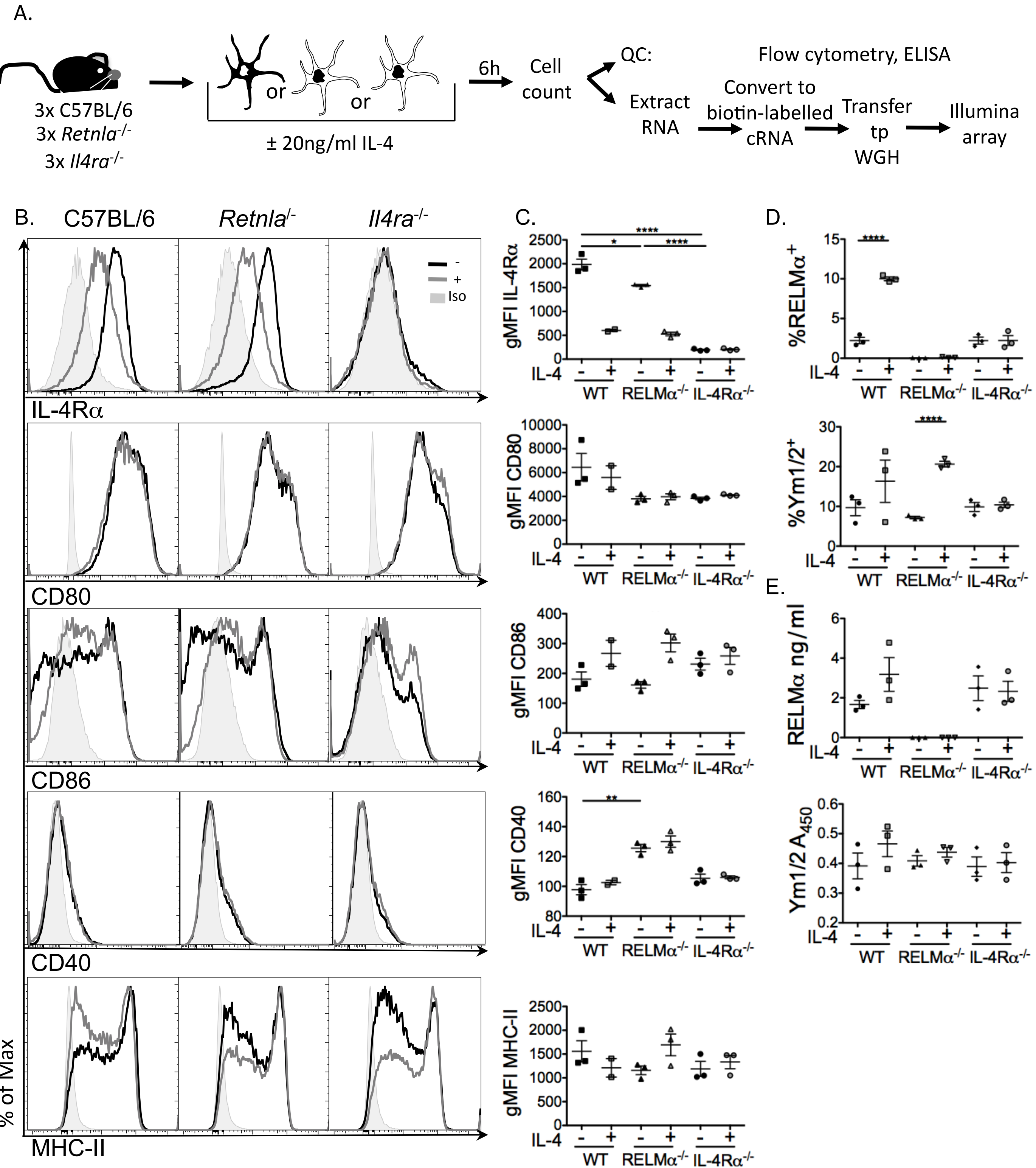
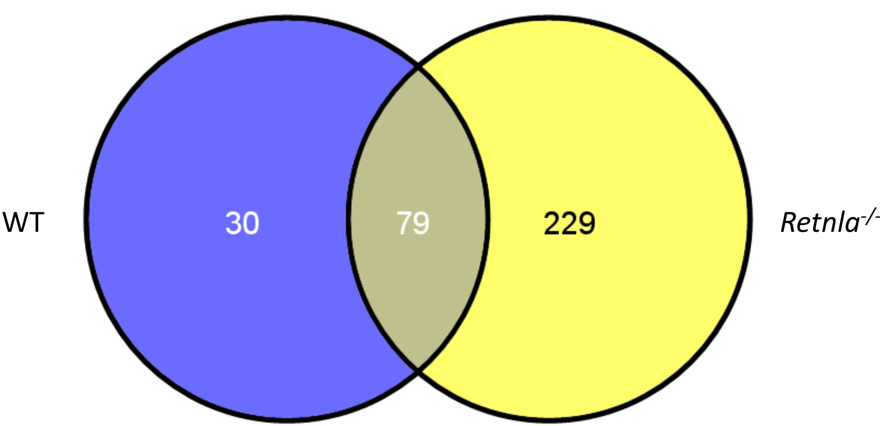


Figure 4.14. IL-4 treatment of RELMα deficient DCs significantly alters transcription of more genes than IL-4 treatment of WT DC. WT, *Il4ra-/-* or *Retnla-/-* GM-DCs were cultured for 6h with IL-4, cells were assessed for activation status by flow cytometry and ELISA (B-E), RNA was extracted and an illumina microarray carried out, genes significantly changed upon IL-4 were assessed (F-H) Data is from one microarray experiment, 3 biological replicates per genotype *=P<0.05 **= P<0.01 ***=P<0.001 ****=P<0.0001.

F.



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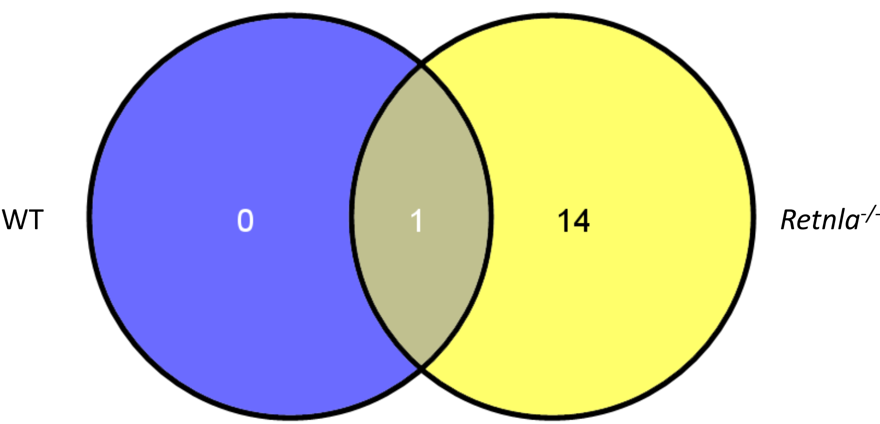

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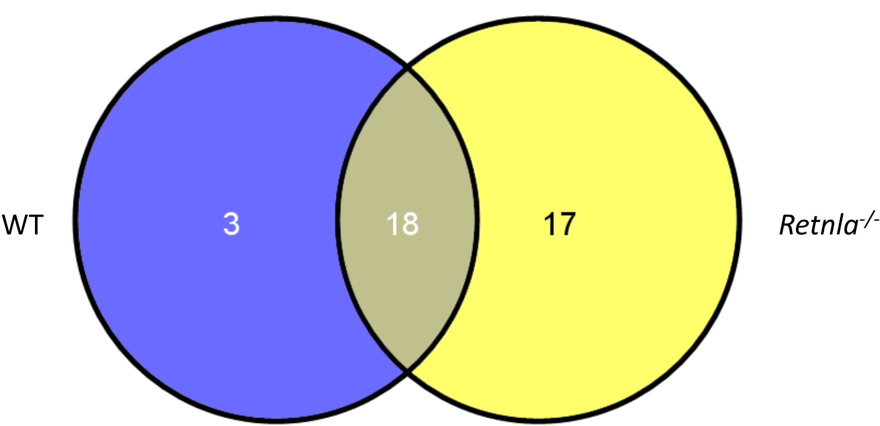


Figure 4.14 continued

Table 4.3.1 Genes significantly down ≤0.5 fold in IL-4 treated WT and Retnla$^{-/-}$ DC				
Symbol	**Synonym**	**Name**	**Putative function**	**FC**
Thbs1	TSP-1	thrombospondin 1	Cell adhesion	0.31

Table 4.3.2 Genes significantly down ≤0.5 fold only in IL-4 treated Retnla$^{-/-}$DC				
Symbol	**Synonym**	**Name**	**Putative function**	**FC**
Il1rn	IL-1ra	interleukin 1 receptor antagonist	Inhibits IL-1 signalling	0.44
Dusp6	MKP3	dual specificity phosphatase 6	Negative regulator of ERK1/2	0.50
Pilra	FDF03	paired immunoglobin-like type 2 receptor alpha	Inhibitory receptor, phosphatase recruitment	0.47
Lpcat2	AYTL1	lysophosphatidylcholine acyltransferase 2	Synthesis of PAF & Glycerophospholipid precusors	0.41
Clec4d	Mpcl	C-type lectin domain family 4, member d	Phagocytic receptor, Activates Syk kinase	0.35
Arhgef3	GEF3	Rho guanine nucleotide exchange factor (GEF) 3	Guaning exchange, regulation of iron uptake	0.38
LOC638301		similar to Interferon-activatable protein 204 (Ifi-204)	Unknown	0.41
Tlr2	TLR2	toll-like receptor 2	Lipopeptide recognition	0.29
Pilra	FDF03	paired immunoglobin-like type 2 receptor alpha	Inhibitory receptor, phosphatase recruitment	0.50
Klhl6	KLHL6	kelch-like 6 (Drosophila)	BCR signal transduction	0.47
Rsad2	Viperin	radical S-adenosyl methionine domain containing 2	moDC induction of Th2 IFN inducible	0.44
Serpinb2	PAI-2	serine (or cysteine) peptidase inhibitor, clade B, member 2	Suppression of Th1, promotion of Th2	0.38
Fpr2	Fprl1	formyl peptide receptor 2	Leukocyte (CD11c+) recruitment/ migration	0.35
Pid1	PID1	phosphotyrosine interaction domain containing 1	Pre-adipocyte proliferation	0.35
Clec4e	Mincle	C-type lectin domain family 4, member e	CTL/mycobacterium + necrotic self recognition	0.29

Table 4.3.3 Genes significantly up ≥ 2 fold in IL-4 treated WT and Retnla$^{-/-}$ DC				
Symbol	**Synonym?**	**Name**	**Putative function**	**FC**
Ccl24	Eotaxin-2	chemokine (C-C motif) ligand 24	Eosinophil recruiment	16
Cxcr2	IL8RB	chemokine (C-X-C motif) receptor 2	IL-8R Neutrophil recruitment	3.48
Aqp3	GIL	aquaporin 3	Pinocytosis cell volume control	3.25
Rnf19b	Ibrdc3	ring finger protein 19B	E3 ubiquitin ligase	2.14
Casp6	MCH2	caspase 6	Apoptosis	3.25
Ms4a6d	?	membrane-spanning 4-domains, subfamily A, member 6D	Unknown	2.64
Hemk1	?	HemK methyltransferase family member 1	Unknown	2.14
Pim3	?	proviral integration site 3	Cell proliferation	2.46
Aldh1a2	RALDH2	aldehyde dehydrogenase family 1, subfamily A2	Conversion of Retinal to Retinoic Acid (RA)	2.64
Tfrc	TfR1	transferrin receptor	Iron homeostasis	2.14
Pdlim1	CLP-36	PDZ and LIM domain 1 (elfin)	Unknown	3.03
Ak2	ADK2	adenylate kinase 2	Mitochondrial component	2.14
Gm8221	?	apolipoprotein L, 3-like pseudogene	Unknown	2
Myo1e	MYO1C	myosin IE	Molecular motor, Clathrin mediated endocytosis	2.83
Chi3l4	Ym2	chitinase 3-like 4	Th2 promotion?	4.29
Irf4	IRF4	interferon regulatory factor 4	TF, DC development	2.14
Chi3l3	Ym1	chitinase 3-like 3	Th2 promotion?	4
Flt1	VEGFR1	FMS-like tyrosine kinase 1	Clearance of VEGF	3.48

Table 4.3.4 Genes significantly up ≥ 2 fold only in IL-4 treated Retnla$^{-/-}$DC				
Symbol	**Synonym?**	**Name**	**Putative function?**	**FC**
Edn1	ET1	endothelin 1	Vascular homeostasis	2.30
Plekhf1	LAPF	pleckstrin homology domain containing, family F (with FYVE domain) member 1	Apoptosis	2.14
Hist1h1c	H1c	histone cluster 1, H1c	Chromatin compaction	2
Sema4b	SEMAC	semaphorin 4B	Negative regulation basophil Th2	2.46
Mgll	MagL	monoglyceride lipase	FFA release from lipids	2.83
Batf3	BATF3	basic leucine zipper transcription factor, ATF-like 3	TF, CD8+ DC development	2.64
Rufy4	?	RUN and FYVE domain containing 4	Unknown	2.30
Btbd11	Unknown	BTB (POZ) domain containing 11	Unknown	2.64
Atf3	ATF3	activating transcription factor 3	Regulation of immune & metabolic function	2.64
Prps1	ARTS	phosphoribosyl pyrophosphate synthetase 1	Purine metabolism	2.14
Cdh1	E-cadherin	cadherin 1	Cell adhesion, epithelial.	2.64
Lad1	LadA	ladinin	Epithelial attachment to mesenchyme	2.00
Tgm2	TG2/TGC	transglutaminase 2, C polypeptide	Protein crosslinking	2.14
Atf5	ATF5	activating transcription factor 5	TF	2
Sdc4	SDC4	syndecan 4	Proteoglycan receptor Activates PKC	3.03
Serpina3g	Spi2A	serine (or cysteine) peptidase inhibitor, clade A, member 3G	Inhibition of cathepsin, protection from apoptosis	2.46
Plau	ATF, URK	plasminogen activator, urokinase	Degradation of ECM	2.14

Table 4.3.5 Genes significantly up ≥ 2 fold only in IL-4 treated WT DC				
Symbol	**Synonym**	**Name**	**Putative function**	**FC**
Hfe	HLA-H	hemochromatosis	Iron homeostasis, regulation of Transferrin Rs	2.14
Retnla	RELMα	resistin like molecule alpha	Regulation of Th2	3.73
Apol7c	?	apolipoprotein L 7c	Unknown	2.83

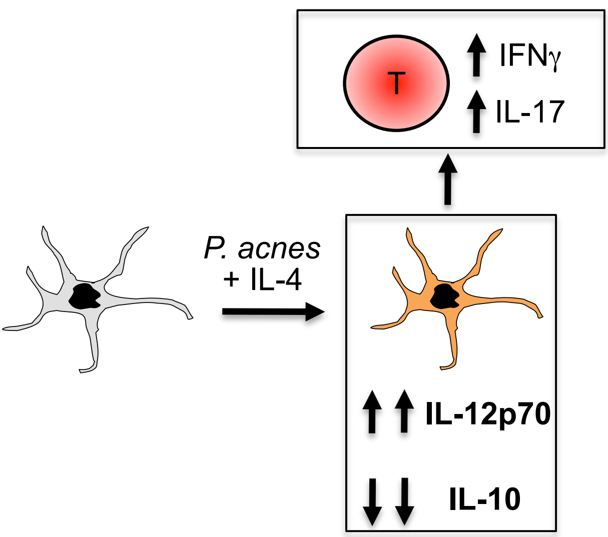
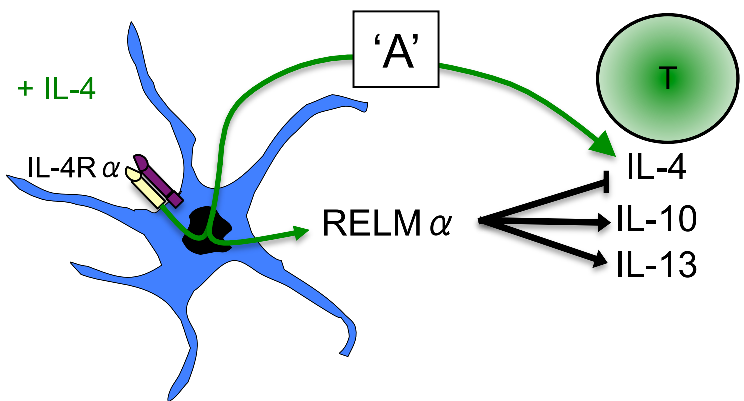


Figure 4.15. Model of IL-4 and bacterial agonist mediated changes in DC function. In the presence of Pa and IL-4, DCs increase IL-12p70 and decrease IL-10 secretion, which *in vivo* results in enhanced IFN γ and IL-17 production, confirmed using *Il4ra*$^{-/-}$ DC transfer (Fig. 4.7C).

A.



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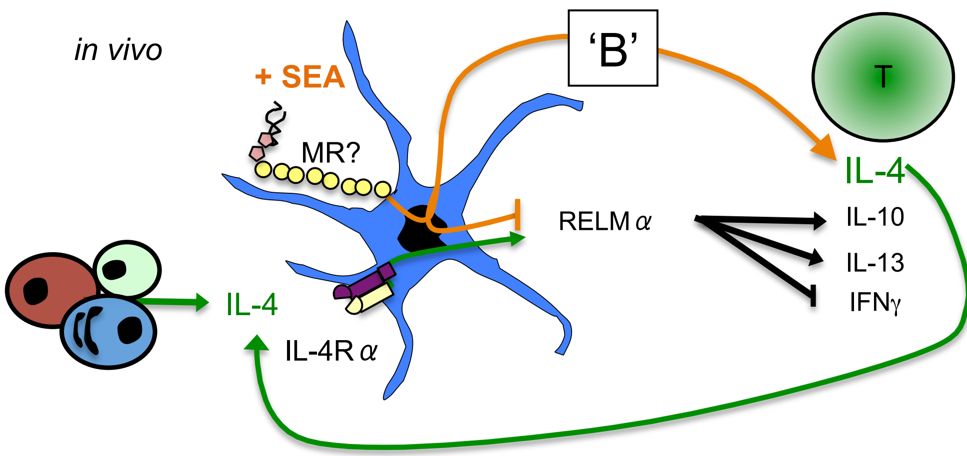


Figure 4.16. Models of IL-4 and helminth mediated changes in DC function.

(A) *In vitro* IL-4 treatment of DCs promotes RELMα production (Fig. 3.2), RELMα leads to enhanced priming of T-cell IL-10 and IL-13, and reduced IL-4 (Fig. 4.10), IL-4Rα dependent factor 'A' promotes T-cell IL-4 (Fig. 4.8C).

(B) *In vivo* SEA pulsed DCs polarise a Th2 response via unknown factor 'B', IL-4 from T-cells and other innate cell sources signal to DCs via IL-4Rα, promoting RELMα. RELMα inhibits IL-4 and promotes IL-10 and IL-13 (Fig. 4.10). However, SEA inhibits IL-4 driven DC RELMα (Fig. 4.5), releasing the RELMα dependent inhibition of T-cell IL-4 and promotion of IL-13 by WT DCs, thus limiting the impact of IL-4Rα deficiency following SEA DC transfer (Fig. 4.7) in which no significant difference in priming of IL-4 or IL-13 is found between WT and *Il4ra*$^{-/-}$ DCs, in contrast to the *in vitro* DC:T-cell co-culture (Fig. 4.8), in which no SEA is present.

Chapter 5. How do IL-4 and Vitamin A Metabolites Alter DC Function?

5.1 INTRODUCTION

At the end of chapter 4 (Table 4.3.3) results of the IL-4 DC microarray highlighted that one of the genes significantly upregulated greater than 2 fold in both WT and *Retnla*$^{-/-}$ DCs was *Aldh1a2,* a gene that encodes the retinal dehydrogenase enzyme RALDH2 (Zhao, McCaffery *et al.* 1996). RALDH2 converts retinal to retinoic acid, a bioactive metabolite with important roles in the immune system (see section 1.8). We decided to interrogate further the relationship between IL-4 and vitamin A metabolism in DCs.

The importance of dietary vitamin A (retinol) in health is now well established, with multiple studies showing significantly reduced all-cause mortality in children 6 months to 5 years of age following dietary supplementation (Sommer 2008). The specific impact of vitamin A signalling within the immune system has begun to be elucidated during the past decade (Iwata, Hirakiyama *et al.* 2004; Coombes, Siddiqui *et al.* 2007; Hall, Grainger *et al.* 2011; Jaensson-Gyllenback, Kotarsky *et al.* 2011; Broadhurst, Leung *et al.* 2012).

Recent work has highlighted a role for retinoic acid (RA), the transcriptionally active metabolite of vitamin A, in the development and promotion of CD4^{+} T-cell responses (Hall, Cannons *et al.* 2011; Pino-Lagos, Guo *et al.* 2011). It had previously been shown that RA expression by gut associated lymphoid tissue (GALT) DCs imprints intestinal specificity on T-cells via the upregulation of homing receptors α4β7 and CCR9 (Iwata, Hirakiyama *et al.* 2004; Benson, Pino-Lagos *et al.* 2007). Furthermore, CD103^{+} gut resident steady state DCs have been shown to express *Aldh1a2* (RALDH2) (Coombes, Siddiqui *et al.* 2007), which encodes an aldehyde dehydrogenase enzyme responsible for the conversion of retinal into retinoic acid (Duester 2000). DC expression of RALDH2 was shown to promote extra-thymic conversion of naïve T cells into Foxp3^{+} regulatory T cells in a TGF-β and RA dependent manner (Coombes, Siddiqui *et al.* 2007; Sun, Hall *et al.* 2007).

Interestingly, it is becoming clear that vitamin A signalling is required for T helper cell function outwith the promotion of Foxp3 expression (Hall, Cannons *et al.* 2011; Pino-Lagos, Guo *et al.* 2011). A requirement for RA in the elicitation of the proinflammatory CD4$^+$ T-cell response to *Toxoplasma gondii* infection (Hall, Cannons *et al.* 2011) has been shown, as has a role for RA in mediating T-cell migration and effector function following allogeneic skin graft (Pino-Lagos, Guo *et al.* 2011). However, the impact of vitamin A metabolism on the immune system in the context of Th2 settings is underrepresented within the literature.

CD11c$^+$ DCs are both necessary and sufficient for the induction of Th2 responses in models of helminth infection (Phythian-Adams, Cook *et al.* 2010; Smith, Hochweller *et al.*). The archetypal Th2 cytokine IL-4 has been shown to promote DC expression of *Aldh1a2* (Elgueta, Sepulveda *et al.* 2008; Yokota, Takeuchi *et al.* 2009; Stock, Booth *et al.* 2011). As we have shown in previous chapters that IL-4 stimulates the alternative activation of DCs, we sought to determine whether IL-4 also regulates DC vitamin A metabolism and the impact of the vitamin A metabolite RA on DC APC function.

5.2 SPECIFIC AIMS:

1. To confirm the role of IL-4 in modulating vitamin A metabolism within DCs.
2. To understand the interactions that occur between signalling pathways involved in alternative activation and vitamin A metabolism.
3. To dissect the impact of retinoic acid and IL-4 on DC function.

5.3 RESULTS

5.3.1 *Aldh1a2* and *Ak2* are significantly upregulated in IL-4 treated DCs

KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/) is a database resource that assists in the understanding of biological functions, by compiling associations onto which high throughput genomic information can be mapped. The KEGG PATHWAY is a collection of manually drawn pathway maps representing the current knowledge of molecular interactions and reaction networks for various biological pathways. Analysis of IL-4 treated WT DC illumina expression array data (as carried out in Fig. 4.14) found that the KEGG pathway with which the IL-4 treated WT DC sample data had the highest number of associations was metabolic pathways, with which 14 significantly altered genes were associated (Fig. 5.1A&B). In order to determine genes that may have more prominent roles in modulating cellular function following treatment with IL-4, we chose to use a 2 fold change cut off, ensuring that only transcripts which were doubled in response to IL-4 were being investigated at this stage. When using the stringent 2FC cutoff, only 2 metabolic genes remained: *Ak2* (adenylate kinase 2) a mitochondrial protein, and *Aldh1a2* (Aldehdye dehydrogenase 1a2) (Fig. 5.1 and Table 5.3.1).

5.3.2 IL-4 induces *Aldh1a2* expression and aldehyde dehydrogenase activity in GMDC and FLT3L DCs treated with GM-CSF

IL-4 has previously been shown to induce enhanced metabolism of retinal to retinoic acid in DC via upregulation of the aldehyde dehydrogenase RALDH2 (Elgueta, Sepulveda *et al.* 2008; Yokota, Takeuchi *et al.* 2009); we were interested to relate this role for IL-4 to the role we had described in chapters 3 and 4. In chapter 3 it was shown that IL-4 increased expression of alternative activation associated molecules (including RELMα and Ym1/2) in DC both *in vitro, in vivo* and *ex vivo*. In chapter 4 DC derived RELMα was shown to be required for induction of T-cell IL-10 and regulation of T-cell IL-4. Using GMDCs, we first confirmed that, in

agreement with published data (Elgueta, Sepulveda *et al.* 2008; Yokota, Takeuchi *et al.* 2009; Stock, Booth *et al.* 2011), IL-4 enhanced *Aldh1a2* transcript and aldehyde dehydrogenase activity in DCs. Overnight culture with IL-4 resulted in significant upregulation of *Aldh1a2* message in GMDCs compared to media only controls (Fig. 5.2A&B). In addition, we confirmed that the enhanced *Aldh1a2* transcript level correlated with increased aldehyde dehydrogenase (ALDH) activity using the aldefluor assay (Fig. 5.2C). This assay utilizes a BODIPY-conjugated aldehyde substrate allowing a fluorescent readout of ALDH activity in individual cells (Stock, Booth *et al.* 2011; Broadhurst, Leung *et al.* 2012). Activity levels were compared to control DCs that had been exposed to a specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB) (Fig. 5.2C). In contrast to GMDCs, DCs generated using FLT3L did not have enhanced aldefluor activity when cultured with IL-4 (Fig. 5.2D&E). However, overnight culture of FLDCs with GM-CSF and IL-4 did increase their aldefluor activity (Fig. 5.2D&E), as has been reported previously (Yokota, Takeuchi *et al.* 2009). This highlights a requirement for colony stimulating factors for DC retinoic acid production. As FLT3L DCs do not become alternatively activated in our hands (Fig. 3.6) we did not further pursue this angle of research as our aim was to compare the impact of IL-4 on DC alternative activation to their metabolism of vitamin A.

5.3.3. *Schistosoma mansoni* infection does not enhance DC aldehyde dehydrogenase activity, but highlights a potential requirement for growth factors

As CD11c^{+} DCs are known to be required for mounting Th2 responses to the parasite *Schistosoma mansoni* (Phythian-Adams, Cook *et al.* 2010), we investigated whether DCs from the spleens of schistosome infected mice were able to synthesise retinoic acid via the action of aldehyde dehydrogenases. Splenic DCs isolated from animals at the peak of the Th2 response, 8 weeks post infection with *S. mansoni* (Fig. 5.3A) were defined by flow cytometry as being live singlets that were SSC^{mid}Gr1^{-}CD19^{-}CD11c^{+} (Fig 5.3B&C); the % of CD11c^{+} cells in the spleen was reduced following infection, while the total number of DCs was increased in the spleens of infected animals due to infection dependent splenomegaly (Fig. 5.3D). As expected, the CD11c^{+} cells had higher levels of MHC-II than CD11c^{-} cells (Fig. 5.3E). Within the gated DC population, there was a significant reduction in the % of aldefluor positive cells and the geometric mean fluorescence intensity of aldefluor

activity was also significantly reduced (Fig. 5.3F), however due to the increased size of the spleen during infection, there was no significant difference in the total number of aldefluor^{+}CD11c^{+} cells in each spleen between naïve and infected animals (Fig. 5.3G). We were initially surprised by the reduced expression of ALDH activity in the CD11c compartment due to the high levels of IL-4 known to be induced by 8 weeks following infection with this helminth (Jenkins, Perona-Wright *et al.* 2008). Due to the results shown in Fig. 5.2 we wondered whether the levels of colony stimulating factors were changed in the spleen during *S. mansoni* infection and if this could be responsible for the altered aldehyde dehydrogenase activity in infection. To assess this we performed qPCR on RNA extracted from naïve or *S. mansoni* (*S.m.)* infected spleens (Fig. 5.3H-J). We first confirmed that *Il4* was expressed at a high level in the spleen during infection, and were not surprised to find that, in keeping with data for GMDCs (Fig. 3.1), during infection where there is high level IL-4 that *Il4ra* was down regulated (Fig. 5.3H). We next assessed the transcript levels of the colony stimulating factors *Gmcsf* and *Mcsf* (Fig. 5.3.I). Though not significant, there was a trend for a reduction in the level of *Gmcsf,* and a significant reduction in *Mcsf* during infection (Fig. 5.3I). We then assessed the level of the DC differentiation factor *Flt3l,* which was also found to be significantly decreased in the spleen after 8 weeks of infection with *S. mansoni* (Fig. 5.3J).

5.3.4. RA does not enhance GMDC aldehyde dehydrogenase activity in the absence of IL-4

Having confirmed that IL-4 was able to induce enhanced BMDC RALDH activity (Fig. 5.2), we asked whether the product of RALDH2 activity (RA) could regulate its own induction within GMDCs. The presence of RAREs in the genes of RA receptors has been shown, suggesting that RA can modulate the ability of cells to receive an RA signal (Leroy, Nakshatri *et al.* 1991; Bastien and Rochette-Egly 2004). To assess this point, we first tested whether exogenous all-trans retinoic acid (RA) was able to enhance DC RALDH activity, as has been published previously (Feng, Cong *et al.* 2010; Jaensson-Gyllenback, Kotarsky *et al.* 2011; Stock, Booth *et al.* 2011). We were surprised to find that overnight culture with RA alone did not enhance RALDH activity in GMDCs (Fig. 5.4A&B). In order to determine whether this disparity was due to the dose of RA, or timing of exposure, we replicated the experimental conditions used in the Jaensson-Gyllenbäck 2011 report, namely using 20nM RA for a longer time period (39h). This also failed to induce a higher

percentage of aldeflour positive cells with RA (Fig. 5.4C). We do not know why we were unable to repeat the finding of Jaensson-Gyllenbäck *et al.* However, we propose that the disparity may due to the differentiation stage of the cells used within the assay. Our study used GMDC following 10 days of differentiation, whereas Jaensson-Gyllenbäck *et al* used cells differentiated for only 6 days. Cultures differentiated for this length of time will still contain non-DC populations (Lutz, Kukutsch *et al.* 1999), which may well display different responses to RA. Additional reports detailing the ability of RA to induce RALDH activity in BMDC also used DCs differentiated for less than 10 days (Feng, Cong *et al.* 2010; Stock, Booth *et al.* 2011). Furthermore, RA was included in the culture during differentiation rather than being added as an additional stimulus post-differentiation, making it hard to directly compare their findings with those in our study (Feng, Cong *et al.* 2010; Stock, Booth *et al.* 2011).

We next assessed the impact of combined RA and IL-4 treatment on GMDC ALDH activity (Fig. 5.4D). Overnight culture in combination with RA enhanced the levels of RALDH activity within the BMDC culture further than the enhancement seen on addition of 20ng/ml IL-4 alone (Fig. 5.4E), a finding we believe not to have been reported previously in the literature.

5.3.5 DC RALDH activity inversely correlates with expression of DC alternative activation markers, with the exception of *Retnla*

As IL-4 is known to have diverse effects upon DCs (Hochrein, O'Keeffe *et al.* 2000) (Sriram, Biswas *et al.* 2007; Cook, Jones *et al.* 2012)(Chapters 3 & 4 of this thesis) we sought to determine whether the ability of DCs to alternatively activate was changed as a consequence of enhanced RALDH activity. GMDCs were cultured overnight in the presence of media alone or media supplemented with 20ng/ml IL-4, surface stained for CD11c and FACS was used to separate the cells into three populations (negative, low, high) based on aldefluor activity, as compared to a DEAB control (Fig. 5.5A&B). RNA was extracted from each population, converted to cDNA and expression of *Aldh1a2* (RALDH2)*, Chi3l3* (Ym1/2), *Mrc1* (MR), *Pparg* (PPARγ), *Retnla* (RELMα) and *Ccl24* (CCL24) assessed by quantitative PCR (Fig. 5.5C-G). High level aldefluor activity correlated with significantly enhanced *Aldh1a2* transcript (Fig. 5.5C), implicating RALDH2 as the enzyme responsible for the IL-4 enhanced aldefluor activity. As would be predicted

from the results presented in chapter 3 (Fig. 3.4), IL-4 significantly enhanced expression of *Chi3l3*, *Mrc1*, *Ccl24* and *Retnla*, but not *Pparg* (Fig. 5.5C-F). Surprisingly, DC RALDH activity inversely correlated with Ym1/2 expression, with aldefluor negative cells having significantly more *Chi3l3* mRNA than aldefluor high cells (Fig. 5.4D). This inverse relationship between high level aldefluor and low level mRNA expression was also the case for MR and PPARγ (Fig. 5.4D&E). Strikingly, however, this pattern was not observed with RELMα, where no significant difference in *Retnla* transcript levels was found between any of the IL-4 exposed sorted populations (Fig. 5.4F). Although there was a trend for a correlation between *Ccl24* transcript and aldefluor activity, this did not reach significance (Fig. 5.5G). This data highlights again a disparity in the regulation of RELMα as compared to other alternative activation related proteins such as Ym1/2, and adds in the additional complexity of a role for vitamin A metabolism, or RA signalling in modulating response downstream of the IL-4Rα in DCs.

5.3.6 RA and IL-4 synergise to enhance DC RELMα but do not impact Ym1/2

Having shown that *Aldh1a2* expression levels and ALDH activity correspond with decreased mRNA expression of alternative activation markers, with the exception of *Retnla*, we hypothesised that such a disparity may be due to the product of RALDH activity (retinoic acid, RA) acting endogenously to promote *Retnla* message and thus increase DC RELMα production and/or secretion. To test this hypothesis, we exposed GMDCs to exogenous RA in the presence of IL-4 and assessed the induction of *Retnla* and *Chi3l3* message and intracellular and secreted protein production (Fig. 5.6A). The addition of RA alone had no significant impact on DC expression of the alternative activation markers RELMα or Ym1/2 (Fig. 5.6B-F). However, following overnight culture in the presence of both RA and IL-4, a significantly higher proportion of BMDCs expressed RELMα protein than cells exposed to IL-4 alone, whereas exogenous RA did not alter DC expression of Ym1/2 (Fig. 5.6B&C). These protein expression profiles were mirrored at the transcript level, with combined RA and IL-4 treatment significantly enhancing *Retnla* but not *Chi3l3* (Fig. 5.6D). Confusingly, there was no comparable significant increase in the quantity of secreted RELMα detected by ELISA in the presence of combined RA and IL-4 treatment (Fig. 5.6E), and no change was seen in the secreted levels of Ym1/2 (plotted as absorbance due to on plate standard failure) (Fig. 5.6E).

5.3.7 RA does not synergise with IL-4 to enhance alternative activation in BMM

As macrophage alternative activation has been more widely investigated than that of DCs (Gordon and Martinez 2010), we also assessed whether M-CSF derived bone marrow macrophages (BMMs) responded to combined RA and IL-4 in the same way as BMDCs (Fig. 5.7A), by upregulating RELMα but not Ym1/2. Interestingly, overnight culture with combined RA and IL-4 did not significantly affect intracellular expression (Fig. 5.7B&C) or secretion (Fig. 5.7D) of alternative activation markers RELMα or Ym1/2 by BMMs (Fig. 5.7). As addition of exogenous RA to BMMs did not result in enhanced IL-4 driven alternative activation and taking into account our data showing that RA does induce enhanced DC alternative activation (Fig. 5.6) we propose (in line with a mechanism proposed in a recent study for macrophage derived RA driving DC RALDH activity (Broadhurst, Leung *et al.* 2012)) that *in vivo*, in the context of helminth infection, macrophage derived RA may act upon DCs to enhance their alternative activation.

5.3.8 RAR signalling regulates IL-4 driven DC alternative activation

Due to the enhanced RELMα production from IL-4 exposed DCs upon addition of RA (Fig. 5.6) we sought to determine whether the presence of RA within the culture media was required for IL-4 driven alternative activation. Initially we chose to inhibit RA signalling within GMDC cultures by using a PanRAR antagonist LE540. LE540 is a chemical antagonist that blocks all RAR signalling but does not however impact other RA receptors. We added LE540 and IL-4 simultaneously to GMDC cultures for 18h and assessed the alternative activation status of these cells by intracellular staining and ELISA (Fig. 5.8A). LE540 was able to limit the ability of IL-4 to drive RELMα protein within GMDCs when compared to cells that were not treated with the RAR antagonist and, surprisingly, LE540 was found to enhance intracellular IL-4 driven Ym1/2 (Fig. 5.8B&C). LE540 also significantly reduced IL-4 driven secretion of both RELMα and the chemokine CCL24 by GMDC (Fig. 5.8D). Again slightly confusingly, although there was a trend for enhanced secretion of Ym1/2 in the presence of both IL-4 and LE540 this did not reach significance in any of the experimental repeats (Fig. 5.8E). This result highlights a potential disconnect between intracelluar RELMα and Ym1/2 production and secretion, similar to that seen in Fig. 5.6C&E, where enhanced intracellular RELMα was not detected at the level of secretion. The impact of LE540 on IL-4 induced RELMα in this experimental

set up was both more significant and more reproducible than any impact upon Ym1/2. The ability of LE540 to differentially modulate the secretion of RELMα as compared to Ym1/2 suggests that the point at which RA signalling intersects the intracellular cascade downstream of IL-4 binding to the IL-4Rα may be after the point at which the pathways leading to RELMα and Ym1/2 have diverged, enabling an inhibition of IL-4 driven RELMα but not Ym1/2.

5.3.9 RARα signalling inhibits IL-4 driven RELMα and promotes IL-4 driven Ym1/2

As antagonising all RAR signalling significantly reduced some components of IL-4 driven DC alternative activation (RELMα/CCL24) whilst promoting others (Ym1/2) (Fig. 5.8) we sought to determine the relative contribution of one of the 3 retinoic acid receptors (Blomhoff and Blomhoff 2006), RARα. RARα was chosen for our studies as it is the most commonly expressed RAR in the myeloid compartment (Nagy, Szanto *et al.* 2012), and the effects of RA in DCs have previously been attributed to this receptor (Iwata, Hirakiyama *et al.* 2004). RARα was also chosen as two reports from 2011 had shown that this receptor mediates broad regulation of T-cell polarisation (Hall, Cannons *et al.* 2011; Pino-Lagos, Guo *et al.* 2011).

GMDCs with a specific defect in expression of RARα were differentiated from CD11c^{Cre}RARα$^{fl/fl}$ bone marrow and the ability of IL-4 to alternatively activate these cells was compared with GMDCs differentiated from littermate control BM (CD11c^{Wt}RARa$^{fl/-}$) that was able to express the RARα (Fig. 5.9A). In stark contrast to blocking all RAR signalling (Fig. 5.8), blocking signalling only through RARα resulted in a significant enhancement of IL-4 driven RELMα and a significant reduction in Ym1/2 (Fig. 5.9B&C). Both the enhancement of RELMα and the reduction in Ym1/2 were also seen basally in the absence of IL-4 signalling, with CD11c^{Cre}RARα$^{fl/fl}$ GMDCs displaying significantly more intracellular RELMα than CD11c^{Wt}RARa$^{fl/-}$ cells (Fig. 5.9B; top left compared to bottom left panels). The same trend for increased RELMα and reduced Ym1/2 was also apparent at the level of secreted protein both in the absence and presence of IL-4 (Fig. 5.9D). These results suggest that RARα may function as a regulatory receptor, to limit the impact of RA at least in the presence of IL-4, as addition of exogenous RA (Fig. 5.6) or absence of the RARα (Fig. 5.9) caused the same change in IL-4 driven DC derived RELMα. This result is in contrast to the inhibition of IL-4 driven RELMα seen when all RAR signalling was

inhibited with LE540 (Fig. 5.8), suggesting that signalling via the other RARs (β and γ) may promote IL-4 driven RELMα. This data suggests that the cumulative effect of RA signalling via various RARs in one cell is not equivalent to the outcome of RA signalling via individual RARs. The cumulative outcome of blocking signalling via all RARs (Fig. 5.8) induced the opposite response following IL-4 treatment as removing signalling via RARα (Fig. 5.9), suggesting that RARα may constrain the response induced following binding to other RARs.

5.3.10 RAR signalling modulates IL-4 driven aldehdye dehydrogenase activity in GMDC

Although addition of exogenous retinoic acid was not found to induce aldehyde dehydrogenase activity in our hands (Fig. 5.4), we decided that, as inhibition of RA signalling altered IL-4 driven DC alternative activation, we should confirm or exclude a role for RA signalling in GMDC aldehyde dehydrogenase activity. GMDCs were cultured overnight in the presence of the panRAR antagonist LE540 with and without IL-4 (Fig. 5.10A). Addition of IL-4 upregulated aldefluor activity, and such upregulation was impaired in the presence of LE540 (Fig. 5.10B). To provide more firm evidence that RA signalling regulates IL-4 dependent production of retinoic acid (via RALDH2 activity), we chose to assess the aldefluor activity of IL-4 treated $CD11c^{Wt}Rara^{fl/-}$ *or* $CD11c^{Cre}Rara^{fl/fl}$ DCs which cannot signal via RARα (Fig. 5.10C). $CD11c^{Cre}Rara^{fl/fl}$ GMDC displayed a much higher basal level of aldefluor activity when compared to $CD11c^{Wt}Rara^{fl/-}$ controls, and this significantly higher level of activity could be further enhanced by addition of IL-4 (Fig. 5.10D). The finding that RARα deficient cells have enhanced basal aldefluor activity (Fig. 5.10) and lower basal levels of Ym1/2 protein when compared to control cells (Fig. 5.9) helps to confirm the existence of an inverse relationship between DC *Aldh1a2* and *Chi3l3* a novel result discussed earlier in this chapter (Fig. 5.5). This data further highlights a regulatory role for RARα, this time in modulating the ability of IL-4 to induce aldehyde dehydrogenase activity.

5.3.11 RA and IL-4 alter DC co-stimulatory molecule and cytokine secretion

Having determined that changing the ability of DCs to respond to RA altered their alternative activation status, and having previously shown that DC expression of both the IL-4Rα and RELMα changes the ability of a DC to polarise T-cell responses (Chapter 4 and (Cook, Jones *et al.* 2012)) we sought to determine how

concurrent exposure of DCs to RA and IL-4 affects their ability to activate T-cells (Fig. 5.11). As DC expression of MHC, co-stimulatory molecules and production of cytokine are major factors in their ability to prime T-cell responses, we determined DC surface phenotype and cytokine production following culture with IL-4 and RA (Fig. 5.11A). Inclusion of RA in the overnight culture did not significantly alter the % of CD11c$^+$ DCs nor the gMFI of CD11c (Fig. 5.11B). In the presence of both IL-4 and RA the surface expression of CD40 was significantly decreased compared to IL-4 treatment alone (Fig. 5.11C). RA had no further impact on CD80 or MHC-II than IL-4 alone (Fig. 5.11C), unfortunately the antibody for CD86 was not available for inclusion in the staining panels at the time these experiments were carried out. As shown in Fig. 3.1, IL-4 reduced the basal secretion of IL-6 and IL-12p40 by GMDCs (Fig. 5.11D) Culture of GMDC with RA resulted in enhanced basal secretion of the pro-inflammatory cytokine IL-6, in the absence of antigenic stimulation and this enhancement was abrogated in the presence of IL-4 (Fig. 5.11D). As reported previously, RA decreased DC secretion of IL-12p40 (Wada, Hisamatsu *et al.* 2009) (Fig. 5.11D). However, in the absence of antigenic stimulation, the amount of cytokine produced in all conditions was very low and no IL-10 or IL-12p70 was detected.

5.3.12 RA and IL-4 alter T-cell polarisation in the context of DC

The cytokine data from Fig. 5.11 suggest that RA and IL-4 exposed DCs may be less capable of polarising a Th1 response or a Th2 response (reduced CD40 (MacDonald, Straw *et al.* 2002)). The reduced IL-12p40 found in the presence of RA and IL-4 may result in reduced availability of IL-12p40 to form bioactive IL-12p70 heterodimers, or could be construed to result in decreased levels of IL-12p40 homodimers which have been shown to limit the action of IL-12p70 (Hochrein, O'Keeffe *et al.* 2000). Thus the impact of RA and IL-4 on DC polarisation of T-cells could not be predicted from this data alone. Previous studies have shown that RA can influence T-cell polarisation, by promoting IL-4 secretion under Th2 conditions (Hoag, Nashold *et al.* 2002; Iwata, Eshima *et al.* 2003), or not promoting IL-4, but limiting IFNγ from already polarised Th1 cells, (Stephensen, Rasooly *et al.* 2002). Given the contrasting reports on RA and IL-4 regulation of T-cell polarisation by DCs and as a result of our finding of reduced CD40 and IL-12p40 expression we decided to carry out a polarisation assay to determine whether DC exposure to RA and IL-4 can modulate T-cell cytokine production. As RA and IL-4 were shown to

enhance DC RELMα production (Fig. 5.6), and *Retnla*$^{-/-}$ DCs supported reduced IL-10 secretion both following *in vivo* transfer post SEA exposure (Fig. 4.9) and *in vitro* in co-culture with polyclonally stimulated T-cells (Fig. 4.10) we investigated the induction of T-cell IL-10 by DCs exposed to RA in combination with IL-4.

To investigate the role of RA and IL-4 in T cell polarisation we undertook *in vitro* DC:T-cell co-culture assays incorporating 4Get (IL-4GFP (Mohrs, Shinkai *et al.* 2001)) or TIGR (IL-10eGFP (Kamanaka, Kim *et al.* 2006)) reporter T-cells and WT GMDCs. This experiment allowed us to determine if DC exposed to RA and IL-4 had an altered capacity to polarise polyclonally activated T-cells. αCD3 was used to stimulate FACS GFP negative T-cells (not currently expressing IL-4 or IL-10) in the presence of DC, with or without IL-4 and RA, for 4 days (Fig. 5.12A). Following culture, as previously presented (Fig. 3.13), addition of IL-4 alone enabled DC induction of enhanced CD4^{+}T-cell IL-10, as assessed by GFP expression and IL-10 protein secretion (Fig. 5.12B&D). IL-10 was increased further by concurrent addition of RA (Fig. 5.12B&D). The presence of RA within the culture wells reduced transcription of *Il4* mRNA in CD4^{+} T-cells as assessed by GFP expression (Fig. 5.12C). Due to the addition of exogenous recombinant IL-4 in this experimental system, it was not possible to accurately measure DC induced T-cell IL-4 secretion, however IL-13 was measured and found to accurately represent the IL-4GFP result, with significantly reduced IL-13 secretion in the presence of RA and IL-4 as compared to IL-4 alone (Fig. 5.12E). Very low levels of IFNγ were detected in the supernatant, and none was detected in the presence of RA regardless of IL-4 presence (Fig. 5.12F).

We have previously shown that DC derived RELMα not only promotes CD4^{+} T-cell IL-10 but also limits T-cell IL-4 (Fig. 4.10 &(Cook, Jones *et al.* 2012)). Thus, in addition to the observed changes in cytokine and co-stimulatory molecule expression (Fig. 5.11), the altered T-cell response could be in part due to the enhanced RELMα produced within DCs in the presence RA and IL-4 (Fig. 5.6). However, given the inability to detect increased RELMα by ELISA in Fig. 5.6, the impact of enhanced RELMα would have to be indirect via RELMα modulation of another cell intrinsic factor which then alters DC APC function .To confirm a role for RELMα in the RA and IL-4 dependent induction of T-cell IL-10, this experiment will have to be repeated with the use of RELMα deficient DCs.

5.3.13 The absence of *RARα alters DC co-stimulatory molecule expression and cytokine secretion*

Having revealed a role for exogenous RA in the ability of IL-4 treated GMDCs to activate and polarise T-cell responses (Fig. 5.12), and having determined that blocking all RAR receptors differentially alters AADC phenotype (Fig. 5.8) compared to blocking only RARα (Fig. 5.9), we next decided to address what the absence of RARα would do to DC APC function in the context of IL-4.

We initially chose to interrogate the phenotype of RARα deficient GMDCs more thoroughly than previously (where we only assessed their ability to alternatively activate (Fig. 5.9)) to ensure that these cells differentiate comparably to WT DCs. *$CD11c^{Wt}Rara^{fl/-}$ or $CD11c^{Cre}Rara^{fl/fl}$* GMDC were cultured overnight with IL-4 and expression of surface markers and cytokine secretion were assessed (Fig. 5.13A). Following overnight culture, $CD11c^{Cre}RAR\alpha^{fl/fl}$ DCs appeared to have a reduced percentage of $CD11c^{+}$ cells/population and reduced per cell expression (as assessed by gMFI) this could not be confirmed statistically due to the low sample number for the medium controls (Fig. 5.13B), interestingly, following culture with IL-4 there was no significant difference in the expression of CD11c between RARα deficient and control DCs (Fig. 5.13B).

As shown in Fig. 3.1, control *$CD11c^{WT}Rara^{fl/-}$* DCs also had reduced expression of MHC-II and CD80 following culture with IL-4, however significance could not be assessed as only 2 samples were available for the medium control population (Fig. 5.13C). *$CD11c^{Cre}Rara^{fl/fl}$* GMDC had lower basal level co-stimulatory molecule expression than control *$CD11c^{Wt}Rara^{fl/-}$* GMDCs, and IL-4 did not alter this response, however due to there only being 2 control wells, statistical significance between control and RARα deficient cells is not included on the graphs (Fig. 5.13C); the lack of impact of IL-4 on co-stimulatory molecule expression in *$CD11c^{Cre}Rara^{fl/fl}$* DCs in contrast to the control cells may be due to a much reduced level of IL-4Rα being expressed by RARα deficient cells, even in the absence of IL-4 (Fig. 5.13D). *$CD11c^{Cre}Rara^{fl/fl}$* GMDCs did however respond to IL-4, as shown by altered cytokine secretion (Fig. 5.13E), suggesting that the reduced levels of IL-4Rα present were still functional, or that the receptor may only have been down regulated in the latter stages of the 18 hour culture. A time course experiment

assessing the expression of IL-4Rα at early time points post culture would be informative to address these possibilities.

CD11c^{Cre}*Rara*$^{fl/fl}$ DC also displayed enhanced basal IL-6 secretion and reduced IL-12p40 compared to control cells that were able to express RARα (Fig. 5.13E). This result was very similar to the result following culture of WT DCs with RA in Fig. 5.11, in which addition of RA resulted in enhanced IL-6 and reduced IL-12p40 secretion. The addition of RA (Fig. 5.6) or removal of RARα (Fig. 5.9C) also induced the same response from IL-4 treated cells in terms their expression of RELMα and Ym1/2, in which both addition of RA or removal of DC RARα enhanced RELMα but inhibited Ym1/2. The enhanced IL-6 and reduced IL-12p40 upon addition of RA (Fig. 5.11) or removal RARα (Fig. 5.13E) again suggests that in DCs, in the presence of IL-4, RARα functions as a regulatory receptor, to limit the impact of RA.

5.3.14 The absence of RARα alters DC ability to polarise T-cell responses

In order to assess the role of DC RARα in activating T-cell responses, we undertook *in vitro* DC:T-cell co-culture assays similar to those described above (Fig 5.11), but this time incorporating reporter T-cells from KN2 (IL-4huCD2) x TIGR (IL-10eGFP) (Mohrs, Wakil *et al.* 2005; Kamanaka, Kim *et al.* 2006) animals. These T-cells report both IL-4 protein synthesis (rather than *Il4* transcription as in 4Get animals) and IL-10 transcription within the same cells, reducing the number of assays required to acquire comparable information as using T-cells that report individual cytokine synthesis. *CD11c*^{Cre}*Rara*$^{fl/fl}$ GMDC were used to determine if DC expression of RARα altered the capacity of IL-4 exposed DCs to polarise polyclonally activated T-cells. αCD3 was used to stimulate flow sorted CD4^{+}GFP negative T-cells (not currently expressing IL-10) in the presence of *CD11c*^{Cre}*Rara*$^{fl/fl}$ or control *CD11c*^{Wt}*Rara*$^{fl/-}$ DC, with or without IL-4, for 3 days (Fig. 5.14A). When DCs were RAR-sufficient (CD11c^{Wt}*Rara*$^{fl/-}$), IL-4 was able to induce high levels of T-cell IL-4 and IL-10, as assessed by anti-huCD2 staining and GFP expression, respectively (Fig. 5.14B). When DCs were RARα deficient (CD11c^{Cre}*Rara*$^{fl/fl}$), IL-4 was able to induce higher levels of T-cell IL-4, but reduced levels of T-cell IL-10, as compared to control cultures with RARα sufficient cells (Fig 5.14B). RARα deficient GMDC also supported reduced IL-4 driven T-cell IL-10 and IL-13 protein secretion, but did not influence T-cell IFNγ secretion, in this experimental system (Fig 5.14C).

Given the comparable results in terms of RELMα induction and cytokine secretion following culture of DCs with RA and IL-4 (Fig. 5.6, 5.11) or in the absence of DC RARα expression in the presence of IL-4 (Fig. 5.9, 5.12), the lack of similiarity in T-cell cytokine induction following addition of RA or removal of RARα was surprising (Fig. 5.12 Vs. Fig. 5.14). Addition of exogenous RA, which can signal through multiple RARs (Blomhoff and Blomhoff 2006), supported enhanced T-cell IL-10 and reduced T-cell IL-4 and IL-13 (Fig. 5.12), whereas removal of RA signalling via RARα resulted in reduced IL-10 and IL-13 induction and enhanced IL-4 (Fig. 5.14).

5.4 SUMMARY

- IL-4 significantly upregulated 14 genes within the KEGG metabolic pathway (Fig. 5.1 and Table 5.3.1): of these only 2 were upregulated greater than 2 fold in WT GMDCS.
- IL-4 significantly upregulates *Aldh1a2* mRNA, and enhances aldehyde dehydrogenase activity, in GMDCs, as assessed by use of the aldefluor assay (Fig. 5.2).
- *Schistosoma mansoni* infection results in the down regulation of aldehyde dehydrogenase activity within splenic CD11c^{+} populations, when compared to uninfected controls (Fig. 5.3).
- Retinoic acid alone does not promote enhanced aldehyde dehydrogenase activity in GMDCs, but does synergise with IL-4 to drive higher aldefluor activity (Fig. 5.4).
- Aldefluor activity correlates with *Aldh1a2* transcript in GMDCs (Fig. 5.5.C)
- *Aldh1a2* inversely correlates with some facets of alternative activation, but not *Retnla* (Fig. 5.5).
- Retinoic acid and IL-4 synergise to promote RELMα in GMDCs, but not in M-CSF derived BMM (Fig. 5.6 & Fig. 5.7).
- Blocking all RAR signalling results in reduced IL-4 driven RELMα in GMDCs, and elevated IL-4 driven Ym1/2 (Fig. 5.8), confirming a role for RA signalling in promoting DC RELMα (Fig. 5.6).
- The RA receptor alpha is a regulatory receptor that constrains RELMα production whilst promoting Ym1/2 (Fig. 5.9).
- RA and IL-4 change the ability of DCs polarise T-cell responses by altering co-stimulatory molecule expression (Fig. 5.11A), cytokine secretion (Fig 5.11B) and RELMα production (Fig. 5.6 and Fig. 5.11H).
- RA exposed, IL-4 stimulated, DCs induce increased T-cell IL-10 and reduced T-cell IL-4 upon co-culture *in vitro* (Fig. 5.12).
- Signalling via RARα in GMDCs promotes T-cell IL-10 and IL-13 and limits T-cell IL-4 following co-culture *in vitro* (Fig. 5.14).

5.5 DISCUSSION

5.5.1 How do RA and IL-4 alter DC activation?

As presented in a proposed model (Fig. 5.15), attempting to summarise some of the key results detailed in this chapter, we have found that RA modulates IL-4 driven DC alternative activation (Fig. 5.5 (1)), with RA promoting IL-4 dependent RELMα, as shown by exogenous addition of RA (Fig. 5.6) and blocking of all RAR receptors, resulting in decreased RELMα (Fig. 5.8). Signalling through the RARα was found to inhibit RELMα production, even in the absence of IL-4, as DCs unable to signal via RARα displayed higher levels of IL-4 dependent and IL-4 independent RELMα (Fig. 5.9). In contrast, RA signalling via all RARs did not significantly alter IL-4 driven Ym1/2 when exogenous RA was added to the cells (Fig. 5.6), but a subtle yet significant increase in IL-4 driven Ym1/2 was found when panRAR signalling was blocked using LE540 (Fig. 5.8). Interestingly, removing only the RARα from DCs significantly reduced expression of Ym1/2 (Fig. 5.9). These data suggest that RA signalling via RARα promotes Ym1/2 and this counteracts the inhibitory impact of RA signalling via the other RARs, resulting in no significant change overall upon addition of exogenous RA to GMDCs (Fig. 5.6).

Work in this chapter has shown that IL-4 promotes DC expression of *Aldh1a2*, resulting in enhanced RALDH2 activity (Fig. 5.15 (2)), as has previously been reported (Elgueta, Sepulveda *et al.* 2008; Yokota, Takeuchi *et al.* 2009; Stock, Booth *et al.* 2011) (Figs. 5.2B & C). GM-CSF was also shown to promote aldehyde dehydrogenase activity in FLDCs (Fig. 5.2E), as previously reported (Yokota, Takeuchi *et al.* 2009). RA signalling via all RARs promoted IL-4 driven RALDH activity, but not IL-4 independent RALDH, as shown in Fig. 5.4 and confirmed in Fig. 5.10B, as inclusion of the panRAR inhibitor LE540 impaired the ability of IL-4 to upregulate aldefluor activity, but did not inhibit basal RALDH2 (Fig. 5.10B). Conversely, interfering with signalling via RARα alone enhanced both IL-4 dependent and IL-4 independent RALDH2 (Fig. 5.10D). The finding that RA does not promote RALDH2 in our system is in contrast to previous reports (Feng, Cong *et al.* 2010; Jaensson-Gyllenback, Kotarsky *et al.* 2011), a difference that we believe must be dependent upon the source, differentiation and activation status of the DCs under investigation.

Cumulative RA signalling following addition of exogenous RA promotes IL-6 and inhibits IL-12p40 (Fig. 5.11D; Fig. 5.15 (3)). A similar cytokine profile was evident with RARα deficient DCs, indicating that, although cumulative RA signalling promotes IL-6 and inhibits IL-12p40, (Fig. 5.11D), RARα signalling alone in fact does the exact opposite of this, limiting pro-inflammatory IL-6 and promoting IL-12p40 (Fig. 5.13E). In contrast, signalling via RARα is required for DC surface marker activation, as in the absence of this receptor, there are much lower basal levels of MHC-II and co-stimulatory molecules (Fig. 5.13B&C; Fig 5.15 (4)).

This data would therefore suggest an overall role for RA in boosting pro-inflammatory cytokine production in the context of GMDCs, and a specific role for RARα in limiting these cumulative effects of RA signalling through all the RARs on cytokine production. Indeed, this may be a direct inhibition of RA signalling via the other RARs, a theory which would require further experimentation to confirm. Further, our data would suggest that although RARα signalling limits some aspects of GMDC cytokine production, it promotes GMDC surface molecule expression. This dual role for RARα in GMDCs suggests, once again, that the impact of RA on DC function in general will be determined by context, location and the balance of signalling via the other RARs.

5.5.2 Why didn't we detect enhanced RELMα secretion?

An issue that we did not address fully in this chapter was the inability to detect secreted RELMα from GMDCs following culture with RA and IL-4, in contrast to the high levels detected intracellularly by flow cytometry and at the level of transcript (Fig. 5.6). An explanation for this is not really apparent, unless some influence of the combination of RA and IL-4 on the cells is modulating their ability to secrete protein, RELMα in particular. If this was the case, and RA is somehow blocking RELMα egress, it might be the case that RA does not in fact promote IL-4 driven RELMα but rather causes an accumulation of the IL-4 induced protein within the cells. However, this is contradicted by the upregulation of *Retnla* mRNA in the presence of RA and IL-4 (Fig. 5.6D). To try and address this issue, a time course could be carried out to assess the levels of RELMα intracellularly and secreted in the presence of both RA and IL-4.

5.5.3 Is DC expression of RALDH dependent upon colony stimulating factors?

We found that FLDC bulk cultures (including both cDC and pDCs) did not increase expression *Aldh1a2* in response to IL-4, unless we added in exogenous GM-CSF (Fig. 5.2). The dependence of FLDC on GM-CSF for RALDH activity had already been reported by Yokota *et al* who initially described a role for IL-4 in driving DC expression of *Aldh1a2 (Yokota, Takeuchi et al. 2009).* Indeed, in the Yokota study mice deficient in the signalling partner for the GM-CSFR Beta-c, (through which IL-3 and IL-5 also signal) were found to have reduced aldehyde dehydrogenase activity in mLN and PP DCs.

Intestinal sites are well documented as having high levels of DC RALDH activity in the steady state (Iwata, Hirakiyama *et al.* 2004; Coombes, Siddiqui *et al.* 2007) (Sun, Hall *et al.* 2007). Furthermore, we have highlighted a potential role for growth factors in the induction of RALDH during infection, as we found reduced levels of aldehyde dehydrogenase in splenic DCs following schistosome infection, at a time when high levels of IL-4 were present. In these experiments, reduced DC aldefluor activity correlated with significantly reduced total splenic mRNA for *Mcsf* and *Flt3l* (Fig. 5.3). As transcript does not always directly correlate with protein levels, in future work it would be necessary to assess the protein levels of these factors in the spleen during infection. Additionally, a study to more closely interrogate the DC phenotype in the spleen both in naïve settings and during infection would be advised, given reports that splenic DCs cannot metabolise retinol unless they are released from the inhibitory effects of prostaglandins (Stock, Booth *et al.* 2011). Now that the number and quality of fluorochromes available to use simultaneously during flow cytometry has increased and the technology available to detect multiple fluorochromes has advanced, it should be possible to include more markers than were available at the time of this study, to more thoroughly assess the *in vivo* DC subsets that have both RA producing capacity and are alternatively activated (as investigated in chapter 3).

It is possible that colony stimulating factors are not responsible for limiting aldehyde dehydrogenase activity in DCs during *S. mansoni* infection. One possibility is that the parasite itself may release factors which block aldehyde dehydrogenase activity. For example, prostaglandins have previously been reported to restrain aldehyde dehydrogenase activity (Stock, Booth *et al.* 2011), and *S. mansoni* is known to express prostaglandin homologues (Fusco, Salafsky *et al.* 1985;

Ramaswamy, Kumar *et al.* 2000). Indeed *S. mansoni* cercarial prostaglandins, and the induction of host PGE_2 and IL-10 by cercarial secretions, are required for the regulation of the inflammatory immune response in the skin, to expediate parasite migration (Ramaswamy, Kumar *et al.* 2000). Thus there is potential at sites of worm transit, and potentially from worm secretions, for local production of prostaglandins which may then restrain DC *Aldh1a2* induction (Stock, Booth *et al.* 2011).

To further complicate matters, the parasites also encode their own retinol converting enzymes (as is predicted by homology to human RALDHs) that may either increase RA in infection settings or, as has actually been suggested in humans (Friis, Mwaniki *et al.* 1997), result in a depletion of vitamin A from the host and thus reduce the substrate for DC RALDH.

As the liver is a storage organ for retinol, and a major site of pathology during schistosomiasis, this may be the most relevant site for investigating the impact of schistosomes on retinol metabolism. Indeed, it was recently shown that macrophages in the livers of *S. mansoni* infected mice express enhanced levels of RALDH enzymes compared to their uninfected counterparts (Broadhurst, Leung *et al.* 2012).

5.5.4 What is the *in vitro* retinoid source?

One issue not addressed in this chapter is the source of the vitamin A metabolites *in vitro* that are converted by DCs in order for the detection of aldefluor activity. As we can detect aldefluor activity without the addition of exogenous retinol or retinal, this suggests that the media we use (RPMI-1640, Sigma, supplemented with 10% FCS) is supplying these to the GMDCs. In order to control for the levels of natural retinoids in the FCS there are a few different approaches that we could take: 1) irradiate the FCS to destroy any endogenous vitamin A metabolites 2) use a different type of media which does not depend on FCS for protein supply, for example XVIVO medium. Both of these approaches have been used previously to address how mLN DCs *in vitro* are able to induce CCR9 expression on co-cultured T-cells (Jaensson-Gyllenback, Kotarsky *et al.* 2011). 3) Alternatively, we could try and quantify the level of endogenous retinoids within the media that we use, or that is taken up by each DC (Kawaguchi, Yu *et al.* 2007) using high pressure liquid chromatography (HPLC). In the proposed experiments in which we remove retinoids from our media, we would have to control for DC phenotype, as RA has been

suggested to be involved in normal DC development (Szatmari, Pap *et al.* 2006; Wang, Villablanca *et al.* 2011). As the previous studies isolated mLN DCs following their natural differentiation in the presence of the *in vivo* cytokine/growth factor milieu, prior to depletion of retinoids from their culture medium, it may be possible to differentiate the DCs for 10 days in the presence of FCS, and modify the retinoid level only for the overnight incubation with our antigens/cytokines of choice (e.g. IL-4). These approaches would also enable us to determine whether vitamin A is required for the ability of IL-4 to trigger alternative activation within our system.

The absence of signalling via RARα alone during the *in vitro* differentiation of CD11c^{Cre}RARa^{flfl} bone marrow, as compared to blocking all RAR activity only at the time of exposure of fully differentiated DCs to IL-4 with addition of the panRAR antagonist LE540, are hard systems to compare experimentally. However the development of a retinoid free system for differentiating DCs, would help address this issue.

5.5.5 The role of RA in T-cell polarisation by DCs

In the work detailed in this chapter, we have shown that addition of exogenous RA or removal of RARα signalling both resulted in the same measured changes to DC cytokine secretion (Figs. 5.11D & 5.13E), with enhanced IL-6 secretion and decreased IL-12p40. Addition of IL-4 at the same time as RA (Fig. 5.11D) or treatment of RARα deficient DCs with IL-4 (Fig. 5.13E) decreased IL-6 secretion more in line with the levels secreted in medium control cells. Similarly, IL-4 addition also reduced DC IL-12p40 secretion such that the lowest levels of IL-12p40 were induced in the presence of RA and IL-4 (Fig. 5.11D) or IL-4 in the absence of DC RARα signalling (Fig. 5.13E). An RA analog has previously been shown to reduce LPS induced IL-12p40 secretion by human monocyte derived DCs differentiated in the presence of GM-CSF and IL-4 (Wada, Hisamatsu *et al.* 2009). IL-4 in the steady state significantly reduces IL-6 secretion in GMDCs, (first shown in Fig. 3.1D).

IL-4 reduction of basal levels of DC IL-6, in the absence of antigen, is potentially a mechanism to help ensure that in the presence of Th2 cytokines (potentially in steady state) that a Th17 response is not induced, as IL-6 in combination with TGFβ is known to polarise T-cell IL-17 secretion (Harrington,

Hatton *et al.* 2005). It is a little surprising that RA induces the upregulation of IL-6 secretion by GMDCs, as intestinal associated DC provision of RA via RALDH activity is known to promote TGFβ dependent Treg induction whilst limiting the IL-6 and TGFβ dependent induction of IL-17 (Mucida, Park *et al.* 2007). However, RA induction of IL-6 from pig monocyte derived DCs has previously been shown (Saurer, McCullough *et al.* 2007). In further work it would be interesting to characterise the secretion of TGFβ by RA and IL-4 exposed GMDCs, particularly in light of their high levels of aldehyde dehydrogenase activity (Fig. 5.4E).

The contrasting roles for exogenous RA vs. DC deficiency in RARα in supporting IL-4 dependent T-cell cytokine production (Figs. 5.12 & 5.14), in spite of inducing a similar activation phenotype in DCs with respect to RELMα production (Figs. 5.6 & 5.9), CD11c, MHC-II and co-stimulatory molecule expression as well as cytokine secretion (Figs. 5.11 & 5.13), can be explained in several ways: 1) The enhanced T-cell IL-10 and reduced IL-4 in the RA+IL-4 DC:T-cell co-cultures is a result of high levels of exogenous RA acting directly on the T-cells within the culture, and not necessarily via alteration of DC phenotype. 2) Alternatively, the differential T-cell polarisation results could suggest a cumulative role for RA signalling in DCs to limit Th2 responses and promote regulation, and a dominant role for RARα in this process. 3) As IL-4 is able to directly induce T-cell IL-4, and $CD11c^{Cre}Rara^{fl/fl}$ GMDC had reduced levels of IL-4Rα following overnight culture compared to control GMDCs (Fig 5.12C), this would suggest that there may be more IL-4 free in the culture well in the presence of $CD11c^{Cre}Rara^{fl/fl}$ GMDCs, which could explain the higher levels of huCD2. However, we have shown previously that IL-4 is able to induce high levels of IL-10 from T-cells cultured with WT DCs (Figs. 4.8 & 4.10), and this was not enhanced in the absence of RARα expression (Fig. 5.14).

CD11c^{Cre}*Rara*$^{fl/fl}$ GMDCs had reduced levels of IL-12p40 secretion following overnight culture than control GMDCs (Fig. 5.13), suggesting that these DCs may also produce less bioactive IL-12p70 (though this was below detection limit in these experiments) and so could be less efficient at polarising Th1 type responses. However, there was no difference in the levels of IFNγ detected within the DC:T-cell co-culture supernatants, regardless of RARα expression. It should be noted that this polyclonal system was optimised to address Th2 induction, and there was no antigenic stimulus within this assay. To properly assess the influence of DC RARα

expression on their ability to modulate Th1 induction, a system using a bacterial stimulus or IL-12 addition could be used.

In future studies it will be necessary to determine whether RA and IL-4 exposed DC:T-cell co-culture assays result in increased T cell Foxp3 expression, and what the role for RARα may be in this system. Given that RA and IL-4 increase the activity of DC aldehyde dehydrogenases (Fig. 5.4E), and that upon co-culture with T-cells they induce increased secretion of IL-10, we would predict that they would be able to induce a regulatory phenotype within the T-cell population. Indeed RALDH2 positive macrophage populations elicited following injection of Thioglycollate and IL-4 i.p. when used in co-culture were recently shown to induce a regulatory T cell phenotype, that was dependent upon RA production by the Thio+IL-4 macrophage population (Broadhurst, Leung *et al.* 2012).

In addition we should determine whether RA and IL-4 exposed GMDCs induce T-cell upregulation of intestinal homing markers such as the integrin α4β7 and the chemokine receptor CCR9, as has previously been reported for GALT $CD103^+$ DCs (Iwata, Hirakiyama *et al.* 2004; Johansson-Lindbom, Svensson *et al.* 2005; Coombes, Siddiqui *et al.* 2007; Sun, Hall *et al.* 2007).

To develop this area of research further, it will be important to relate the results achieved using GMDCs to *in vivo* DC populations. To initially address this, we propose to use injection of exogenous IL-4c i.p. (as in Fig. 3.8) in combination with and without RA, into control and $CD11c^{Cre}RAR\alpha^{fl/fl}$ animals, followed by assessment of DC alternative activation status and aldehyde dehydrogenase activity. To relate this to a more physiological induction of a Th2 type response, helminth antigens such as SEA or *S. mansoni* eggs themselves could be used in place of IL-4c.

In this chapter we showed for the first time an interaction between RA and IL-4 within DCs resulting in enhancement of both aldehyde dehydrogenase activity and RELMα production. Furthermore, a differential requirement for RARs in directing responses downstream of IL-4 and RA binding was revealed, with RARα constraining RELMα, but promoting Ym1/2. We concluded by showing that addition of exogenous RA or removal of DC RARα resulted in altered DC APC function, with

addition of RA inducing T-cell IL-10 and limiting IL-4, and removal of DC RARα limiting T-cell IL-10 and promoting T-cell IL-4.

A.

B.

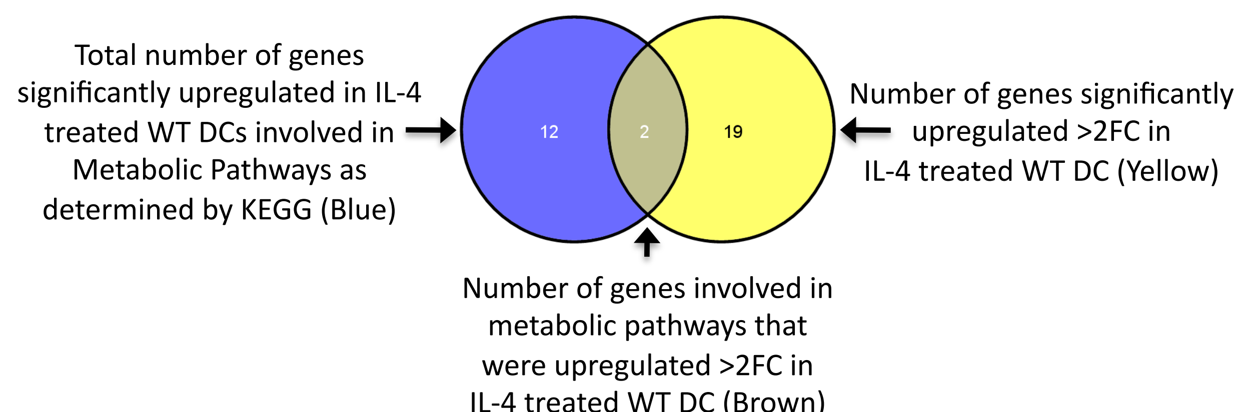


Figure 5.1 Genes involved in metabolic pathways are significantly upregulated by IL-4 in WT GMDC. C57BL/6 WT GMDCs were cultured with IL-4 for 6 hours, RNA was extracted and an illumina microarray carried out, KEGG pathway analysis was carried out highlighting metabolic pathways (KEGG pathway mmu:01100, (A)) as the most altered upon IL-4 treatment (B; blue) with 14 genes significantly changed (highlighted in red in A), of these 14 genes only 2 (B; overlap/brown) were significantly upregulated with a FC greater than 2 (B;Yellow). Data is from the same microarray experiment, as shown in Fig 4.12.

Table 5.3.1 Metabolic pathway genes significantly increased by IL-4 in WT GMDC				
Symbol	**Synonym**	**Name**	**Putative function**	**FC**
Aldh1a2	RALDH2	aldehyde dehydrogenase family 1, subfamily A2	Conversion of Retinal to RA	2.64
Ak2	ADK2	adenylate kinase 2	Mitochondrial component	2.14
Prps1	ARTS	phosphoribosyl pyrophosphate synthetase 1	Purine metabolism	1.91
Mgll	MagL	monoglyceride lipase	FFA release from lipids	1.88
Xdh	XDH	xanthine dehydrogenase	Purine metabolism	1.65
Stt3b	SIMP	STT3, subunit of the oligosaccharyltransferase complex, homolog B	Post-translational N-glycosylation	1.58
Ptgs1	COX1	prostaglandin-endoperoxide synthase 1	Constitutive prostaglandin synthesis	1.56
Pfkp	PFK-C	phosphofructokinase, platelet	Glycolysis	1.44
Mpi	PMI	mannose phosphate isomerase	Frustose-6P production for glycolysis	1.42
Pla2g12a	sPLA2	phospholipase A2, group XIIA	Arachidonic acid release from phospholipids	1.39
Aldh3a1		aldehyde dehydrogenase family 3, subfamily A1	B-cell development	1.26
Gart	PGFT	phosphoribosylglycinamide formyltransferase	Purine biosynthesis	1.20
B3galt4	GALT2/ GALT4	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4	Unknown	1.16
Alg8	ALG8	asparagine-linked glycosylation 8 homolog	Alginate biosynthesis	1.12

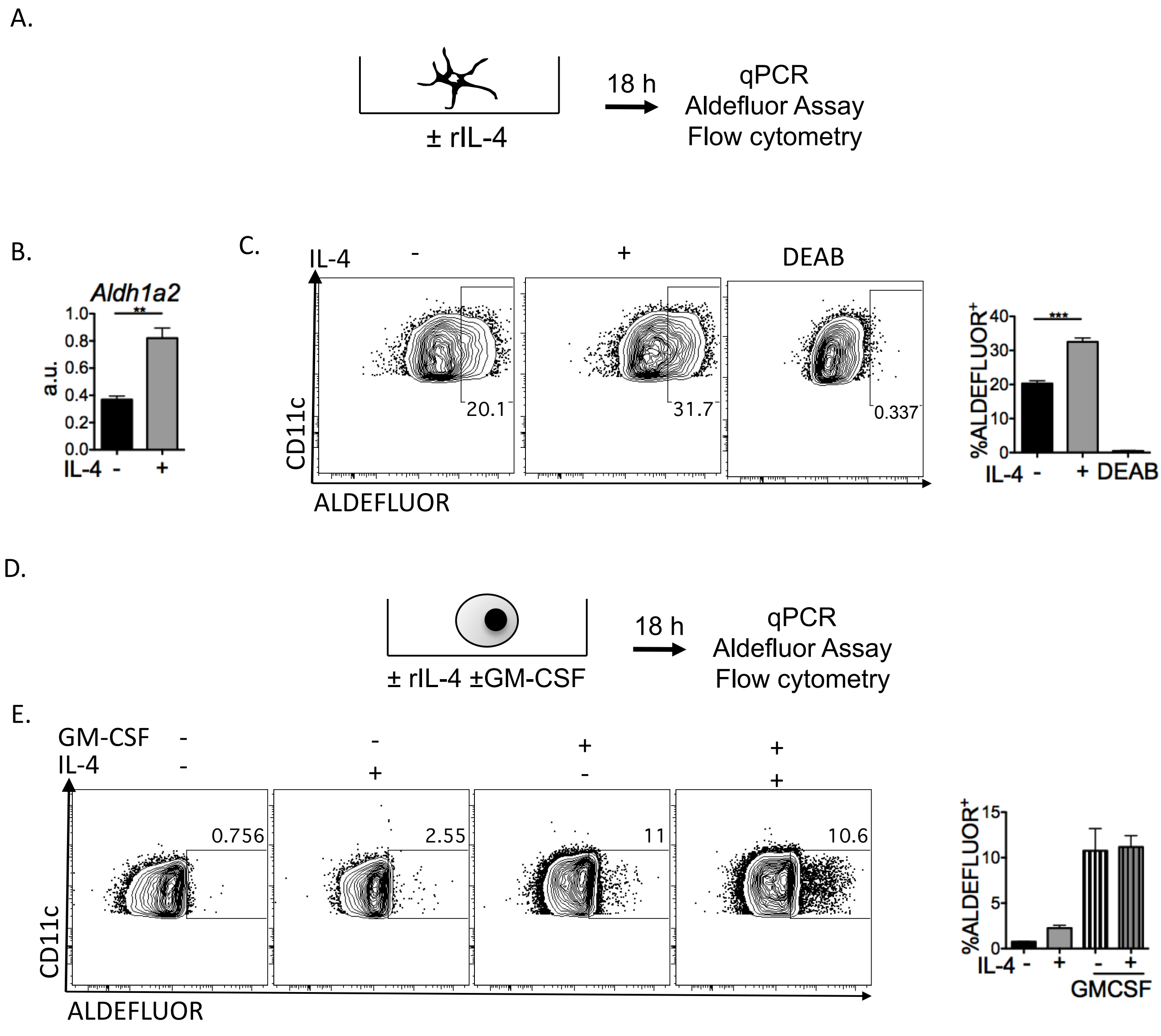


Figure 5.2 IL-4 induces *Aldh1a2* expression and aldehyde dehydrogenase activity in GMDC and FLDCs treated with GM-CSF. GMDC (A-C) and FLDC (D+E) were cultured overnight with IL-4 and GM-CSF and aldehyde dehydrogenase activity was assessed (aldefluor activity). Data is representative of 5(A+B) or 1(E) independent experiments, error bars represent triplicate culture wells, a.u.= arbitrary units as compared to *Hprt* expression. **= P<0.01 ***P<0.001.

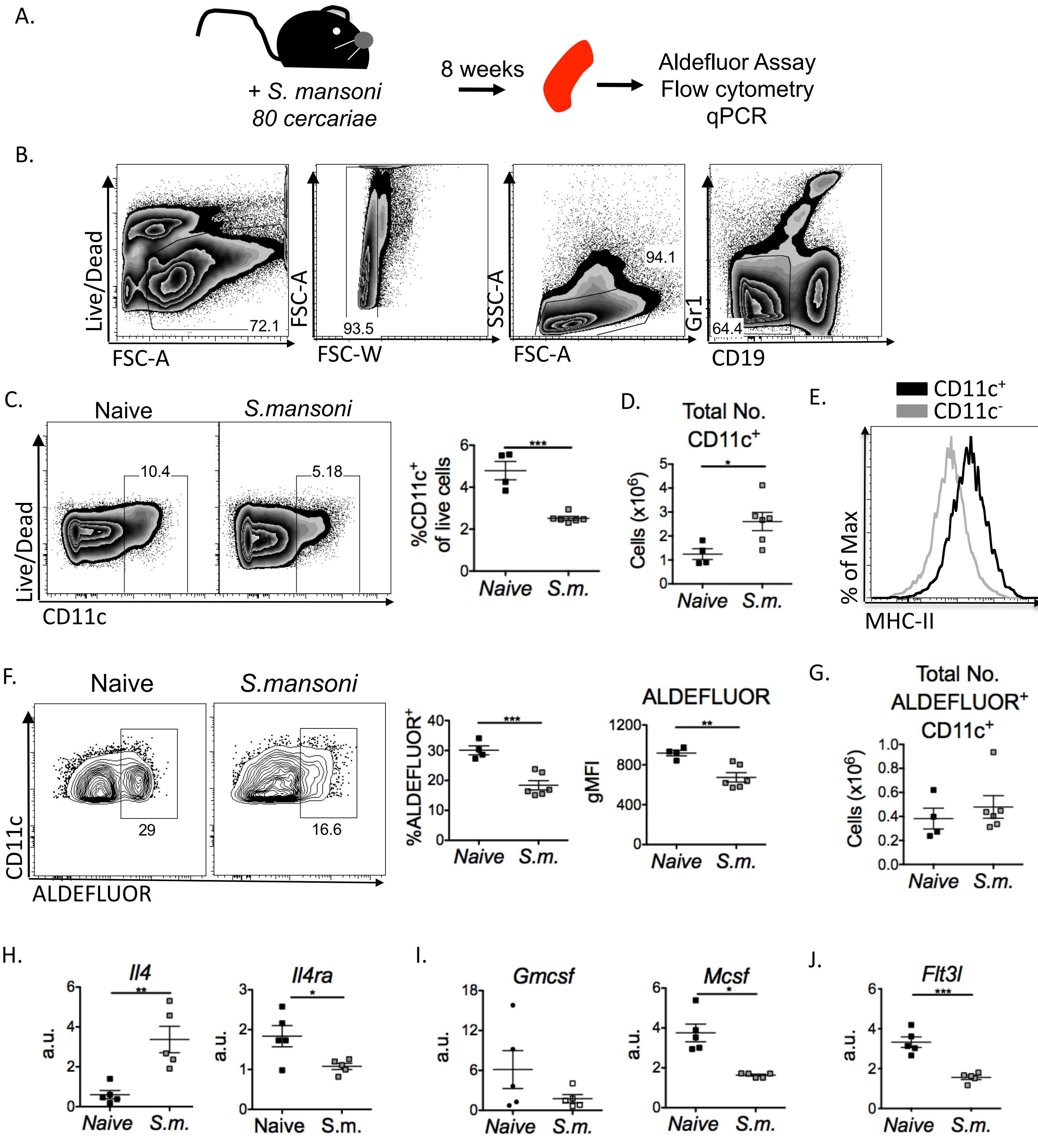


Figure 5.3. *Schistosoma mansoni* infection does not enhance DC aldefluor activity, but highlights a potential requirement for growth factors. Spleens were harvested from naïve or *S.mansoni* infected animals 8 weeks post infection, spleens were digested, RBC lysed and surface stained (A+B) prior to assessment of CD11c (C) and MHC-II (D) expression and aldefluor activity (E). In B+C *Naïve*=3 pooled spleens per symbol, *S.m.*= individual infected spleen samples. RNA was extracted from whole spleens prior to qPCR analysis of *Il4* and *Il4ra* (F), *Gmcsf, Mcsf* (G) and *Flt3l* (H) expression, Data is representative of 2 independent experiments, a.u.=arbitrary units relative to *Gapdh*. *=P<0.05 **= P<0.01 ***P<0.001 ****P<0.0001.

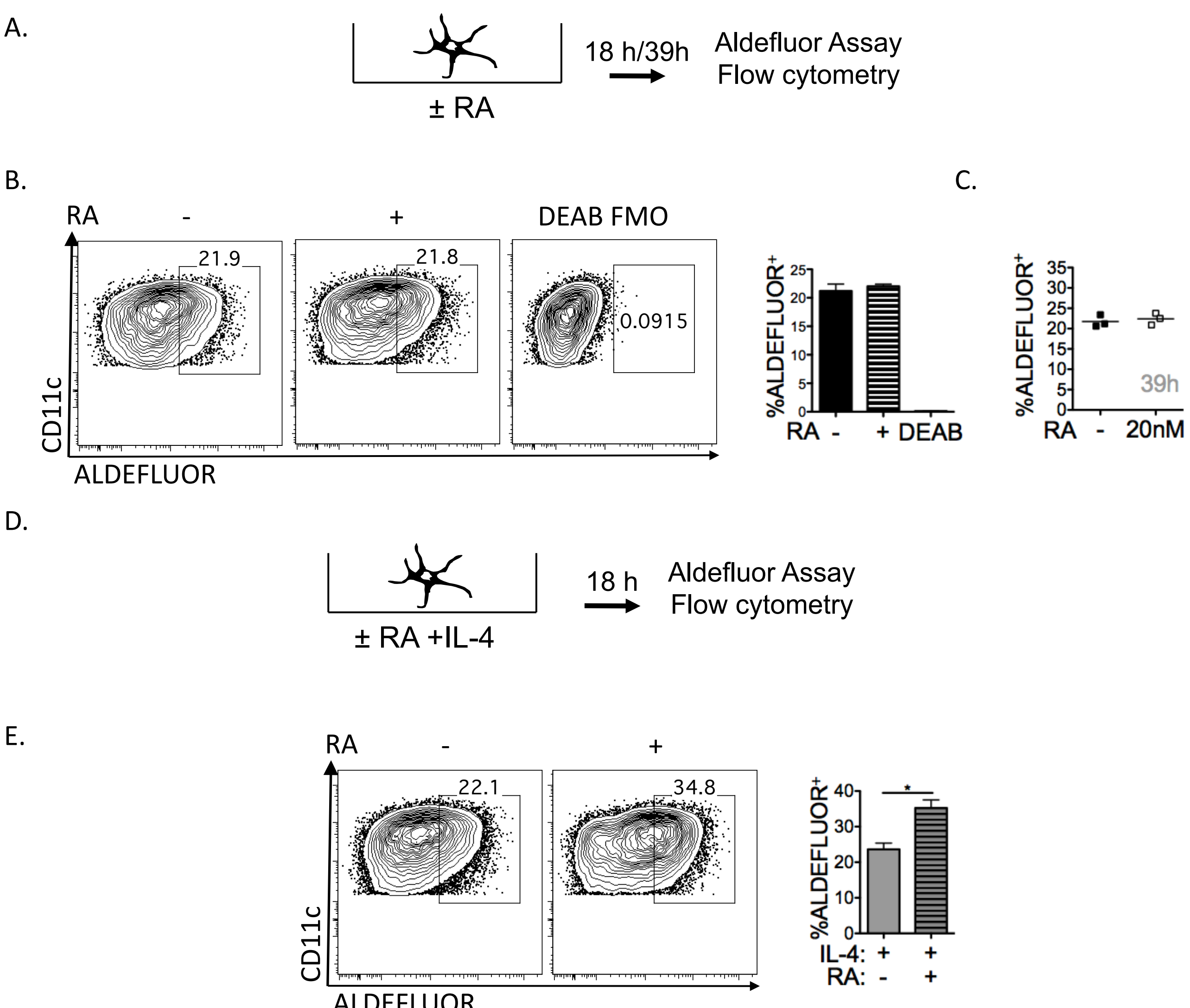


Figure 5.4. RA does not enhance GMDC aldefluor activity in the absence of IL-4. GMDCs were cultured overnight with or without 10 μM RA (A) or for 39h with or without 20nM RA (C) or overnight with 10 μM RA and 20ng/ml IL-4 (D+E) and aldehyde dehydrogenase activity was assessed (aldefluor assay). Data is representative of 5(B+E) or 1(C) independent experiments, error bars represent triplicate culture wells. *=P<0.05.

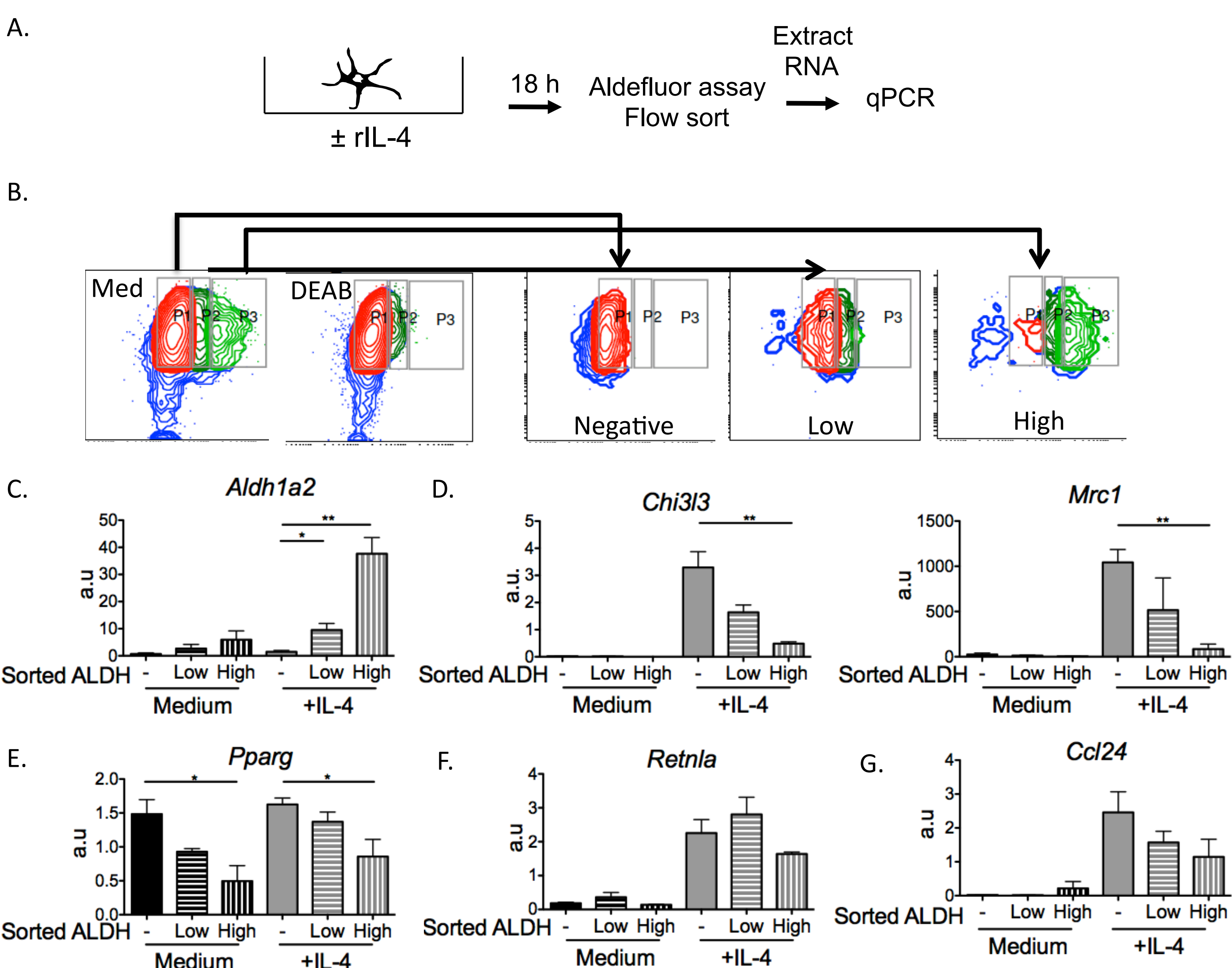


Figure 5.5 IL-4 driven DC RALDH activity correlates with reduced expression of markers of alternative activation but not *Retnla.* Following overnight culture with IL-4, GMDC populations were flow sorted based upon Aldefluor activity (A+B) and qPCR was used to assess expression of *Aldh1a2* (C) *Chi3l3, Mrc1* (D)*, Pparg* (E), *Retnla* (F) and *Ccl24* (G) within aldefluor negative, low or high expressing populations. Data is representative of 4-5 independent experiments, error bars represent triplicate culture wells, a.u.= arbitrary units as compared to *Hprt* expression. *=P<0.05 **= P<0.01.

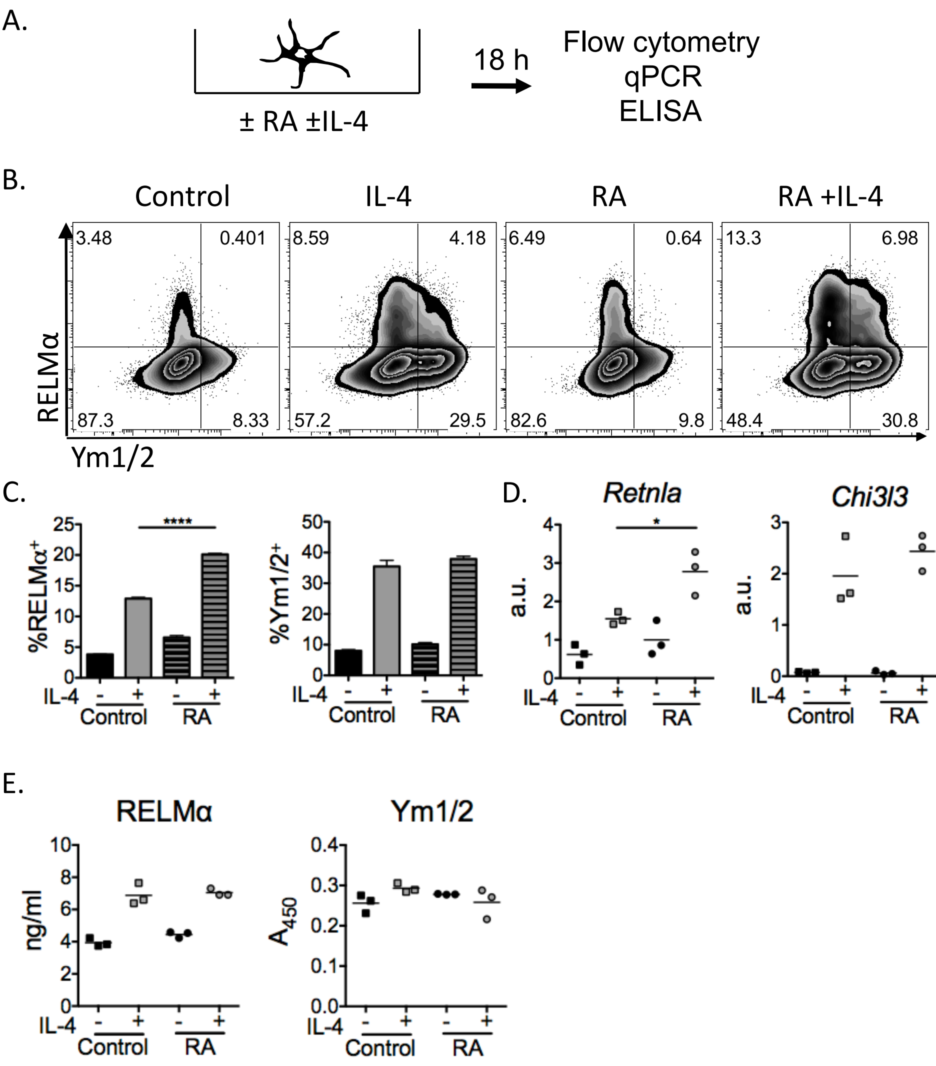
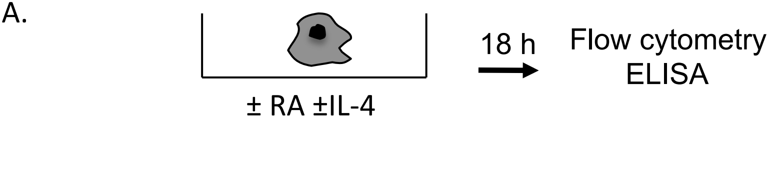


Figure 5.6 RA enhances IL-4 driven RELMα in GMDC. GMDC were cultured overnight with or without 20ng/ml IL-4 and 10μM RA (A). Production of intracellular RELMα and Ym1/2 protein (B+C) *Retnla* and *Chi3l3* transcript (D) and secreted protein (E) were assessed via intracellular staining (B+C), qPCR (D), and ELISA (E). Data is representative of 3 independent experiments, error bars represent triplicate culture wells, a.u.= arbitrary units as compared to *Hprt* expression. *=P<0.05, ****P<0.0001

A.



B.

C.

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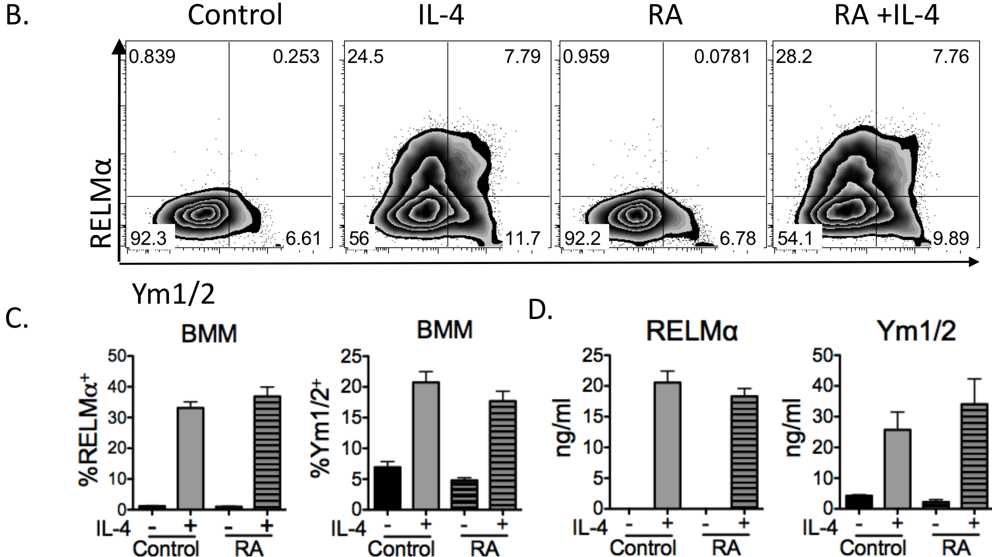

Figure 5.7 RA does not enhance IL-4 driven RELMα in BMM. BMM were cultured overnight with or without 20ng/ml IL-4 and 10μM RA. Production of RELMα and Ym1/2 protein was assessed via intracellular staining (B+C) and ELISA (D). Data is representative of 2 independent experiments, error bars represent SEM of triplicate culture wells.

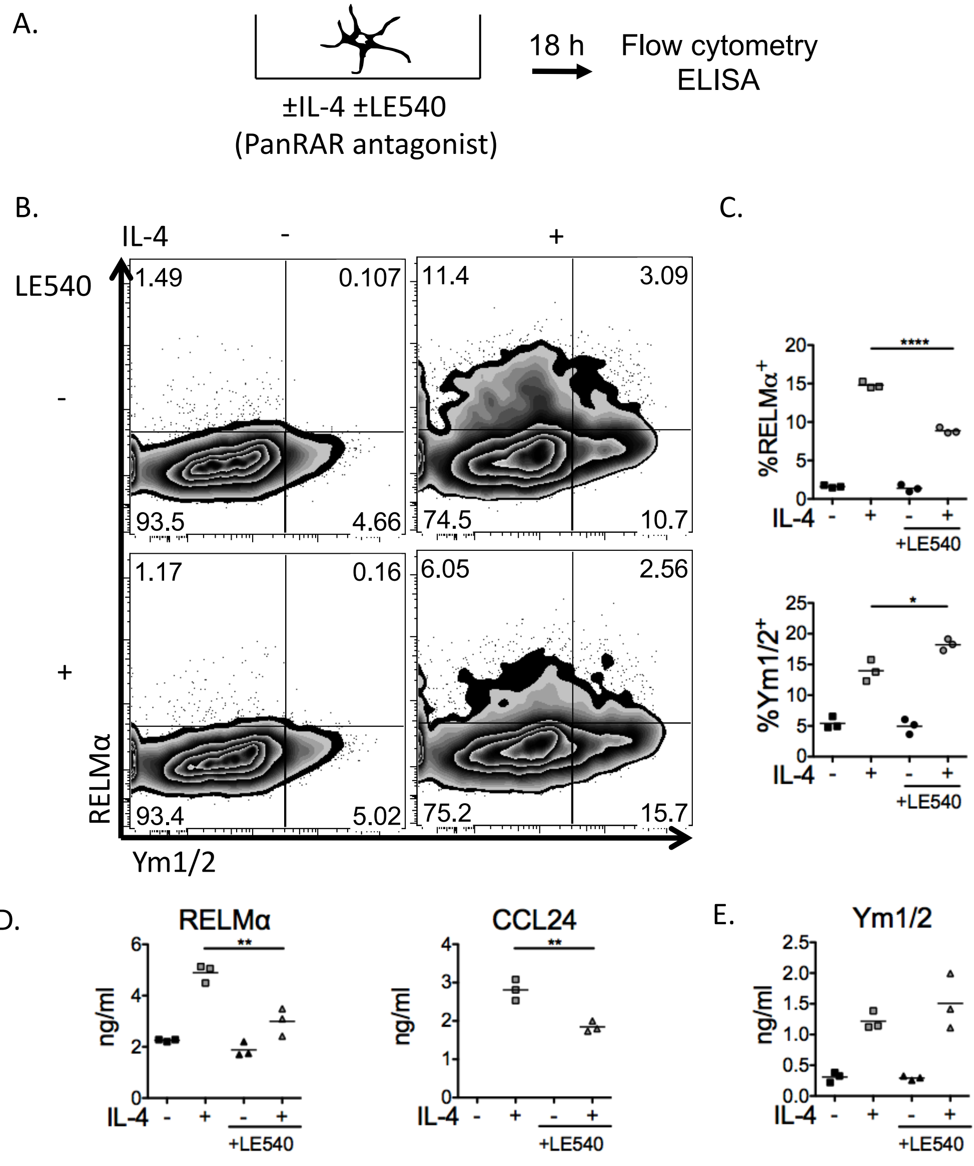

Figure 5.8 RAR signalling regulates IL-4 driven DC alternative activation. GMDC were cultured overnight with or without 20ng/ml IL-4 and the PanRAR antagonist LE540 (10μM) (A). RELMα and Ym1/2 protein production was assessed by intracellular staining (B+C). RELMα, CCL24 and Ym1/2 secretion was assessed by ELISA (D+E). Data is representative of 3 independent experiments, error bars represent triplicate culture wells. *=P<0.05, **= P<0.01, ****P<0.0001

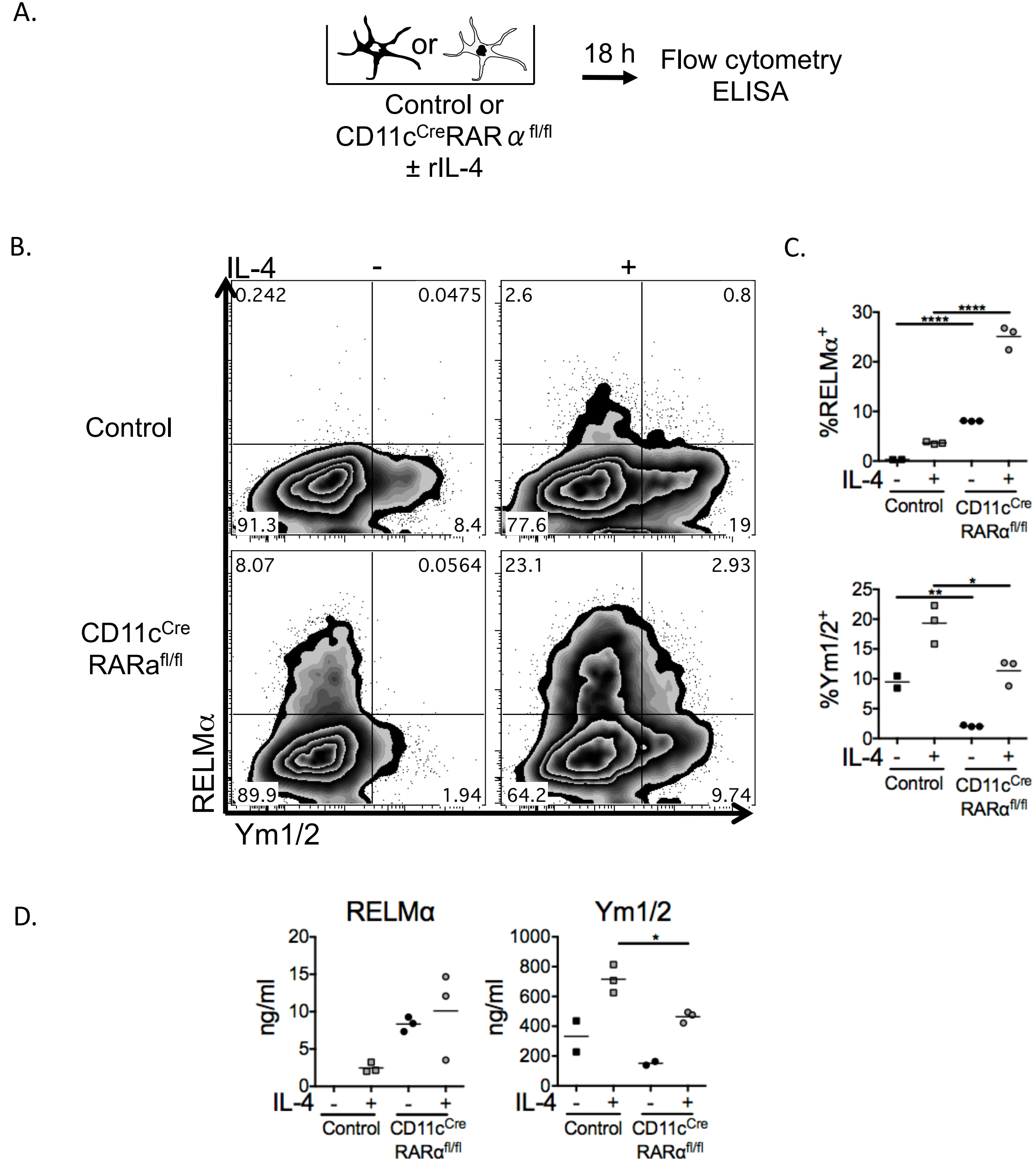

Figure 5.9 RARα signalling inhibits IL-4 driven RELMα and promotes IL-4 driven Ym1/2. *CD11c^{Wt}Rara$^{fl/-}$ or CD11c^{Cre}Rara$^{fl/fl}$* GMDC were cultured overnight with or without 20ng/ml IL-4 (A) RELMα and Ym1/2 protein production was assessed by intracellular staining (B +C) and ELISA (D). Data is representative of 1 independent experiment, error bars represent triplicate culture wells. *=P<0.05, **= P<0.01, ****P<0.0001.

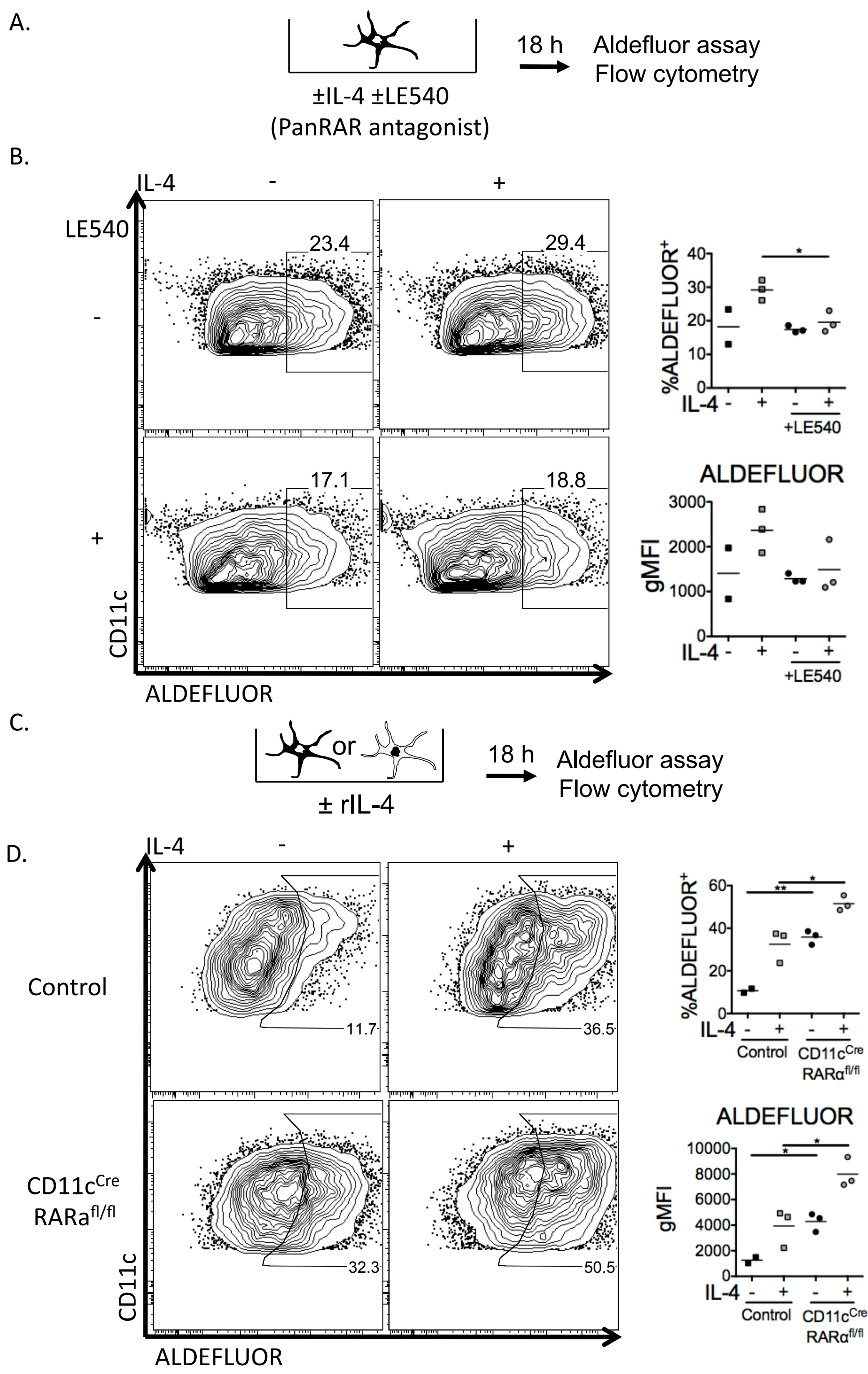

Figure 5.10 RAR signalling modulates IL-4 driven aldehdye dehydrogenase activity in GMDC. *WT* (A+B*) or CD11c*^{Wt}*Rara*$^{fl/-}$ *and CD11c*^{Cre}*Rara*$^{fl/fl}$ GMDC (C+D) were cultured overnight with or without IL-4 and LE540 (A+B only), aldefluor activity was assessed by flow cytometry (B+D). Data is representative of 3 independent experiments (A+B) or one independent experiment (C+D), error bars represent triplicate culture wells. *=P<0.05, **= P<0.01.

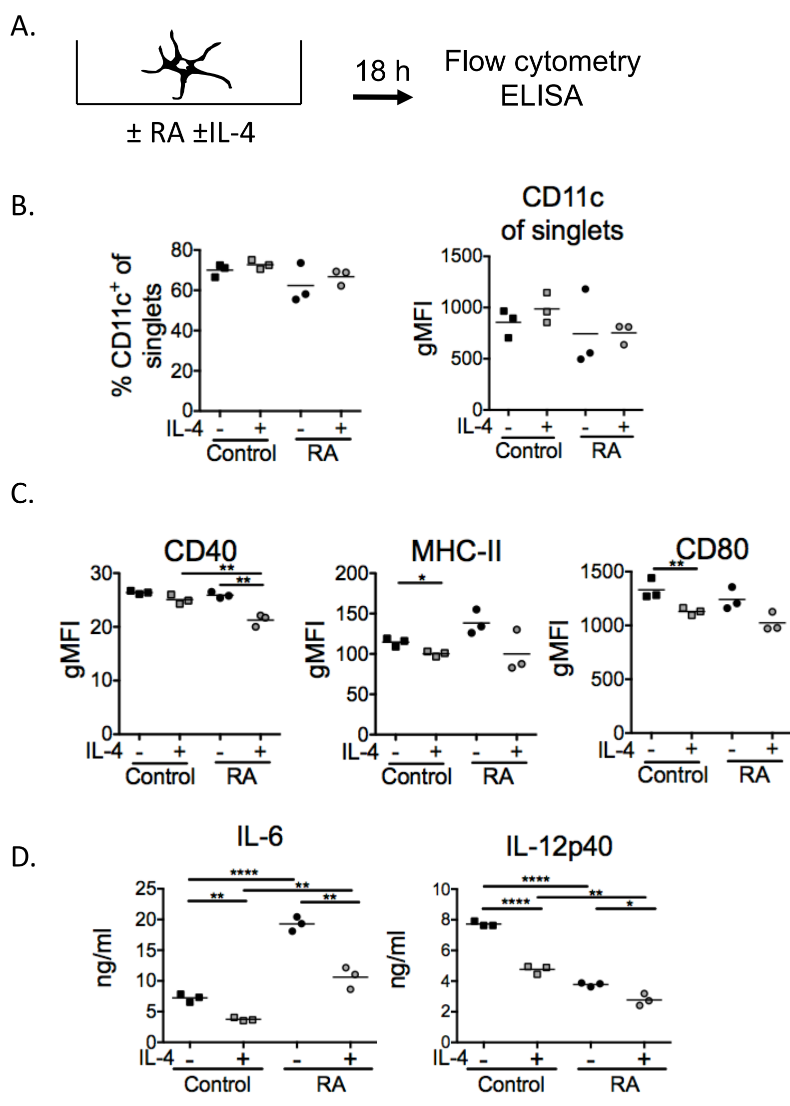

Figure 5.11 RA and IL-4 alter DC co-stimulatory molecule and cytokine secretion. GMDC were cultured overnight with or without IL-4 and RA (A); CD11c expression (B), co-stimulatory molecule and MHC-II expression (C) and cytokine secretion (D) were assessed. Data is representative of 5 independent experiments, N=3 replicate wells per condition. *=P<0.05 **= P<0.01 ***P<0.001 ****P<0.0001.

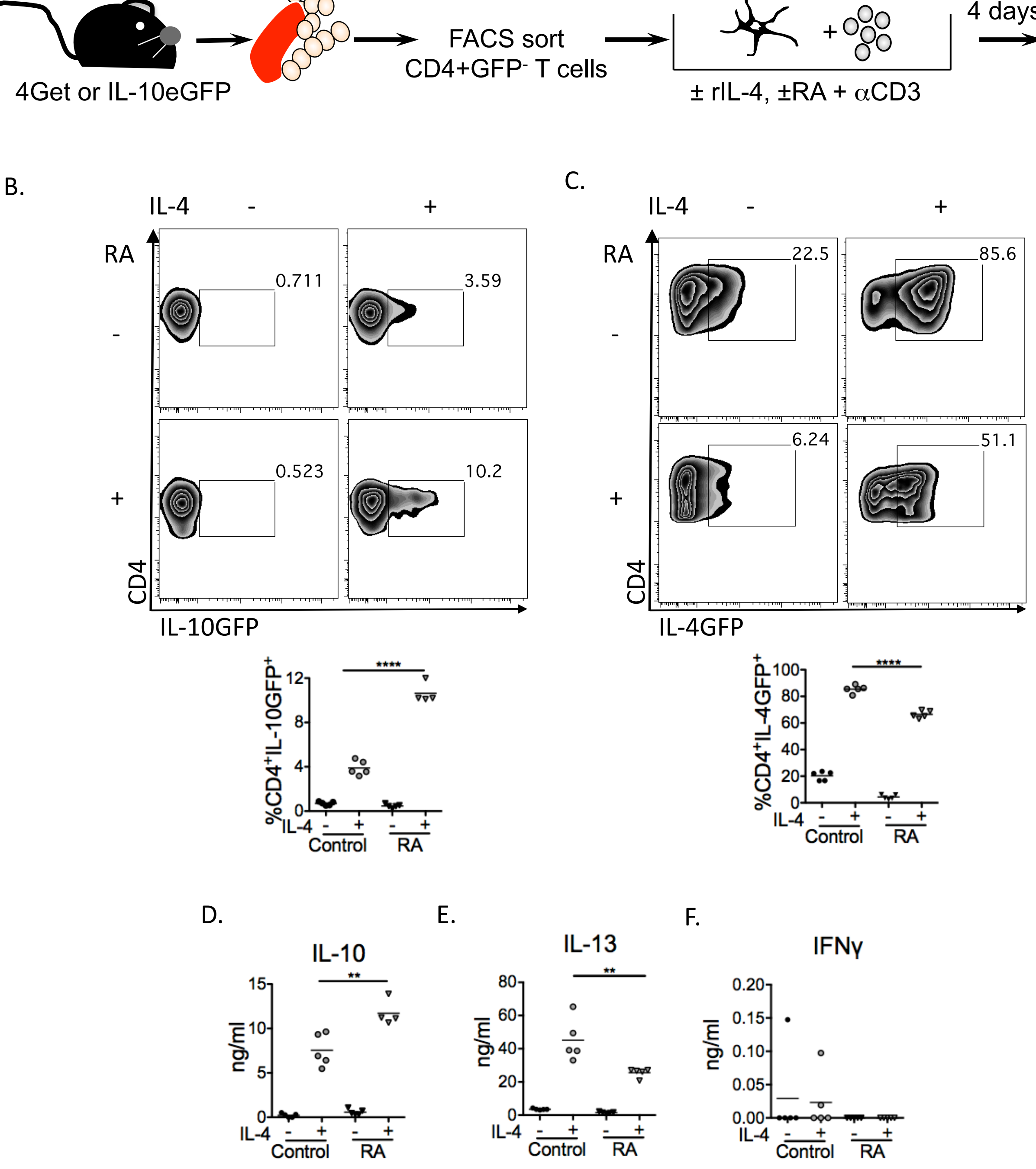

Figure 5.12 RA and IL-4 alter DC APC capacity. IL-10eGFP⁻CD4+ or 4Get⁻CD4^{+} T-cells, were cultured for 3 days with WT GMDCs, anti-CD3 mAb, with or without 20ng/ml IL-4, and 10 μM RA (A) and assessed for IL-10 or IL-4 mRNA expression by flow cytometry (B +C) and IL-10 (D), IL-13 (E) and IFN γ (F) secretion by ELISA. Data is representative of 2 (B+C) or 4 (D-F) independent experiments, N=4-5 replicate wells per condition. *=P<0.05 **= P<0.01 ***P<0.001 ****P<0.0001

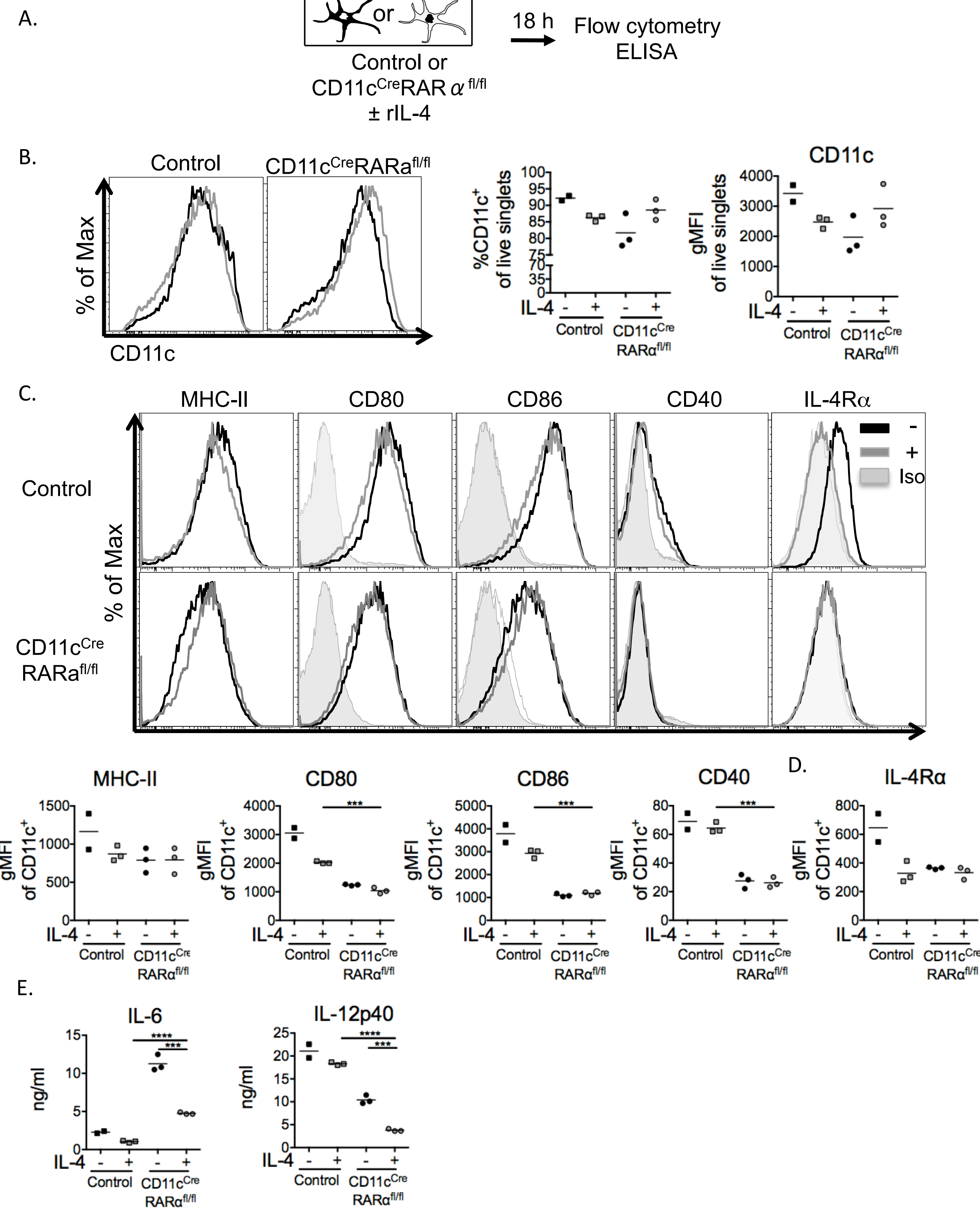

Figure 5.13 RARα alters DC co-stimulatory molecule expression and cytokine secretion. *CD11c^Wt^Rara^fl/-^ and CD11c^Cre^Rara^fl/fl^* GMDC were cultured overnight with or without 20ng/ml IL-4 (A). CD11c expression (B), co-stimulatory molecule expression (C) and IL-4Rα expression (D) were assessed by flow cytometry. Cytokine secretion was assessed by ELISA (E). Data is from 1 independent experiment, N=3 replicate wells per condition. *=P<0.05, ***P<0.001, ****P<0.0001.

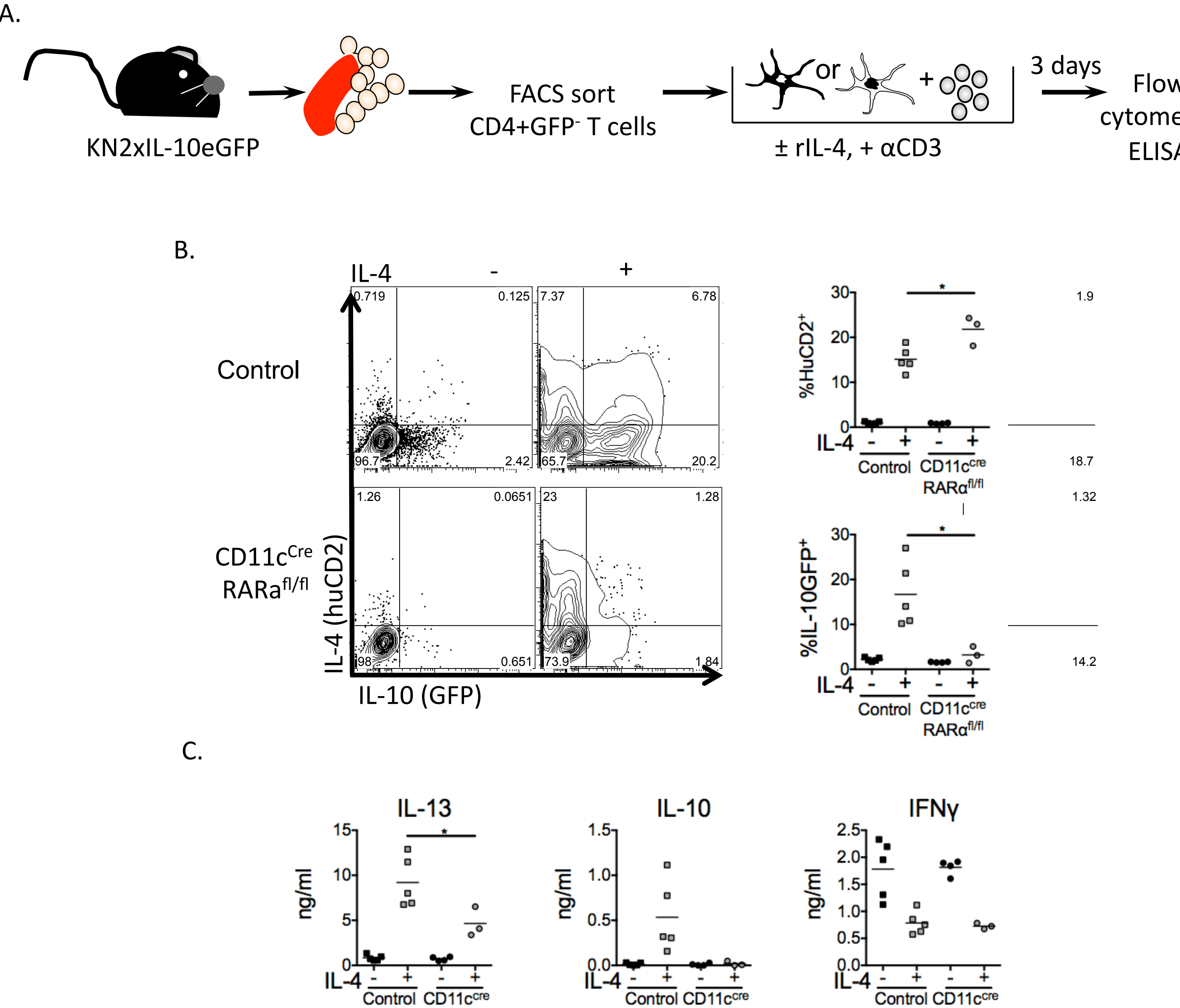

Figure. 5.14 The absence of RAR α alters DC ability to polarise T-cell responses. IL-10eGFP⁻CD4⁺T-cells sorted from KN2xIL10eGFP animals, were cultured for 3 days with *$CD11c^{Wt}Rara^{fl/-}$ or $CD11c^{Cre}Rara^{fl/fl}$* GMDC and anti-CD3 mAb, with or without 20ng/ml IL-4 (A) and assessed for IL-4 protein (HuCD2) or IL-10 mRNA expression by flow cytometry (B) and IL-10, IL-13 and IFN γ secretion by ELISA (C). Data is representative of 1 independent experiment, N=3-5 replicate wells per condition. *=P<0.05.

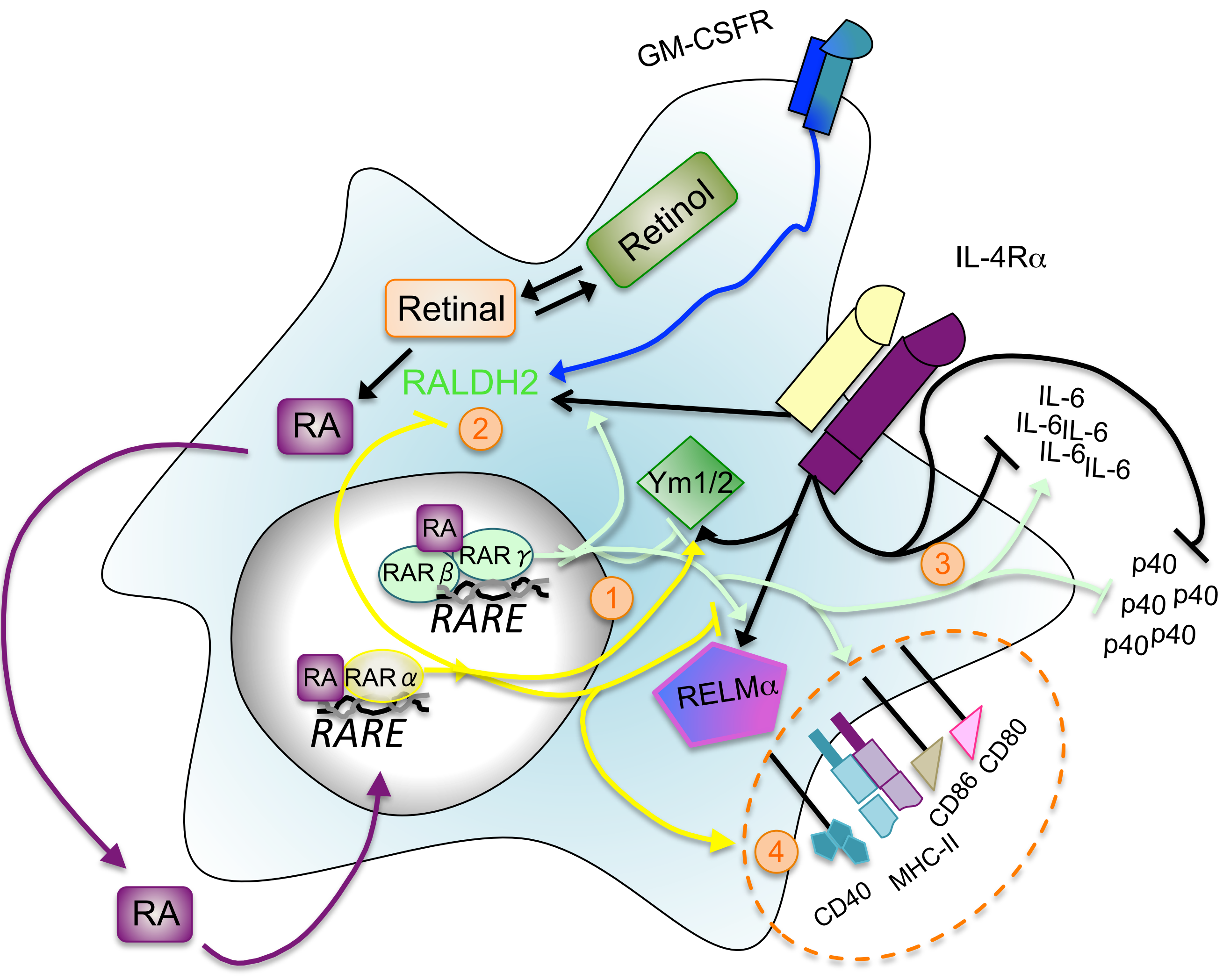

Figure. 5.15 The impact of IL-4 and RA signalling in DCs. This figure depicts the modulation of DC alternative activation (1) , RALDH2 production (2), cytokine secretion (3) and co-stimulatory molecule expression (4) directed by RAR α (yellow lines), all RARs (green lines), IL-4R α and GM-CSF (blue lines). Arrows represent promotion, straight lines represent inhibition.

Chapter 6. How do IL-4 and TLR2 Signalling Pathways Integrate to Influence DC Activation?

6.1 INTRODUCTION

In the previous chapters of this thesis we have shown that IL-4 can induce the alternative activation of DCs, both *in vitro, in vivo* and *ex vivo* (chapter 3), upregulating a similar but not identical profile to that seen in AAM (chapter 3). IL-4 exposed GMDCs express high levels of RELMα, Ym1/2 and CCL24 (Fig. 3.2-3.4 & Table 4.3.3) but do not significantly upregulate arginase activity in response to IL-4 (Fig. 3.5). In chapter 4 a microarray analysis of IL-4 treated GMDCs showed that multiple genes were also down regulated by IL-4, for example, TLR2 (Table 4.3.2). Furthermore in chapter 5 we showed that IL-4 induced DC expression of aldehyde dehydrogenase activity (Fig. 5.2B&C), and that the product of such enzymatic activity, RA, was able to synergise with IL-4 to enhance DC RELMα (Fig. 5.6) and to modulate IL-4 dependent T-cell polarisation (Fig. 5.12).

The TLR2 ligands zymosan and Pam3CSK4 (P3C) are known to modulate DC IL-10 to IL-12 balance, by inducing IL-10 and impairing IL-12p70 (Agrawal, Agrawal *et al.* 2003; Dillon, Agrawal *et al.* 2006; Slack, Robinson *et al.* 2007; Wang, Villablanca *et al.* 2011). In chapter 4 of this thesis IL-4 was shown to modulate DC IL-10 and IL-12 production in response to the defined TLR4 and TLR9 ligands, LPS and CpG, inducing an opposite response to the published outcome of TLR2 ligation (Dillon, Agrawal *et al.* 2004) by enhancing secreted IL-12p70 and reducing secreted IL-10 (Fig. 4.2). We hypothesised that TLR2 and IL-4 signalling pathways may converge at the level of IL-10 and IL-12 regulation and so were interested to investigate how DCs would respond following exposure to both IL-4 and TLR2 ligation simultaneously.

Zymosan has also been reported to modulate vitamin A metabolism in a TLR2 dependent manner (Manicassamy, Ravindran *et al.* 2009). We and others (Chapter 5) (Yokota, Takeuchi *et al.* 2009; Stock, Booth *et al.* 2011; Agace and Persson 2012; Broadhurst, Leung *et al.* 2012) have reported that IL-4 can enhance DC aldehyde dehydrogenase activity. Furthermore, we have shown that the product of such enzyme activity, retinoic acid, is able to modulate IL-4 driven DC alternative activation by increasing IL-4 driven RELMα (Fig. 5.6).

In the work detailed in this chapter we have characterised how IL-4 influences the phenotype of DCs exposed to the synthetic triacylated lipopeptide P3C, a defined TLR2 ligand (Oliveira-Nascimento, Massari *et al.* 2012), and a mixed antigenic preparation of the *Saccharomyces cerevisiae* fungal cell wall, zymosan, which contains ligands recognized by multiple PRRs including TLR2 (Di Carlo and Fiore 1958; Gazi, Rosas *et al.* 2011). We have determined the impact of TLR2 and IL-4R ligation on DC aldehyde dehydrogenase activity, cytokine secretion, and alternative activation. Using inhibitor studies and microarray analysis we have begun to understand the signalling pathways downstream of IL-4R and TLR2 engagement which specifically modulate DC RELMα production, highlighting a substantial role for the MAPK target ERK1/2 in mediating IL-4 modulation of DC RELMα.

6.2 SPECIFIC AIMS:

1. To determine the relationship between TLR2 agonists, IL-4 treatment, RALDH activity and alternative activation of DCs.
2. To investigate the ERK signalling pathway downstream of TLR2 and IL-4Rα ligation.

6.3 RESULTS

6.3.1 TLR2 agonists enhance IL-4 induced aldehyde dehydrogenase activity in GMDC

P3C is a synthetic bacterial triacylated lipopeptide that is recognised immunologically by the PRR heterodimer of TLR2 and TLR1 (Oliveira-Nascimento, Massari *et al.* 2012). Zymosan is a mixed antigenic preparation of the *Saccharomyces cerevisiae* cell wall, rich in β-glucans and mannan, which are recognised by a combination of receptors, including the c-type lectin receptor (CLR) dectin-1 (Brown, Taylor *et al.* 2002; Taylor, Brown *et al.* 2002), the heterodimer pairing of TLR2 and TLR6 (Ozinsky, Underhill *et al.* 2000) and an unknown pathway that is Syk dependent but that is independent of dectin-1 (Rogers, Slack *et al.* 2005; Manicassamy, Ravindran *et al.* 2009).

TLR2 agonists have previously been shown to enhance DC expression of Aldh1a2 (Manicassamy, Ravindran *et al.* 2009; Wang, Villablanca *et al.* 2011). *Aldh1a2* encodes RALDH2, a retinol conversion enzyme previously confirmed as being induced by IL-4 (Yokota, Takeuchi *et al.* 2009; Stock, Booth *et al.* 2011; Agace and Persson 2012) (Fig. 5.2). We sought to determine whether IL-4 and the TLR2 agonists P3C and zymosan interact synergistically to enhance aldehyde dehydrogenase activity within DCs.

GMDC were cultured for 18h with media alone, P3C, or zymosan with or without addition of exogenous IL-4 (Fig. 6.1A). $CD11c^+$ cells were gated and the highest level of aldehyde dehydrogenase activity was induced in the presence of IL-4 together with P3C, as assessed by use of the aldefluor assay (Fig. 6.1B&C). Zymosan enhanced aldefluor expression above the level induced basally (medium alone), and the presence of IL-4 with either P3C or zymosan resulted in a significantly higher percentage of $CD11c^+$ cells expressing activity than in the presence of IL-4 alone (Fig. 6.1B&C). However, per $CD11c^+$ cell expression of

aldefluor activity, when compared to IL-4 controls, only increased significantly in the presence of zymosan (Fig. 6.1D). The transcript encoding RALDH2 was significantly enhanced by IL-4, and by zymosan alone (Fig. 6.1D), but there was no synergistic increase in *Aldh1a2* mRNA expression in the presence of P3C, or a combination of IL-4 and either TLR2 agonist. This could suggest that the regulation of enzyme activity may not be via de novo transcription, or that the time point at which the cells were sampled did not correlate with the point at which a change in transcript levels *for Aldh1a2* occurred, a time course could be carried out to test this possibility.

6.3.2 TLR2 agonists activate GMDCs, IL-4 regulates zymosan directed secretion of IL-10 and IL-12

To competently activate T-cells, DC must express co-stimulatory molecules and secrete cytokines. We were interested to assess how either TLR2 agonist was able to modulate DC activation status, and whether this was altered by the presence of IL-4. GMDC were cultured for 18h with either media alone, P3C or zymosan with or without addition of exogenous IL-4, and surface expression of co-stimulatory markers was assessed by flow cytometry (Fig. 6.2B). Zymosan capably induced high level expression of MHC-II (Fig. 6.2C), CD86 (Fig. 6.2D), CD80 and CD40 (Fig. 6.2E). P3C enhanced expression of MHC and the co-stimulatory molecules assessed. However, at the concentrations of P3C used, the per cell expression of MHC-II (Fig. 6.2C) and CD86 (Fig. 6.2D) was much lower than that induced by zymosan (Fig. 6.2B-F). P3C induced more comparable expression of CD80 and CD40 (Fig. 6.2E). The low level induction of CD86 by P3C is in accordance with similar previous work carried out using human monocyte derived DCs (Agrawal, Agrawal *et al.* 2003). The surface phenotype induced by either TLR2 agonist was not significantly changed by the simultaneous addition of IL-4 (Fig 6.2 B-E). These findings are similar to those seen in Fig. 4.1 in which ligation of TLR9 and TLR4 enhanced DC co-stimulatory molecule expression, and only a subtle impact of IL-4 treatment on co-stimulatory molecule expression was seen, with a slight enhancement of LPS stimulated CD86 being apparent.

In a similar manner to the ability of P3C to differentially enhance DC co-stimulatory molecules, very low level IL-10 and no IL-12p70 was secreted by GMDCs cultured overnight with P3C (Fig. 6.2F). However, both TNFα and IL-6 were secreted in significant amounts when compared to medium only controls (Fig. 6.2G).

No IL-10 was detected in supernatants in which both IL-4 and P3C had been included in the overnight culture, whereas no impact of IL-4 was seen on the ability of P3C exposed DCs to secrete IL-12p70, TNFα or IL-6 (Fig. 6.2F&G). Zymosan induced the highest levels of all cytokines assessed (Fig. 6.2F&G). Interestingly, IL-4 regulated the secretion of IL-10 and IL-12p70 by zymosan exposed DCs (Fig. 6.2F) but did not significantly impact TNFα or IL-6 secretion (Fig. 6.2G). In the presence of IL-4, zymosan induced significantly less IL-10 and significantly more IL-12p70 secretion than it was able to provoke alone, a similar result to that seen with TLR9 and TLR4 agonists in the presence of IL-4 (Fig. 4.1). The absence of secreted IL-12p70 from DC in the presence of P3C differs from published work using splenic DCs, in which similar levels of IL-12p70 were found following culture with either P3C or zymosan (Dillon, Agrawal *et al.* 2006). Interestingly, however, work from the same group had previously found only very low level IL-12p70 induction from human monocyte DCs in response to P3C (Agrawal, Agrawal *et al.* 2003). The differences in cytokine secretion between these studies are probably due to antigen dose, as in this work we used P3C at 250ng/ml, the 2003 study used P3C at 20μg/ml (Agrawal, Agrawal *et al.* 2003) and the 2006 study at 100μg/ml (Dillon, Agrawal *et al.* 2006). Work using anti-IL-10R and IL-10 deficient DCs has previously shown that zymosan induction of DC IL-10 down modulates zymosan driven DC secretion of IL-12, TNFα, IL-6 (Manicassamy, Ravindran *et al.* 2009).

6.3.3 P3C and zymosan limit IL-4 driven RELMα and Ym1/2 to different extents

Having shown that TLR2 agonists modulate DC aldehdye dehydrogenase activity (Fig. 6.1), and that IL-4 differentially modulates DC responsiveness to the TLR2 agonists P3C and zymosan (Fig. 6.2), we next investigated whether the presence of P3C and zymosan modulated IL-4 induced DC alternative activation. GMDC were cultured for 18h with either media alone, P3C or zymosan, with or without addition of exogenous IL-4 and flow cytometry, ELISA and qPCR were used to assess RELMα and Ym1/2 expression (Fig. 6.3A). The TLR2 specific agonist P3C was able to completely abrogate intracellular IL-4 induced RELMα (Fig. 6.3 B&C), RELMα secretion (Fig. 6.3D), and *Retnla* transcript (Fig. 6.3E), with DCs present in P3C + IL-4 culture wells having the same level of RELMα expression as control DCs cultured in medium only (Fig. 6.3 B-E). P3C did not inhibit IL-4 induced Ym1/2 and there was a trend for reduced intracellular Ym1/2 protein, although this did not reach significance (Fig. 6.3 B&C). P3C did significantly inhibit Ym1/2

secretion (Fig. 6.3D) and *Chi3l3* transcript (Fig. 6.3E), but did not reduce expression to basal levels, as was evident with RELMα. Zymosan completely abrogated IL-4 induced expression of both RELMα and Ym1/2 at the level of intracellular and secreted protein (Fig. 6.3 B-D) and transcript (Fig. 6.3E).

The differential inhibition of RELMα and Ym1/2 expression following treatment with IL-4 and P3C was different to the impact seen when culturing DC with any other defined TLR ligand (CpG, LPS, zymosan) at the same time as IL-4, where the inhibition of both RELMα and Ym/12 followed roughly the same pattern (Fig. 4.3 & Fig. 6.3). However, a difference in inhibition of IL-4 induced RELMα but not Ym1/2 was also seen when the mixed helminth antigenic preparation SEA was used to stimulate DCs in combination with IL-4 (Fig. 4.5). This may suggest that the component of SEA that is responsible for RELMα inhibition may also be signalling via the same TLR as P3C (eg. TLR2 and the co-receptor TLR1).

6.3.4 Zymosan inhibits arginase and promotes iNOS in GMDC

To further investigate the impact of TLR2 agonists on GMDC activation, we assessed the regulation of arginine converting enzymes by P3C, zymosan and IL-4. As discussed in previous chapters, IL-4 does not enhance GMDC arginase expression in contrast to its ability to enhance BMM arginase (Fig. 3.12D). As ligation of either TLR9 or TLR4 significantly induced DC expression of *Nos2* but not *Arg1* (Fig. 4.3 E&F), we were interested to determine whether the TLR2 ligands would influence expression of these arginine conversion enzymes and whether TLR2 ligation would then alter the ability of IL-4 to drive arginase activity.

GMDC were cultured for 18h with media alone, P3C or zymosan with or without addition of exogenous IL-4. Arginase (*Arg1)* and inducible nitric oxide synthase (*Nos2*) levels were assessed by qPCR and production of nitric oxide (NO) was determined using the Griess reaction (Fig. 6.4D). Though not significant, there was a trend for reduced expression of *Arg1* and enhanced *Nos2* in GMDC cultured overnight with P3C, a pattern that was not altered by presence of IL-4 (Fig. 6.4B&C). Zymosan was able to significantly inhibit *Arg1* and significantly enhance *Nos2* expression, independently of IL-4 presence (Fig. 6.4B&C). Nitric oxide production detected by the Griess reaction is a direct readout of inducible nitric oxide synthase activity (Tsikas 2007). Zymosan significantly enhanced NO

production by GMDC as compared to medium alone controls, while IL-4 caused a subtle but significant reduction in the level of zymosan induced NO (Fig. 6.4D).

6.3.5 P3C and zymosan modulate chemokine production by IL-4 treated GMDCs

As TLR2 ligands were found to modulate AADC expression of RELMα and Ym1/2, we investigated whether the same ligands influenced the expression of chemokines that we had found to be enhanced in GMDCs exposed to IL-4 (Fig. 3.4D&E, 4.3E&F). GMDC were cultured for 18h with either media alone, P3C or zymosan, with or without addition of exogenous IL-4 (Fig. 6.5A). P3C and zymosan both significantly reduced transcripts for *Ccl17* below the basal level found in the presence of media alone (Fig. 6.5B). Following the addition of IL-4, neither P3C or Zymosan altered expression of *Ccl17,* levels of which were the same as in the presence of IL-4 alone (Fig. 6.5B). In the absence of IL-4, P3C and Zymosan had no impact on the transcription (Fig. 6.5C) or secretion (Fig. 6.5D) of CCL24. In the presence of IL-4 there was an interesting contrasting effect of P3C versus zymosan. With P3C, CCL24 secretion was significantly higher than following culture with IL-4 alone, whereas zymosan significantly inhibited IL-4 driven CCL24 (Fig. 6.5D). We had previously found no impact of TLR9 or TLR4 ligation on the modulation of IL-4 induced CCL24 (Fig. 4.3E&F). These data suggest that signalling via a TLR2 agonist is more closely related to changes downstream of IL-4 signalling, than is signalling via TLR9 or TLR4.

6.3.6 IL-4 and TLR2 counter-regulate one another in GMDCs

As the data in this chapter indicated several examples of interaction between the different TLR2 agonists and exposure to IL-4, we decided to assess whether IL-4 could directly regulate TLR2 expression. GMDC were cultured overnight with IL-4 and their expression of *Tlr2* was assessed by qPCR (Fig. 6.6A). Following overnight culture, IL-4 significantly downregulated mRNA transcript for TLR2 (Fig. 6.6B). We next assessed whether the presence of the IL-4Rα modulated GMDC TLR2 expression. WT or *Il4ra*$^{-/-}$ GMDC were cultured for 6 hours with or without IL-4 and *Tlr2* expression was assessed by qPCR (Fig. 6.6C). After only 6 hours of culture with IL-4, WT GMDC *Tlr2* expression was not reduced (Fig. 6.6D) in contrast to the reduction seen by 18 hours (Fig. 6.6B). Suggesting that the inhibition of TLR2 expression by IL-4 may not be a direct effect, and thus require alterations in a

cascade of transcription and translation before a decline in the levels of *Tlr2* mRNA was apparent. Additionally, the level of *Tlr2* message at 6 hours may not correlate with the translated protein for TLR2, an added complexity that we cannot detect using this qPCR system.

IL-4Rα$^{-/-}$ GMDC displayed significantly enhanced basal levels of *Tlr2* mRNA relative to WT GMDCs and, reassuringly, this was not changed by addition of IL-4 to the culture (Fig. 6.6D). Suggesting the presence of the IL-4Rα is required for the inhibition of TLR2 in the steady state in the absence of exogenous IL-4.

As IL-4 and TLR2 ligands have been implicated in the modulation of DC aldehyde dehydrogenase activity (Manicassamy, Ravindran *et al.* 2009; Yokota, Takeuchi *et al.* 2009), we investigated whether aldehyde dehydrogenase activity level correlated with TLR2 expression. GMDCs were cultured overnight in the presence of media alone or media supplemented with 20ng/ml IL-4, surface stained for CD11c and flow sorted into three populations (negative, low, high) based on aldefluor activity as compared to a DEAB control (Fig. 6.6E & Fig. 5.5). RNA was extracted from each population, converted to cDNA and expression of *Tlr2* was measured using quantitative PCR, normalised to the housekeeping gene *Hprt* (Fig. 6.6F). *Tlr2* mRNA expression inversely correlated with aldefluor activity, with aldefluor high cells having significantly lower *Tlr2* expression (Fig. 6.6F). IL-4 was found to decrease *Tlr2* expression (confirming the result in Fig. 6.6B) and, even in the presecnce of IL-4, the cells with highest aldehyde dehydrogenase activity had lowest Tlr2 expression (Fig. 6.6F). This suggests that a close interplay exists between the expression of aldehyde dehydrogenas activity and *Tlr2*, with IL-4 treatment downregulating Tlr2 expression, whilst simultaneously enhancing *Aldh1a2* expression and RALDH activity as shown in Figure 5.5.

6.3.7 Tlr2 limits IL-4 driven RELMα in GMDC, and is responsible for P3C inhibition of alternative activation

Having shown that TLR2 ligation simultaneous with IL-4 exposure can synergistically enhance the ability of IL-4 to promote DC aldefluor activity (Fig. 6.1), but that the same agonists can also inhibit other facets of IL-4 dependent DC stimulation (alternative activation Fig. 6.3), we wondered whether the impact of P3C and zymosan upon alternative activation was mediated via TLR2. In order to confirm a role for TLR2 in mediating the inhibitory effect of P3C and zymosan on IL-

4 induced RELMα and Ym1/2 (Fig. 6.3), we assessed the impact of the TLR2 agonists and IL-4 on GMDC deficient in TLR2 expression. WT or *Tlr2*$^{-/-}$ GMDCs were cultured overnight in the presence of medium, P3C or zymosan with or without the addition of IL-4 (Fig. 6.7A). In the absence of TLR2, GMDC expressed higher intracellular levels of IL-4 induced RELMα but no significant change in IL-4 induced Ym1/2 (Fig. 6.7B-D). The ability of P3C to inhibit IL-4 driven RELMα and Ym1/2 was entirely dependent upon TLR2 expression, as P3C did not limit IL-4 driven intracellular expression or secretion of either alternative activation marker in the absence of TLR2 (Fig. 6.7B-D). Surprisingly, the zymosan inhibition of IL-4 driven DC alternative activation was independent of TLR2, as zymosan addition resulted in abrogation of both RELMα and Ym1/2 in the *Tlr2*$^{-/-}$ GMDCs at the level of both intracellular and secreted protein (Fig. 6.7 B-D). As zymosan can signal via the CLR dectin-1 as well as TLR2 (Brown 2006), it is possible that dectin-1 is responsible for the ability of zymosan to inhibit IL-4 driven alternative activation.

6.3.8 IL-4 inhibition of zymosan driven phospho-ERK1/2 is dependent upon TLR2

Previous studies have highlighted that the mitogen activated protein kinase (MAPK) signalling cascade is activated downstream of TLR2 signalling, with phosphorylation of the extracellular signal regulated kinases (ERK1/2; MAPK3/1) being a frequently reported outcome (Dillon, Agrawal *et al.* 2004; Dillon, Agrawal *et al.* 2006; Slack, Robinson *et al.* 2007; Adhikary, Sun *et al.* 2008; Manicassamy, Ravindran *et al.* 2009; Wang, Villablanca *et al.* 2011). We aimed to investigate whether in our hands, ERK phosphorylation was a downstream outcome of P3C and zymosan binding, and to what extent IL-4 was able to impact such signalling events. Phosphorylation of ERK occurs within a much shorter time frame than the 18 hour overnight culture used as standard in our assay so, in order to assay these early signalling events, a 15 minute time point was assessed. GMDCs were surface stained for CD11c prior to 15 minute incubation with either P3C, zymosan or the phorbol ester PdBU (which acts as a positive control for ERK activation) in the presence or absence of IL-4. Following culture, cells were harvested, fixed and permeabilised prior to staining with an anti-pERK antibody (Fig. 6.8A). To provide a control for the absence of ERK activation, the MEK1/2 inhibitor (MEK1/2 is the kinase upstream of ERK1/2) U0126 was used to treat control GMDCs prior to addition of the antigen and IL-4 combinations. Experiments were only considered to

have worked if addition of the inhibitor U0126 was able to limit all phosphorylation induced by PdBU. IL-4 alone did not induce ERK1/2 activation in DCs (Fig. 6.8), P3C induced a small but significant increase in ERK phosphorylation and zymosan induced dramatic activation of ERK, equal to the level of phosphorylation seen with the positive PdBU treated control wells (Fig. 6.8B&C). Interestingly, IL-4 clearly interfered with zymosan induced ERK activation and this inhibition was not evident in TLR2$^{-/-}$ DCs (Fig. 6.8.B&C). Furthermore IL-4 was able to inhibit P3C induced ERK phosphorylation in WT DCs (Fig. 6.8). In this section we have shown that both TLR2 ligation and IL-4 integrate at the level of ERK phosphorylation, as IL-4 is able to limit pERK induced in response to either P3C or zymosan, surprisingly, this inhibition is dependent upon TLR2, while zymosan induction of pERK is independent of TLR2.

6.3.9 IL-4 differentially modulates components of the MAPK signalling cascade in WT and RELMα deficient DCs

Having identified a role for the MAPK signalling pathway in IL-4 modulation of the GMDC response to TLR2 ligation, we next decided to re-assess our microarray data from WT and RELMα deficient DCs carried out in earlier chapters (Fig. 4.14, Fig. 5.1), this time specifically focussing on signalling pathways. Our initial analyses (chapter 4, section 14) had only investigated genes significantly changed ($P < 0.05$) within a high fold change threshold (>2FC enhancement or reduced by more than 0.5FC). In this new signalling focused analysis of the data, all genes significantly changed by IL-4 treatment ($P < 0.05$) were assessed for involvement in signalling pathways. 26 genes that encode components of signalling pathways or proteins that directly interact with such pathways were found to be significantly upregulated by IL-4 within either WT or RELMα deficient DCs (Fig. 6.9). Interestingly, 17 of these signalling genes were only changed by IL-4 in cells deficient in RELMα, with 10 of the 17 known to interact with or regulate the MAPK signalling pathway.

The bias toward RELMα dependence in regulation of MAPK signalling gave our first indication that the differential regulation of IL-4 dependent RELMα as compared to Ym1/2 downstream of PRR (SEA, Fig. 4.5), TLR2 (P3C, Fig. 6.3) and RA (Fig. 5.5 & 5.6) signalling may be dependent upon components of this pathway.

6.3.10 ERK signalling inhibits IL-4 driven RELMα in GMDC

Having determined that IL-4 can regulate pERK signalling within DCs following TLR2 ligation (Fig. 6.8), that the TLR2 agonist P3C can phosphorylate ERK and abrogate IL-4 driven RELMα production in DC (Fig. 6.3 & 6.7) and that RELMα is involved in regulating the IL-4 induced MAPK signalling capacity of DCs (Fig. 6.9) we hypothesised that DC expression of alternative activation markers may be dependent upon ERK signalling. To test this, GMDCs were cultured in the presence or absence of the MEK inhibitor U0126, prior to addition of IL-4 (Fig. 6.10A). GMDC alternative activation status was then assessed by ELISA and qPCR. In the presence of U0126, IL-4 exposed GMDCs secreted significantly enhanced levels of RELMα protein when compared to IL-4 exposed cells in the absence of inhibitor (Fig. 6.10B). This elevated RELMα secretion correlated with significantly increased *Retnla* transcript (Fig. 6.10B), suggesting that baseline ERK signalling is responsible for inhibiting IL-4 induced RELMα. In contrast to RELMα, Ym1/2 was not significantly increased on addition of U0126 (Fig. 6.10C), with a trend for reduction at both the protein and transcript level. Similarly, IL-4 induced CCL24 was significantly inhibited by the addition of U0126, highlighting a dependence on ERK signalling for IL-4 upregulation of this chemokine (Fig. 6.10D). This result suggests that ERK signalling is directly involved in DC alternative activation, being particularly responsible for controlling the expression levels of various IL-4 induced gene products, inhibiting RELMα but promoting CCL24.

6.4 SUMMARY

- IL-4 synergises with TLR2 agonists P3C and zymosan to enhance GMDC aldehyde dehydrogenase activity (Fig. 6.1).
- IL-4 modulates zymosan dependent IL-10 and IL-12p70 secretion by GMDCs but does not alter zymosan induced TNFα or IL-6 (Fig. 6.2F&G).
- IL-4 does not alter P3C induction of cytokine secretion by GMDCs (Fig. 6.2F&G).
- P3C abrogates IL-4 induced RELMα, but only partially inhibits Ym1/2 in GMDCs (Fig. 6.3).
- Zymosan significantly inhibits IL-4 induced RELMα and Ym1/2 in GMDCs (Fig. 6.3).
- Zymosan inhibits *Arg1* but promotes *Nos2* and nitric oxide production in GMDCs (Fig. 6.4).
- P3C and IL-4 synergise to enhance CCL24, whereas zymosan inhibits IL-4 driven CCL24, in GMDCs (Fig. 6.5D).
- IL-4 downregulates *Tlr2* expression in GMDCs, and *Tlr2* expression inversely correlates with aldefluor activity: cells with higher aldefluor activity have lower levels of *Tlr2* expression (Fig. 6.6).
- P3C abrogation of IL-4 induced RELMα and Ym1/2 in GMDCs is dependent upon TLR2, whereas zymosan inhibition of IL-4 induced RELMα and Ym1/2 is independent of TLR2 (Fig. 6.7B-D).
- P3C and zymosan induce intracellular phosphorylation of ERK1/2 in GMDCs: P3C activation of ERK1/2 is TLR2 dependent whereas zymosan activation of ERK1/2 is independent of TLR2 (Fig. 6.8C).
- IL-4 inhibits only TLR2 dependent P3C and zymosan induction of pERK1/2 in GMDCs (Fig. 6.8D).
- IL-4 significantly alters multiple components of the MAPK signalling pathway in GMDCs, particularly in *Retnla*$^{-/-}$ deficient cells (Fig. 6.9).
- ERK signalling differentially affects different aspects of GMDCs alternative activation: IL-4 induced RELMα is inhibited by ERK signalling (Fig. 6.10B), whereas IL-4 induced CCL24 is promoted by ERK signalling (Fig. 6.10D).

6.5 DISCUSSION

6.6.1 The TLR2 and IL-4 dependence of RALDH2

In the work described in this chapter, we found that inclusion of IL-4 and either a defined agonist of TLR2 (P3C) or an antigenic mixture containing TLR2 ligands (zymosan) resulted in the greatest induction of aldefluor activity within GMDCs. This result indicates a synergistic interaction between TLR2 ligation and IL-4 signalling in the control of DC RA synthesis (Figs. 6.1 & 6.11).

Zymosan induction of *Aldh1a2* has previously been shown to be partially dependent upon TLR2 expression in splenic DCs, as TLR2 deficiency significantly decreased zymosan dependent *Aldh1a2* transcription but did not totally abrogate the response to the level seen in medium control treated DCs (Manicassamy, Ravindran *et al.* 2009). The remaining induction (independent of TLR2) was found to also be independent of zymosan signalling via dectin-1, and the authors proposed that the rest of the response to zymosan was dependent upon signalling mediated via unidentified Syk dependent receptor although this data was not published (Manicassamy, Ravindran *et al.* 2009). As we have shown that RA and IL-4 synergise to enhance DC *Aldh1a2* (Fig. 5.4E), elevated TLR2 and IL-4 dependent aldehyde dehydrogenase activity may result in a feed-forward loop via production of RA to promote even more enhancement of RALDH activity (Fig. 6.11). To determine the relative contributions of TLR2, dectin-1 and IL-4Rα signalling in RALDH activity, DCs deficient in *Tlr2, Clec7a* and *Il4ra* should be compared with regard to their aldefluor activity both basally and in the presence of IL-4 and TLR2 agonists.

6.6.2 TLR2 and IL-4 modulation of DC cytokine production

P3C has previously been shown to induce much higher levels of pERK than LPS, and the enhancement of ERK signalling was shown to alter the ability of these TLR agonists to induce the cytokines IL-10 and IL-12 (Agrawal, Agrawal *et al.* 2003; Dillon, Agrawal *et al.* 2004). Deficiency in ERK1 in DCs, or inhibition of ERK signalling using the upstream inhibitor of MEK1/2 U0126, resulted in increased secretion IL-12p70 and reduced secretion of IL-10 by LPS or P3C stimulated DCs (Dillon, Agrawal *et al.* 2004). In later work, the same authors also assessed the impact of zymosan on both murine and human monocyte derived DC cytokine secretion (Dillon, Agrawal *et al.* 2006), finding that zymosan acting via TLR2 and

dectin-1 resulted in increased secretion of IL-10 and reduced IL-12p70, as compared to LPS stimulation (Dillon, Agrawal *et al.* 2006). The zymosan induction of IL-10 could be inhibited in a U0126 dependent manner, and blocking of dectin-1 β-glucan recognition of zymosan using laminarin during stimulation of *Tlr2*$^{-/-}$ DCs totally abrogated phosphorylation of ERK (Dillon, Agrawal *et al.* 2006). More recent studies in human monocyte derived DCs have highlighted a role for DC expression of cyclooxygenase-2 in response to zymosan resulting in autocrine prostaglandin E2, which enhanced *Il10* transcription (Alvarez, Municio *et al.* 2009). This recent work may be particularly relevant to our results, given the 2011 paper from Stock *et al* which found that PGE_2 limited the induction of DC *Aldh1a2* at extra-intestinal sites (Stock, Booth *et al.* 2011). Taking these two studies together, the expression of COX2 in response to zymosan may result in a feedback loop to inhibit further induction of *Aldh1a2*.

We propose that the IL-4 dependent inhibition of pERK seen following culture with zymosan (Fig. 6.8) is responsible for the reduced IL-10 and enhanced IL-12p70 detected in the supernatant following overnight culture with both zymosan and IL-4 (Fig. 6.2F). In addition, if P3C was used at higher dose, we predict that the same outcome (enhanced IL-12p70 and reduced IL-10 following the addition of IL-4) would result. Moreover, we would anticipate that DCs expressing elevated levels of IL-12p70 and reduced IL-10 would be prone to polarise naïve T-cells towards a Th1 type phenotype, as shown in summary Figure 6.11.

6.6.3 Interactions between IL-4, TLR2 and ERK signalling

We highlighted in Fig. 6.6 that IL-4 is able to down modulate transcription of *Tlr2* by 18h in murine GMDCs and that, in the absence of IL-4Rα there was an enhanced level of *Tlr2* in GMDCs. The ability of IL-4 to modulate *Tlr2* expression has previously been reported in human monocytes (Staege, Schaffner *et al.* 2000) and human monocyte derived DCs (Krutzik, Ochoa *et al.* 2003). It was also shown by Staege *et al* that IL-4 down regulated monocyte expression of TLR4 following 24h of culture. IL-4 modulation of TLR4 may be an important mechanism in modulating the DC response to LPS, which we showed in chapter 4. For this reason, the levels of TLR4 following LPS and IL-4 treatment of GMDCs should be assessed and related to this previous work (Staege, Schaffner *et al.* 2000). No apparent role for IL-10 or GM-CSF in down regulating TLR2 was found in the study

by Krutzik *et al,* which is an important result as TLR2 ligation promotes DC IL-10 secretion (Dillon, Agrawal *et al.* 2004; Dillon, Agrawal *et al.* 2006; Alvarez, Municio *et al.* 2009) (Fig. 6.2E).

Furthermore, DCs with the highest level of aldefluor activity (which also had the highest level of *Aldh1a2* transcript (Fig. 5.5)) were found to have the lowest expression level of *Tlr2* (Fig. 6.6F). TLR2 agonists were shown to promote RALDH activity in GMDCs (Fig. 6.1 and discussed above), as has been reported previously by Manicassamy *et al* (Manicassamy, Ravindran *et al.* 2009). The ability of RA to down modulate monocyte TLR2 expression has also been shown previously (Liu, Krutzik *et al.* 2005). As enhanced aldehyde dehydrogenase activity should result in enhanced RA, this may account for the low level *Tlr2* found in cells with highest aldefluor activity (Fig. 6.6F), with RA acting in an autocrine manner. In the report by Liu *et al*, the authors failed to locate any RAR binding elements in the TLR2 upstream region, suggesting that the transcriptional regulation of TLR2 by RA is indirect (Liu, Krutzik *et al.* 2005). It would be interesting to assess within sorted populations of aldefluor negative, low and high activity, the level of ERK activity, although this would be technically difficult since post sort levels may have declined such that this activity would be undetectable.

Our work investigating TLR2 modulation of IL-4 dependent DC alternative activation has shown that P3C inhibited RELMα and Ym1/2 in DCs in a TLR2 dependent manner whereas zymosan limited DC alternative activation in a TLR2 independent manner (Fig. 6.7). Thus, the zymosan inhibition of IL-4 induced RELMα must occur via a receptor other than TLR2. As zymosan can signal via the CLR dectin-1 (Brown, Taylor *et al.* 2002) it is possible that dectin-1 is responsible for the ability of zymosan to inhibit IL-4 driven alternative activation. In order to test this, the soluble *β*-glucan laminarin could be used to block signalling through dectin-1 in WT GMDCs. Alternatively, GMDCs could be grown from bone marrow derived from *Clec7a*$^{-/-}$ mice, and these then compared to similarly stimulated WT cells. However, the zymosan impact upon *Aldh1a2* expression has previously been proposed to be independent of both TLR2 and dectin-1, but dependent upon Syk, leading the authors to propose a novel and currently undefined Syk dependent signalling pathway (Rogers, Slack *et al.* 2005). Furthermore, induction of IL-10 by zymosan has been shown to be dependent upon ERK signalling, but zymosan was unable to

activate ERK in *Syk*$^{-/-}$ GMDCs (Slack, Robinson *et al.* 2007). Signalling to ERK via the TLR2 agonist P3C was shown to be entirely MyD88 dependent (Slack, Robinson *et al.* 2007). It will be important to confirm or exclude a role for Syk in the modulation of DC alternative activation. *Syk*$^{-/-}$ GMDCs could be used for these studies, or use of an inhibitor of a small molecule inhibitor of Syk, for example, R406, as has been used in previous GMDC studies (Ritter, Gross *et al.* 2010)

IL-4 alone did not induce ERK1/2 phosphorylation in GMDCs (Fig. 6.8), which is in contrast to the previously published effect of IL-13 in lung tissue (Lee, Zhang *et al.* 2006). P3C induced a small but significant increase in ERK phosphorylation, and zymosan induced dramatic activation of ERK (Fig. 6.8), findings which have been reported previously (Agrawal, Agrawal *et al.* 2003; Dillon, Agrawal *et al.* 2004; Dillon, Agrawal *et al.* 2006; Alvarez, Municio *et al.* 2009). IL-4 was able to significantly inhibit some, but not all, of the zymosan induced ERK phosphorylation (Fig. 6.8). Interestingly, given the TLR2 independence of zymosan modulation of DC alternative activation, the IL-4 inhibition of zymosan driven pERK was not evident in *Tlr2*$^{-/-}$ DCs (Fig. 6.8 B&C). This result suggests that some component of zymosan signalling to the DCs is acting via TLR2, even though zymosan inhibition of alternative activation is not (Fig. 6.7), and that IL-4 only inhibits TLR2 dependent pERK, not dectin-1 (or an unknown syk dependent receptor) induced pERK. Zymosan has previously been shown to activate ERK1/2 signalling in both human and murine monocyte derived DCs in a TLR2 and dectin-1 dependent manner, as shown by the use of *Tlr2*$^{-/-}$ murine DCs and blocking of dectin-1 with laminarin (Dillon, Agrawal *et al.* 2006). Furthermore zymosan depleted of TLR2 ligands (dZ), such that it signals only via dectin-1, has recently been confirmed to phosphorylate ERK in BMM (Eberle and Dalpke 2012). In our work, IL-4 also inhibited the induction of pERK by P3C in a TLR2 dependent fashion (Fig. 6.8). As a next step, the use of ERK deficient animals, or specific ERK inhibitors, would enable determination of the requirements for ERK activation in modulating the DC response to IL-4.

6.6.4 Interactions between IL-4, RELMα and ERK

As discussed in section 6.6.3, *Tlr2*$^{-/-}$ DCs were used to confirm that P3C abrogation of IL-4 induced *Retnla/*RELMα and P3C inhibition of *Chi3l3/*Ym1/2 (Fig. 6.3) was dependent upon TLR2. No significant reduction was found between

medium + IL-4, or P3C + IL-4 treatment, when DCs were deficient in TLR2 (Fig. 6.7). In addition, IL-4 was found to inhibit P3C phosphorylation of ERK (Fig. 6.8). Furthermore, results of microarray analysis of GMDCs exposed to IL-4 highlighted that many genes involved in modulating signalling cascades were significantly altered (up or downregulated) in *Retnla*$^{-/-}$ DCs but not in WT cells following 6 hours of culture with IL-4 (Fig. 6.9). For example, TLR2 was actually found to be more significantly reduced by IL-4 treatment of RELMα deficient cells than WT cells, a finding that we need to confirm with TLR2 surface staining by flow cytometry. There were 5 different *Dusp*/MKP genes significantly modulated by IL-4 treatment. *Dusps* encode dual specificity phosphatases that remove phosphate groups from MAPKs to inhibit their action (Caunt and Keyse 2012). DUSP6 is a specific phosphatase of ERK1/2 signalling (Nunes-Xavier, Tarrega *et al.* 2010; Zhang, Kobayashi *et al.* 2010; Caunt and Keyse 2012), and has been described to function in a feedback loop regulated by the transcription factor encoded by *Ets2* (Nunes-Xavier, Tarrega *et al.* 2010)*,* another gene significantly downregulated by IL-4 treatment of *Retnla*$^{-/-}$ but not WT DCs (Fig. 6.9). The frequency of genes involved in MAPK signalling, specifically in the regulation of ERK1/2 signalling, that were altered significantly in RELMα deficient DCs (Fig. 6.9), coupled with our finding that IL-4 regulated pERK activation in GMDCs (Fig. 6.8), leads us to propose that ERK signalling may regulate RELMα and/or vice versa (Fig. 6.11).

We addressed this possibility by using a specific inhibitor of MEK1/2, the upstream kinase of ERK1/2 (Fig. 1.3), to demonstrate that ERK1/2 differentially modulates alternative activation products in GMDCs (Fig. 6.10). Inhibition of ERK1/2 by pre-culture with U0126 significantly enhanced IL-4 induced RELMα at the level of protein secretion and *Retnla* transcription (Fig. 6.10B), highlighting that ERK1/2 activation would usually function to inhibit IL-4 induction of RELMα. In contrast, there was no significant change in the expression of *Chi3l3* transcript or Ym1/2 (Fig. 6.10B), a significant reduction in CCL24 protein and a trend for reduced *Ccl24* transcript (though this did not reach significance)(Fig. 6.10C). Thus, ERK1/2 regulates several key aspects of IL-4 dependent alternative activation in DCs (Fig. 6.11).

This finding does not discount the possible role for other kinases in the regulation of DC alternative activation. For example, several studies have suggested

functional roles for both PI3K and AKT in modulating AAM (MacKinnon, Farnworth *et al.* 2008; Ruckerl, Jenkins *et al.* 2012). It was reported that inhibition of PI3K in a human monocytic cell line (THP-1) resulted in abrogation of IL-4 induced alternative activation, as assessed by MR expression (MacKinnon, Farnworth *et al.* 2008). In the same study, inhibition of PI3K limited IL-4 phosphorylation of AKT (also known as PKB), but not STAT6. Furthermore, IL-4 was shown to promote activation of AKT (MacKinnon, Farnworth *et al.* 2008). Another study published in 2012 showed that inhibition of AKT with the inhibitor triciribine *in vivo* significantly reduced IL-4 induced peritoneal macrophage proliferation and RELMα expression, but did not significantly modulate Ym1/2 (Ruckerl, Jenkins *et al.* 2012). PI3K is activated downstream of IL-4Rα signalling by IRS1/2, and PI3K then activates AKT (Nelms, Keegan *et al.* 1999)(Fig. 1.8). ERK can also be phosphorylated downstream of IL-4Rα, via the action of IRS1/2 (Fig. 1.8). Thus, IRS1/2 should be investigated with regard to the differential involvement of ERK in regulating DC RELMα expression in response to IL-4.

RELMα itself has also been shown to phosphorylate AKT in pulmonary microvascular smooth muscle cells in a PI3K dependent manner (Teng, Li *et al.* 2003). In this study, the authors also annotated the *Retnla* gene, finding putative transcription factor binding consensus sequences (Teng, Li *et al.* 2003); 6 C/EBP binding motifs (which had been identified as an IL-4 dependent STAT6 binding partner for the induction of RELMα (Fizz1) prior to the Teng report (Stutz, Pickart *et al.* 2003)), 5 NF-κB motifs, an AP-1 site in the 3'UTR, a putative IRF site and two GAS elements were located (Teng, Li *et al.* 2003). One of the GAS sites had already been identified as the site at which STAT6 and C/EBP regulated the IL-4 dependent induction of RELMα in the BMnot cell line (Stutz, Pickart *et al.* 2003). The number of binding sites available for different transcription factors within the cloned murine *Retnla* gene highlights the possible range of complex interactions that could regulate the transcription of this gene within mammalian cells (Teng, Li *et al.* 2003). Interestingly, this article did not report any RARE within the *Retnla* gene (Teng, Li *et al.* 2003), suggesting that the combined impact of RA and IL-4 on RELMα transcription (Fig. 5.6) will not be direct.

In this chapter we revealed a disparity in the ability of TLR2 agonists to limit IL-4 induced alternative activation in DCs. We went on to identify a novel role for

pERK in modulating IL-4 dependent responses in GMDCs. pERK was found to differentially modulate facets of DC alternative activation, inhibiting IL-4 induction of RELMα, promoting IL-4 induced CCL24 and having no impact upon Ym1/2.

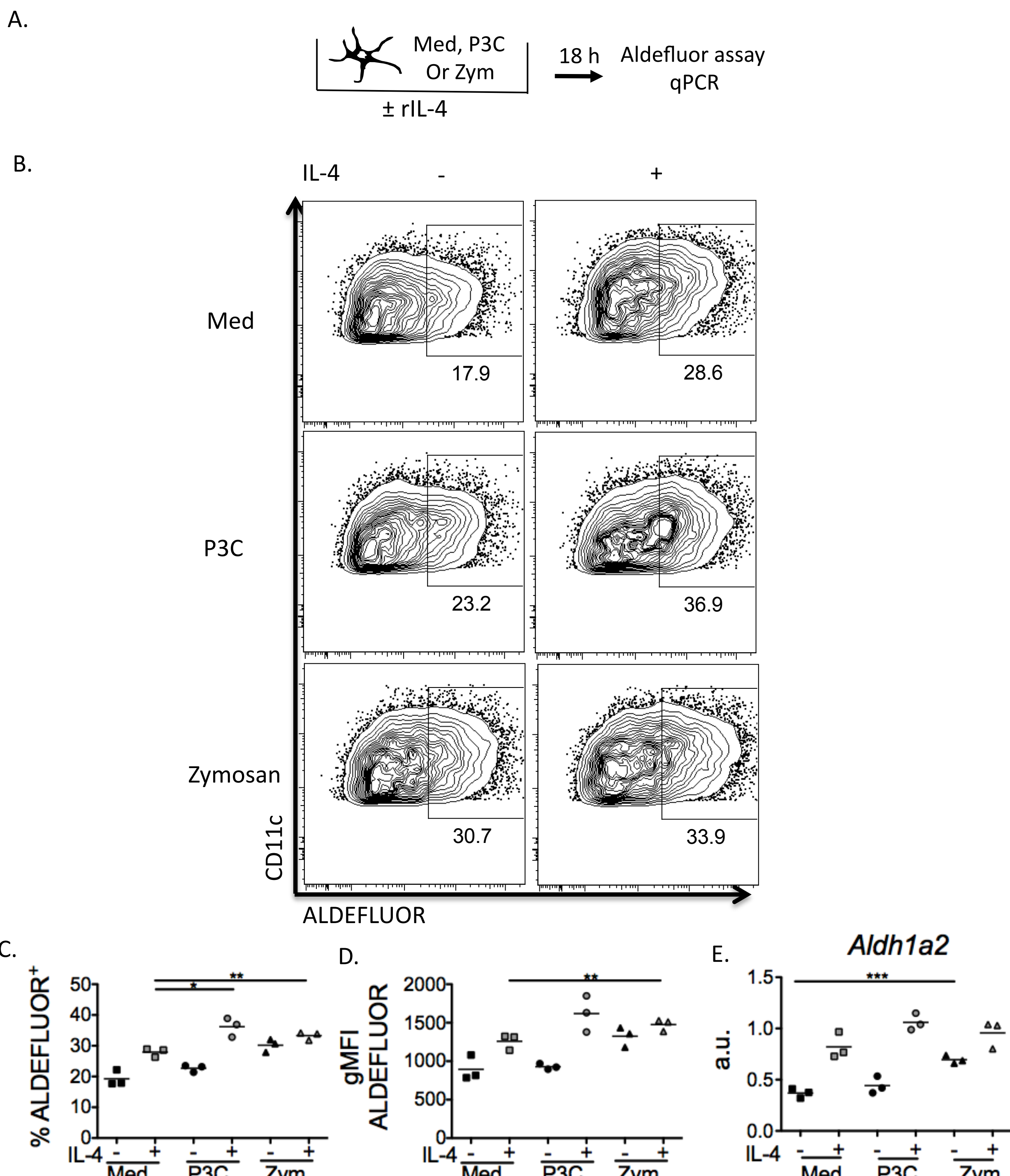

Figure 6.1 TLR2 agonists enhance IL-4 driven aldehyde dehydrogenase activity in GMDCs. WT GMDC were cultured overnight with or without addition of 20ng/ml IL-4, Pam3Csk4 (20ng/ml P3C) or zymosan (100 μg/ml, Zym) (A). DCs were gated as CD11c+, prior to assessment of both the % level of aldehyde dehydrogenase activity within the population (B +C) and the gMFI of aldefluor activity within the total CD11c+ population (D). Expression of *Aldh1a2* transcript was also quantified (E). Data is representative of 3 independent experiments, error bars represent triplicate culture wells, a.u.= arbitrary units as compared to *Hprt* expression. *= P< 0.05 **= P<0.01 ***P<0.001.

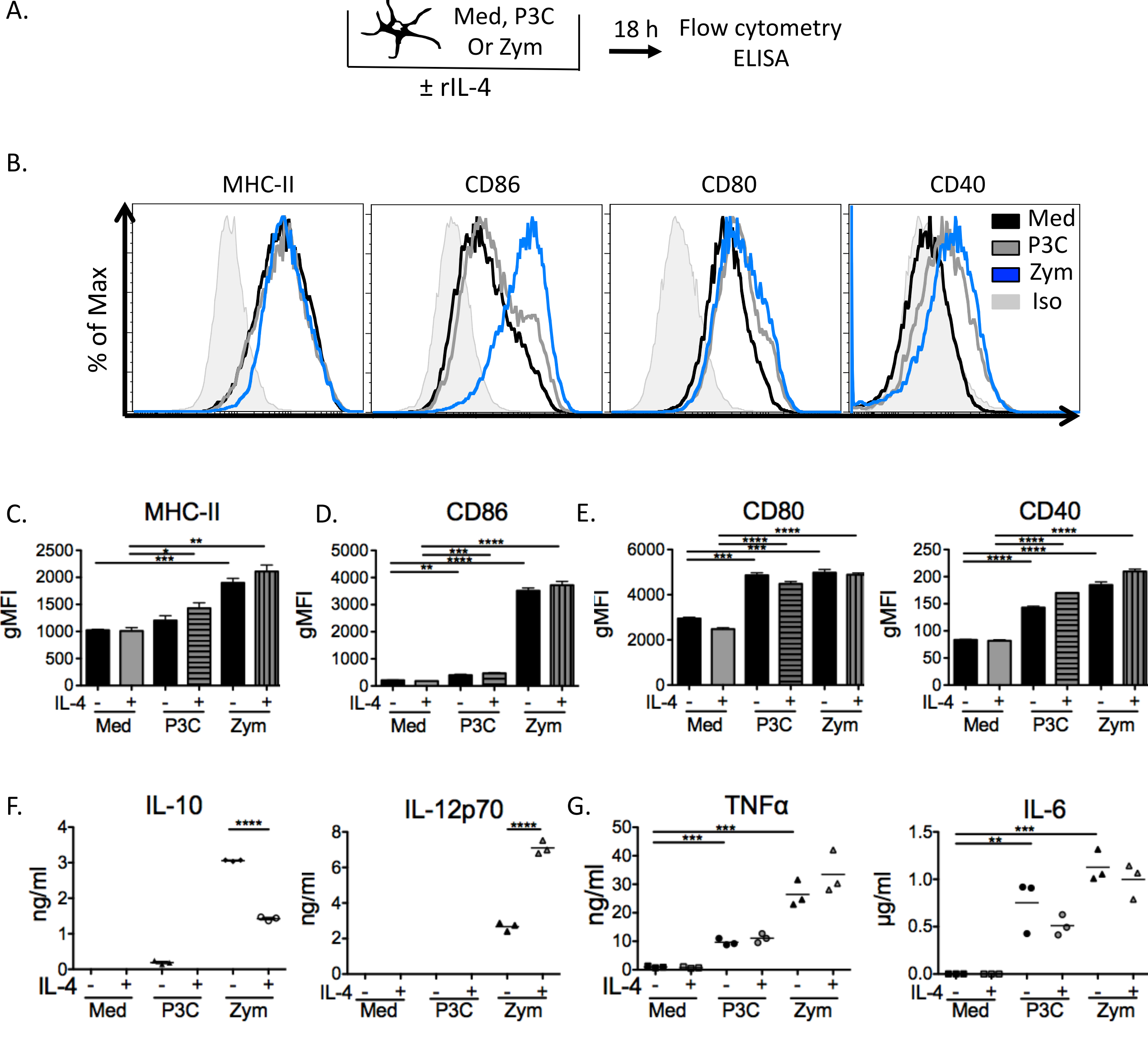

Figure 6.2 TLR2 agonists activate GMDCs, IL-4 regulates Zymosan directed secretion of IL-10 and IL-12. WT GMDC were cultured overnight with or without addition of 20ng/ml IL-4, 250ng/ml Pam3Csk4 (P3C) or 100 µg/ml zymosan (Zym) (A). Co-stimulatory marker expression (B-E) and cytokine secretion were determined (F+G). Histograms in B are representative plots of triplicate culture wells, black line=medium, grey line=P3C, blue=Zym, shaded= isotype control. Graphs in C-E show geometric mean fluorescence intensity (gMFI). Data is representative of 5 independent experiments, error bars represent triplicate culture wells, *= P< 0.05, **= P<0.01, ***P<0.001, ****=P<0.0001.

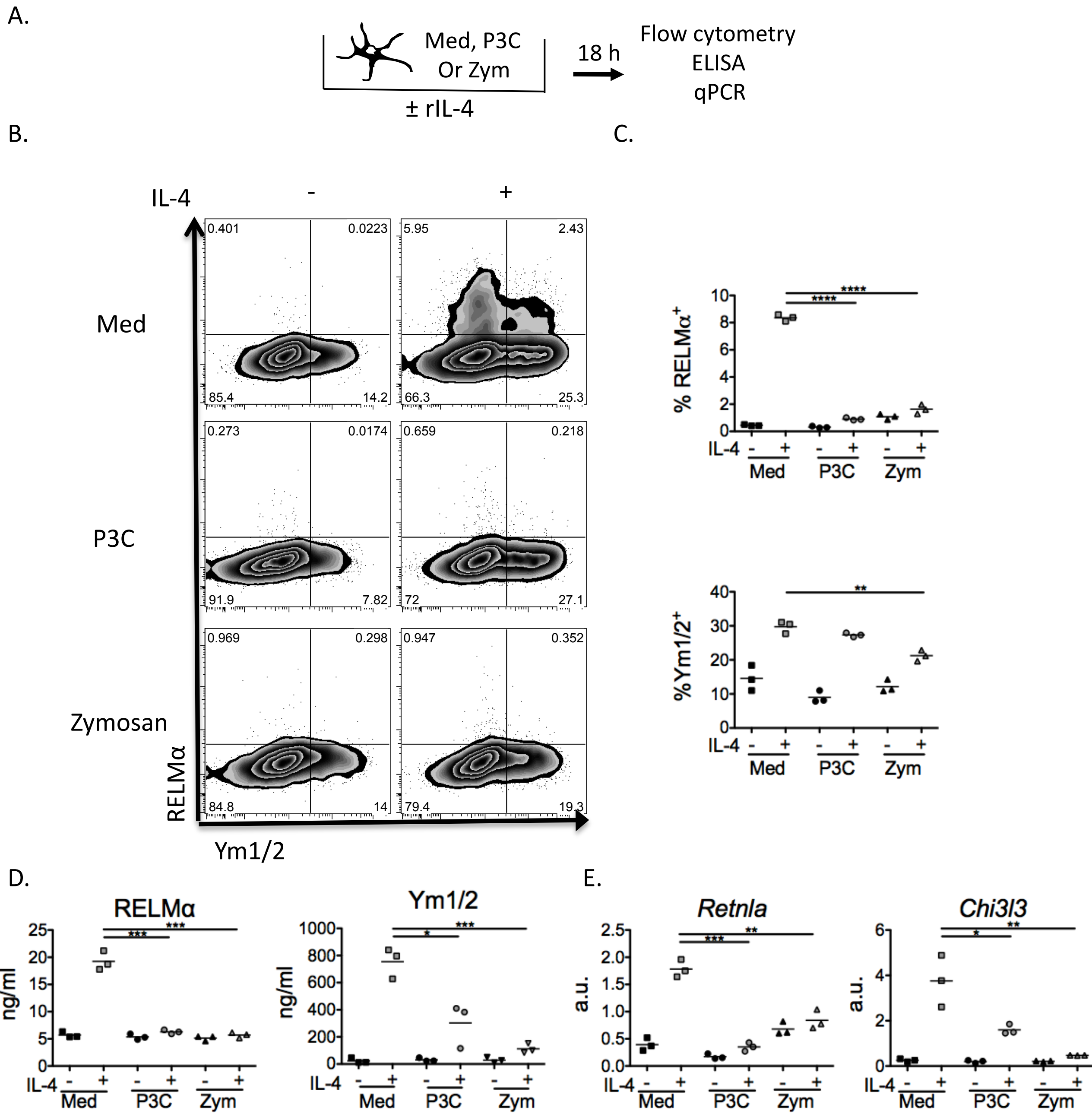

Figure 6.3 P3C and zymosan limit IL-4 driven RELMα and Ym1/2 to different extents. WT GMDC were cultured overnight with or without addition of 20ng/ml IL-4, 250ng/ml Pam3Csk4 (P3C) or 100μg/ml zymosan (Zym) (A). Intracellular protein (B+C), secreted protein (D) and mRNA levels of RELMα and Ym1/2 were detected by flow cytometry, ELISA and qPCR respectively. Data is representative of 5 independent experiments, a.u.= arbitrary units as compared to *Hprt* expression, error bars represent triplicate culture wells, *= P< 0.05 **= P<0.01 ***P<0.001 ****P<0.0001.

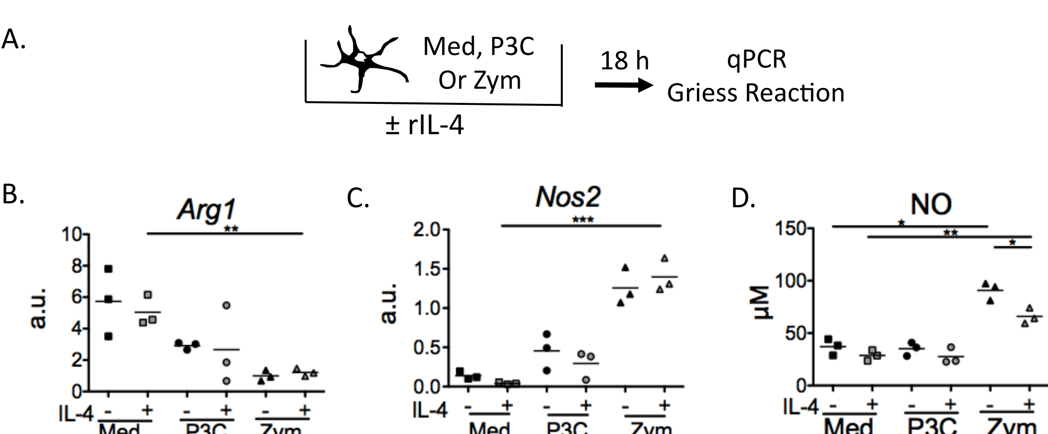

Figure 6.4 Zymosan inhibits *Arg1* and promotes iNOS in GMDC. WT GMDC were cultured overnight with or without addition of 20ng/ml IL-4, 250ng/ml Pam3Csk4 (P3C) or 100 μ g/ml zymosan (Zym) (A). mRNA for the arginase converting enzymes Arginase 1 (*Arg1: B*) and iNOS (*Nos2; C)* were assessed by qPCR as was the production of NO by the cells (D). Data is representative of 3 independent experiments, a.u.= arbitrary units as compared to *Hprt* expression, error bars represent triplicate culture wells, *= P< 0.05 **= P<0.01 ***P<0.001.

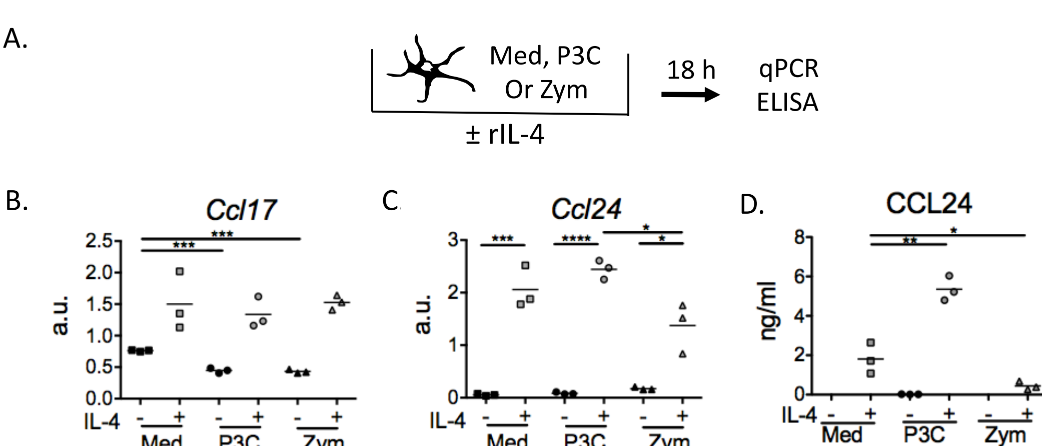

Figure 6.5 P3C and zymosan modulate chemokine production by IL-4 treated GMDCs. WT GMDC were cultured overnight with or without addition of 20ng/ml IL-4, 250ng/ml Pam3Csk4 (P3C) or 100 μ g/ml zymosan (Zym) (A). mRNA for the chemokines CCL17 (*Ccl17: B*) and CCL24 (*Ccl24; C)* were assessed by qPCR as was the secretion of CCL24 protein (D). Data is representative of 3 independent experiments, a.u.= arbitrary units as compared to *Hprt* expression, error bars represent triplicate culture wells, *= P< 0.05 **= P<0.01, ***=P<0.001, ****=P<0.0001.

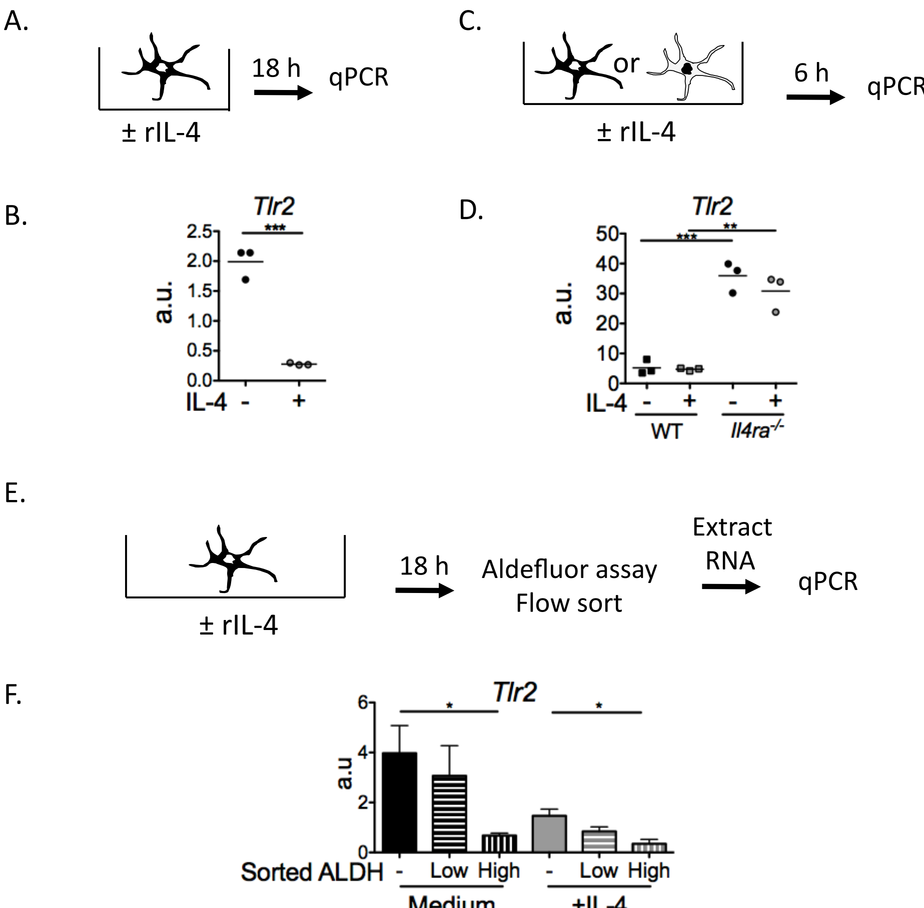

Figure 6.6 IL-4 and TLR2 counter-regulate one another. WT GMDC were cultured overnight with or without addition of 20ng/ml IL-4 (A). mRNA for *Tlr2 was assessed by qPCR (B*); WT or *Il4ra*$^{-/-}$ GMDC were cultured with IL-4 for 6h (C), expression of *Tlr2* was determined by qPCR (D). Following overnight culture with IL-4, WT GMDC populations were flow sorted based upon aldefluor activity and qPCR was used to assess expression of *Tlr2* within aldefluor negative, low or high expressing populations (F). Data is representative of 2 independent experiments, a.u.= arbitrary units as compared to *Hprt* expression, error bars represent triplicate culture wells, *= P< 0.05 **= P<0.01 ***=P<0.001.

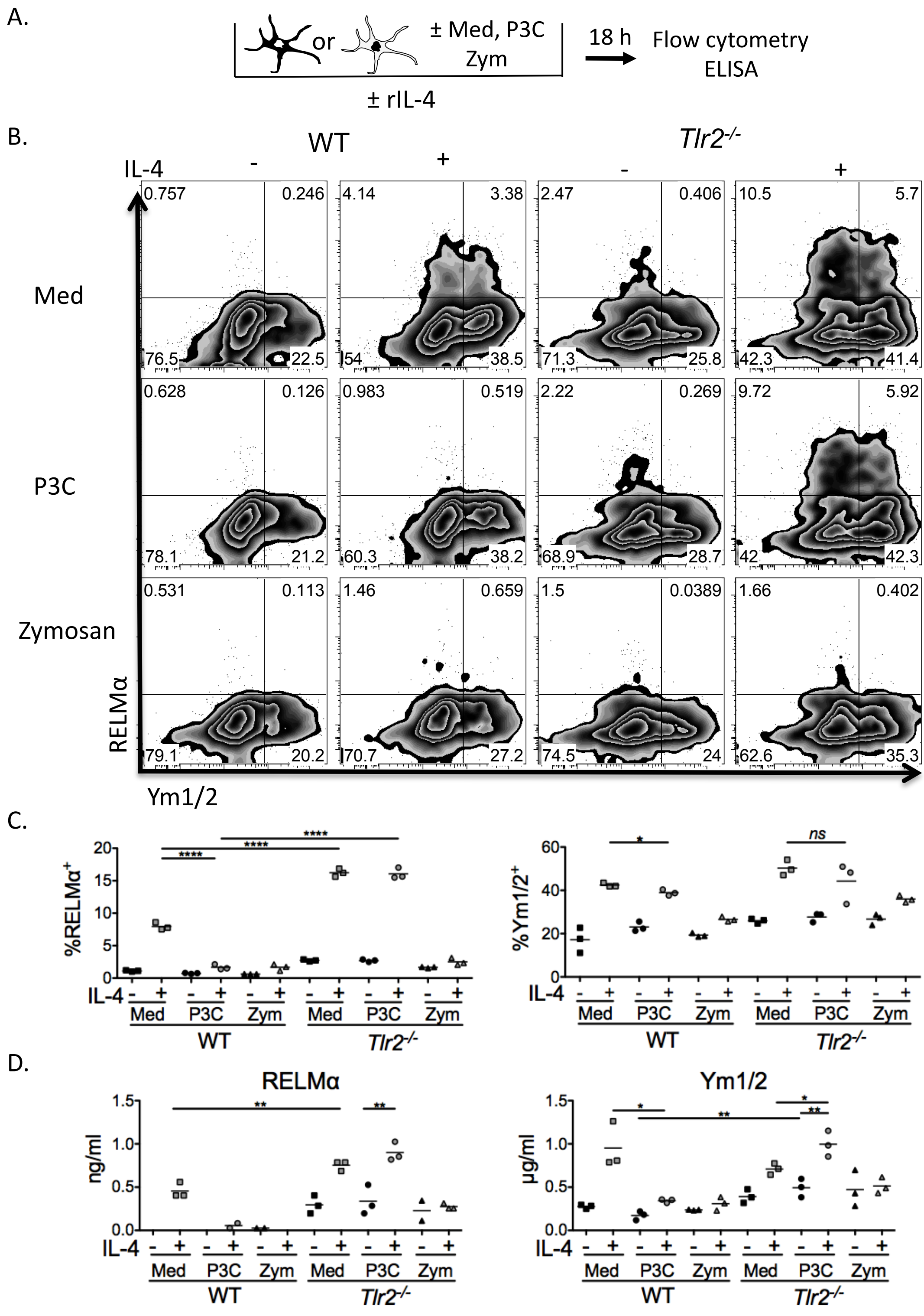

Figure 6.7 TLR2 limits IL-4 driven RELMα in GMDC, and is responsible for P3C inhibition of alternative activation. WT or *Il4ra*$^{-/-}$ GMDC were cultured with or without addition of 20ng/ml IL-4, Pam3Csk4 (250ng/ml P3C) or Zymosan (100μg/ml Zym) (A). RELMα and Ym1/2 production was assessed by intracellular staining (B+C) or ELISA (D). Data is representative of 3 independent experiments, error bars represent triplicate culture wells, *= P< 0.05 **= P<0.01 ****=P<0.0001.

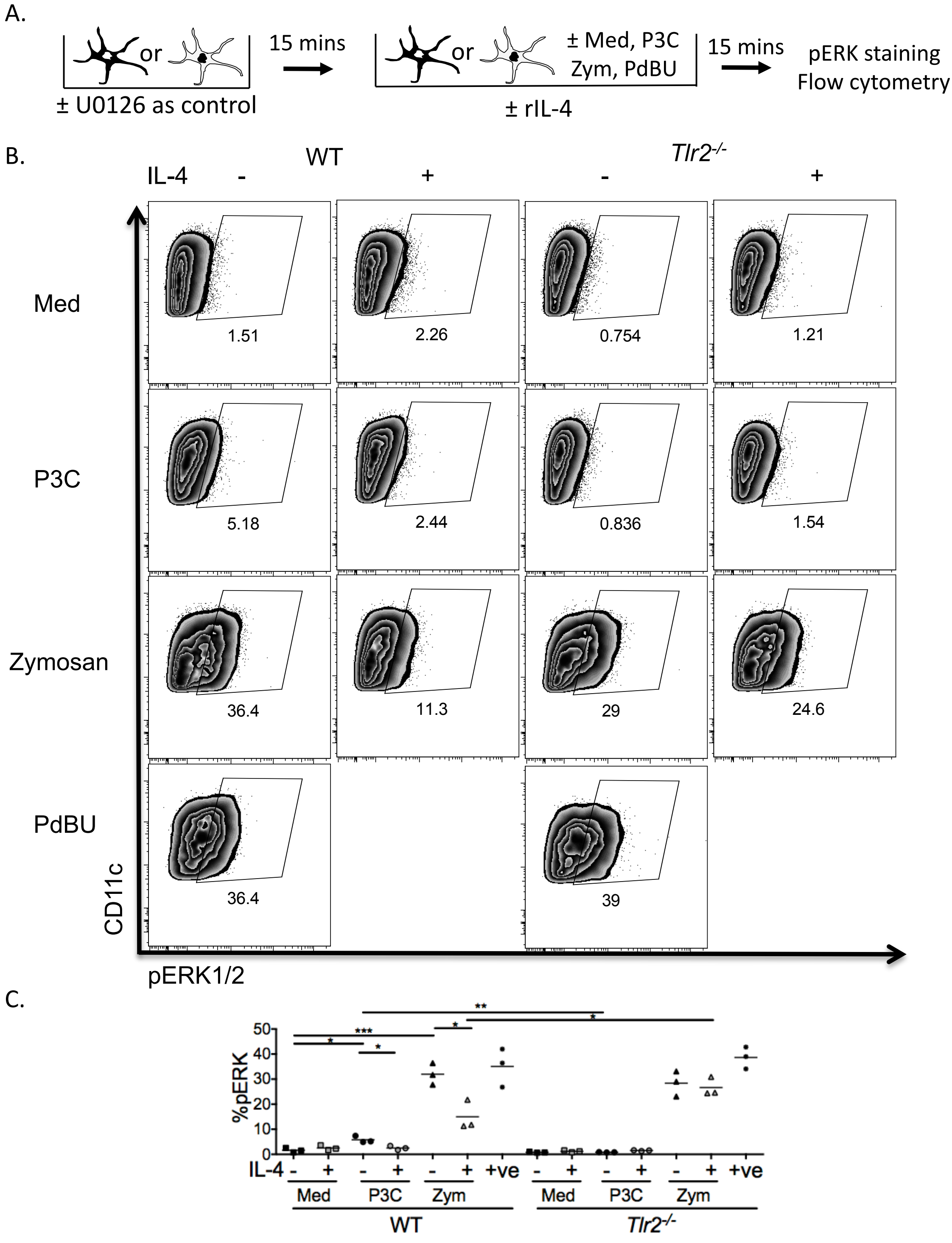

Figure 6.8 IL-4 inhibition of zymosan driven phospho-ERK1/2 is dependent upon TLR2. WT or *Tlr2*$^{-/-}$ GMDC were cultured for 15 minutes with an inhibitor or ERK1/2 signalling (U0126, 10 μM) or media, prior to addition of 250ng/ml Pam3Csk4 (P3C), 100 μg/ml zymosan (Zym) or 100nM phorbol 12, 13-dibutyrate (+ve) with or without 20ng/ml IL-4 for a further 15 minutes (A). Phosphorylation of ERK was determined by intracellular staining (B +C). Data is representative of 3 independent experiments using WT GMDCs, 1 independent experiment using *Tlr2*$^{-/-}$ GMDCs, error bars represent triplicate culture wells, *= P< 0.05 **= P<0.01 ***=P<0.001.

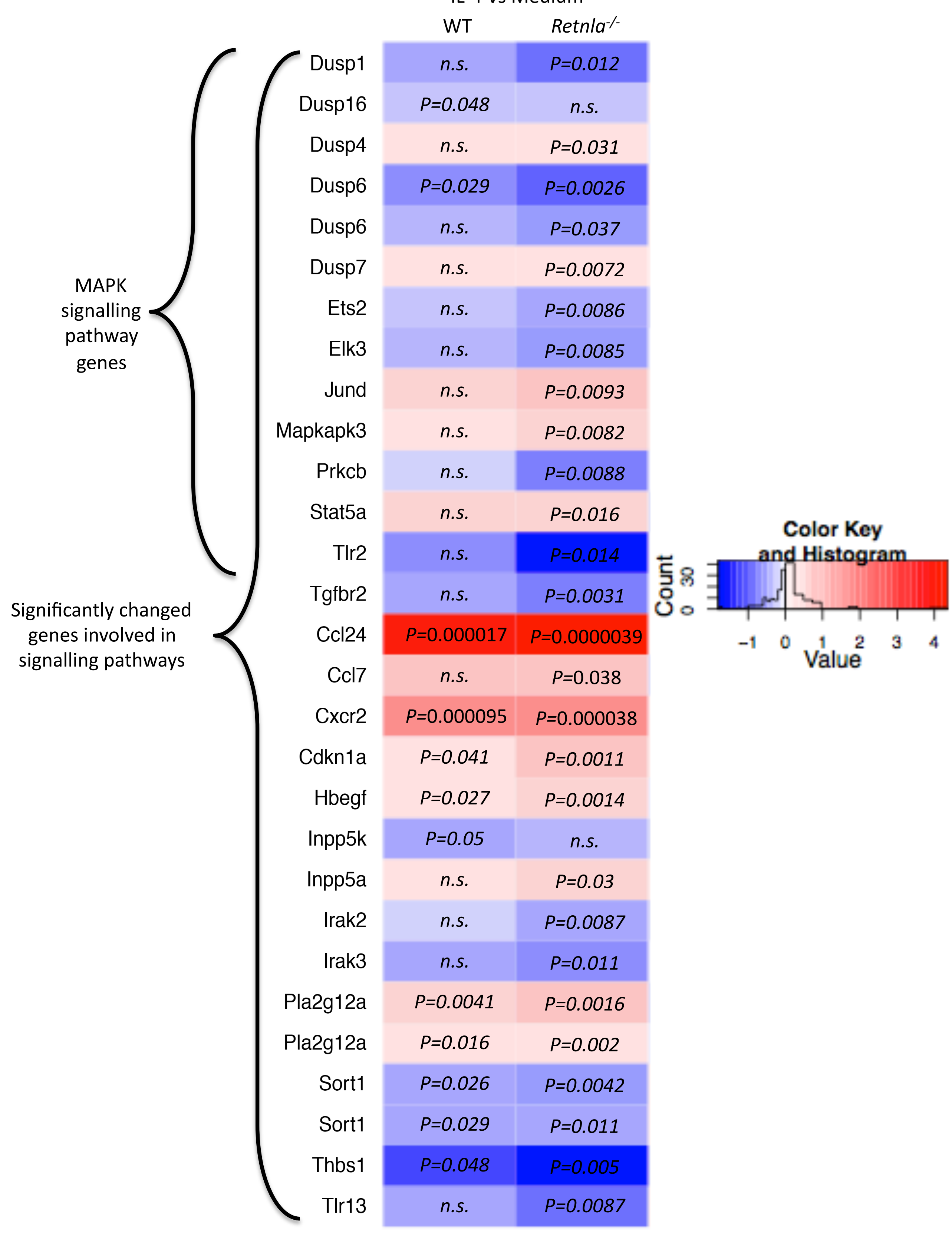

Figure 6.9 IL-4 differentially modulates components of the MAPK signalling cascade in WT and RELMα deficient DCs. WT or *Retnla*$^{-/-}$ GMDCs were cultured for 6h with 20ng/ml IL-4, RNA was extracted and an illumina microarray carried out (same experiment as Fig. 4.12). Heat map of transcripts significantly altered following IL-4 treatment, limited to genes involved in signalling pathways. Data is from one microarray experiment, 3 biological replicates per genotype, following normalisation comparisons were performed using linear modelling. p= Adj. p significance values assessed using Bayesian analysis, corrected using Benjamini & Hochberg method for multiple testing (Al Ivens, CIIE Bioinformatics support).

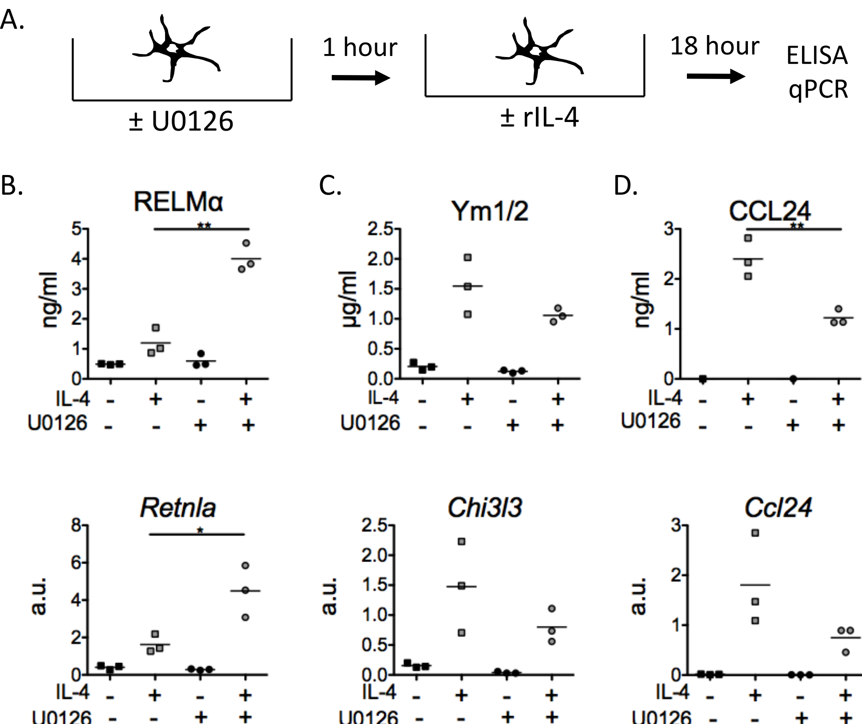

Figure 6.10 ERK signalling inhibits IL-4 driven RELMα in GMDC. GMDC were cultured for 1 hour with an inhibitor of ERK1/2 signalling (10μM U0126) or media, prior to addition of 20ng/ml IL-4 or media to the culture well for a further 18 hours (A). Alternative activation was determined by secretion and expression of RELMα (B), Ym1/2 (C) and CCL24 (D). Data is representative of 2 independent experiments, error bars represent triplicate culture wells, *= P< 0.05 **= P<0.01.

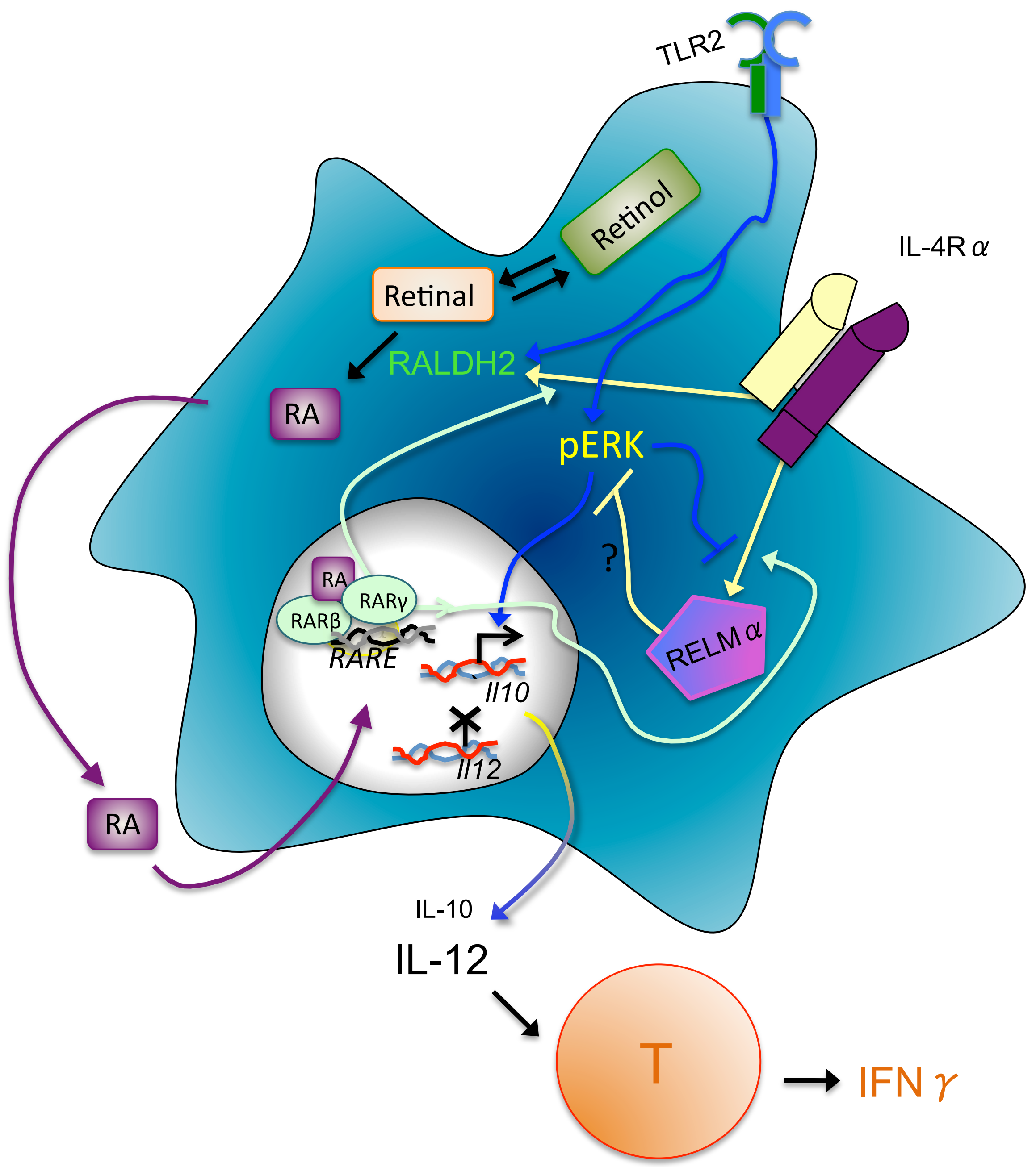

Figure 6.11. Model of how TLR2, IL-4 and RA signalling integrate in DCs. IL-4 promotes DC RELMα, and limits pERK, pERK restrains IL-4 induced RELMα, and IL-4 can limit TLR2 induced pERK, which may be via RELMα. RALDH activity is promoted by TLR2 signalling and IL-4, RALDH activity results in RA production, RA signalling enhances IL-4 driven RALDH and RELMα. ERK signalling is known to promote DC secretion of IL-10 and inhibit IL-12p70, IL-4 limits TLR2 stimulated DCs secretion of IL-10 and promotes IL-12p70, which based on data in chapter 4, we propose will polarise naïve T-cells to secrete IFNγ. Blue lines represent TLR2/ERK, yellow lines represent IL-4R and green lines represent RA dependent changes. Arrows = promotion, lines = inhibition.

Chapter 7. Concluding Remarks and Future Directions

Work in this thesis has shown that DCs can be alternatively activated by IL-4, both *in vivo* following provision of exogenous IL-4c, or in response to a potent Th2 inducing parasite. *Ex vivo* following isolation of splenic DC populations, and *in vitro* in the presence of the Th2 cytokines IL-4 and IL-13. We confirmed that DCs are more superior at activating naïve T-cells than macrophages *in vivo*, and that AADCs are different flavours to AAMs, showing differential expression of the alternative activation products RELMα, Ym1/2 and arginase-1. The lack of IL-4 driven arginase in DCs highlights what we believe may be different biological roles for alternative activation in DCs as compared to macrophages, with AADCs fine tuning the priming of T-cells in the context of a type-2 immune setting.

A functional role for DC RELMα in the promotion of T-cell cytokine responses was highlighted, with RELMα being required for the optimal induction of T-cell IL-10 and IL-13 and modulation of IL-4 and IFNγ. In future work it will be necessary to determine if DC responsiveness to IL-4 is important for health and as such whether AADCs are required for animals to survive disease. $CD11c^{Cre}Il4ra^{fl/fl}$ animals should be infected with Th2 driving pathogens such as *S. mansoni*, to confirm whether alternative activation of $CD11c^{+}$ cells is required for survival, as is the case with IL-4Rα positive macrophages (Herbert, Holscher *et al.* 2004). In addition, the data presented in chapter 4 of this thesis would suggest that the ability of animals deficient in IL-4Rα expression within their CD11c compartment to survive bacterial and viral infections should also be investigated further. The expectation being that such animals would be less capable of mounting a Th2 supported Th1 response and thus less competent to clear a Th1 infection.

The notion that a Th2 immune response characterised by alternative activation can support survival following a severe actue infection such as bacterial sepsis, is supported by the theory that Th2 immunity evolved to repair damage. For example, the Th2 immune response may serve to promote a Th1 response following wounding, such as the damage caused as a pathogen breaches a mucosal barrier (Allen and Wynn 2011). To address whether this theory holds true, it would be informative to investigate whether animals unable to be alternatively activated, for

example those genetically deficient in STAT6 or IL-4Rα, are worse at surviving bacterial or viral infections.

RA was revealed as a factor able to promote IL-4 driven RALDH2 and RELMα in GMDCs. In contrast, RA was not shown to promote IL-4 dependent macrophage RELMα. In chapter 4 we highlighted that in the absence of IL-4R signalling to bacterially stimulated DCs there is an enhanced level of IFNγ and IL-17 induced *in vivo*. It would be interesting to determine whether following *in vivo* transfer bacterially stimulated WT and *Il4ra*$^{-/-}$ DCs produce different levels of *Aldh1a2* and thus potentially produce enhanced levels of local RA.

Through the use of panRAR inhibitors, addition of exogenous RA and GMDCs deficient in RARα, regulated signalling between RARα, and all other RARs was highlighted as a mechanism via which RA controlled the alternative activation of DCs. To confirm whether RARα expression by DCs is required for surviving chronic Th2 infections, CD11c^{Cre}RARa$^{fl/fl}$ animals should be infected with a Th2 polarising parasite such as *S. mansoni.* To address the importance of our TLR2/IL-4 interaction story, a pathogen that signals via TLR2, such as the fungus *Aspergillus fumigatus* should be used to infect IL-4, IL-4Rα or STAT6 deficient animals.

Excitingly, we have highlighted for the first time a role for pERK signalling in the inhibition of DC responses to IL-4, specifically limiting RELMα but promoting CCL24, whether ERK signalling regulates the IL-4 dependent production of RELMα by other cells such as macrophages and eosinophils should also be investigated.

Data within this thesis would suggest that secretion of RELMα from DCs does not following a standard secretion pathway, as the use of golgistop did not enhance intracellular levels and we were unable to detect enhanced RELMα secretion in the presence of RA and IL-4 in spite of an increase intracellularly. The secretory pathway of RELMα will be investigated further, by initially determining whether RELMα may be released contained within exosomes.

Multiple avenues for future work were revealed through the use of a microarray assessment following IL-4 treatment of GMDCs. Further assessment of

the function of these novel targets of IL-4 in DC, will help in the search for the elusive signal 3, in DC Th2 induction.

The results in this thesis would suggest that alternative activation of DCs, primarily determined via the IL-4 dependent induction of RELMα expression is an endogenous biological process with functional relevance to DC APC function, further studies will be required to address whether DCs express RELMα at sites other than the spleen. Based on the data presented here it may be expected that DC populations within intestinal sites at which vitamin A uptake occurs may in fact become alternatively activated at steady state, it will be interesting to determine which pre-defined DC subsets (Merad and Manz 2009) are responsible for RELMα production, and whether there is a role for RA in alternative activation *in vivo*.

We believe that with further work it will be possible to more closely interlink all strands of this thesis, determining how signalling pathways downstream of GM-CSF, RA, IL-4 and TLR2 ligands integrate in their regulation of DC function, specifically with regard to their impact upon and requirements for DC alternative activation.

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Appendix 1

Alternatively activated dendritic cells regulate CD4^{+} T-cell polarization in vitro and in vivo

Peter C. Cook, Lucy H. Jones, Stephen J. Jenkins, Thomas A. Wynn, Judith E. Allen, and Andrew S. MacDonald.

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Alternatively activated dendritic cells regulate CD4$^+$ T-cell polarization in vitro and in vivo

Peter C. Cook[a,1], Lucy H. Jones[a,1], Stephen J. Jenkins[a], Thomas A. Wynn[b], Judith E. Allen[a], and Andrew S. MacDonald[a,2]

[a]Institute of Immunology and Infection Research, Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JT, Scotland, United Kingdom; and [b]Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Edited by Ruslan Medzhitov, Yale University School of Medicine, New Haven, CT, and approved May 1, 2012 (received for review December 22, 2011)

Interleukin-4 is a cytokine widely known for its role in CD4$^+$ T cell polarization and its ability to alternatively activate macrophage populations. In contrast, the impact of IL-4 on the activation and function of dendritic cells (DCs) is poorly understood. We report here that DCs respond to IL-4 both in vitro and in vivo by expression of multiple alternative activation markers with a different expression pattern to that of macrophages. We further demonstrate a central role for DC IL-4Rα expression in the optimal induction of IFNγ responses in vivo in both Th1 and Th2 settings, through a feedback loop in which IL-4 promotes DC secretion of IL-12. Finally, we reveal a central role for RELMα during T-cell priming, establishing that its expression by DCs is critical for optimal IL-10 and IL-13 promotion in vitro and in vivo. Together, these data highlight the significant impact that IL-4 and RELMα can have on DC activation and function in the context of either bacterial or helminth pathogens.

antigen presenting cells | T lymphocytes | innate immunity | adaptive immunity

Activation of dendritic cells (DCs) with bacterial or viral antigen (Ag) results in production of proinflammatory cytokines and enhanced ability to stimulate Th1/Th17 responses (1). In addition, a range of cytokines influence DC activation, with IL-12 and IL-10 playing critical roles in either promotion or regulation of DC maturation and function (2, 3). The hallmark of allergic disease and helminth infection is induction of a CD4$^+$ Th2 response, characterized by secretion of cytokines such as IL-4 and IL-13 (4). Although DCs themselves are not thought to produce IL-4 (5), this canonical Th2 cytokine can be secreted rapidly in direct response to pathogen stimulation by a variety of other innate cells (4). As such, DCs encountering Ag or pathogen stimulation in Th2 infection or disease settings will likely be simultaneously exposed to IL-4. However, the impact of IL-4 on the functional capability of DCs remains relatively unexplored.

In comparison with DCs, the effect of IL-4 on activation of macrophage (MΦ) populations has been much more thoroughly addressed, with IL-4/IL-13 treatment resulting in "alternatively" activated macrophages (aaMΦs) (6) characterized by the expression of Arginase-1, chitinase-like molecule Ym1, resistin-like molecule α (RELMα, also known as FIZZ1), and C-type lectin receptors such as mannose receptor (MR) and Dectin-1 (6, 7). Although the individual and cumulative function of these IL-4–induced products is not clear, aaMΦs are thought to play vital roles in helminth infection and tissue remodeling, and are capable of suppressing T-cell responses (8–10). However, only a limited number of studies have addressed whether DCs express markers associated with aaMΦs (11–14), and it remains unclear how expression of IL-4–induced molecules may influence DC function in Th2 settings. Somewhat surprisingly, previous work has indicated that IL-4 can enhance production of IL-12p70 by DCs stimulated with bacterial LPS in vitro via inhibition of IL-10 (15–18). This suggests that IL-4 may facilitate DC priming of Th1 responses in ongoing, counterregulatory, Th2 settings (15, 16, 19).

We set out to delineate the role of IL-4 in influencing DC activation and function, both in vitro and in vivo. We show that treatment of DCs with IL-4 in vitro resulted in robust expression of a wide range of alternative activation markers at both the transcript and protein level, with a more selective expression of alternative activation markers RELMα and Ym1 in vivo. We further demonstrate a key role for DC-derived RELMα in promotion of optimal Th2 responses that contrasts with the previously suggested regulatory function of this alternative activation product (20–22). Together, these data reveal that the ability to respond to IL-4 is critical for optimal Th1 or Th2 priming by DCs through modulation of IL-12/IL-10 or RELMα production, respectively.

Results

IL-4 Induces DC Expression of RELMα in Vivo. The phenotypes of aaMΦ populations, isolated from a variety of tissues in Th2 inflammatory settings, have been well characterized, with one hallmark being the abundant expression of markers RELMα and Ym1 (6). RELMα has also previously been identified in cells other than aaMΦs, including eosinophils and epithelial cells (10, 20, 22). To investigate whether DCs respond to Th2 environments in a manner similar to MΦs, mice were injected i.p. with rIL-4 complexed with anti–IL-4 mAb (IL-4c), which ensures slow release of cytokine for 2–3 d (23). Peritoneal exudate cells (PEC) were isolated 4 d after mice received a single dose of IL-4c or PBS. For flow-cytometric analysis, granulocytes, monocytes, and B cells were excluded (based on cell size, Gr1, and CD19 staining), MΦs were defined as F4/80$^+$FSC$^{int/hi}$MHC-II$^{lo/int}$ and DCs as F4/80$^-$FSC^{lo}CD11c^{hi}MHC-II^{hi} (Fig. 1*A*). IL-4c did not significantly alter the number of DCs in the PEC but, as recently reported (23), significantly increased accumulation of MΦs ($P < 0.05$; Fig. 1*A*), suggesting that, in contrast to MΦs, DCs do not proliferate in response to IL-4c injection. To assess whether IL-4 administration caused alternative activation of these populations, we performed intracellular staining for RELMα and Ym1/2. As expected, MΦs isolated from IL-4c–injected mice displayed striking levels of RELMα and Ym1/2 expression compared with MΦs isolated from PBS control animals (Fig. 1*B*). Furthermore, the expression of these proteins was not uniform, with RELMα$^+$Ym1/2$^-$, RELMα$^+$Ym1/2$^+$, and RELMα$^-$Ym1/2$^+$ MΦ populations all being identified. In contrast to the effect on MΦs, predominantly single positive RELMα−expressing DCs were observed at a low dose of IL-4c (0.6 μg) with little change in Ym1/2 evident. However, at a higher dose of IL-4c (4 μg), a significant proportion of RELMα-positive DCs expressed Ym1/2. In addition, the ability to respond to IL-4 in vivo was not restricted to peri-

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[1]P.C.C. and L.H.J. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: Andrew.MacDonald@ed.ac.uk.

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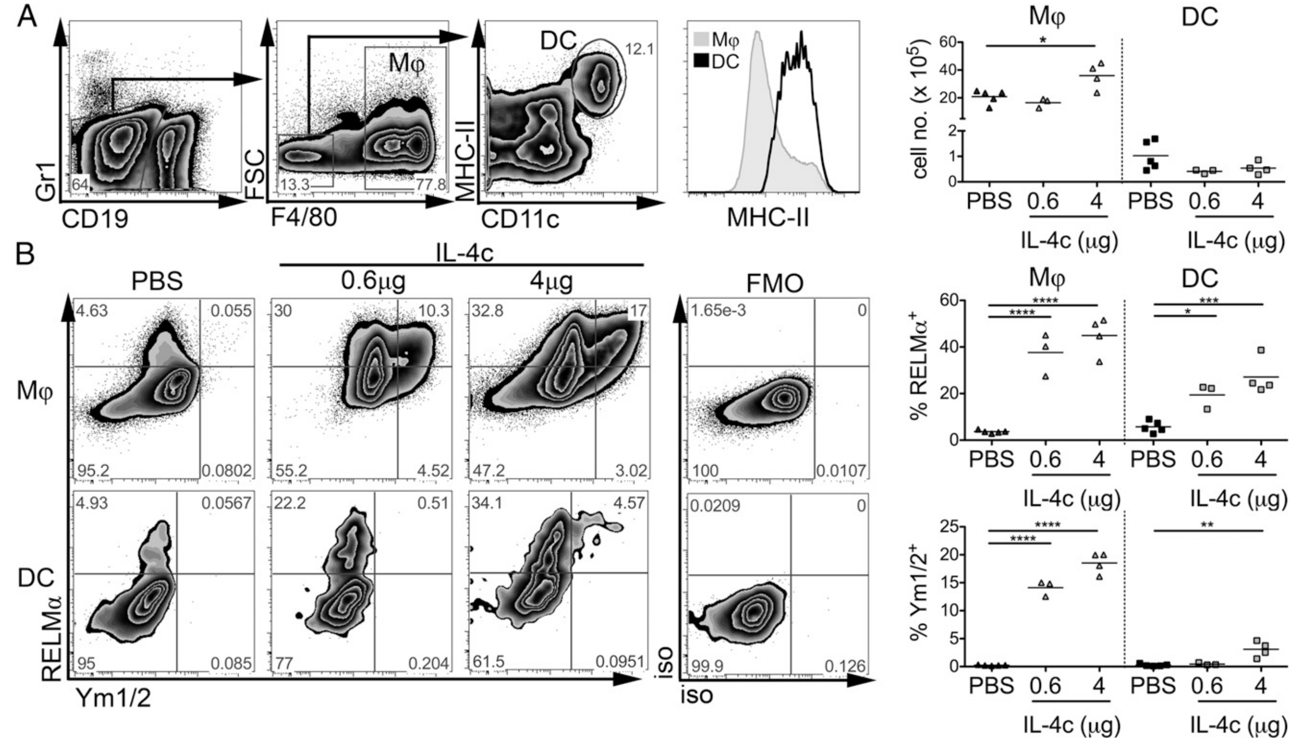

Fig. 1. IL-4c induces MΦ and DC expression of RELMα in vivo. MΦs and DCs in PEC from PBS- or IL-4c–treated mice were assessed by flow cytometry (*A*). MΦs and DCs as defined in (*A*) stained for intracellular expression of RELMα and Ym1/2 (*B*). Data are representative of five experiments. Graphs show percent expression for individual mice, three to five per group. FMO, fluorescence minus one control.

toneal cavity DCs, as splenic MHCII^{hi}CD11c^{hi} DCs also significantly up-regulated RELMα and Ym1/2 expression following IL-4c injection (Fig. S1).

To determine the general capacity of DCs to alternatively activate in vivo in response to stimuli other than IL-4c, we assessed RELMα and Ym1/2 production by DCs from mice infected with *Litomosoides sigmodontis* or *Schistosoma mansoni* (Fig. S2), parasitic helminths that promote strong Th2 responses. Pleural cavity (Fig. S2*A*) or splenic (Fig. S2*B*) MHCII^{hi}CD11c^{hi} DCs from infected mice displayed significantly increased expression of RELMα in comparison with naive control animals. Ym1/2 expression was less dramatically influenced by infection (Fig. S2). Thus, DCs from diverse tissue sites can express markers associated with aaMΦs in vivo, in response to injection with IL-4c or in the more complex setting of helminth infection.

IL-4 Up-Regulates DC Expression of Multiple Markers of Alternative Activation. To carefully dissect the influence of IL-4 on a well-characterized population of cells, we generated DCs from murine bone marrow (BM) using GM-CSF, then exposed the DCs to recombinant IL-4 in vitro. As shown previously, DCs expressed IL-4Rα (Fig. 2*A*) (18), and IL-4 did not substantially alter MHC-II or the costimulatory molecules CD40, CD80 and CD86 (Fig. S3). However, IL-4 significantly increased DC mRNA levels and protein secretion of both RELMα (*Retnla*) and Ym1/2 (*Chi3l3*) (Fig. 2 *B* and *C*). Intracellular staining showed that production of these

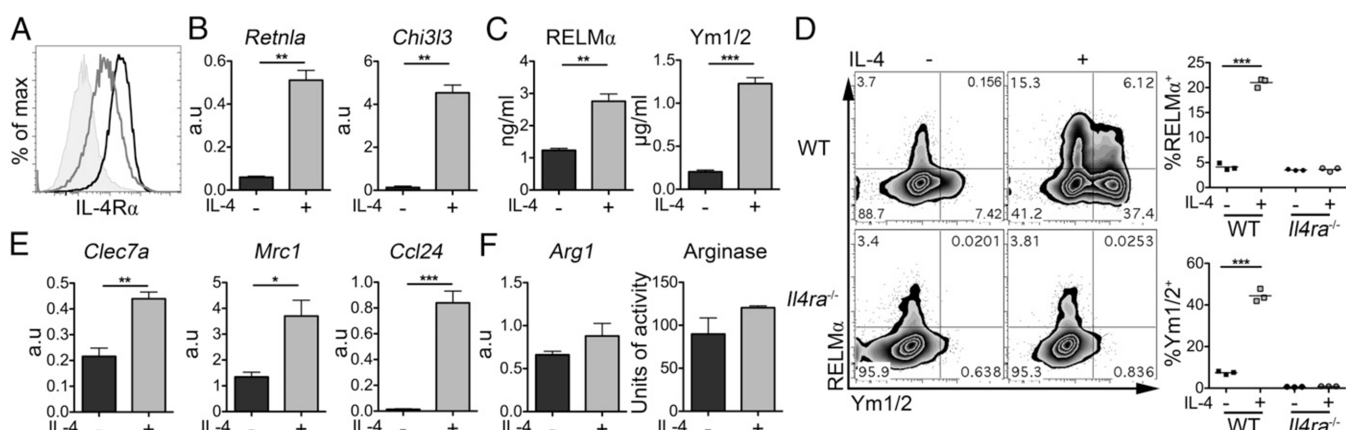

Fig. 2. IL-4 stimulates RELMα and Ym1 expression by BMDCs. IL-4Rα expression by BMDCs (*A*) (shaded portion = isotype control; black line = media; gray line= +IL-4). WT or *Il4ra*$^{-/-}$ BMDCs were cultured overnight with or without IL-4 and were assessed for markers of alternative activation (*B*–*F*) (black = without IL-4; gray = 20 ng/mL IL-4). Data are representative of more than three experiments. Error bars indicate SEM of triplicate wells. a.u., Arbitrary units.

proteins was not limited to a single population, with both RELMα and Ym1/2 single and double-positive DCs clearly distinguishable (Fig. 2*D*: WT). Verifying that DC up-regulation of RELMα and Ym1/2 was a consequence of IL-4 responsiveness, *Il4ra*$^{-/-}$ DCs did not up-regulate RELMα and Ym1/2 in response to IL-4 (Fig. 2*D*: *Il4ra*$^{-/-}$). In addition, in WT DCs, IL-4 increased transcripts for Dectin-1 (*Clec7a*), *Mrc1* (MR) and *Ccl24* (Fig. 2*E*), markers previously associated with aaMΦs (6, 7). However, Arginase-1, another signature molecule of aaMΦs (6), was not significantly increased in terms of either transcription or enzymatic activity in IL-4–treated DCs (Fig. 2*F*). Increased transcript expression of *Retnla*, *Clec7a*, and *Ccl24* was also seen following in vitro exposure of FACS-purified splenic cDCs from naive mice to IL-4 (Fig. S4). Together, these data reveal that murine DCs can respond to IL-4 in a manner similar to that previously described for aaMΦs by significantly up-regulating RELMα and Ym1/2 and other aaMΦ markers, with the notable exception of Arginase-1.

Signaling to DCs via IL-4Rα Alters Their Response to Inflammatory Stimuli and Is Important for Their Ability to Induce IFNγ and IL-17. Previous studies have shown that IL-4 can boost DC proinflammatory cytokine production upon engagement of specific pattern recognition receptors, and suggested that this may represent a mechanism to enable DC induction of adaptive Th1 responses in IL-4–rich environments (15, 19). We assessed the influence of IL-4 on DCs exposed to IL-4 concurrently with complex Ag from pathogens and individual toll like receptor (TLR) ligands. We found that DC exposure to ligands of TLR4 (LPS), TLR9 (CpG), or a heat-killed preparation of the Gram-positive bacterium *Propionibacterium acnes* (Pa) boosted IL-12p70 secretion, and inhibited production of IL-10, RELMα, and Ym1/2, in response to IL-4 (Fig. S5 *A*–*D*). Production of proinflammatory cytokines IL-1β and IL-6 was not significantly altered by IL-4 treatment (Fig. S5*E*). In contrast to Pa and TLR ligands, DCs exposed to soluble extracts from the egg stage of *S. mansoni* [soluble egg Ag (SEA)] in the presence or absence of IL-4 did not alter secretion of either RELMα or Ym1/2, indicating that SEA has no potential to drive or block alternative activation of DCs (Fig. S5 *C* and *D*). Similarly, unlike Pa- and TLR-pulsed DCs, IL-4 treatment did not alter production of proinflammatory or regulatory cytokines by SEA-exposed DCs. Thus, the influence of IL-4 over DC activation is context dependent: IL-4 can enhance TLR-driven IL-12p70 production, but engagement of these pattern recognition receptors by defined TLR ligands or bacteria can also dramatically inhibit alternative activation markers ordinarily induced by IL-4. In contrast, the helminth Ag SEA did not impede this process.

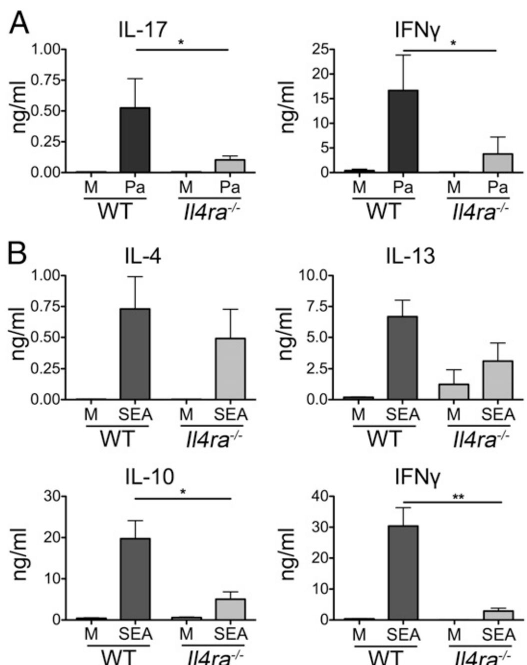

Fig. 3. *Il4ra*$^{-/-}$ DCs are less able to induce IFNγ and IL-17 responses. WT or *Il4ra*$^{-/-}$ BMDCs were cultured overnight in medium alone (M), with Pa (*A*) or SEA (*B*), harvested, and injected s.c. into WT mice. Seven days later, the draining pLN were harvested, cells were restimulated for 72 h with Pa (*A*) or SEA (*B*), and cytokine secretion was assessed by ELISA. Data are representative of five experiments. Error bars indicate SEM of three to five mice per group.

Having demonstrated that DCs can express molecules associated with aaMΦs in an IL-4Rα–dependent manner, and that IL-4 can boost TLR-mediated proinflammatory cytokine production, we next addressed the relevance of DC responsiveness to IL-4 for T cell priming, one major characteristic used to distinguish DCs from MΦs. We have previously shown that Pa-activated DCs generate a strong mixed Th1/Th17 response following transfer into naive recipient mice (24). In contrast, we have established that DCs pulsed with SEA are capable inducers of Th2 responses both in vitro and in vivo, despite failing to up-regulate traditional markers of activation (5). Although it has been debated recently whether DCs are needed to prime Th2 responses (4), we have definitively shown that Th2 priming to schistosomes is dependent on CD11c^{+} DCs (25).

To investigate whether DC responsiveness to IL-4 alters priming of naive T cells, we generated WT or *Il4ra*$^{-/-}$ DCs, exposed them to Pa or SEA in vitro, then adoptively transferred them into WT naive recipient mice, and compared Ag-specific immune responses in the draining LN (dLN). Transfer of Pa pulsed *Il4ra*$^{-/-}$ DCs resulted in significantly lower amounts of Ag-specific IL-17 and IFNγ in recipient mice, in comparison with WT Pa DCs (Fig. 3*A*). This demonstrates that DC IL-4Rα signaling is required to promote optimal T-cell responses even in a Th1/Th17 polarizing environment with low levels of available Th2 cytokine. The deficiency in IL-17 priming is unlikely to be due to alteration in DC production of Th17-promoting cytokines IL-1β and IL-6, as IL-4 did not significantly influence secretion of either of these cytokines by DCs responding to Pa in vitro (Fig. S5*E*).

In the context of helminth- rather than bacterial Ag–conditioned DCs, *Il4ra*$^{-/-}$ SEA DCs induced similar levels of Ag-specific IL-4 and IL-13, but significantly lower amounts of IL-10 and IFNγ in comparison with WT SEA DCs (Fig. 3*B*). This indicates that DC expression of IL-4Rα is not an absolute requirement for Th2 priming, but is needed for the induction of IFNγ and IL-10 responses against SEA. In keeping with previous work (26), the level of IL-17 induced by SEA-activated C57BL/6 DCs was negligible, and was not influenced by the presence or absence of IL-4Rα on the transferred DCs.

The reduced ability of *Il4ra*$^{-/-}$ DCs to promote Pa-specific IL-17 and IFNγ, and SEA-specific IL-10 and IFNγ, was not due to a fundamental defect in Ag uptake, processing, or presentation by these cells. *Il4ra*$^{-/-}$ DCs capably took up Ag in the form of FITC-labeled dextran (Fig. S6), and displayed enhanced ability to stimulate proliferation of OTII TCR Tg T cells in vitro, following exposure to ovalbumin (OVA) peptide or protein, compared with WT DCs (Fig. S7).

IL-4 Alters the Response of DCs to CD40 Ligation. CD40 expressed by DCs binds CD154 on the surface of activated T cells, this interaction enhancing DC activation and cytokine production which is often crucial for their ability to prime Th1, Th2 and Th17 responses (24, 27). To address whether the IL-4 induced alteration in the balance of IL-12, IL-10, RELMα, and Ym1/2 production by DCs might be influenced by interaction with T cells we examined the impact of CD40 ligation on DC cytokine production following IL-4 exposure. To mimic DC–T-cell interaction in vitro, we analyzed cytokine secretion by DCs that had previously been exposed to Pa or SEA in the presence of absence of IL-4, after stimulation with agonistic αCD40 mAb. IL-4 treatment of Pa-pulsed DCs significantly enhanced pro-

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duction of IL-12p40, IL-6, and IL-12p70, and reduced secretion of IL-10, following addition of αCD40 mAb (Fig. S8*A*). However, in keeping with previously published data (28), control (medium) DCs or SEA DCs did not produce significant amounts of IL-12p70 or IL-10 following stimulation with anti-CD40, and this was not influenced by IL-4 exposure (Fig. S8*A*). Therefore, the influence of IL-4 on cytokine secretion by DCs responding to bacterial stimulation was not transient, and extended to secondary activation via CD40 ligation.

Previous studies have suggested that RELMα and Ym1/2 may have a role in regulating or promoting T-cell responses, respectively (14, 21, 22), and it is conceivable that during T-cell priming, CD154 expressed by T cells could engage DC CD40 and influence secretion of these molecules. However, IL-4–treated DCs stimulated with αCD40 mAb produced similar levels of RELMα and Ym1/2 compared with isotype controls (Fig. S8*B*), indicating that CD40 stimulation does not regulate IL-4–driven expression of these alternative activation products either in control or SEA DCs. Furthermore, in the case of Pa stimulated DCs, bacterial inhibition of RELMα and Ym1/2 was maintained throughout culture, and was not overcome by CD40 ligation (Fig. S8*B*). These CD40 ligation experiments also illustrate that DCs previously exposed to IL-4 still produced substantial amounts of RELMα and Ym1/2 up to 36 h after initial cytokine treatment (Fig. S8*B*). Thus, production of RELMα and Ym1/2 by DCs is not transient and requires neither the continued presence of IL-4 nor subsequent CD40 ligation.

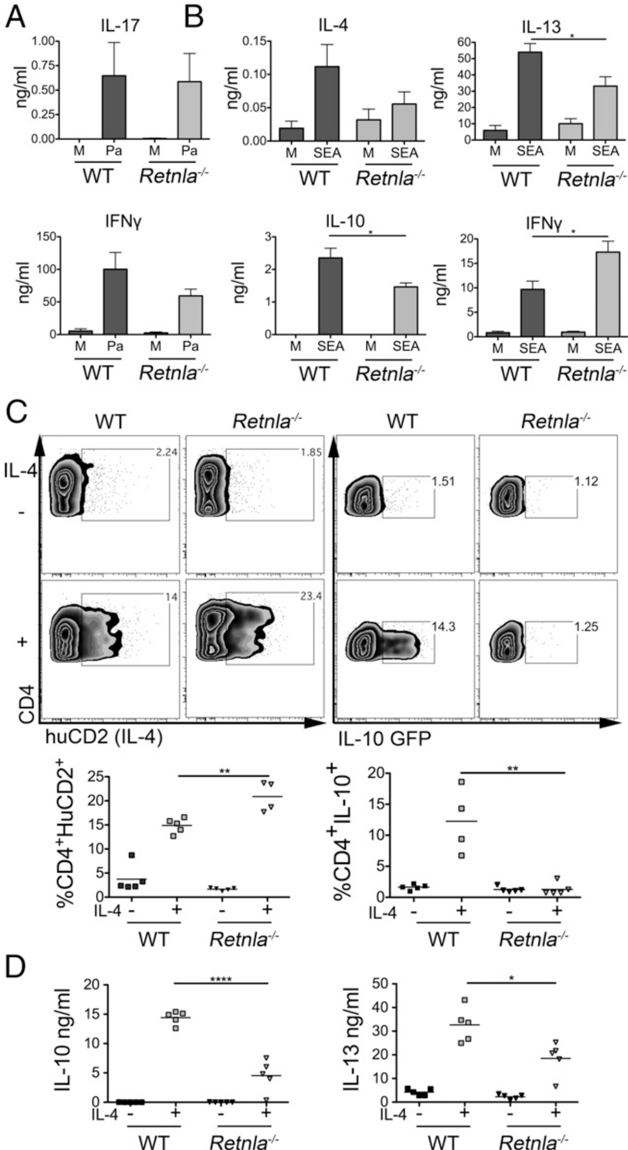

Fig. 4. DC expression of RELMα during Th2 priming regulates IFNγ and promotes IL-10 and IL-13 production. WT or *Retnla*$^{-/-}$ BMDCs were cultured overnight in medium alone (M), Pa (*A*), or SEA (*B*) and injected s.c. into WT mice. Next, 5–7d later, pLN were harvested, cells were restimulated for 72 h with Pa (*A*) or SEA (*B*), and cytokine secretion was assessed by ELISA. CD4$^+$ cells sorted from KN2 IL-4 reporter mice, or IL-10eGFP$^-$-CD4$^+$ cells, were cultured for 4 d with WT or *Retnla*$^{-/-}$ BMDCs and anti-CD3 mAb with or without IL-4 and assessed for IL-4 protein production or IL-10 mRNA expression by flow cytometry (*C*) and cytokine secretion by ELISA (*D*). Data are representative of two (*A* and *C*) or four (*B* and *D*) experiments. Error bars indicate SEM of three to five mice (*A* and *B*) or four to five replicate wells (*C* and *D*) per group.

DC Secretion of RELMα During Th2 Priming Regulates IFNγ and Promotes Th2 Responses. Although our data demonstrate that DC sensitivity to IL-4 plays an important role in priming of T-cell IL-10, IL-17, and IFNγ (Fig. 3 *A* and *B*), it is not clear whether this is a consequence of alternative activation marker expression or some other facet of the diverse influence of IL-4 on DCs (Fig. 2 and Fig. S5). To specifically address the role of a defined alternative activation product in T-cell, priming we generated DCs from *Retnla*$^{-/-}$ mice (20), which were similar to WT in terms of numbers and phenotype. We adoptively transferred SEA- or Pa-pulsed WT or *Retnla*$^{-/-}$ DCs into WT-naive recipient mice and assessed the resulting Ag-specific immune response.

Retnla$^{-/-}$ Pa DCs induced similar IFNγ and IL-17 responses to WT DCs in the draining LN following transfer (Fig. 4*A*), demonstrating that DC-derived RELMα neither promotes nor regulates T-cell differentiation in a bacterial Th1/Th17 setting. Thus, impaired priming by *Il4ra*$^{-/-}$ DCs exposed to bacteria (Fig. 3*A*) is RELMα independent. The lack of a major role for RELMα in Th1/17 priming also fits with the observed inhibition of IL-4–induced RELMα expression by DCs following bacterial or TLR ligand exposure (Fig. S5).

To directly assess the importance of DC RELMα secretion in a Th2 setting, we transferred *Retnla*$^{-/-}$ DCs pulsed with SEA into naive recipients. In repeated experiments, both WT and *Retnla*$^{-/-}$ SEA DCs stimulated similar levels of Ag-specific IL-4, but mice that received *Retnla*$^{-/-}$ DCs displayed significantly reduced levels of IL-10 and IL-13 compared with WT SEA DCs (Fig. 4*B*). Furthermore, *Retnla*$^{-/-}$ SEA DCs induced significantly greater amounts of IFNγ compared with WT SEA DCs (Fig. 4*B*). This suggests that DC production of RELMα is required for optimal Th2 cell priming by helminth-activated DCs, promoting some components of the Th2 response while regulating the level of the Th1 response.

To further dissect requirements for DC RELMα production in promoting optimal T-cell Th2 cytokine secretion, we examined the ability of WT or *Retnla*$^{-/-}$ DCs to induce CD4$^+$ T-cell IL-4, IL-10, and IL-13 in vitro. In this assay, the addition of exogenous IL-4 to WT DCs cocultured with CD4$^+$IL-10eGFP$^-$ T cells purified from IL-10eGFP reporter mice (29), or CD4$^+$ T cells from KN2 mice in which IL-4–producing cells express huCD2 (30), resulted in induction of T-cell expression of eGFP or huCD2, and secretion of IL-10 and IL-13 (Fig. 4 *C* and *D*). In keeping with the lack of a significant impact of RELMα deficiency on IL-4 induction in vivo (Fig. 4*B*), *Retnla*$^{-/-}$ DCs capably provoked huCD2 expression on responding KN2 T cells in vitro (Fig. 4*C*). However, *Retnla*$^{-/-}$ DCs were incapable of promoting T-cell expression of IL-10 mRNA as detected by GFP expression (Fig. 4*C*). Furthermore, analysis of secreted cytokine by ELISA showed that cocultures of CD4$^+$ T cells and WT DCs produced more IL-10 and IL-13 protein in the presence of IL-4 compared with cocultures with *Retnla*$^{-/-}$ DCs (Fig. 4*D*). Interestingly, T cells cultured with WT DCs secreted lower levels of IFNγ protein than their *Retnla*$^{-/-}$ counterparts, supporting observations following in vivo DC transfer of SEA activated DCs (Fig. 4*B*).

Mechanistically, the altered ability of *Retnla*$^{-/-}$ DCs to polarize SEA-specific cytokine responses was not due to defective Ag uptake, processing, or presentation by these cells. Similar to *Il4ra*$^{-/-}$ DCs, *Retnla*$^{-/-}$ DCs competently took up Ag (Fig. S6), and showed enhanced ability to induce proliferation of OTII TCR Tg T cells in vitro (Fig. S7), with either OVA peptide or protein. This increased proliferation is consistent with expectation, given the reduced IL-10 production evident following transfer of *Retnla*$^{-/-}$ DCs in vivo (Fig. 4*B*) or coculture of *Retnla*$^{-/-}$ DCs with T cells in vitro (Fig. 4 *C* and *D*).

These data strongly suggest that DC production of RELMα is an important process both for directing priming of Th2 responses and for control of Th1 responses, and provide mechanistic insight into the function of RELMα during the early stages of T-cell activation.

Discussion

A substantial body of literature documents the ability of IL-4 to generate aaMΦs in multiple settings. However, comparatively few studies have addressed how DCs respond to IL-4, whether they can express markers associated with aaMΦs (11–14), and what the functional relevance of this expression may be. The data that we present here reveal that murine DCs can respond to IL-4 both in vitro and in vivo in a manner similar to that previously described for aaMΦs, by significantly up-regulating RELMα, Ym1/2, and other aaMΦ markers, with the notable exception of Arginase-1. Furthermore, we have identified that DC expression of RELMα plays a critical role in their instruction of T cells to produce IL-10 and IL-13. This demonstrates a previously undescribed role for RELMα, and reveals that DC responsiveness to IL-4 is vital for priming optimal Th2 responses.

Exposure of DCs to IL-4 triggered expression of numerous markers associated with aaMΦ, but one notable difference was the lack of Arginase-1 up-regulation at both the mRNA and protein activity level. Arginase-1 competes with iNOS for L-arginine (6), and one consequence of increased Arginase-1 activity is L-arginine depletion from the local environment. In murine *Leishmania major* infection, increased arginase activity in parasite-infected MΦs depletes the skin of L-arginine, impairing proliferation of T cells in the lesion (31). Furthermore, MΦ-derived Arginase-1 is required to suppress T-cell proliferation during Th2 infection, where it limits pathology (8). The function of DCs is instead mainly to prime naive T cells in the early phase of immune response development. In this context, a high level of Arginase-1 expression by immunogenic DCs that could deplete L-arginine from the local environment might be undesirable.

In addition to RELMα and Ym1/2, we have found that IL-4–treated DCs significantly increased expression of *Mrc1*, *Clec7a*, and *Ccl24*, all of which have previously been associated with aaMΦs (6, 7). MR and Dectin-1 are both C-type lectin receptors that can bind carbohydrates and trigger distinct signaling pathways in DCs (32). MR has been linked mainly to internalization of Ags containing mannose motifs; however, in the absence of an additional stimulus (such as TLR ligation), engagement of the receptor is thought to be unable to mediate proinflammatory responses in DCs (32). In contrast, Dectin-1 recognition of β glucans, which are mainly found in fungal cell walls, initiates proinflammatory responses in DCs even in the absence of TLR ligation (33). Our finding that IL-4 increases DC expression of both these C-type lectin receptors suggests that they will have an enhanced ability to internalize and respond to a wide range of glycosylated pathogen motifs that could further modulate their activation and function. Increased DC expression of CCL24 could also influence this process, as this chemokine can enhance recruitment of CCR3-expressing cells such as eosinophils and basophils, as has been reported for CCL24-producing aaMΦs in the lung (34). These recruited granulocytes could, in turn, assist Th2 priming in certain situations through provision of cytokines such as IL-4.

As well as identifying that DCs can display hallmarks of alternative activation following IL-4 exposure in vitro and in vivo, we have revealed DC expression of alternative activation markers in the context of chronic helminth infection, a subject that has so far barely been addressed in the literature (11). There was some heterogeneity in the levels of specific markers expressed when comparing in vitro and in vivo settings, in particular with Ym1/2. This could reflect basic differences between BMDCs and their in vivo counterparts, or differential regulation of aspects of DC alternative activation in vivo, in line with the well-documented diversity of aaMΦs (6).

Our results also support and extend previously published work addressing how IL-4 might alter the IL-12/IL-10 balance (15, 19), but are unique in showing the impact of IL-4 on T-cell polarization by DCs in vivo. Our data highlight an important feedback loop in DC-mediated priming in vivo, where sources of IL-4/IL-13 will not only promote Th2 responses (4), but will also facilitate DC-mediated priming of Th1 responses through enhancement of IL-12p70 and inhibition of IL-10 production by DCs. Extending our understanding of the cross-talk between microbes, DCs, and their environment, we have demonstrated the ability of defined TLR ligands and heat-killed bacteria to inhibit the ability of IL-4 to prompt RELMα and Ym1/2 production by DCs. In light of our identification of a pro-Th2 role for DC-derived RELMα, inhibition of such by bacteria or their products may be important in some settings to allow optimal Th1/17 immunity to develop.

In peripheral environments, DCs migrate away from the site of Ag exposure toward draining LNs (35). In order for RELMα and Ym1/2 to play a relevant role in T-cell priming in the draining LNs, DC expression of such molecules would have to be prolonged, or responsive to subsequent interaction with T cells. We have shown that production of RELMα and Ym1/2 by DCs is not transient and requires neither the continued presence of IL-4 nor subsequent CD40 ligation. Furthermore, by adoptive transfer of *Retnla*$^{-/-}$ DCs in vivo, we have identified a previously unreported requirement for DC production of RELMα in the initiation of Th2 responses in the LN draining the site of DC introduction. A Th2-promoting role for RELMα contrasts with the down-regulatory role that has previously been described using global *Retnla*$^{-/-}$ mice, which identified RELMα as a negative regulator of ongoing Th2 inflammation against helminth Ag, with *Retnla*$^{-/-}$ mice displaying elevated Th2 responses and greater pathology (21, 22). These reports would suggest that the ultimate role for RELMα produced by multiple cellular sources in vivo (including eosinophils, epithelial cells, and MΦs) is to regulate chronic Th2 pathology by influencing T-cell differentiation and cellular recruitment while regulating wound repair. In the present study, we focused on the early events in Th2 priming by DCs in vitro and in vivo. By restricting RELMα deficiency to DCs alone, our results extend our fundamental understanding of the cellular, temporal, and spatial diversity of RELMα function, demonstrating an alternative function of DC expression of this molecule early in immune response development. Our identification of a critical role for DC-derived RELMα in promotion of T-cell production of IL-10 reveals a mechanism that may, in part, explain the dysregulated immune pathology previously observed in Th2 infection models using global *Retnla*$^{-/-}$ mice (21, 22).

It is clear from our results that RELMα production does not solely account for Th2 induction by DCs, but helps promote specific facets of this response (IL-10 and IL-13). Because we have shown that IL-4 stimulates production or expression of a wide range of alternative activation-related molecules in DCs, and that *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs do not display identical functional ability, it is likely that a combination of IL-4–induced products will work together and in balance to ultimately dictate the character of the resultant Th2 response. For example, as Ym1/2 has previously been suggested to promote some Th2 cytokines, at least in vitro, its production by DCs responding to IL-4 may act in concert with RELMα and other alternative activation products to generate optimal Th2 immunity.

In summary, we have shown that the canonical Th2 cytokine IL-4 can have a profound impact on DC activation and function, triggering both in vivo and in vitro expression of a range of

molecules ordinarily associated with aaMΦs, altering responsiveness to challenge with bacteria and TLR ligands, and dramatically influencing T-cell response induction and polarization. Although the cumulative impact of IL-4 on DC activation and function is complex, in the context of pathogenic stimulation, the over-riding effect of IL-4 appears to be to enable IFNγ induction through enhanced DC IL-12p70 production. Furthermore, DC production of the IL-4 induced protein, RELMα, is important in priming of T-cell IL-10 and IL-13. Together these findings indicate that IL-4 has a more influential and diverse role in directly regulating DC-mediated T-cell priming than has been previously appreciated.

Materials and Methods

Mice and IL-4c Injections. C57BL/6, BALB/c, *Retnla*$^{-/-}$, IL-10eGFPxDOG, KN2 (C57BL/6), *Il4ra*$^{-/-}$, and OTII mice (20, 23, 25, 29, 30) were maintained under specific pathogen-free conditions at the University of Edinburgh. Experiments were conducted under a Project License granted by the Home Office (United Kingdom) in accordance with local guidelines. IL-4/anti-IL-4 mAb complexes (IL-4c) were prepared as described previously (23). Mice were injected i.p. with 50 μL PBS or 0.625–10μg IL-4 complexed to 11B11, and peritoneal exudate cells (PEC) and spleens harvested 4 d later.

Cell Culture and DC Transfer. BM-derived DCs were generated with GM-CSF as previously described (36). Following 10 d of culture, BMDCs were harvested and replated at 2×10^6/mL for 18 h in the presence or absence of rIL-4 (20 ng/mL; Peprotech). In coculture experiments, CD4$^+$GFP$^-$ T cells were sorted from IL-10eGFPxDOG or KN2B6xB6 mice using BD FACs Aria-II and cultured with WT or *Retnla*$^{-/-}$ DCs. For DC transfer, WT, *Il4ra*$^{-/-}$, or *Retnla*$^{-/-}$ BMDCs were cultured as above, with SEA, Pa, or medium alone. BMDCs were injected s.c. into recipient WT mice (2.5×10^5 per foot) and 5–7d later the draining popliteal LNs and restimulated as previously described (24, 36). Supernatants were harvested after 72 h, and cytokine production was assessed by ELISA. Further details are provided in *SI Materials and Methods*.

Flow Cytometry and ELISA. Details of mAb used are given in *SI Materials and Methods*. Samples were acquired using FACS LSR II or FACS Canto II using BD FACSDiva software and analyzed with FlowJo v.9 software (Tree Star). ELISAs were performed on culture supernatants using paired mAb, and recombinant cytokine standards, or DuoSets (eBioscience, BD Pharmingen, R&D Systems, and Peprotech). Arginase assay details are provided in *SI Materials and Methods*.

RNA Isolation and Quantitative PCR. RNA was recovered from cells using TRIzol (Invitrogen), cDNA was generated using SuperScript-III (Invitrogen). Relative quantification of the gene of interest was performed by quantitative PCR. Primer details are given in *SI Materials and Methods*.

Statistical Analysis. Statistical analyses were carried out using GraphPad Prism 5. The Student's *t* test or one-way analysis of variance was used to determine significant differences between sample groups (in figures, $*P < 0.05$ $**P < 0.01$ $***P < 0.001$ $****P < 0.0001$).

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Supporting Information

Cook et al. 10.1073/pnas.1121231109

SI Materials and Methods

Infections. Mice were infected with *L. sigmodontis* for 10 d or with *S. mansoni* for 8 wk, as previously described (1, 2).

BMDC Stimulation and DC: T-Cell Coculture. In selected experiments, DCs were exposed to endotoxin-free soluble egg Ag [SEA, 25 μg/mL, prepared as described elsewhere (3)], heat-killed *Propionibacterium acnes* (Pa, 10 μg/mL), lipopolysaccharide (LPS, 250ng/mL, Sigma) or CpG oligodeoxynucleotide 1826 (ODN 1826, 5 μg/mL, InvivoGen). To assess further activation, CD40 ligation with αCD40 mAb was carried out as previously described (4). In vitro coculture polarization experiments were performed with 50,000 IL10-eGFP^{-}CD4^{+} or KN2 CD4^{+}. T cells were cultured in 96-well plates for 3–4 d with 2,500 RELMα sufficient or *Retnla*$^{-/-}$ BMDCs, 1 μg/mL anti-CD3, with or without IL-4 (20 ng/mL). For CFSE dilution assays, CD4^{+} OT-II TCR Transgenic T cells were purified from spleen and LN using CD4^{+} Dynabeads (Invitrogen) following the manufacturer's protocol. T cells were labeled with 5 μM CFSE (Invitrogen) for 15 min at 37 °C. Excess CFSE was allowed to leach from the cells before culture with 5×10^4 WT, *Retnla*$^{-/-}$, or *Il4ra*$^{-/-}$ BMDCs in the presence of 0.01 μg/mL OVA$_{323–339}$ or 5 μg/mL OVA protein (Sigma), which had been endotoxin depleted in-house. Cultures were incubated at 37 °C for 4 d before assessment of CFSE dilution by flow cytometry.

Flow Cytometry. In some experiments, cells were first stained with LiveDead aqua (Invitrogen). Following FcR-Block (2.4G2), cells were surface stained using the following mAb: CD4-PE, CD11c-APC/eFluor780, MHCII-PerCP/Cy5.5, CD11b-eFluor450, CD80-APC, CD40-PE, CD19-AlexaFluor700, CD86-FITC, Dectin1-APC, IL-4Rα-biotin, SiglecF-PE, F4/80-PE/Cy7, Gr1-PerCP/Cy5.5, and HuCD2-APC. For intracellular staining, cells were fixed in 1% PFA, permeabilized using Cytoperm (BD PharMingen), and stained with anti-RELMα, and anti-Ym1 biotin followed by AlexaFluor 488–conjugated anti-rabbit Ab and streptavidin-APC. To assess Ag uptake, 2×10^5 BMDCs were incubated with 200 μg FITC-conjugated dextran (Sigma) for 30 min at 37 °C or on ice before acquisition.

Splenic DC Purification. Splenic DCs were enriched from five to six naive C57BL/6 mice using NycoDenz density gradient separation and CD11c^{hi}B220^{-} cDCs were sorted from B220^{+}CD11c^{mid} pDCs using a BD FACs Aria II. After sorting, 5×10^4 DCs were cultured overnight with 20 ng/mL IL-4, before RNA extraction, reverse transcription, and quantification of transcript levels using quantitative PCR.

Arginase Activity Assay. Arginase activity was measured as described previously (5). One unit of arginase enzyme activity is defined as the amount of enzyme that catalyzed the formation of 1 μmol urea per minute at 37 °C.

Quantitative PCR. Relative quantification of the gene of interest was performed by qPCR analysis using Roche Light Cycler 480, with LightCycler SYBR Green I Master mix, compared with a serially diluted standard of pooled cDNA. Expression was normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*).

Primers were as follows:

Hprt-F: 5′-TCCTCCTCAGACCGCTTTT-3′,
Hprt-R: 5′-CCTGGTTCATCATCGCTAATC-3′,
Retnla-F: 5′-TATGAACAGATGGGCCTCCT-3′,
Retnla-R: 5′-GGCAGTTGCAAGTATCTCCAC-3′,
Chi3l3-F: 5′-GAACACTGAGCTAAAAACTCTCCTG-3′,
Chi3l3-R: 5′-GACCATGGCACTGAACGAG-3′,
Clec7a-F: 5′-ATGGTTCTGGGAGGATGGAT-3′,
Clec7a-R: 5′-GCTTTCCTGGGGAGCTGTAT-3′,
Mrc1-F: 5′-TCATTGGAAGATCCACTCTGG-3′,
Mrc1-R: 5′-CAGCGCTTGTGATCTTCATTATAG-3′,
Arg1-F: 5′-GTCTGTGGGGAAAGCCAAT-3′,
Arg1-R: 5′-GCTTCCAACTGCCAGACTGT-3′.
Ccl24-F: 5′-GCAGCATCTGTCCCAAGG-3′,
Ccl24-R: 5′-GCAGCTTGGGGTCAGTACA-3′.

1. Jenkins SJ, et al. (2011) Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* 332:1284–1288.
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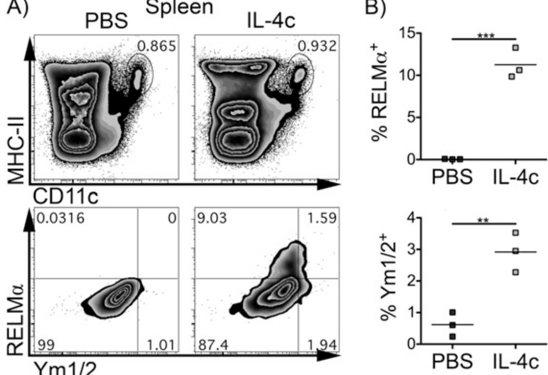

Fig. S1. IL-4c injection induces alternative activation of splenic DCs. Splenic DCs (MHC-II$^+$ CD11c^{hi}) from PBS- or IL-4c–treated mice (2×5 μg, d 0 and d 2) were harvested at d 4 and stained for intracellular expression of RELMα and Ym1/2 (*A* and *B*). Data are representative of at least three experiments. Graphs show percent expression for individual mice, three per group.

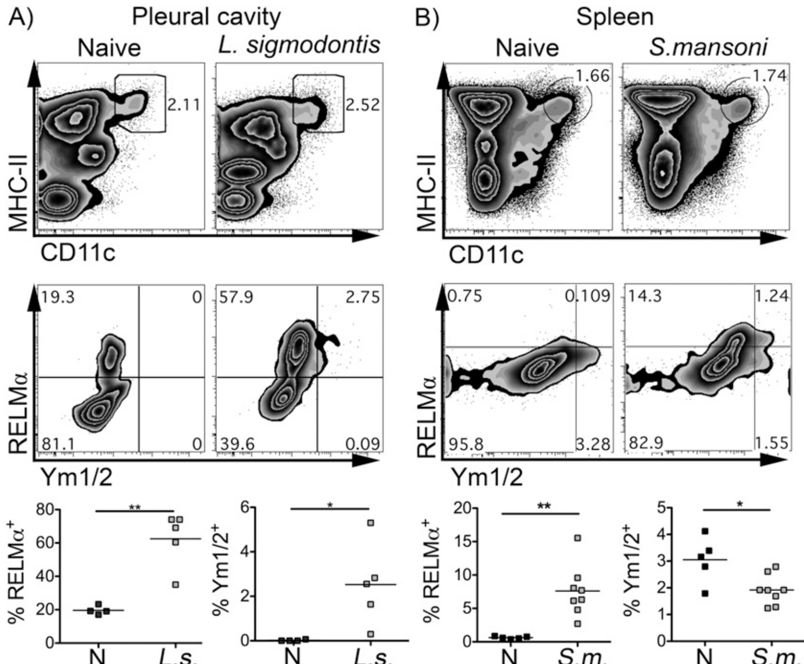

Fig. S2. Parasitic helminth infection induces alternative activation of DCs at multiple tissue sites. Pleural cavity (*A*) or splenic (*B*) DCs (MHC-II$^+$ CD11c^{hi}) from naive mice infected with *L. sigmodontis* (*L.s.*) (*A*) or *S. mansoni* (*S.m.*) (*B*) were stained for intracellular expression of RELMα and Ym1/2. Data are representative of three to six experiments. Graphs show percent expression for individual mice, four to eight per group.

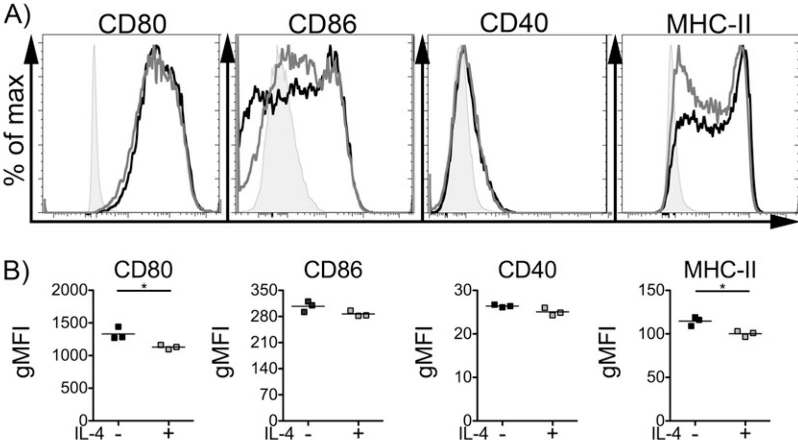

Fig. S3. IL-4–stimulated BMDCs do not dramatically alter their activation phenotype. WT BMDCs were stimulated overnight with 20 ng/mL IL-4, and their expression of costimulatory molecules was assessed by flow cytometry (*A*). Shaded area indicates isotype control; black line indicates media; gray line indicates +IL-4. Geometric mean fluorescence intensity (MFI) of marker expression by CD11c⁺ cells (*B*). Data are representative of more than five experiments. Error bars represent SEM of triplicate wells.

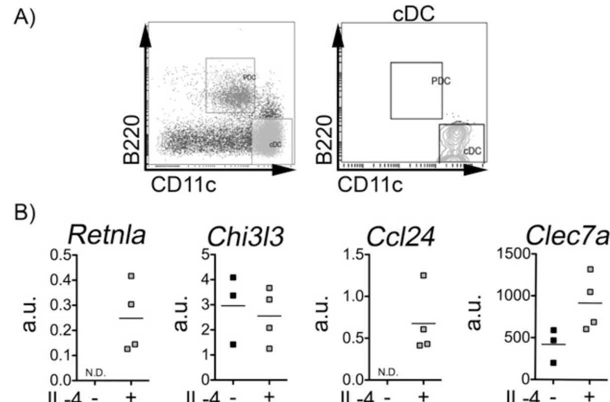

Fig. S4. IL-4 alternatively activates splenic ex vivo DCs in vitro. Following enrichment for DC populations, splenic cDCs ($CD11c^{hi}B220^{-}$) from naive mice were separated from pDCs ($B220^{+}CD11c^{mid}$) (*A*) and cultured overnight in the presence of 20 ng/mL IL-4. IL-4 increased mRNA expression of alternative activation markers (*B*). Data are representative of two experiments, with three to four replicate wells per group. a.u., Arbitrary units; N.D., not detected.

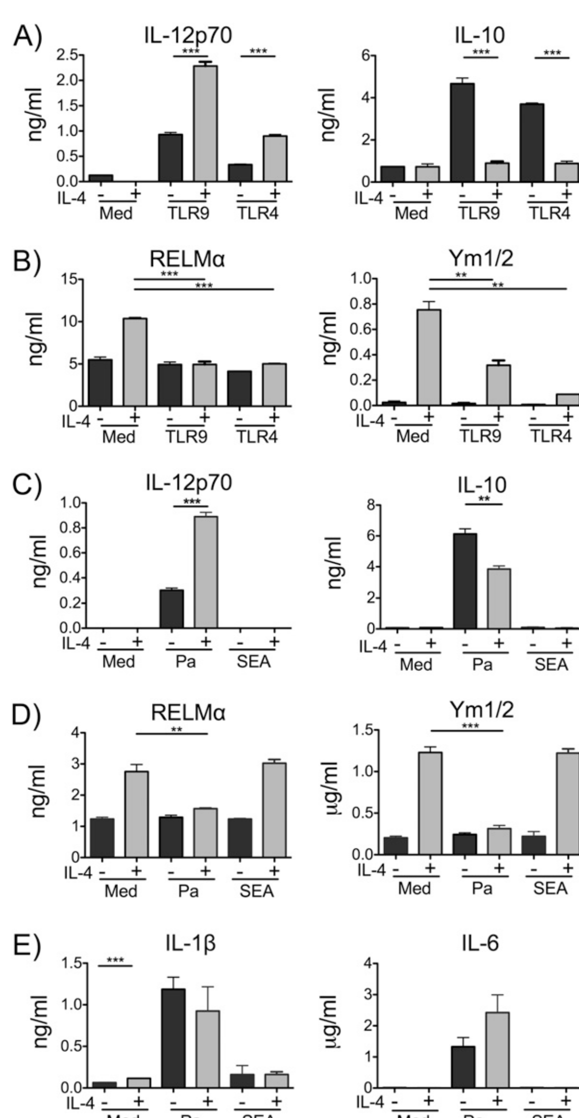

Fig. S5. Defined TLR ligands and pathogen products modulate IL-4–driven RELMα and Ym1 expression by BMDCs. BMDCs were cultured overnight in the presence or absence of IL-4 and TLR agonists CpG 1826 (TLR9) or LPS (TLR4) (*A* and *B*) or Pa or SEA (*C*–*E*). IL-12p70, IL-10, RELMα, Ym1/2, IL-1β, and IL-6 secretion was assessed by ELISA. Error bars represent SEM of triplicate wells. Data are representative of four (*A*–*D*) or two (*E*) experiments.

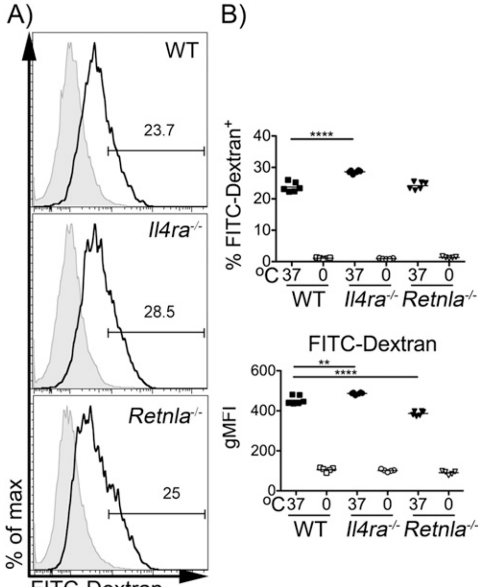

Fig. S6. WT, *Il4ra*$^{-/-}$, and *Retnla*$^{-/-}$ DCs are equally capable at antigen uptake. WT, *Il4ra*$^{-/-}$ or *Retnla*$^{-/-}$ BMDCs were incubated with FITC-conjugated dextran at 37 °C, and Ag uptake was assessed by flow cytometry (*A* and *B*). Shaded area indicates 0 °C; black line indicates 37 °C. Data are representative of two experiments, five to six replicate wells per group.

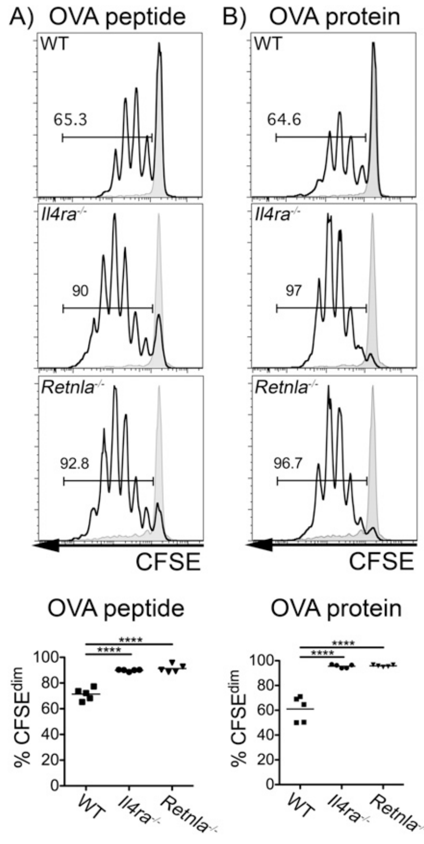

Fig. S7. Ag processing and presentation is unimpaired in *Il4ra*$^{-/-}$ and *Retnla*$^{-/-}$ DCs. WT, *Il4ra*$^{-/-}$, or *Retnla*$^{-/-}$ BMDCs were cultured for 4 d with CFSE-labeled OT-II TCR Tg T-cells in the presence of OVA peptide (*A*) or protein (*B*), and the ability to stimulate proliferation was assessed by flow-cytometric analysis of CFSE dilution. Shaded area indicates no peptide/protein; black line indicates 0.01 μg/mL OVA peptide (*A*) or 5 μg/mL OVA protein (*B*). Data are representative of two experiments, five replicate wells per group.

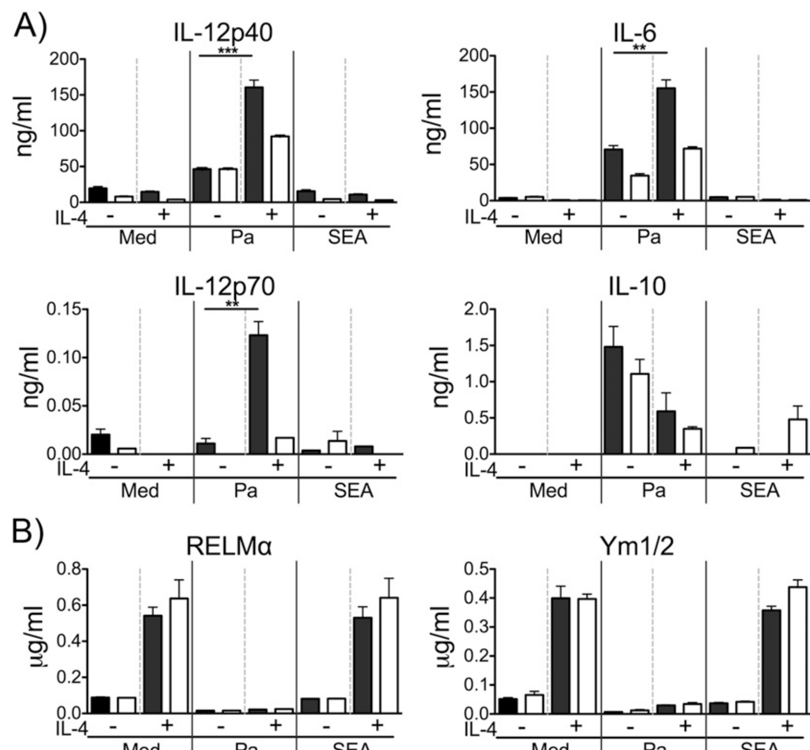

Fig. S8. IL-4 alters the response of DCs following CD40 ligation. BMDCs cultured overnight with IL-4 and Pa or SEA were washed and recultured with agonistic anti-CD40 (■) or control antibody (□) for another 24 h, and cytokine (*A*), RELMα, and Ym1/2 (*B*) secretion was assessed by ELISA. Data are representative of three experiments. Error bars represent SEM of triplicate culture wells.