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# **Regulation of innate and adaptive immunity by the vaccine adjuvant alum**

**Ewa Oleszycka M.Sc.**

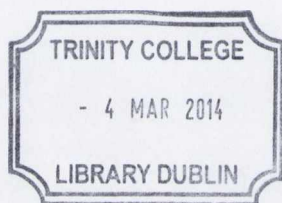


A PhD thesis submitted to Trinity College Dublin  
as a completion of the degree of Doctor of Philosophy

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**2013**



*Thesis 10273*

## **Declaration of authorship**

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I feel also very fortunate that I had a chance to study here with my friends from Kraków, Anna S. and Anna M. I would especially like to thank Anna S. who was the very first person to introduce me to the concept of science in Ireland many years



ago. She was the first undergraduate student from Kraków, who came to Ireland for a summer scholarship and later advertised this among other students. She was also my guide in Dublin when I started my PhD as she was already here doing hers. She introduced me to many people who I now consider friends. I am also very grateful to Anna M. who had so many coffees and conversations with me. I feel also very fortunate that I stayed in touch with a few interesting people who I studied with for 5 years in Kraków and who also decided to move abroad to do a PhD. Unfortunately, we did not manage to go to the same city, but I think that has given us the chance to discuss science beyond our PhD topics. Particularly, Kasia in Heidelberg and Anna in Edinburgh contributed to my “knowledge exchange” process.

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## Abstract

While many licensed vaccines consist of whole or inactivated pathogens, there is a move toward vaccines based on purified antigens which although safer are generally less immunogenic and therefore require adjuvants to trigger protective immunity. Alum, the most common adjuvant, has a record of successful use in vaccines, where an antibody-mediated immune response can confer protective immunity. However, alum is a poor inducer of cellular immune responses. The mechanism underlying this selective enhancement of humoral responses is still not well understood. Here, to gain an improved insight into its mode of action, innate immune responses to alum and their impact on adaptive immune responses were studied.

Alum strongly regulates innate immune response, in part by promoting the release of cytokines. It is demonstrated here that alum induced necrosis and promoted release of the damage-associated molecular pattern (DAMP), IL-33, which controlled eosinophil and IL-4-producing innate cell influx. Furthermore, both alum-driven IL-1 $\alpha$  and IL-1 $\beta$  promoted neutrophil infiltration in an inflammasome-independent manner, while a product of caspase-1 or caspase-11 induced inflammatory monocyte recruitment. Although, IL-1 and IL-18 did not significantly modulate alum-driven adaptive immune responses, ST2, the IL-33 receptor, regulated the type of antigen-specific antibody isotype induced by alum. Alum-driven TH1-related IgG2c secretion was enhanced in ST2<sup>-/-</sup> mice, while IgE production was reduced.

Recent studies have shown that the adjuvant properties of alum can be enhanced by incorporating additional immunostimulators, including the TLR ligands, CpG and the LPS derivative, MPL. However, it is demonstrated here that alum inhibits the induction of the TH1-polarising cytokine IL-12 in dendritic cells. This is due to selective inhibition of the IL-12p35 subunit and the inhibitory effect was the result of adjuvant-induced PI3 kinase signalling. Moreover, IL-12 inhibition was not restricted to aluminium-containing adjuvants, but was also seen with a number of other particulate adjuvants. Furthermore, alum promoted secretion of IL-10, a potent anti-inflammatory cytokine, by DCs. Alum injection enhanced early production of IL-10 by draining lymph node cells. Moreover, deficiency in IL-10 resulted in enhanced TH1 responses after immunisation with OVA and alum, while production of antigen-specific antibodies were intact.

Overall, these results strongly indicate that alum promotes necrosis at the site of injection followed by IL-1 and IL-33 dependent sterile inflammation, which is independent of inflammasome formation. Moreover, this is the first time that IL-10 and ST2 are identified as potent modulators of alum-induced adaptive immune responses.



## **Publications**

Mori A\*, **Oleszycka E\***, Sharp FA, Coleman M, Ozasa Y, Singh M, O'Hagan DT, Tajber L, Corrigan OI, McNeela EA, Lavelle EC. (2012) The vaccine adjuvant alum inhibits IL-12 by promoting PI3 kinase signaling while chitosan does not inhibit IL-12 and enhances Th1 and Th17 responses. *European Journal in Immunology*, Oct;42(10):2709-19 (\*joint first authors)

Yang M, Hearnden CH, **Oleszycka E**, Lavelle EC. (2013) NLRP3 inflammasome activation and cytotoxicity induced by particulate adjuvants. *Methods in Molecular Biology*, 1040:41-63



## Abbreviations

3-MA	3-methyladenine
4Get	IL-4 reporter mouse
Adju-Phos	aluminium phosphate
ADV	adenovirus
Aip1	actin-interacting protein 1
AIM2	absent in melanoma 2
AMP	Adenosine monophosphate
AP1	activator protein 1
APC	antigen-presenting cell
APRIL	a proliferation-inducing ligand
AS	adjuvant system
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine-5'-triphosphate
B220	220kDa isoform of B cells
BAFF	B-cell activating factor
Bcl-6	B-cell lymphoma 6
BCR	B cell receptor
BDCA-2	blood dendritic cell antigen 2
BMDC	bone marrow-derived dendritic cell
bnAb	broad neutralising antibody
BSA	bovine serum albumin
CARD	caspase activation and recruitment domains
CCR	C-C chemokine receptor
CD	cluster of differentiation
cDC	conventional dendritic cell
cDNA	complementary DNA
cGAS	Cyclic GMP-AMP Synthase
CIITA	class II, major histocompatibility complex, transactivator
CLR	C-type lectin receptor
CpG	deoxy-cytidylate-phosphate-deoxy-guanylate
CQ	chloroquine
Ct	cycle threshold
CXCR	C-X-C chemokine receptor
CXR	carboxy-X-rhodamine
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	damage associated molecular pattern
DAP	diaminopimelic acid
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DCIR	DC expressing ITIM-bearing receptor
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DDX	DEAD box helicases



DEAD	Asp-Glu-Ala-Asp motif
DEAH	Asp-Glu-Ala-His motif
DExD/H box	DEAD, DEAH, and the Ski families
DHX	DEAH box helicases
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EAE	experimental autoimmune encephalomyelitis
Ebi3	Epstein-Barr virus induced gene 3
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
EndoOVA	endotoxin-free ovalbumin
ET	extracellular trap
FACS	Fluorescence-activated cell sorting
Fc	fragment crystallisable region
FcR	Fc receptor
FoxP3	forkhead box P3
FSC	forward scatter
GATA	guanine-adenine-thymine-adenine
GC	germinal centre
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	Guanosine monophosphate
gp	glycoprotein
GSK3c	glycogen synthase kinase 3
H1N1	hemagglutinin type 1 and neuraminidase type 1
H5N1	hemagglutinin type 5 and neuraminidase type 1
HBV	hepatitis B virus
HET-E	incompatibility locus protein from <i>Podospora anserina</i>
HIN200	hematopoietic IFN-inducible nuclear protein with the 200 amino acid repeat
HIV	human immunodeficiency virus
HK	heat-killed
HMGB-1	High Mobility Group Box 1
HPV	human papilloma virus
HRP	horseradish peroxidase
HSA	human serum albumin
HSV	herpes simplex virus
iBMM	immortalised bone marrow-derived macrophages
ICOS	Inducible costimulator
ICOSL	Inducible costimulator ligand
IFI16	interferon-inducible myeloid differentiation transcriptional activator
IFN	interferon
Ig	immunoglobulin
IGIF	IFN- $\gamma$ inducing factor





IL	interleukin
IL-1R	Interleukin-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RaP	Interleukin-1 receptor accessory protein
IL-4R $\alpha$	Interleukin-4 receptor alpha
IL-7R	Interleukin-7 receptor
IL-10R	Interleukin-10 receptor
IL-12R	Interleukin-12 receptor
IL-15R	Interleukin-15 receptor
IL-18BP	Interleukin-18 binding protein
IL-18R	Interleukin-18 receptor
IL-18RaP	Interleukin-18 receptor antagonist
IL-23R	Interleukin-23 receptor
IL-36R	Interleukin-36 receptor
IL-36Ra	Interleukin-36 receptor antagonist
Ilh2	type 2 innate lymphoid helper cells
IRAK	Interleukin-1 receptor-associated kinase
IRES	internal ribosome entry site
IRF	interferon regulatory factor
iNOS	inducible nitric oxide synthase
ISCOM	Immune stimulating complex
iTreg	inducible regulatory T cell
KC	keratinocyte-derived chemokine
LC	light chain
LGP2	laboratory of genetics and physiology-2
LPS	lipopolysaccharide
LRR	leucine-rich repeat
mAb	monoclonal antibody
Mal	MyD88-adaptor-like
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MDSC	myeloid-derived suppressor cell
MDA5	melanoma differentiation-associated protein-5
MDP	muramyl dipeptide
MHC	major histocompatibility complex
MICL	myeloid C-type lectin-like receptor
MIP-1 $\alpha$	macrophage inflammatory protein 1 alpha
MPL	monophosphoryl lipid A
M-MLV	Moloney murine leukemia virus
mLN	mediastinal lymph node
MSU	monosodium urate
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response gene 88
NACHT	NAIP, CIITA, HET-E and TP1



NAD	NACHT-associated domain
NAIP	NLR family, apoptosis inhibitory protein
NF- $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLR	Nod-like receptor
NLRC4	NLR family CARD domain-containing 4
NLRP3	NLR family, pyrin domain-containing 3
NOD	nucleotide oligomerisation domain
nTreg	natural regulatory T cells
ODN	Oligonucleotide
OPD	o-Phenylenediamine dihydrochloride
OVA	ovalbumin
PAMP	pathogen associated molecular patterns
PBS	phosphate buffered saline
PD-1	programmed death 1
PD-L1	programmed death ligand 1
pDC	plasmacytoid DC
PerC	peritoneal exudate cell
PGE2	prostaglandin E2
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinases
PLG	poly (D,L-lactide)-co-glycolide
PMA	phorbol 12-myristate 13-acetate
Poly I:C	polyinosinic:polycytidylic acid
PR3	proteinase 3
PRR	pattern recognition receptor
PTGES	PGE synthase
PYD	pyrin domain
QS21	<i>Quillaja saponaria</i> fraction 21
Rag2	recombination activating gene 2
RIG	retinoic acid-inducible gene
RIP	ribosome inactivating protein
RLR	RIG-I-like receptor
ROR $\gamma$ t	retinoid-acid receptor-related orphan receptor gamma t
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	reverse transcription
SCW	<i>Streptococcus pyogenes</i> cell wall
SIGIRR	single immunoglobulin IL-1R-related molecule
ssRNA	single-stranded RNA
SSC	side scatter
sST2	soluble ST2
ST2	suppression of tumourigenicity 2
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes



Syk	spleen tyrosine kinase
TB	tuberculosis
T-bet	T-box transcription factor
TC	cytotoxic T cell
TCM	central memory T cell
TCR	T cell receptor
TE	Tris and EDTA buffer
TEM	effector memory T cell
Tfh	follicular T cell
TGF- $\beta$	transforming growth factor beta
TH	helper T cell
TIR	Toll/Interleukin-1 receptor
TIRAP	domain-containing adaptor protein
TIV	trivalent inactivated influenza virus vaccine
TLR	Toll-like receptor
TMB	Tetramethyl benzidine TNF
TP1	telomerase-associated protein
Treg	regulatory T cell
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon- $\beta$
TSLP	thymic stromal lymphopoietin
ULK	uncoordinated -51-like kinase
VACV	vaccinia virus
YVAD-fmk	carbobenzoxy-tyrosyl-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone
Zbtb46	zinc finger and BR-C, ttk and bab (btb) domain containing 46
ZVAD-fmk	carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone



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**APPENDIX**



**CHAPTER 1**  
**INTRODUCTION**



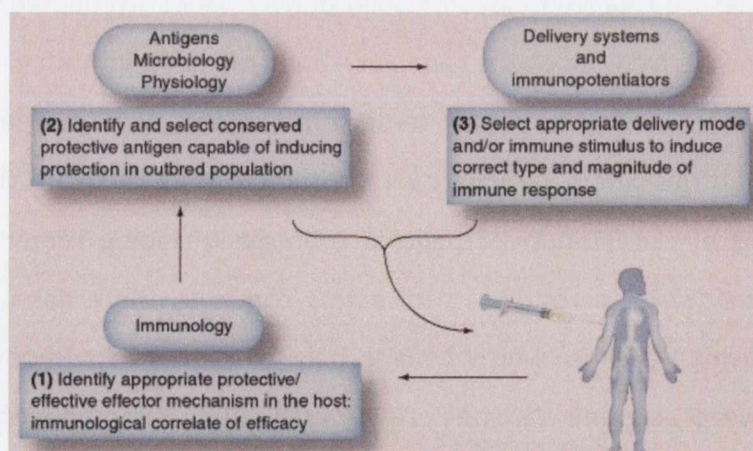
## 1.1.Vaccines

Vaccines are ideally designed to be non-toxic, but at the same time induce a specific, long lasting, preferably lifelong, protection against pathogens. The history of vaccination started in the 18th century with attempts to deal with rampant smallpox epidemics. It was Edward Jenner who introduced the concept of vaccination in 1796 when he demonstrated that infection with the cowpox virus could induce protective immunity against smallpox (1). In the 20<sup>th</sup> century, Louis Pasteur developed many vaccines using attenuated pathogens, including cattle anthrax (in the 1870s) and rabies (in 1885) (2). This attenuation approach has been highly productive in providing the basis for a number of successful vaccines. It later led to the development and widespread introduction to human population of vaccines against measles, mumps, rubella, yellow fever and polio (3). Furthermore, a second polio vaccine was also prepared from inactivated virus and this approach of killing pathogens for vaccination was also applied to pathogens including *Bordetella pertussis*. However, even though these vaccines were highly effective, development of vaccines using live, attenuated or inactivated pathogens is not applicable to all diseases due to safety concerns. For instance, the inactivated pertussis vaccine was demonstrated to induce undesirable reactogenicity at the injection site and some systemic reactions (4). Furthermore, in the 1950s some batches of the inactivated poliovirus vaccine caused poliomyelitis in children, although this was found to be a result of incorrect vaccine preparation (5). Even now, there are reported cases of polio derived from attenuated oral poliovirus vaccine. Vaccine derived poliovirus can cause paralytic polio in humans which can be further transmitted through person-to-person contact. This can lead to serious problems in countries where vaccine coverage is low. For instance, in 2011 alone there were outbreaks of vaccine-derived polio in countries including Yemen, Mozambique and Madagascar (6). As a result of such safety concerns, there has been a drive to develop non-living vaccines which consist only of pathogen components (subunit vaccines). However, subunit vaccines are generally poorly immunogenic and require the addition of an adjuvant to induce protection and to date there are only a few adjuvants in clinical use (Section 1. 12).

**Table 1.1. Childhood vaccination in Ireland.** \*6-in-1 vaccine. Adapted from (7).

Live and attenuated vaccines	Subunit vaccines
Measles	Pertussis*
Mumps	Tetanus*
Rubella	Diphtheria*
Tuberculosis	Hepatitis B virus*
	Haemophilus influenzae type B*
Inactivated vaccines	Pneumococcal disease
Polio*	Meningococcal C disease
	Human Papillomavirus

Overall, despite encountering some problems and the initial slow progress, vaccines are one of the most important developments in medicine over the past centuries. They have led to the eradication of smallpox and dramatically decreased the incidence of other infectious diseases including poliomyelitis, tetanus and pertussis. However, there is a pressing need to develop new vaccines, particularly against HIV, malaria and tuberculosis. There are also some drawbacks to existing vaccines which need to be addressed. For instance, some vaccines do not induce lifelong protection or induce only partial protection, which requires additional, booster vaccinations. Other vaccines protect only against some pathogen strains, e.g. pneumococcal and meningococcal vaccines (8). Importantly, to address these problems, there is a need to understand our immune responses toward pathogens to determine the nature of protective immunity, which will allow for the development of new and improved vaccines (Figure 1. 1).



**Figure 1. 1. Rational vaccine design.** Vaccine design should start with comprehensive understanding of protective immune responses against pathogens, followed by selecting appropriate antigens and delivery systems to induce appropriate immune responses. Figure from (9).

## **1.2.Immune responses**

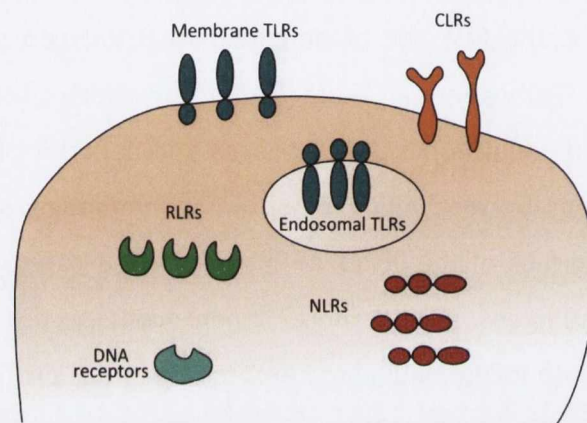
The immune system evolved to enable organisms to recognise and combat pathogens, which can be harmful or even deadly to their host. However, pathogens firstly encounter anatomical barriers, including protective layers of the skin and epithelial cells, which are armed with enzymes and anti-microbial peptides (10). If pathogens breach these barriers, there are obstacles placed by the immune system to overcome. The immune system can be divided into two components: innate and adaptive immunity. Importantly, these systems are linked and both are essential in preventing infections.

The innate immune system is older evolutionarily and found in all forms of plants and animals. It is the first line of defence and is induced shortly following pathogen invasion (11). Pathogens can be recognised by effector cells of the innate immune system including neutrophils, macrophages and dendritic cells (DCs). These cells can clear pathogens by recognition of opsonins (antibodies and complement proteins) coating the surface of the invader. This interaction results in phagocytosis and killing of the pathogens. Furthermore, innate cells secrete cytokines and chemokines which leads to inflammation and in some cases this process can promote the induction of adaptive immunity (12). In contrast, the adaptive immune response depends on antigen recognition by lymphocytes and is directed at specific pathogen-derived antigens. The induction of adaptive immune responses occurs a number of days after the first contact with pathogen. However, the next contact with the same pathogen can lead to rapid recognition and clearance due to immune memory (13).



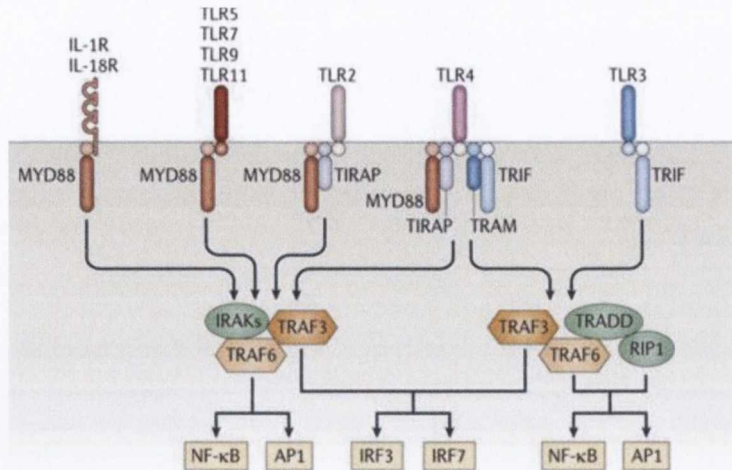
### 1.3. Recognition of infection and cell death

The innate immune response depends on the recognition and distinction of self from non-self using sets of pathogen recognition receptors (PRRs), sensing a limited range of evolutionarily conserved motifs expressed by pathogens, pathogen associated molecular patterns (PAMPs), which are crucial for pathogen survival, and thus cannot be mutated (14). PRRs comprise four main families: Toll-like receptors (TLRs), retinoic acid-inducible gene RIG-like receptors (RLRs), Nod-like receptors (NLRs) and C-type lectin receptors (CLRs) (15-17). Moreover, there are also various cytoplasmic DNA receptors, which belong to different protein families (18) (Figure 1.2 and Table 1.2).



**Figure 1. 2. Pathogen-recognition receptors.** PRRs can be either membrane-associated receptors such as Toll-like receptors (TLRs) or C-type lectin receptors (CLRs) or cytosolic proteins such as Nod-like receptors (NLRs), retinoic acid-inducible gene RIG-like receptors (RLRs) and the emerging family of cytoplasmic DNA receptors. Taken together, PRRs provide a complex and tight system of sensing pathogens and danger by innate immune cells.

TLRs were discovered as the first set of receptors recognising PAMPs, and remain the best-characterized PRRs. TLRs are responsible for sensing invading pathogens outside cells and in intracellular endosomes. The membrane TLRs, TLR1, TLR2, TLR4, TLR5 and TLR6 detect microbial membrane components e.g. lipids, proteins and lipoproteins, while a set of endosomal TLRs, TLR3, TLR7, TLR8, TLR9 recognise nucleic acids derived from pathogens (19). Recently, also it has been demonstrated that mouse TLR11, TLR12 and TLR13 are endosomal receptors which recognise profilin and flagellin and bacterial 23S rRNA (20).



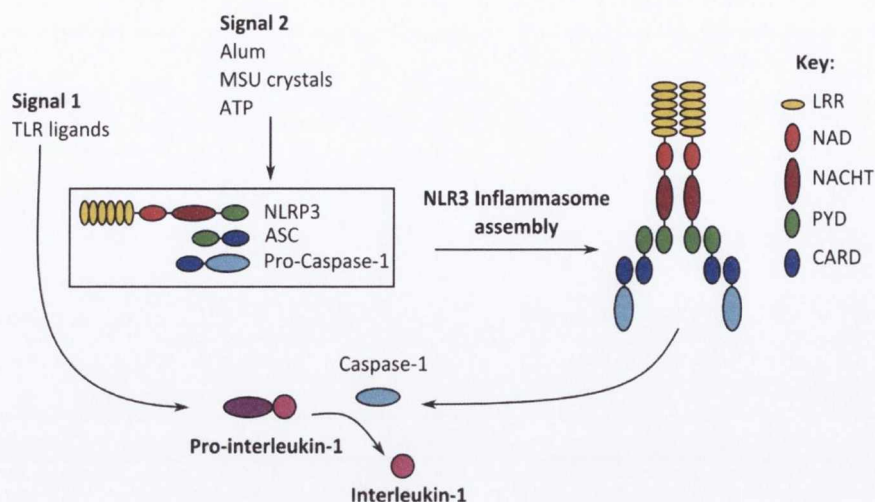
**Figure 1. 3. TLR signalling pathways.** Following ligand binding, the Toll-IL-1 resistance (TIR) domain of TLRs, IL-1R or IL-18R engage TIR domain-containing adaptor protein, myeloid differentiation primary-response protein 88 (MyD88), TIR domain-containing adaptor protein/MyD88-adaptor-like protein (TIRAP/Mal), TIR-domain-containing adaptor protein inducing IFN- $\beta$  (TRIF) or TRIF-related adaptor molecule (TRAM). Engagement of these adaptor proteins leads to activation of downstream kinases such as IL-1R-associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs), which lead to activation of the transcription factors, nuclear factor- $\kappa$ B (NF- $\kappa$ B), the interferon-regulatory factors (IRFs), and activator protein 1 (AP1). TLR signalling induces pro-inflammatory cytokines, and in the case of the endosomal TLRs promotes type I interferon (IFN). Figure from (21).

Another PRR family comprises the CLRs, which can recognise carbohydrates on pathogens, including mannose, fucose and glucan structures. Interestingly, some CLRs are capable of modulating TLR signalling, but do not induce gene expression by themselves (16). One of the best characterized members of CLR family is dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), which can recognise a wide range of pathogens, including HIV, *Mycobacterium tuberculosis* and *Helicobacter pylori* (22). Another, Dectin-1, is involved in recognising fungal pathogens, including *Candida albicans* and *Aspergillus fumigates* (23). There are also orphan receptors, including dendritic cell expressing ITIM-bearing receptor (DCIR), myeloid C-type lectin-like receptor (MCL) and blood dendritic cell antigen 2 (BDCA-2) (16).

Cells can sense pathogen-derived RNA and DNA in a TLR-independent manner due to the presence of DNA and RNA sensors in the cytoplasm. Of these, two nucleic acid sensor families, RNA receptors, RLRs, are better characterized. The RLR family is

composed of retinoic acid-inducible gene I (RIG-I), laboratory of genetics and physiology-2 (LGP2) and melanoma differentiation-associated protein-5 (MDA5). They recognise dsRNA viruses or dsRNA generated during replication of ssRNA viruses (24). It was also proposed that DNA is transcribed into RNA by RNA Polymerase III followed by RNA recognition by RLRs (25). One of the critical components in cytosolic DNA-mediated innate immunity is stimulator of interferon genes (STING) (26). STING is a crucial adaptor protein in inducing type I interferons after recognition of different types of cytosolic DNA. It has been demonstrated that it has a pivotal role in immune responses to bacteria, viruses, self DNA in autoimmune disorders and DNA vaccines (27). However, it is still inadequately understood how different types of cytoplasmic DNA can be detected. In the last few years there have been many reports describing novel cytoplasmic DNA receptors, including DNA-dependent activator of interferon regulatory factor (DAI) (28), interferon gamma-inducible protein 16 (IFI16) (29), DExD/H-box helicases, including DHX9, DHX36, DDX41 and DDX60 (18), and cyclic GMP-AMP (cGAMP) synthase (cGAS) (30, 31). However, while these different sensors interact with cytoplasmic DNA and induce STING-mediated signalling *in vitro*, for most of them, there is a lack of data on whether they are relevant for DNA and virus recognition *in vivo*.

NLRs are cytoplasmic sensors, which recognise not only microbial products but also endogenous danger signals associated with inflammation. The NOD1 and NOD2 proteins were the first NLRs found to drive pro-inflammatory responses following detection of the bacterial peptidoglycan-derived molecules, diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively. In contrast, following activation of cells, some members of the NLR family share the ability to form multiprotein complexes (inflammasomes) which are able to process proforms of IL-1 $\beta$  and IL-18 (32). For instance, on assembly of the NLR family, pyrin domain containing protein 3 (NLRP3) inflammasome, pro-caspase-1 is cleaved producing active caspase-1 which is involved in proteolytic activation of pro-IL-1 $\beta$  and pro-IL-18 (33) (Figure 1. 4). NLRP3 can sense a wide variety of molecules and compounds, including ATP, microbial toxins, including nigericin and  $\alpha$ -hemolysin, silica and monosodium urate crystals and aluminium adjuvants (33).



**Figure 1. 4. Activation of the NLR3 inflammasome.** Activation of NLRP3 by various stimuli leads to a conformational change which allows NLRP3 to oligomerise and facilitates recruitment of ASC and caspase-1; subsequently caspase-1 is activated and can then process pro-IL-1 $\beta$  and pro-IL-18 into their active forms. Figure adapted from (34).

However, it remains unclear how these diverse and unrelated stimuli can trigger NLRP3 inflammasome formation. It has been hypothesised that there is a common intracellular event induced by these stimuli which activates NLRP3. This may be mediated by mitochondrial damage, reactive oxygen species production, and lysosomal damage, formation of large pores in the membrane or potassium efflux from cells. However, most of these events do not occur following exposure of cells to various NLRP3 activators. To date, only efflux of potassium from the cytoplasm has been demonstrated to be a common event during NLRP3 activation and, moreover, reduction in intracellular potassium concentrations alone can induce NLRP3-induced IL-1 $\beta$  secretion (35). Interestingly, it is not only members of the NLR family which are involved in activation of caspase-1 and IL-1 $\beta$ . Absent in melanoma 2 (AIM2), a member of HIN200 family, can also form an inflammasome, in response to dsDNA from *Francisella tularensis*, vaccinia virus or mouse cytomegalovirus (36-38).

**Table 1. 2. PRRs and their ligands** Adapted from (14, 16, 20).

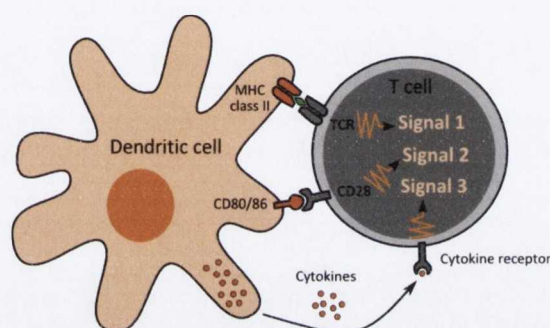
PRRs	Ligand	Example of the ligand source
<b>TLRs</b>		
<b>TLR1/TLR2</b>	Triacetylated lipopeptides	Gram(-) bacteria, mycoplasma
<b>TLR3</b>	dsRNA	Viruses
<b>TLR4</b>	Lipopolysaccharide	Gram(-) bacteria
<b>TLR5</b>	Flagellin	Bacteria
<b>TLR6/TLR2</b>	Triacetylated lipopeptides	Gram(+) bacteria, mycoplasma
<b>TLR7 and TLR8</b>	ssRNA, short RNA	Virus, bacteria, self
<b>TLR9</b>	CpG	Virus, bacteria, protozoa, self
<b>TLR11</b>	Profilin and flagellin	Apicomplexan parasites and bacteria
<b>TLR12</b>	Profilin	Apicomplexan parasites
<b>TLR13</b>	23S rRNA	Bacteria
<b>CLRs</b>		
<b>DC-SIGN</b>	Mannose, Fucose, Glucan	Virus, bacteria, fungi
<b>Dectin-1</b>	B-1,3-Glucans	Fungi
<b>Dectin-2</b>	Mannose	Fungi
<b>RLRs</b>		
<b>RIG-I</b>	Short dsRNA	Virus
<b>MDA5</b>	Long dsRNA	Virus
<b>Cytoplasmic DNA receptors</b>		
<b>AIM2</b>	dsDNA	Virus, bacteria
<b>cGAS</b>	dsDNA	Virus
<b>NLRs</b>		
<b>NOD1</b>	DAP	Bacteria
<b>NOD2</b>	MDP	Bacteria
<b>NLRP3</b>	ATP, MSU crystals, alum, asbestos, silica, $\alpha$ -hemolysis	Damage, endogenous and exogenous particles
<b>NLRC4</b>	Flagellin, Type III secretion system	Bacteria

#### 1.4.Dendritic cells bridge innate and adaptive responses

DCs are a heterogeneous population of innate cells which are involved in shaping immune responses. They are antigen-presenting cells (APCs), which initiate adaptive immune responses by presenting foreign antigens to highly diverse T cell receptors (TCRs) on the surface of T cells. Due to their essential role in initiating adaptive responses, DCs are armed with multiple PRRs, which enable them to sense the external environment. Ligation of PAMPs triggers DC activation and activated DCs migrate to draining lymph nodes, where they present antigen-derived peptides, which bound to MHC class I or II can be recognised by antigen-specific T cells. In response to engagement of PRRs, DCs upregulate co-stimulatory molecules on their surface,

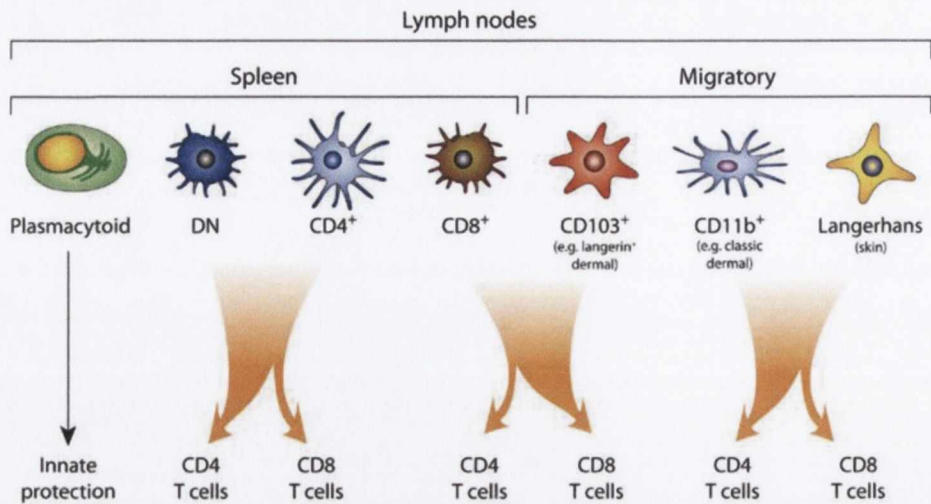
including CD80, CD86 and CD40. Additionally, DCs secrete specific cytokines, which influence the polarisation of T cell responses. In contrast, in the absence of activation, DCs do not upregulate co-stimulatory molecules and thus, presentation of self antigens induces anergy in T cells, which can result in tolerance (39).

Due to their essential role as the initiators of adaptive responses, DCs can be found in the tissues throughout the entire body, especially at sites where pathogen invasion can occur, the mucosal surfaces and skin. However, DCs are heterogeneous and there are distinct populations with different properties regarding their potential for stimulation of an immune response.



**Figure 1. 5. The polarisation of CD4<sup>+</sup> T cells requires three signals.** DCs induce activation and polarisation of naïve CD4<sup>+</sup> T cells via three stimulatory signals. Signal 1 requires pathogen-derived antigen presentation through MHC class II on DCs, which is recognised by an antigen-specific T-cell receptor (TCR). Mature DCs express co-stimulatory molecules including CD80 and CD86, which serve as a signal 2, recognised by complementary ligands on T cells. Furthermore, stimulated T cells require third signal, which will polarise them into specific T helper cell subclasses.

DCs can be subdivided into 2 main subtypes: plasmacytoid DCs (pDCs) and conventional DCs (cDCs). They differ in surface markers and function, as cDCs function mainly in initiation of the adaptive response (40). In contrast, pDCs selectively express TLR7 and TLR9 and have the capacity to produce large amounts of type I IFNs in response to viruses or host-derived nucleic acid-containing complexes (41). However, this division is not sufficient, because cDCs can also be subdivided (Figure 1. 6). For instance, mouse splenic DCs differ in their capacity for antigen processing, CD8<sup>+</sup> DCs are specialised in cross-presentation (presenting extracellular antigens in the context of MHC class I) while CD8<sup>-</sup> DCs specialise in presenting antigen via MHC class II (42).



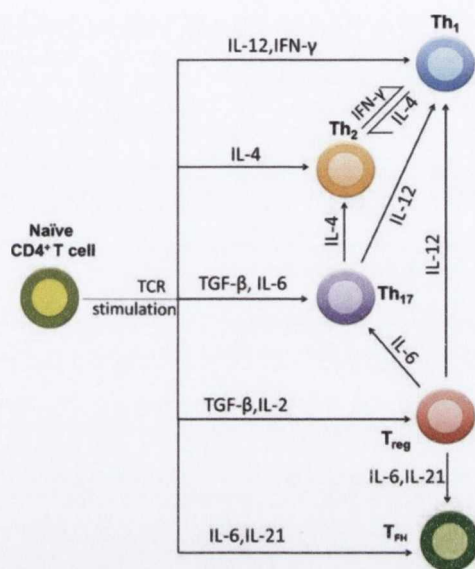
**Figure 1. 6. Dendritic cell subsets.** DCs can be subdivided based on their surface phenotype markers and their localisation. pDCs are involved in innate immune responses against pathogens, while cDCs including resident and migratory DCs are engaged in the adaptive immune responses. In the mouse spleen and lymph node, resident DCs can be divided into three subsets depending on CD4 and CD8 expression, double negative (DN) DCs, CD4<sup>+</sup> DCs and CD8<sup>+</sup> DCs. Furthermore, the lymph nodes also contain migratory DCs, such as CD103<sup>+</sup> DC, CD11b<sup>+</sup> DC, and Langerhans cells. DC subsets can specialise in driving either CD4<sup>+</sup> T cell or CD8<sup>+</sup> T cell adaptive immune responses. Figure from (43).

Interestingly, despite the fact that there is a substantial amount of knowledge about cDC biology and function, a cDCs specific transcription factor, Zbtb46, has only recently been identified (44, 45). Zbtb46 is an important transcriptional repressor in steady-state cDCs, which prevents their activation during homeostasis (46). Until the discovery of Zbtb46, CD11c, which is shared by many cells, including DCs, was used for depletion studies. However, depletion of CD11c<sup>+</sup> cells results in disappearance of, not only cDCs, but also pDCs and some populations of monocytes and macrophages, whereas during depletion of Zbtb46-expressing cells, only cDCs are missing (44). Overall, this novel approach will give significant insights into the contribution of cDCs alone, but not all CD11c-expressing cells in immune responses.

### 1.5. T cells regulate and orchestrate adaptive immune responses

The immune system generates non-specific and antigen-specific responses to ensure clearance of pathogens. Both are essential for host protection, but to develop sustained immunity, antigen-specific memory responses are needed. T cells play a crucial role in adaptive immune responses as effector cells. They can recognise

specific antigens via TCRs and drive immune responses through activation of other cells. DCs can present antigen to two distinct populations of T cells: CD4<sup>+</sup> and CD8<sup>+</sup> T cells via MHC class II and I, respectively (47). Furthermore, antigen-specific CD4<sup>+</sup> T cells can become polarised into subtypes including TH1, TH2, TH17, T follicular helper cells and T regulatory cells (48). These cells drive either cell-mediated immunity, humoral immunity or tolerance.



**Figure 1. 7. Differentiation and plasticity of effector CD4<sup>+</sup> T cells.** Naive T cells differentiate into TH1, TH2, TH17, Treg or Tfh cells depending on cytokines present during differentiation. T helper cells can change their effector phenotype due to cellular plasticity. Figure from (49).

Cell-mediated immune responses provide defence against intracellular pathogens, while humoral immune responses are based on antibody production, which protects against extracellular pathogens and their toxins. Interestingly, differentiated T helper cells can be converted into another effector subset, due to the fact that these subsets retain some degree of plasticity (49).

### 1.5.1. T helper cells

Initially, it was proposed by Coffman and Mossman that CD4<sup>+</sup> T cells can be subdivided into TH1 and TH2 cells, which mediate different types of adaptive immune responses through the cytokines they secrete (50). TH1 cells secrete IFN-γ and TNF-β allowing them to activate macrophages, NK cells and antigen-specific CD8<sup>+</sup> T cells,



which drive cell-mediated immunity. Conversely, TH2 cells produce IL-4, IL-5 and IL-13. These cytokines are responsible for promoting B cell expansion, antibody production and innate immune response, including eosinophil infiltration and mast cell degranulation, the key events in humoral immune responses. Due to this strong polarisation of immune responses, it was proposed that an imbalance in TH1/TH2 responses can result in pathology, including TH2-mediated allergy and asthma or TH1-mediated autoimmunity (51). However, in recent years other CD4<sup>+</sup> T cell subtypes have been discovered, which also impact the outcome of immune response, including TH17 and Tfh cells (52). Interestingly, TH17 cells have been implicated in the pathogenesis of autoimmune diseases, including rheumatoid arthritis and multiple sclerosis. However, these cells also have an important function in combating infections, particularly at mucosal surfaces, where TH17 cells are abundant. The signature cytokine produced by TH17 cells is IL-17A, but these cells can also produce other cytokines, including IL-17F and IL-22 (53).

Naïve CD4<sup>+</sup> T cells polarise into different effector T cells depending on the nature of “signal 2” and “signal 3” provided by DCs, namely co-stimulatory molecules and secreted cytokines. IL-12p70 is a key cytokine produced by antigen-presenting cells, which drive the polarisation of naïve T cells into the TH1 subset. In contrast, the suppression of IL-12 production and expression of OX40 ligand by DCs can induce TH2 polarisation (39). Induction of TH2 responses is also enhanced by DC-independent factors, including IL-4, TSLP, IL-25 or IL-33 (54). TH17 cells require IL-23, TGF- $\beta$  and IL-6 for differentiation (55). Moreover, IL-23 together with IL-1 $\beta$  is essential for the maintenance and amplification of TH17 responses (56). Furthermore, polarisation of T cells depends on specific transcription factors, which are triggered by polarising cytokines secreted by APCs. TH1 development is dependent on IL-12-induced activation of STAT4 and IFN- $\gamma$ -induced STAT1 and their master transcription factor is T-bet. TH2 polarisation is driven by IL-4-induced STAT6 signalling and the master transcription factor GATA3. Interestingly, the transcription factors, T-bet and GATA3 inhibit polarisation to TH2 and TH1 cells, respectively. TH17 cells differentiate through upregulation of the transcription factor ROR $\gamma$ t and this process also requires STAT3 signalling (48).

**Table 1. 3. Polarising and signature cytokines and transcription factors of T helper cell subtypes.** Adapted from (49, 51, 57)

CD4+ T cell	Polarising cytokines	Signalling pathway activated	Transcription factors	Signature cytokines
TH1	IL-12	STAT4	T-bet	IFN- $\gamma$
TH2	IL-4	STAT6	GATA3	IL-4, IL-5, IL-13
TH17	IL-6, IL-23, TGF- $\beta$	STAT3	ROR $\gamma$ t	IL-17A
Tfh	IL-21	STAT3	Bcl-6	IL-21
Treg	TGF- $\beta$	STAT5	FoxP3	IL-10, TGF- $\beta$

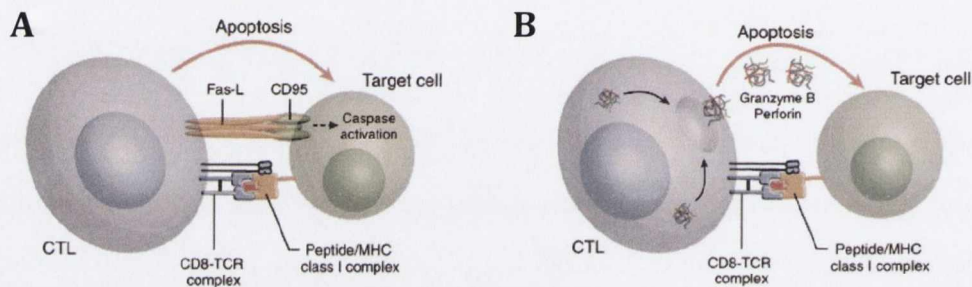
### 1.5.2. T regulatory cells

In contrast to the T helper cells described above, T regulatory (Treg) cells are immunosuppressive lymphocytes, which are essential in maintaining the balance between immunity and tolerance toward various antigens. Indeed, they protect the host from a variety of autoimmune diseases, immunopathology and allergic diseases. However, tolerance can also be dangerous, as in the case of tumours, where regulatory mechanisms compromise their eradication. The anti-inflammatory properties of Treg cells can be mediated by secretion of IL-10, TGF- $\beta$  and IL-35. Furthermore, their development is dependent on STAT5 signalling and their master transcription factor is forkhead box P3 (FoxP3). Treg cells can be subdivided into two subsets based on their origin: natural Treg (nTreg) and inducible Treg (iTreg). nTreg cells develop in the thymus during TCR affinity selection, due to recognition of self-antigens. nTreg are cytokine-independent, constitutively express high levels of CD25 and FoxP3 and represent 5-10% of total CD4+ T cells. On the other hand, iTreg cells need to be polarised into CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells from naïve CD4+ T cells under tolerogenic conditions, such as the presence of TGF- $\beta$  or IL-10 (58). Despite the fact that nTreg and iTreg cells develop independently, both are defined as CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> expressing cells and they cannot be distinguished based on these markers (59). However, recently, it has been demonstrated that nTreg cells express neuropilin-1, while iTreg cells do not (60, 61), which provides an excellent opportunity to establish a role of nTreg versus iTreg in various pathogenic conditions. Interestingly, although the majority of Treg cells are CD25<sup>+</sup>, there are also subsets of CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>-</sup> Treg cells, which have suppressive effects at mucosal surfaces,

including the lungs and intestine (62, 63). However, their origin is still not fully understood.

### 1.5.3. CD8<sup>+</sup> T cells

CD8<sup>+</sup> T cells are cytotoxic cells, which are important players of the adaptive immune responses that control intracellular infections and tumours by killing infected or transformed cells. CD8<sup>+</sup> T cells are able to recognise peptides presented by MHC class I on most cells, followed by direct cytolysis of target cells. Antigen-specific CD8<sup>+</sup> T cells kill cells by inducing apoptosis (Figure 1. 8). Furthermore, CD8<sup>+</sup> T cells can produce cytokines, including IFN- $\gamma$ , which have an impact on polarisation of T helper cells (64).

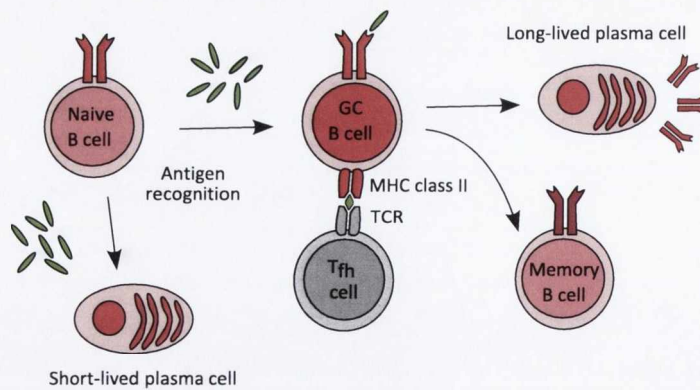


**Figure 1. 8. CTL-mediated cytotoxicity.** Cytotoxic T lymphocytes can kill target cells by inducing apoptosis via triggering death receptors (A) or by release of granzyme B and perforins (B). Figure from (65)

Although CD8<sup>+</sup> T cells predominantly produce IFN- $\gamma$  (66), when cultured under certain conditions, for instance, in the presence of IL-2 and IL-4, they can produce IL-4 (67). Therefore, cytotoxic T cells have been subdivided into cytotoxic T cells type 1 and type 2 (68). This division has been supported by identifying cytotoxic T cells producing IL-4 in leprosy patients (69), patients with chronic obstructive pulmonary disorder (70), asthma (71) and different types of cancer (72, 73). Interestingly, it has been observed in experimental models of tumour challenge that only tumour-specific Tc1, but not Tc2, can protect against tumour growth, which suggests ineffectiveness of Tc2 cells in promoting cell-mediated immunity (74). Importantly, due to their cytotoxic functions CD8<sup>+</sup> T cells are crucial players in mediating protection against viruses and intracellular bacteria and therefore, their activation is essential for developing vaccines against many diseases (75).

## **1.6. Induction of humoral immune responses**

Plasma cells are a key player in the humoral immune responses due to their ability to produce antigen-specific antibodies, which can neutralise toxins and some pathogens. Plasma cells develop from naïve B cells, which encounter foreign antigen. Depending on the type and strength of the interaction between antigen and the BCR, B cells can differentiate into short-lived antibody-secreting plasma cells or germinal centre (GC) B cells. GC B cells require help from follicular helper T (Tfh) cells to undergo somatic hypermutation, isotype switching and affinity-based selection which results in the generation of memory B cells or long-lived plasma cells (Figure 1. 9) (76). T follicular helper cells are defined by high expression of the inhibitory receptor PD-1, CXCR5 and secretion of the signature cytokine IL-21 (77). Recently, it has been shown that Bcl-6 is an essential regulator of Tfh cell development. Overexpression of Bcl-6 in T cells leads to upregulation of CXCR5 and PD-1, while repressing expression of the master transcription factors of other T helper cell subsets (78). Furthermore, Tfh cell-derived IL-21 is required for differentiation of GC B cells (79, 80). Differentiated plasma cells home to the bone marrow, where they produce antibodies for prolonged periods, in some cases even for the entire life span. Plasma cell-derived antibodies are divided into different isotypes according to the differences in the constant region of the heavy chain. There are 5 classes of heavy chain constant domains; IgM, IgG, IgA, IgD, and IgE. Furthermore, IgG can be split into 4 subclasses, IgG1, IgG2, IgG3, and IgG4 in humans and IgG1, IgG2a or IgG2c (depending on genetic background), IgG2b, and IgG3 in mice, each with their own specific properties, which have been attributed to their distinct capacity to interact with Fc receptors (81).



**Figure 1. 9. Differentiation of naïve B cells into plasma cells.** Naïve B cells have to encounter antigen in the lymph node. Upon antigen recognition by its BCR, B cells differentiate into short-lived plasma cells which produce antibodies rapidly after antigen encounter or germinal centre B cells. GC B cells require interaction with T follicular helper cells for additional signals to generate long-lived plasma cells or memory B cells.

IgG subclasses play diverse and important roles in immune responses, including induction of cytotoxicity, phagocytosis and cytokine production. Interestingly, IgG2a/c and IgG2b are considered to be most potent for activating effector responses and dominate in autoimmune (82) and antiviral immunity (83, 84). IgG class switching is influenced by multiple factors, including the cytokine environment and the nature of antigen. IgG1 switching is induced in the presence of the TH2-associated cytokine, IL-4, which also promotes IgE (85), whereas IgG2a/c, IgG2b and IgG3 switching is influenced by the TH1-associated cytokine, IFN- $\gamma$  (86). Additionally, IgG2b switching can be promoted by TGF- $\beta$  (87). Furthermore, protein antigen elicit IgG1, IgG2a/c and IgG2b expression, while carbohydrate antigens induce IgG3 antibody production (88).

While the isotype and subclass of an antibody dictate its effector functions, light chain re-arrangement facilitates antigen-specific recognition. This specificity is crucial, because the immune system can mediate protective responses by production of neutralising antibodies. Induction of neutralising antibodies is seen as the key correlate of protective immunity for vaccines against many diseases, including tetanus, pertussis, diphtheria, polio, hepatitis B and measles (89). However, some pathogens, including HIV, can rapidly change their antigenic profile as a result of their high mutation rate during replication, which allows them to escape immune responses in the host (90). In the case of influenza, the development of a universal vaccine is very challenging, not only because there are multiple strains and subtypes, but also these

vary every year (91). However, there are increasing efforts to induce broadly neutralising antibodies (bnAbs), which bind to conserved motifs on viral proteins. This would potentially diminish the challenge of antigen shift and multiple viral strains (92). Such bnAbs have been identified in patients and engineered *in vitro* in the case of HCV, HIV and influenza with the help of phage display technology and B cell cloning (92). Broadly neutralising monoclonal antibodies target epitopes on the envelope proteins of HIV and influenza, which are critical for virus evasion. In the case of HIV, bnAbs target different sites on the envelope protein gp120 (93, 94) while surprisingly, influenza cross-reactive antibodies bind to the conserved region of the hemagglutinin stem, which is suggested to be important for membrane fusion (95). However, despite increasing knowledge, there is still a lack of immunogens and vaccination technologies capable of inducing bnAb *in vivo* in animal models or humans.

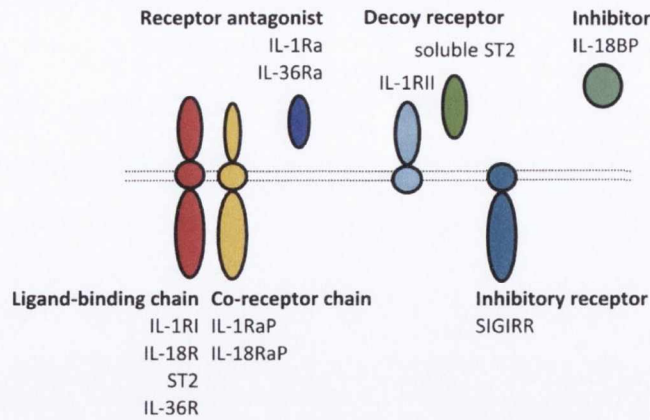
### **1.7.The IL-1 family regulates immune responses**

The IL-1 family consists of 11 members of which IL-1 $\alpha$ , IL-1 $\beta$ , IL-1Ra, IL-18 and IL-33 are the best characterised (Table 1. 4) (96). It has become apparent that these cytokines play a substantial role in shaping both innate and adaptive immune responses and are implicated in the pathogenesis of many diseases. IL-1 family members share a conserved gene structure, which suggests that they derive from a common ancestral gene. However, IL-1 family members differ significantly in their processing, secretion and biological functions. Interestingly, although they are all extracellular cytokines, only IL-1Ra possesses a signal peptide which enables its secretion via the endoplasmic reticulum and Golgi apparatus (97). The secretion mechanism is poorly understood in the case of the other members. Interestingly, members of the IL-1 family can be processed by various enzymes, which can change their biological functions. For instance, precursors of IL-1 $\beta$  and IL-18 require cleavage to become biologically active (98, 99). On the other hand, IL-1 $\alpha$  and IL-33 processing modulates their functions, but is not necessary for their activation (100).

**Table 1. 4. Members of IL-1 family** Adapted from (101).

Cytokines	Family name	Processing required for activity	Receptor	Inhibitors
<b>IL-1<math>\alpha</math></b>	IL-1F1	no		
<b>IL-1<math>\beta</math></b>	IL-1F2	yes	IL-1RI/IL-1RaP	IL-1RII, IL-1Ra, SIGIRR
<b>IL-18</b>	IL-1F4	yes	IL-18R/IL-18RaP	IL-18BP, SIGIRR
<b>IL-33</b>	IL-1F11	no	ST2/IL-1RaP	sST2, SIGIRR
<b>IL-36<math>\alpha</math></b>	IL-1F6			
<b>IL-36<math>\beta</math></b>	IL-1F8	?	IL-36R/IL-1RaP	IL-36Ra, SIGIRR
<b>IL-36<math>\gamma</math></b>	IL-1F9			
<b>IL-37</b>	IL-1F7	?	Unknown	Unknown
<b>IL-38</b>	IL-1F10	?	Unknown	Unknown
<b>IL-1Ra</b>	IL-1F3	no	Blocks IL-1RI	n/a
<b>IL-36Ra</b>	IL-1F5	?	Blocks IL-36R	n/a

IL-1 family members signal through closely related receptor complexes, which contain a ligand binding chain and co-receptor. IL-1 family receptors belong to the Toll like/IL-1 receptor superfamily, members of which share an intracellular TIR domain (102). After cytokine binding to its corresponding ligand, a co-receptor is recruited and signalling is initiated by TIR domain interactions between receptor subunits. The resulting receptor complex can recruit adaptor proteins, such as MyD88, followed by activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), which ultimately leads to production of cytokines and chemokines (Figure 1. 3) (102). Due to their potency, IL-1 family cytokine signalling is extensively regulated. For instance, IL-1Ra and IL-36Ra compete with IL-1 and IL-36, respectively, for binding sites on their corresponding receptors (97, 103). Furthermore, the receptor IL-1RII which lacks a TIR domain, a soluble version of IL-33 receptor ST2 (sST2), and IL-18 binding protein (IL-18BP) can bind IL-1 $\beta$ , IL-33 and IL-18, respectively, to block their interaction with agonist receptors (104-106). Apart from these mechanisms targeting specific members of the IL-1 family, there is also an inhibitory receptor, SIGIRR (single immunoglobulin IL-1R-related molecule), which interacts with IL-1, IL-18, IL-33 and IL-36 receptors and inhibits their intracellular activation (107).



**Figure 1.10. IL-1 family members signalling.** IL-1 family members signal through a set of agonist receptors, which are tightly regulated by various mechanisms. Adapted from (96).

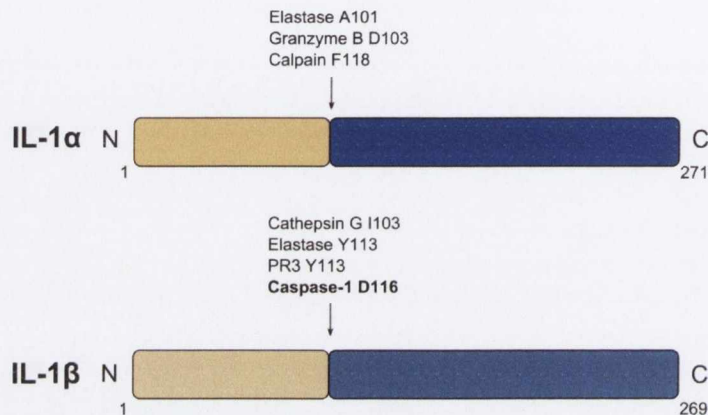
### 1.7.1. IL-1 $\alpha$ and IL-1 $\beta$

IL-1 was described as the first interleukin in 1979 (108) and had been studied under various names, including endogenous pyrogen, lymphocyte activating factor and catabolin, reflecting its multiple functions when administered systemically (109). Both, IL-1 $\alpha$  and IL-1 $\beta$  are potent inducers of innate immune responses. IL-1 induces swelling, pain and fever, which are the physiological outcomes of the upregulation of pro-inflammatory cyclooxygenase type 2 (COX2) and inducible nitric oxide synthase (iNOS), leading to nitric oxide production, prostaglandins and platelet activation factor release (96). Furthermore, IL-1 promotes chemokine release and upregulation of adhesion molecules by endothelial cells, which leads to infiltration of inflammatory cells, particularly neutrophils (96). Interestingly, apart from inducing innate responses, IL-1 also plays a substantial role in regulating adaptive immune responses. For instance, IL-1 is required for the generation of TH17 cells (56) (Section 1. 5. 1). Although IL-1 $\alpha$  and IL-1 $\beta$  share the same receptor complex and can induce the same signalling pathways, their distinct expression, processing and release impacts on their biological functions *in vivo*.

IL-1 $\beta$  secretion is mostly restricted to innate immune cells, including monocytes, macrophages and DCs. IL-1 $\beta$  requires a two steps activation process to become fully active. There has to be a signal to promote expression of pro-IL-1 $\beta$  in the cytoplasm. However, pro-IL-1 $\beta$  requires cleavage by caspase-1 to become bioactive (99) and commonly inflammasomes are engaged in this process (110) (Section 1. 3).



Interestingly, there have been reports showing the involvement of other enzymes in IL-1 $\beta$  cleavage. For instance, neutrophil and macrophage-derived serine proteases, such as elastase, cathepsin G or proteinase-3 can cleave the IL-1 $\beta$  precursor into a bioactive form (111). Furthermore, caspase-8 can form a non-canonical inflammasome, which can also process IL-1 $\beta$  following fungal or mycobacterial sensing (112) or Fas, a TNF family receptor, activation (113).



**Figure 1. 11. Processing of IL-1 $\alpha$  and IL-1 $\beta$ .** Intracellular and extracellular proteases can cleave IL-1 $\alpha$  and IL-1 $\beta$ . IL-1 $\alpha$  can be processed by elastase, calpain and granzyme B, while IL-1 $\beta$  can be processed by cathepsin G, elastase, PR3 and caspase-1. The amino acid recognition sites are indicated beside enzymes. The IL-1 domain is represented in blue. Adapted from (100).

IL-1 $\alpha$  is mainly retained in the nuclei of cells, because it contains a nuclear retention signal sequence (114). In contrast to IL-1 $\beta$ , it can be constitutively expressed, for instance in the nuclei of keratinocytes, endothelial cells, fibroblasts and mucosal epithelial cells (115). It has been suggested that nuclear IL-1 $\alpha$  can participate in transcription (116). Furthermore, in some cell types, such as monocytes and B cells, it can also be expressed on the cell surface (117). The mode of IL-1 $\alpha$  release from cells has not been fully elucidated. However, IL-1 $\alpha$  is considered an endogenous danger signal, which is widely expressed and retained in cells during homeostasis, but released passively in its full length form during necrosis to induce inflammation (118, 119). For instance, injection of mice with necrotic cells promoted neutrophil infiltration, dependent solely on IL-1 $\alpha$ . This was shown by demonstrating that anti-IL-1 $\alpha$  antibodies abolished neutrophil influx but, anti-IL-1 $\beta$  or deficiency in caspase-1 did not (118).

Interestingly, IL-1 $\alpha$  has intact biological function in its precursor form, but it has been demonstrated that IL-1 $\alpha$  processing can modulate its activity. For instance, granzyme B, calpain or elastase can enhance the potency of IL-1 $\alpha$  (120).

The potency of IL-1 as a pro-inflammatory cytokine is reflected in its pathological role in a number of diseases. IL-1-related diseases are diverse, but are often induced by the accumulation of endogenous or exogenous particles leading to sterile inflammation. Indeed, the neurodegenerative disease, Alzheimer's disease, the pulmonary disease, asbestosis, the cardiovascular disease, atherosclerosis and the joint disease, gout, are induced by deposition of the crystals, amyloid- $\beta$ , asbestos and silica, cholesterol crystals, and monosodium urate crystals, respectively (121). Interestingly, IL-1 can be also pathogenic in ischemic diseases, such as stroke (122) or myocardial infarction (123), where resolution of the inflammation is crucial to maintain tissue homeostasis. Furthermore, some diseases can be triggered by gain-in-function mutations which lead to spontaneous release of IL-1 $\beta$ . For instance, mutations in the NLRP3 protein, which lower the threshold for inflammasome assembly, have been associated with rare genetic disorders, hereditary periodic fever syndromes including, Muckle-Wells syndrome, familial cold autoinflammatory syndrome and chronic infantile neurological cutaneous and articular syndrome (124).

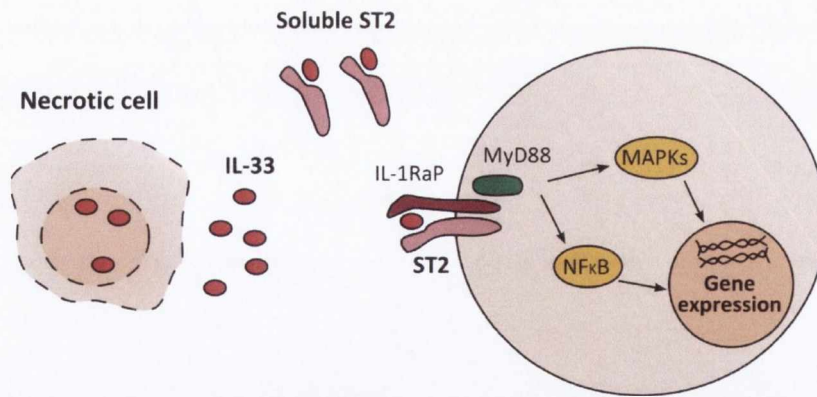
### **1.7.2. IL-18 modulates cellular immune responses**

IL-18, a pro-inflammatory cytokine, which promotes IFN- $\gamma$  production, was originally named IFN- $\gamma$  inducing factor (IGIF) (125). Similarly to IL-1 $\beta$ , IL-18 is synthesised as a precursor which needs to be processed by caspase-1 in order to become bioactive (98). However, there is a significant difference between these two cytokines. Unlike IL-1 $\beta$ , the IL-18 precursor is constitutively expressed by monocytes, macrophages and the barrier cells, keratinocytes and epithelial cells (126-128). IL-18 secretion is tightly regulated. IL-18-binding protein (IL-18BP) is a highly expressed soluble factor, unrelated to any other member of the IL-1 family and it has high affinity for soluble IL-18 (129). However, once the IL-18 is in excess, it can bind to its unique receptor subunit, IL-18R which associates with IL-18RaP (signalling pathway on Figure 1. 3). In contrast to IL-18, expression of IL-18R is limited. For instance, it is expressed by

TH1, but not TH2 cells (130). However, it is dispensable for initial TH1 polarisation, but provides an amplifying signal for proliferation and cytokine production (131). Interestingly, regulatory T cells can also express IL-18R. However, in contrast to TH1 cells, IL-18 suppresses their function, rather than activating them (132). Furthermore, natural killer cells express IL-18R and similarly to TH1 cells, IL-18 enhances IFN- $\gamma$  production in the presence of IL-12 (133). IL-18 also augments the cytolytic properties of NK cells by induction of Fas ligand (134) and perforins (135), which induce death in target cells.

### **1.7.3. IL-33 modulates humoral immune responses**

IL-33 is a more recently discovered member of the IL-1 family, which is suggested to play an important role in TH2 polarisation (136). Similarly to IL-1 $\alpha$ , IL-33 is expressed in the nucleus of many cells, predominantly by barrier cells e.g. skin keratinocytes, epithelial and endothelial cells (137). Although IL-33 is expressed abundantly, its mechanism of release is still unclear. Initially, it was proposed that IL-33, similarly to IL-1 $\beta$  and IL-18, was cleaved and activated by caspase-1. However, it was later observed that IL-33 is not cleaved by physiological concentrations of caspase-1 or the other inflammatory caspases, caspase-4 or caspase-5. Moreover, the apoptosis associated caspases, caspase-3 and caspase-7, can efficiently process IL-33, which leads to attenuation of its biological activity (138). IL-33 does not have a secretory sequence and one possibility is that IL-33 is released from cells during necrosis as an active, full-length cytokine. Furthermore, it has been demonstrated that IL-33 activity can be enhanced by neutrophil-released cathepsin G and elastase. Therefore, during inflammation, when IL-33 is released and neutrophils are recruited, a highly active form of IL-33 may be present (139).



**Figure 1. 12. The IL-33-ST2 signalling pathway.** IL-33 released during cell necrosis can bind to the ST2 receptor or, its soluble version, sST2. IL-33-ST2/IL-1RaP interaction facilitates the activation of the MyD88 dependent pathway, activating (NF- $\kappa$ B) and MAP kinases, which leads to expression of inflammatory cytokines. Figure adapted from (140).

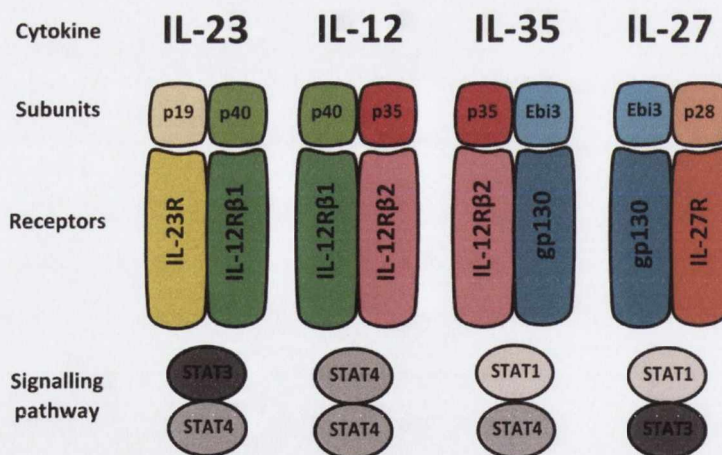
IL-33 is a ligand for the heterodimeric receptor composed of a unique receptor subunit ST2 and IL-1RaP, which is expressed by a limited number of cells, including granulocytes, type 2 innate lymphoid cells, TH2 cells and CD8<sup>+</sup> T cells. The role of IL-33 was firstly investigated in T cells, because ST2 is selectively expressed on TH2 cells, but not on TH1 cells (141). Indeed, naïve T cells polarised *in vitro* into TH2 cells and stimulated with IL-33 produce more IL-5 and IL-13. Moreover, *in vitro*, IL-33 can polarise naïve T cells toward IL-5 and IL-13-producing T cells, in an IL-4 independent manner (142). It has also been demonstrated that IL-33 is potent inducer of CD8<sup>+</sup> cytotoxic T cells responses in mice infected with viruses (143).

In addition to its role in T cell responses, the ST2 receptor is also expressed by many innate cells, thus IL-33 may have an impact on very early immune responses, before antigen-specific T cells are involved. Lineage-negative c-kit<sup>+</sup> nuocytes and innate type 2 helper cells which express ST2 were recently observed (144, 145). IL-33 has been demonstrated to induce proliferation and robust IL-13 production by these type 2 innate lymphoid helper (Ilh2) cells. However, there are only a few reports describing these IL-13-producing cells and further work is needed to dissect their role in humoral and cellular immune responses. ST2 is also expressed by mast cells (146), eosinophils (147) and basophils (148) and IL-33 is potent stimulator of these cells. For instance, mast cells stimulated *in vitro* with IL-33 produce the pro-inflammatory cytokines IL-1, IL-6 and TNF- $\alpha$ , and the chemokines MCP1 and MIP-1 $\alpha$  (146). IL-33 also stimulates mast cell degranulation, maturation and survival. Furthermore, IL-33 triggers an anaphylactic response in mice, in a mast cell-dependent manner (149). In addition, IL-

IL-33 can directly enhance eosinophil activation and survival *in vitro* and it induces airway eosinophilia even in Rag2-deficient mice (150). Furthermore, the effect of IL-33 was investigated in asthma, where mast cells, eosinophils and basophils play a crucial role. Indeed, IL-33 is upregulated in the lungs of asthmatic patients and in mouse models of asthma (151, 152) and administration of IL-33 to the lungs of naïve mice enhanced airway inflammation (142). Consistent with this observation, blocking antibodies to IL-33 or ST2 during airway challenge attenuated inflammation (153). Recent reports suggest a role for IL-33 in regulating neutrophil functions. IL-33 modulated the migration of activated neutrophils in two mouse models of disease, sepsis and rheumatoid arthritis (154, 155). However, IL-33 is not a chemoattractant, but instead upregulates expression of the chemokine receptor CXCR2 on neutrophils.

### 1.8. Modulation of cell-mediated immunity by the IL-12 family

The IL-12 family of cytokines is critical for cell-mediated immunity, because it shapes TH1 and TH17 immune responses (156). It is a unique cytokine family, because its members are heterodimeric cytokines, sharing subunits and molecular partners, but concurrently mediating diverse biological functions. The IL-12 family includes, pro-inflammatory (IL-12 and IL-23), immunoregulatory (IL-27) and, anti-inflammatory (IL-35) cytokines.



**Figure 1. 13. IL-12 family members.** Members of the IL-12 family are heterodimeric cytokines, which share p40, p35 and Ebi3 subunits. However, they mediate different immune responses. IL-12, IL-23, IL-27 are involved in initiation of cell-mediated immune responses, while IL-35 suppresses T cell proliferation. Adapted from (157).

Its firstly described member, IL-12 is involved in TH1 cell polarisation and promoting IFN- $\gamma$  production. IL-12 is preferentially secreted by APCs, including DCs in response to PAMPs. IL-12 is biologically active as a heterodimer composed of two subunits, p35 and p40, which are shared with IL-35 and IL-23, respectively (158). The other pro-inflammatory member of the IL-12 family, IL-23 is also secreted by APCs and takes part in polarising immune responses. In contrast to IL-12, IL-23 takes part in the polarisation of TH17 responses. IL-23 shares its IL-12p40 subunit with IL-12, but also comprises a unique IL-12p19 subunit (159). IL-27 possesses both pro-inflammatory and regulatory properties. Similarly to IL-12, IL-27 is involved in promoting TH1 responses, but only early in the initiation of polarisation (160). Interestingly, it is also implicated in regulation of TH17 cells by the induction of IL-10 producing T cells during experimental autoimmune encephalomyelitis (EAE) (161). Overall, due to their important roles in cell-mediated immunity, any changes in IL-12, IL-23 and IL-27 expression might change the outcome of immune responses. Interestingly, another member of the IL-12 family, IL-35 has an opposing effect on T cell polarisation when compared to IL-12, IL-23 and IL-27. It has been described as a suppressor of T cell proliferation. Moreover, along with IL-10 and TGF- $\beta$ , IL-35 is expressed by Treg cells and contributes towards their regulatory functions (162).

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases, which catalyse the addition of a phosphate molecule specifically to the inositol ring of phosphoinositides. These enzymes are key players in many cellular responses, including rearrangement of the cytoskeleton, cell growth, chemotaxis and survival (163). There are 4 classes of PI3Ks, Ia, Ib, II and III, each involved in different signalling pathways. Class Ia PI3Ks comprise 3 subclasses,  $\alpha$ ,  $\beta$  and  $\delta$ , which are involved in receptor-mediated signalling during immune responses. One of the most profound examples is the negative regulation of IL-12. It has been observed that DCs stimulated with TLR ligands produce IL-12. However, in the absence of PI3K class Ia signalling, stimulated DCs secrete higher level of IL-12 (164). Furthermore, there are two distinct PI3K-mediated pathways which block IL-12 production. Class Ia PI3Ks activate Akt kinase which has two target molecules, target of rapamycin (mTOR) and glycogen synthase kinase 3 (GSK3). Activation of mTOR leads to IL-10 secretion and IL-10-dependent IL-12 inhibition. GSK3 is involved in PI3K-mediated IL-12 inhibition in a distinct manner, because when not

blocked by Akt it promotes IL-12 production. Overall, PI3Ks regulate IL-12 production by negatively regulating the IL-12 inducer, GSK3, and promoting secretion of anti-inflammatory IL-10 via mTOR signalling (165).

Furthermore, PI3 kinase-deficient mice exhibit impaired immunity due to unbalanced polarisation of immune responses. Infections by protozoan and helminth parasites trigger opposing and highly polarised TH1 and TH2 responses, crucial in maintaining infection by the host. Mice deficient in p85 $\alpha$ , the regulatory subunit for all class Ia PI3Ks, are susceptible to infection with the intestinal nematode *Strongyloides venezuelensis*, due to a lack of gastrointestinal mast cells. Only reconstitution of p85 $\alpha$ -deficient mice with TH2-conditioned p85 $\alpha$ <sup>+/+</sup> mast cells restores anti-parasite immune responses. Non-polarised p85 $\alpha$ <sup>+/+</sup> mast cells do not promote humoral responses, which indicates that the lack of PI3kinase skews immune responses toward TH1 (166). Moreover, PI3 kinase class Ia  $\delta$ -deficient mice have elevated TH1 responses and are able to control *Leishmania major* infection, while wild-type BALB/c mice succumb to infection due to TH2 biased responses (167).

### **1.9. Suppression of immune responses by IL-10**

One of the most important cytokines involved in limiting excessive immune responses is IL-10, which was originally identified by Mosmann *et al.* as a factor produced by TH2 cells which inhibits cytokine production by TH1 cells (168). Since this discovery the list of cells which can produce IL-10 and information on the functions of this cytokine has greatly expanded. IL-10 can be produced by cells of both the innate and adaptive immune system. T cells are a significant source of IL-10, especially Th2 and Treg cells, but it can also be produced by TH1, TH17 and CD8<sup>+</sup> T cells (169). Other cells producing IL-10 include monocytes, macrophages, some DCs, B cells and some granulocytes. There are also non-immune cellular sources of IL-10 including keratinocytes, epithelial cells and some tumour cells (169). Moreover, most immune cells respond to IL-10 as they express the IL-10 receptor (IL-10R) (170).

IL-10 is necessary to maintain the balance between tolerance and immunity and it is crucial in limiting autoimmune pathologies. IL-10 deficient mice are much more prone to autoimmune diseases, e.g. EAE (171). It has been suggested that IL-10

plays a role in the regulation of EAE by suppressing pathogenic TH1 responses. Moreover, during infections, deregulation of IL-10 can cause impaired or exacerbated immune responses toward pathogens. In the case of protozoal infections, mice deficient in IL-10 upregulate pro-inflammatory cytokines, resulting in immunopathology, which is lethal (172, 173). However, over-expression of IL-10 can also be detrimental. In the case of pathogens where TH1 responses are required for protection, such as *Mycobacterium tuberculosis*, this leads to impaired immune responses and chronic infection (174). Some effects of IL-10 can be explained by its impact on shaping and polarising immune responses. A role for IL-10 in promoting a shift from a TH2 to TH1 response was shown in studies where IL-10 or IL-10R blocking antibodies were used. Neutralisation of IL-10R in mice immunised with OVA in the presence of low concentrations of LPS led to an OVA-specific TH1 response. However, the same concentrations of LPS were not sufficient to prime TH1 responses in the absence of blocking antibody (175). This suggests that under these conditions, the induction of TH1 responses requires not only a pathogen-derived molecule, but also neutralisation of the endogenous immunosuppressive cytokine (175). In the case of infections where a TH1 response is crucial for protection, blocking IL-10 signalling by monoclonal antibodies can lead to stronger protective immunity. It was shown in mice that injection of anti-IL-10R antibodies results in better clearance of *Mycobacterium tuberculosis*. However, this effect was observed only in BALB/c mice, but not in C57BL/6 mice, because BALB/c immune responses are biased toward TH2 polarisation and during TB there is significant production of IL-10, which can be reversed by anti-IL-10R antibodies (176).

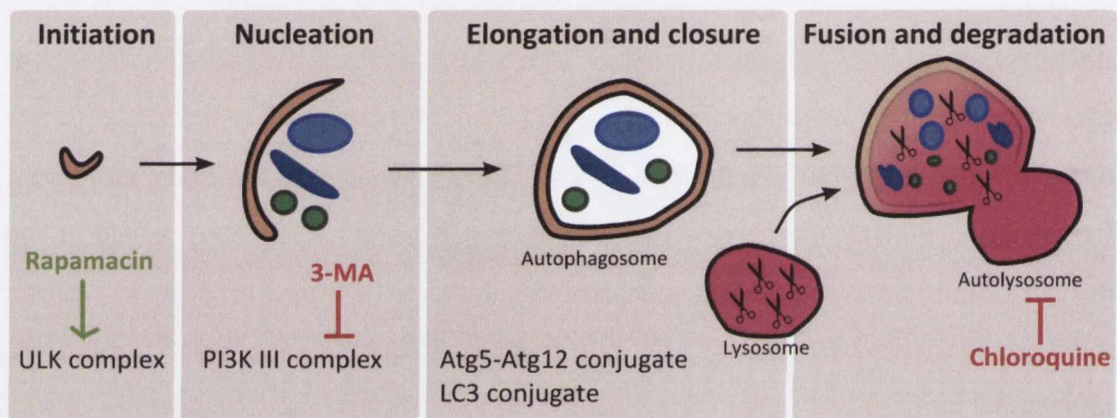
### **1.10. Autophagy can modulate immune responses**

The term “autophagy” originates from the Latin word, which means “self-eating”. It is the catabolic mechanism conserved in eukaryotes which involves degradation of various cellular components to ensure survival. Autophagy can be triggered by a range of stimuli, including starvation, the sensing of damaged organelles, misfolded proteins or intracellular pathogens (177). These various signals promote the formation of a double-layered membrane, which can elongate and engulf



cytoplasmic components. The enclosed isolation membrane forms an autophagosome, which fuses with the lysosome to form the autolysosome. The content of the vesicle is degraded by lysosomal enzymes (178) (Figure 1. 14).

Autophagy plays an important role in host defence. For instance, autophagy can eliminate intracellular pathogens by delivering them to lysosomes. However, some pathogens can prevent their eradication by autophagy and even exploit this process for survival and replication inside the host cell. For instance, bacteria can mask themselves against autophagic recognition, block autophagosome maturation, which delays their lysis, escape autophagosome or develop resistance to the acidic environment in the autolysosome (177).



**Figure 1. 14. The different stages of autophagy.** Autophagy is initiated by inhibition of mammalian target of rapamycin (mTOR) kinase, which can be activated by rapamycin. The next step, vesicle nucleation requires the activation and the formation of the PI3 kinase III complex. The elongation process engages two complexes, conjugation of two proteins, Atg5 and Atg12 and lipid conjugation of phosphatidylethanolamine to LC3 (LC3-I), which leads to accumulation of converted LC3 (LC3-II) on the autophagosomes. LC3-II remains on autophagosomes until their fusion with lysosomes and formation of autolysosomes. Autophagy can be inhibited by various drugs, including 3-methyladenine and chloroquine. Adapted from (179).

It has been proposed that autophagy can directly act as an important negative regulator of inflammatory responses by targeting cytokine production. For instance, autophagy modulates the processing and secretion of the pro-inflammatory cytokine, IL-1 $\beta$ . Autophagy can sequester pro-IL-1 $\beta$  and inflammasome components, leading to their degradation (180, 181). Interestingly, IL-23 is also negatively regulated by autophagy (182) and the regulation of IL-1 and IL-23 production by autophagy may

modulate the IL-17 secretion and the excessive inflammation associated with autoimmune responses (182).

### **1.11. Immunological memory**

The ability to remember past experiences and the induction of rapid responses after re-encounter with the same antigen is the defining feature of the adaptive immune system. This property is crucial in prophylactic vaccines, which aim to mimic primary infection and subsequently give protection upon pathogen encounter. The development of memory T and B cells and antibody-producing plasma cells has been extensively studied for more than 50 years.

It is established that B cell memory is mediated by sustained antibody production by plasma cells and long-lived memory B cells. Both are generated primarily in germinal centres following antigen-specific B cell proliferation, but the underlying molecular mechanisms are not fully understood (76, 183). A number of factors are needed to induce and sustain humoral immune response, including cell-cell interaction between B and T cells (Fas, ICOSL, PD-L1 on B cells) and the cytokines, IL-4 and IL-21, secreted by Tfh cells (77). Memory B cells can be defined as IgG<sup>+</sup> and IgM<sup>+</sup> memory B cells. Emerging data suggest that IgM<sup>+</sup> memory cells are more prone to regenerate GCs, while IgG<sup>+</sup> memory cells tend to generate antibody-forming cells upon restimulation (184). Memory B cells can also be defined by surface marker expression and mutation level, but the mechanistic basis for this difference is unknown (183).

Although antibody production by plasma cells can be maintained for decades, the persistence of plasma cells is not intrinsic, but depends on a specific niche within the bone marrow (185). Stromal cells surrounding plasma cells provide survival signals either by direct contact or secretion of cytokines. For instance, they produce IL-6, a key factor in promoting plasma cell survival. Moreover, hematopoietic cells, including eosinophils, megakaryocytes and myeloid precursors express members of the TNF superfamily, a proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF), which are essential for long-lived plasma cell maintenance (76). However, there is limited space for plasma cells in their niches; therefore plasma cells compete for survival. Thus, newly differentiated plasma cells migrate to the bone marrow and

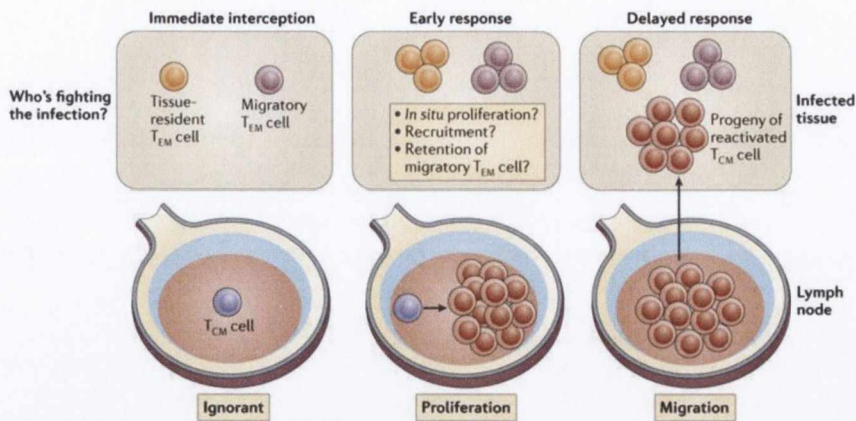
displace resident plasma cells generated during previous immune challenges. This model provides an explanation for a progressive decline of serum antigen-specific antibodies over time (186). Interestingly, there is evidence that long-lived plasma cells can be generated in the absence of germinal centre formation, in a T cell-independent manner. For instance, mice immunised with a *Streptococcus pneumoniae* capsular polysaccharide vaccine generate plasma cells independently of GC formation (187).

T cell memory is maintained by a small pool of memory T cells, which differentiate from a subset of effector T cells when their population contracts following the resolution of infection or vaccination (188). Memory T cells are highly diverse with specialised effector functions, which allows them to play distinct but cooperative roles during re-infection (189).

**Table 1. 5. Major subpopulations of memory T cells.** Adapted from (189).

Subset	Surface markers	Proliferation potential
Tissue-resident T <sub>EM</sub>	CD69, CD103	Little
Circulating T <sub>EM</sub>	CCR5	Little
T <sub>CM</sub>	CD62L, CCR7	High

Memory T cells have been subdivided into central and effector memory T cells (T<sub>CM</sub> and T<sub>EM</sub>), which differ in expression of lymph-node homing receptors, their requirement for re-differentiation into effector cells and proliferation potential (Table 1. 5) (190). Similarly to naïve T cells, T<sub>CM</sub> recirculate through secondary lymphoid organs and upon recognition of antigen give rise to effector T cells, which migrate into the site of infection. In contrast T<sub>EM</sub> circulate in the blood and non-lymphoid tissues and in the event of re-infection, they can rapidly respond to pathogens without proliferation. Interestingly, more recent studies showed that T<sub>EM</sub> cells can be further subdivided into circulating and tissue-resident T<sub>EM</sub> cells (191). Due to their small number, tissue-resident T<sub>EM</sub> have been described only recently.



**Figure 1. 15. Memory T cell subsets play distinct but cooperative roles during re-infection.** Resident  $T_{EM}$  cells immediate reaction to re-infection is crucial as they can eliminate or at least control infection, before circulating  $T_{EM}$  would migrate to the site. Furthermore, if resident and recruited  $T_{EM}$  cells cannot clear infection, after a few days, reactivated  $T_{CM}$  in draining lymph nodes differentiate and proliferate to leading to a new wave of effector cells migrating into the infected tissue Figure from (192).

Specific stimuli are required to maintain memory T cell survival. It has been demonstrated that memory T cells need IL-7 and IL-15 in their environment (193). Interestingly, tissue-resident memory T cells express lower levels of IL-7R and IL-15R, but they are maintained in the tissue by their expression of CD69 and CD103, which sequester them and prevent them from entering the circulation (194). Despite the fact there is increasing knowledge about the effector functions of memory T cells, their differentiation from effector T cells during the contraction phase remains poorly understood.

## 1.12. Adjuvants

Vaccines are ideally designed to be non-toxic and to induce a specific, long lasting immune response against pathogens. There is a move away from traditional live attenuated and killed vaccines toward subunit vaccines. However, safe, subunit antigens are generally less immunogenic than whole organisms. Therefore, there is a need for additional components, adjuvants, in subunit vaccines, which can enhance and/or direct immune responses against antigens (195). The term “adjuvant” originates from the Latin word, *adiuvare*, which means “to help” and adjuvants can be composed of diverse materials with distinct mechanisms of action. The history of adjuvants started in 1925, when Ramon observed that the antitoxin response to tetanus and diphtheria toxoid was enhanced by co-administration of foreign materials, such as agar, saponins, starch oil or breadcrumbs (196). Indeed, adjuvants play a crucial helping role in vaccination by enhancing immune response against even poorly immunogenic antigens. Adjuvants can also provide additional assets for vaccines. They can reduce the dose of the antigen required for protection and increase the efficacy of vaccines in children, older people and immunocompromised individuals (9).

Currently only a few adjuvants are in clinical use, namely, alum, the squalene based oil-in-water emulsions MF59 and adjuvant system 03 (AS03), and adjuvant system 04 (AS04), a combination of alum and the TLR4 ligand, monophosphoryl lipid A (MPLA) (197). Until 1997, alum had been the only adjuvant used clinically in vaccine preparations. Alum proved to be efficient in vaccines for diseases including diphtheria, tetanus and pertussis, where neutralising antibodies are crucial for protection (Table 1. 5) (198). The squalene-based adjuvants, MF59 and AS03, have been incorporated into vaccines against influenza virus to enhance protection (199). The conventional trivalent inactivated seasonal influenza virus vaccine (TIV), contains three virus strains that are expected to affect people during the upcoming winter. However, this vaccine has limitations, mainly in terms of the duration of protection and immunogenicity. TIV does not induce long lasting protective immunity, because influenza viruses include many strains, which are also highly variable. Therefore, people have to be vaccinated every year. Furthermore, vaccination with TIV does not always ensure protection

against influenza and does not provide optimal protection in elderly people (200). Importantly, unexpected outbreaks of the pandemic strains, H1N1 and H5N1, raised a further challenge to vaccine production. To improve on the conventional vaccine and possibly generate a universal influenza vaccine, several strategies have been investigated, in order to induce stronger and broader immune response against influenza. Some of these attempts have led to the licensing of new vaccines. Since 1997, an MF59-adjuvanted subunit vaccine has been available. MF59 improved the immunogenicity of the vaccine and, importantly, allows the induction of optimal protection with a lower dose of antigen, which is crucial for a pandemic emergency, where the generation of sufficient vaccine is difficult (201). Another new licensed influenza vaccine is based on AS03, which is a squalene-based and  $\alpha$ -tocopherol-containing oil-in-water emulsion. An advantage of the AS03-adjuvanted vaccine is the induction of cross-reactive antibody responses against heterologous viral challenge (202). However, it has only been used with the prepandemic H5N1 influenza vaccine, but not in seasonal vaccines.

Another approach to induce a protective immune response is to develop vaccines which combine two or more adjuvants, such as the licensed AS04, a combination of alum and MPLA. Ideally this approach would induce synergistic effects to trigger stronger immune responses than individual adjuvants. This addition of a TLR ligand to alum led to induction of higher antibody titres with longer persistence (203). AS04 is a component of two licensed vaccines, one against hepatitis B virus (HBV), Fenderix, and the other against oncogenic Human Papilloma Virus (HPV), Cervarix (204). Moreover, an AS04-based herpes simplex virus (HSV) vaccine was tested, which unfortunately failed in Phase III clinical trials (205). Interestingly, while AS04 is used in the HPV vaccine, Cervarix, produced by GlaxoSmithKline, alum alone is a component of another HPV vaccine, Gardasil, manufactured by Merck. When immune responses were compared in a randomised trial, higher neutralising antibody production was detected in women immunised with Cervarix (206). However, there is still a lack of data regarding efficacy in preventing cervical cancer for both vaccines since they were only adopted recently.

Overall, the spectrum of the diseases which can be prevented by these licensed adjuvants is broad, but simultaneously limited (Table 1.6), due to the fact that none of

these adjuvants can induce strong cell-mediated immune responses. Thus there is a pressing need to develop new adjuvants and the list of clinically applied adjuvants for vaccination will probably expand.

**Table 1. 6. Adjuvants in clinical use.** Adapted from (198, 201, 202, 204).

Adjuvant	Composition	Vaccines
<b>Alum</b>	aluminium hydroxide aluminium phosphate	Pertussis, Tetanus, Diphtheria <i>Haemophilus influenzae</i> type b Pneumococcus Hepatitis A and B Papilloma virus
<b>MF59</b>	Oil-in-water emulsion	Influenza
<b>AS03</b>	Oil-in-water emulsion	H5N1 Influenza
<b>AS04</b>	Alum + MPLA	Papilloma virus

Adjuvants comprise a wide variety of molecules and formulations, which stimulate immune responses by different mechanisms. They can be categorized based on their physical and chemical properties and they include:

- Particulate adjuvants
  - Mineral salts e.g. alum, Calcium Phosphate (207)
  - Biodegradable microparticles e.g. PLG (208)
  - Emulsions e.g. MF59 (209)
  - Liposomes (210)
  - ISCOMs (Immune stimulating complexes) (211)
  - VLPs (Virus-like particles) (212)
- Immunostimulatory adjuvants
  - Microbial derivatives e.g. LPS derivative MPLA or CpG (213)
  - Bacterial toxins e.g. Cholera toxin, *E. coli* heat-labile toxin (214)
  - Saponins e.g. QS21 (215)
- Combination vaccine adjuvants e.g. AS03 and AS04 (203, 216)

### **1.13. Aluminium adjuvants**

Aluminium-based adjuvants include several preparations, for instance potassium aluminium sulphate, aluminium phosphate, aluminium hydroxide, and Imject alum, a mixture of aluminium hydroxide and magnesium hydroxide. In the literature, these are all commonly referred to as “alum”, which can be misleading. Furthermore, only two of these, aluminium hydroxide (Alhydrogel) and aluminium phosphate (Adju-Phos) are used in licensed vaccine preparations (217, 218). Alum was originally used for protein precipitation, mainly for diphtheria and tetanus toxoids in an attempt to purify them. Interestingly, in 1926 Alexander Glenny and colleagues showed that diphtheria toxoid precipitated with potassium aluminium sulphate induced stronger antibody responses than toxoid alone (219). Since this discovery, alum has been applied in many vaccine formulations (198). However, to standardise the procedure of vaccine preparation, it is now common to adsorb antigens onto the standardised commercially available preparations, Alhydrogel or Adju-Phos (218).

Aluminium adjuvants elicit strong humoral immune responses which are mediated by secreted antigen-specific antibodies, particularly IgG1, both in mice and humans (220, 221). Therefore, alum is applied for vaccines against diseases where neutralising antibodies to bacterial toxins and viruses are required for protection (198). Moreover, alum increases the level of allergy-associated IgE and is used in murine models of allergy and asthma as the inducer of pathological immune response against allergens (222). However, alum is a relatively poor inducer of cell-mediated immune responses and is therefore unsuitable for HIV, malaria or tuberculosis vaccines, where strong cellular immunity is essential (223). It is still not fully understood how alum can induce and mediate immune responses and it is only recently that significant interest has been shown in the mechanism of action of alum.

#### **1.13.1. Depot theory**

Aluminium adjuvants are composed of poorly soluble particles, whose size depends on their exact chemical structure. For instance, Alhydrogel (aluminium hydroxide) particles have an average size of 10 $\mu$ m (a range of 3 to 15 $\mu$ m) (224), while Adju-Phos (aluminium phosphate) is around 2 $\mu$ m (225, 226). However, these particles



are further composed of nanoparticles, which in solution aggregate into microparticles. This complex structure gives aluminium adjuvants attractive adsorption properties (227). While these properties have been well established in laboratory conditions, aluminium adjuvants lose their structure when administered into tissues. It was reported that alum forms nodules at the site of injection (219) and when they are ground up and injected into a naïve animal, they induce immune response against the adsorbed antigen (228). Based on these observations, it was proposed that alum works as an antigen “depot”, retaining the antigen at the injection site and allowing its slow release. However, despite the fact that the “depot” theory was formulated decades ago, the kinetics of development, composition and importance of the alum “depot” or alum nodules were only described recently. Nodules are formed within 4 hours after alum administration due to interaction of the clotting agent fibrinogen with alum (229). However, these nodules do not only contain alum and antigen, but are also composed of extracellular chromatin, histones, extracellular myeloperoxidase and neutrophils (229). The composition of alum nodules is very similar to extracellular traps formed by neutrophils (230). However nodule formation seems dispensable for alum adjuvanticity, because fibrinogen-deficient mice, which do not form an alum depot after vaccination, were found to have similar antigen-specific IgG1 titres compared to wild-type mice (229). Furthermore, to verify whether the alum depot is essential for long-lasting immune responses, nodules were surgically removed as early as 2 hours post injection and the humoral immune response was analysed for up to 35 days post immunization. Surprisingly, this early removal of the alum depot did not affect the magnitude of the antigen-specific IgG1 responses (231). Overall, these findings contradict the initial theory that the aluminium depot is essential for developing antigen-specific immune responses.

### **1.13.2. Recognition of alum by the immune system**

The means by which alum is recognised by the immune system is still not fully elucidated. Recent reports proposed that alum is not recognised by any receptor, but rather sensed by plasma membrane lipids (232). For instance DCs, which are essential in initiating adaptive immune responses, treated with pronase, an enzyme which

cleaves surface proteins, can still interact with alum. Thus, it has been proposed that alum is recognised by surface lipids, which form lipid rafts, rather than protein receptors. Lipid rafts are enriched in sphingolipids and cholesterol, which makes them different from the surrounding cell membrane (233). Interactions of alum with cholesterol and sphingolipids (e.g. sphingomyelin) on the DC surface leads to receptor-independent cell activation dependent on the Syk-PI3 kinase pathway. Furthermore, according to this report, DCs do not phagocytose alum and any internalisation of alum was followed by cell death. In contrast to DCs, macrophages can internalize alum. In agreement with this observation, it was reported that *in vitro*, macrophages are loaded with crystalline aluminium inclusions (234). Moreover, following the injection of an aluminium-containing vaccine, it can be observed that macrophages infiltrating muscle tissue also contain intracellular alum (207). Nevertheless, a direct comparison of the ability of DCs, macrophages and other cell types to interact and internalize alum has not been conducted.

Furthermore, in agreement with the study showing that alum is not recognised by protein receptors, alum does not seem to engage TLRs. Mice deficient in the TLR adaptor proteins MyD88 and TRIF produce comparable levels of antigen-specific antibodies to wild-type mice (235, 236). However, MyD88-deficiency has an effect on alum-induced innate responses. MyD88-deficient mice were found to have impaired recruitment of inflammatory monocytes carrying antigen to the draining lymph nodes following alum injection (237). However, this effect could be explained by the fact that MyD88 is also involved in transducing signals from the IL-1 receptor.

Indeed, apart from lipid interactions, alum can also activate the NLRP3 inflammasome. Upon alum activation, NLRP3 along with ASC and caspase-1 forms an inflammasome which leads to caspase-1 activation and processing of IL-1 $\beta$  and IL-18 from their proforms. DCs and macrophages primed with LPS and stimulated with alum produce IL-1 $\beta$  and IL-18 in a NLRP3-dependent manner (238). However, the involvement of IL-1 and NLRP3 in promoting innate and adaptive immune response to alum is controversial. It has been observed that NLRP3-deficient mice had impaired cell recruitment to the site of alum injection and draining lymph nodes. Local secretion of IL-1 $\beta$  was reduced, which may explain the reduced numbers of neutrophils, eosinophils, monocytes and DCs (239). Furthermore, not only was there a decreased

number of inflammatory monocytes carrying antigen into draining lymph nodes, but their activation was also impaired, as reflected in lower expression of MHC class II and the co-stimulatory molecule CD86 (239). However, these results were not reproduced in IL-1 or caspase-1 deficient mice (240, 241). Moreover, there is also controversy about the involvement of NLRP3 in alum-induced adaptive immune responses. Two studies reported that NLRP3-deficient mice have defects in alum-driven antigen-specific IgG1 (236, 242). However, several other studies have questioned the role of NLRP3 in alum-induced humoral responses (239, 241, 243). One report showed that NLRP3<sup>-/-</sup> and wild-type mice immunised with alum and OVA have similar antigen-specific IgG1 titres. Furthermore, NLRP3<sup>-/-</sup>, caspase-1<sup>-/-</sup> and wild-type mice displayed comparable number of antigen-specific T cells in draining lymph nodes and spleens, following injection with alum and antigen (241). Interestingly, two reports showing contradictory results on the role of NLRP3 in alum-driven IgG1 antibodies, both observed that the absence of NLRP3 impaired alum-induced IgE responses (239, 242).

Alum promotes not only IL-1 $\beta$  and IL-18 *in vitro*, but also prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production (244). Prostaglandins are lipid mediators involved in the induction of inflammatory responses. Furthermore, PGE<sub>2</sub> is involved in shaping immunity by suppressing TH1 responses. Interestingly, PGE<sub>2</sub> is produced only by primed macrophages, but not DCs, which further suggests that there is a difference in alum sensing between these cell types. Furthermore, PGE<sub>2</sub> production is Syk and p38-MAPK-dependent, but NLRP3 inflammasome-independent. In addition, mice deficient in PGE synthase (PTGES), have reduced levels of alum-induced antigen-specific IgE production *in vivo*, but antigen-specific IgG1 titres are intact in Ptges<sup>-/-</sup> mice. However, PGE<sub>2</sub> induction was not measured *in vivo* during alum-driven innate or adaptive responses, and thus it is unclear what the mechanism of PGE<sub>2</sub>-dependent IgE production is.

Although it has been reported that alum can interact with DCs and induce IL-1 $\beta$  and IL-18 processing, it is still not understood how alum initiates adaptive immune responses. Alum does not induce (245) or only weakly induces maturation of DCs *in vitro* (232). In addition, alum-driven production of polarising cytokines by DCs is undetectable. *In vivo* studies are more informative regarding the mechanism by which alum activates DCs. Indeed, at the site of injection, alum induces the recruitment of

inflammatory monocytes and DCs, enhances uptake and processing of antigens and induces DC maturation by increasing MHC class II, CD40 and CD86 expression (237). However, this effect is probably indirect as *in vitro* alum does not directly lead to dendritic cell activation. Furthermore, alum enhances the migration of inflammatory monocytes from the injection site to lymph nodes resulting in an increase in the number of DCs in draining lymph nodes (237).

### **1.13.3. The innate immune response to alum**

Intraperitoneal administration of alum is an established model to determine local innate immune responses due to easy isolation of peritoneal exudate cells. In unimmunised mice, resident macrophages are the major cell population found in the peritoneal cavity (246). Upon alum injection these cells are involved in sensing and shaping immune response (241). However, other cells including mast cells, endothelial cells, fibroblasts and other leukocytes may also play a role in sensing the adjuvant. Alum-induced immune responses are rapidly induced. Just 2 hours post administration, alum induces the production of chemokines, including the neutrophil chemotaxin KC, monocyte chemotactic protein MCP-1 and the eosinophil chemotaxin eotaxin (237) and 2 hours later the cytokines IL-1 $\beta$ , IL-5, IL-6 can be detected at the site of injection (241). Interestingly, the resident mast cells and macrophages disappear from the peritoneal cavity 6h post alum injection. Simultaneously, alum induces a rapid recruitment of leukocytes (237, 241). Neutrophils are the first line of defence and are attracted shortly after injection, followed by inflammatory monocytes (which are precursors of DCs) and also DCs are recruited to the site of injection. Moreover, the number of eosinophils is also increased, but their accumulation is slower after alum administration (241).

Importantly, within the infiltrating cells are IL-4-expressing cells which have an impact on polarisation of alum-induced adaptive immune responses. In the absence of IL-4 and the IL-4 receptor, mice develop similar antigen-specific TH2 responses compared to wild-type mice when immunised with alum and antigen. However, significantly increased levels of antigen-specific (TH1-related) IgG2a and decreased levels of IgE are detected in IL-4 and IL-4R $\alpha$ -deficient mice (247, 248). These data

suggest that IL-4 is involved in suppressing TH1 responses. Further studies showed that the alum-induced IL-4 is antigen-independent and the IL-4 expressing cells are Gr-1<sup>+</sup> innate cells. When Gr-1<sup>+</sup> IL-4-producing cells were sorted they were found to be mainly eosinophils (249). IL-4 producing cells accumulate as early as 1 day post injection at the site of injection, in the peritoneal cavity while their accumulation peaks at day 6 in the spleen. When Gr-1<sup>+</sup> cells are depleted from the peritoneum using an anti-Gr-1 antibody, mice immunised with alum exhibited not only TH2 but also TH1 responses, which further suggests a role for alum-induced IL-4 in modulating cellular immunity (241). While Gr-1<sup>+</sup> IL-4-producing cells resemble eosinophils, Gr-1 is not a specific marker for eosinophils, therefore eosinophil-deficient, Phil mice and GATA1-deficient mice were used to confirm their involvement in blocking TH1 responses (241). Phil mice express diphtheria toxin under the control of the eosinophil peroxidase promoter, which results in a lack of eosinophils, while  $\Delta$ GATA1 mice have a mutation in GATA1, which prevents eosinophil differentiation. However, there was no decrease in alum-induced OVA-specific IgG1 or IgG2a/c in eosinophil-deficient  $\Delta$ GATA1 and Phil mice. The only effect observed was an increase of antigen-specific IgE in  $\Delta$ GATA1 mice.

#### **1.13.4. Endogenous danger signals**

Although alum-induced innate and adaptive immune responses are well described, the underlying question of how alum initiates these events remains. One of the proposed mechanisms is via the induction of endogenous danger signals. Damage-associated molecular patterns (DAMPs) are released by damaged tissues and cells undergoing necrosis. Indeed, intramuscular injection of alum induces necrosis in the surrounding tissue (207). Moreover, cell death is also induced in the peritoneal cavity following injection of alum (250). Therefore, it is very likely that the injection of alum releases DAMPs. To date, uric acid and host DNA have been shown to be released following alum administration (237, 250). Uric acid is released into the peritoneal cavity as early as 2 hours after intraperitoneal alum injection. Uricase treatment of mice injected with alum decreases antigen-specific T cell and humoral responses, which indicates that uric acid is important for alum adjuvanticity. Moreover, uric acid has similar properties to alum. *In vitro* it can induce IL-1 $\beta$

production by primed DCs. Moreover, *in vivo* uric acid induces TH2 responses, which resemble allergic responses (251). Recently, host DNA was described as a new DAMP released after alum injection. It is accumulated in the peritoneal cavity as early as 3 hours after injection. Moreover, treatment of mice immunised with alum with DNase decreased antigen-specific IgG1 and IgE responses and immunisation with dsDNA mimics alum-induced adaptive immune responses. Interestingly, another report showed that alum-induced DNA release is critical for DC-T cell interactions and pre-treatment of mice with DNase impairs these interactions, which leads to partial reduction in antigen-specific IgG1 production. However, the lack of mediators of DNA signalling, STING or IRF3, did not decrease antigen-specific IgG1 titres (250, 252). Only antigen-specific IgE was reduced in the absence of IRF3 (250). Therefore, it still unknown how DNA released during immunisation mediates alum adjuvanticity.

#### **1.13.5. Polarisation of immune responses by alum.**

Alum strongly promotes humoral immunity and TH2 responses, but poorly enhances cellular immune responses. Alum induction of antigen-specific IgG1 antibodies can lead to enhanced protection against bacterial toxins and viruses, as in the case of tetanus, pertussis or HBV vaccines (198). Furthermore, alum is used for induction of allergy and asthma in mice, because it is an effective inducer of IgE antibodies (253). However, alum does not provide protection against intracellular pathogens, such as *Mycobacterium tuberculosis* (254) or HIV (255). Despite decades of use the mechanism underlying this polarisation is not fully understood. However, based on our present knowledge there are a number of candidate cytokines and pathways which may be involved in the selective enhancement of humoral responses and inhibition of cellular immunity. Crucially, the outcome of immune responses depends on the differentiation of naïve CD4<sup>+</sup> T cells into T helper cells. Indeed, when mice are immunised with alum and antigen there is an increase of antigen-specific CD4<sup>+</sup> T cells in draining lymph nodes and spleens (252, 256). Also cells isolated from mice immunised with alum and antigen produce the TH2 signature cytokines, IL-4, IL-5 and IL-13 when restimulated *ex vivo* with antigen (237, 250). TH2 cells, associated with humoral immunity, enhance the production of IgE and IgG1 and overall are crucial not

only in responses against extracellular pathogens, such as parasites, but also in pathologic allergic reactions (48). Surprisingly, in the absence of specific TH2-associated cytokines, alum-induced IgG1 responses were not reduced. For instance, while IL-4 and IL-13 signalling (via STAT6) is involved in modulating alum-induced immune responses, it is dispensable for the development of antigen-specific IgG1 responses (248). Furthermore, alum triggers not only antigen-specific IgG1, but also high titres of IgG2a (TH1-related) in STAT6-deficient mice, while IgE production is reduced. Similar results were obtained in mice deficient in IL-4 (247). In contrast, IL-5-deficient mice respond in the same manner as wild-type mice when immunised with alum, which suggests that IL-5 is not necessary for immunity induced by alum (241, 248). Therefore, the classical TH2-associated cytokines are not required for the induction of protective humoral immune responses during alum vaccination, but they might influence the magnitude of these responses. This suggests that there are other key cytokines and/or pathways regulating alum adjuvanticity.

Although alum is a poor inducer of cellular immune responses, there are reports which suggest that alum can induce antigen-specific memory CD8<sup>+</sup> T cell development (256, 257). However, their generation is limited due to increased expression of the inhibitory PD-1 receptor on their surface. Therefore, it has been suggested that CD8<sup>+</sup> T cells primed using alum require additional stimuli to induce memory CD8<sup>+</sup> T cells, e. g. the LPS-derivative MPL. Furthermore, alum with MPL provided better protection for influenza challenge than alum alone (257). Interestingly, when this experiment was repeated in the same laboratory, alum without MPL induced better protection in mice challenged with influenza (256).

## 1.14. Hypothesis

Alum is an essential adjuvant in vaccines where the induction of neutralising antibodies is required for protection. However, it is a poor inducer of cell-mediated immune responses; therefore, alum is useful only for a limited number of vaccines. Furthermore, the mechanism of alum action has not been fully elucidated and this presents an obstacle in finding enhanced adjuvant strategies. The hypothesis underlying this thesis is that the poor efficacy of alum in promoting cell-mediated immunity is a result of adjuvant-induced immunomodulatory cytokine production.

Alum is a potent inducer of IL-1 $\beta$ . However, while there are numerous reports demonstrating that alum-induced IL-1 $\beta$  secretion is NLRP3-dependent *in vitro*, there are conflicting data about involvement of NLRP3 inflammasome in alum-driven IL-1-dependent innate responses. It is proposed here that the NLRP3 inflammasome has a minimal role in shaping alum-driven immune responses.

Another proposed mechanism of alum adjuvanticity involves alum-induced release of DAMPs at the site of injection. Currently there are only two danger signals associated with alum induced damage, uric acid and host DNA. Here it is hypothesised that alum can induce inflammation-associated necrosis, which releases the endogenous danger signal IL-33 at the site of injection, which impacts on the outcome of the immune response.

Moreover, it is hypothesised that alum also drives immunosuppressive mechanisms during vaccination, which can explain the poor induction of cell-mediated immune responses by alum.



### **1.15. Aims and objectives**

- To comprehensively describe the role of IL-1, IL-18 and the inflammasome components, NLRP3, ASC and caspase-1, in alum-induced innate immune responses
- To characterise the nature of cell death promoted by alum
- To investigate whether alum promotes IL-33 release *in vivo*
- To investigate the role of IL-33 in alum-mediated innate immune responses and its potential to influence the outcome of alum immunisation
- To investigate the effects of alum on TLR-induced cytokines produced by dendritic cells
- To assess the ability of alum to promote IL-10 production, and thereby, compromise TH1 responses *in vivo*

## **CHAPTER 2**

# **MATERIALS AND METHODS**



## 2.1. Materials

All materials were purchased from Sigma-Aldrich unless stated otherwise.

### 2.1.1. General tissue culture materials and treatments for animal studies

#### Complete RPMI 1640 culture medium (cRPMI)

Roswell Park Memorial Institute (RPMI)-1640 medium (Biosera) was supplemented with 8% (v/v) heat-inactivated foetal calf serum (Biosera), 2mM L-glutamine (Gibco) and 50U/ml penicillin and 50µg/ml streptomycin (Gibco). Additionally, cRPMI medium was supplemented with 1mg/ml of selection antibiotic geneticin (Gibco) for J558 cell culture.

#### Complete DMEM culture medium (cDMEM)

Dulbecco's Modified Eagle Medium (DMEM) medium (Biosera) was supplemented with 8% (v/v) heat-inactivated foetal calf serum (Biosera), 2mM L-glutamine (Gibco) and 100µg/ml penicillin/streptomycin (Gibco).

#### 0.88% Ammonium Chloride Buffer

0.88g NH<sub>4</sub>Cl was dissolved in 100ml of endotoxin-free H<sub>2</sub>O (Baxter) and filter sterilised with a 0.22µm syringe-driven filter (Millipore).

#### PBS

Sterile and pyrogen-free (Biosera).

**Table 2. 1. TLR ligands**

TLR ligand	Target	Supplier
LPS from <i>Escherichia coli</i> , Serotype R515	TLR4	Enzo Life Sciences
CpG ODN 1826	TLR9	Oligos etc
Zymosan	TLR2	Invivogen
Heat-killed (HK) <i>E. coli</i> (strain BL21)	Various	n/a

## HK *E. coli*

This work was carried out by Dr Eimear Lambe in the Adjuvant Research Group, School of Biochemistry and Immunology, Trinity College Dublin. BL21 strain of *E. coli* was streaked on a lysogeny broth (LB) agar plate and was left to grow overnight at 37°C. An individual bacteria colony was transferred into 10ml of liquid LB and was left shaking to grow overnight at 37°C. The culture was then transferred into 100ml of liquid LB and was incubated shaking for 2h at 37°C. To determine the number of bacteria within the culture, serial dilutions were spread onto LB agar plates which were subsequently incubated overnight at 37°C. The number of colonies were counted and multiplied by the dilution factor. The number of bacteria in the culture was confirmed by measuring the optical density at 600nm using a spectrophotometer. The *E. coli* were killed by heating for 15 minutes at 70°C and were then spread onto LB agar plates to ensure that no growth occurred. The cells were pelleted by centrifugation and resuspended in PBS to a final concentration of  $1 \times 10^9$  cells/ml.

**Table 2. 2. Adjuvants and endogenous danger signals used *in vitro* and *in vivo*.**

Adjuvants	Source
Alhydrogel (referred to as Alum)	Brenntag Biosector, Frederikssund, Denmark
Adju-Phosphate	Brenntag Biosector, Frederikssund, Denmark
Calcium Phosphate	Brenntag Biosector, Frederikssund, Denmark
Chitosan	Novamatrix, Norway
Polylactide-co-glycolide (PLG) Microparticles	Novartis Vaccines
Polystyrene Microparticles	Corpuscular Microspheres-Nanospheres, USA
Monosodium Urate Crystals	Enzo Life Sciences

## Preparation of PLG microparticles

This work was carried out by Padma Malyala, Manmohan Singh and Derek O'Hagan in Novartis, Cambridge, MA, USA. 1µm microparticles in 0.5% Dioctyl Sodium Sulfosuccinate (DSS) were prepared using a double emulsion solvent evaporation method. Anionic microparticles were prepared by homogenizing 10ml of 6% w/v polymer solution in methylene chloride with 2.5ml of PBS using a 10mm probe (Ultra-Turrax T25 IKA-Labortechnik, Germany) with a water:oil ratio of 1:4. After

homogenizing for 2 minutes, the emulsion formed was added to 50ml of distilled water containing dioctylsulfosuccinate surfactant, and the mixture was homogenized at very high speed with a 20mm probe (ES-15 Omni International, Jarrenton, VA) for 5 minutes in an ice bath. This procedure resulted in the formation of a water-in-oil emulsion that was then stirred at 1000rpm for 12 hours at room temperature; the methylene chloride was allowed to evaporate. The resulting suspension was washed twice and the PLG microparticles were reconstituted to the original volume.

**Table 2. 3. List of reagents used for *in vitro* restimulation assays**

Reagent	Supplier
NA/LE Hamster Anti-Mouse CD3e	BD Pharmingen
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich

**Table 2. 4. List of inhibitors**

Inhibitor	Target	Supplier
CA-074-Me	Cathepsin B	Sigma-Aldrich
3-Methyladenine (3-MA)	Type III PI3 kinases	Sigma-Aldrich
Cytochalasin D	Actin polymerization	Sigma-Aldrich
Chloroquine (CQ)	Increase lysosomal pH	Invitrogen
Wortmannin	PI3 kinases	Calbiochem
YVAD-fmk	Caspase-1	Bachem
ZVAD-fmk	Caspases	Bachem
Uricase	Monosodium Urate	Sigma-Aldrich

**Table 2. 5. Antigens used for *in vivo* studies**

Reagent	Supplier
Ovalbumin, Grade V	Sigma-Aldrich
Human serum albumin (HSA)	Novozymes Biopharma UK Ltd
Endotoxin-free ovalbumin (EndoOVA)	Hyglos

## Ovalbumin

Ovalbumin was used as an antigen in studies examining the adjuvanticity of alum in mice. However, ovalbumin obtained from Sigma contains endotoxins, which can modulate the immune responses induced by vaccination. Therefore, to remove

endotoxins, Detoxi-Gel endotoxin removing columns (Thermo Fisher Scientific) were used. Ovalbumin was resuspended in sterile H<sub>2</sub>O (100mg/ml) and filter sterilised with a 0.22µm syringe-driven filter. Three endotoxin-removing columns were regenerated by washing with five resin-bed volumes (5 x 1ml) of 1% sodium deoxycholate followed by ten resin-bed volumes of endotoxin-free water. The ovalbumin solution was passed three times through each of the columns. The final concentration of ovalbumin was determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

### **2.1.2. Materials for dsDNA concentration analysis**

**Quant-iT PicoGreen dsDNA Kit** (Invitrogen) containing PicoGreen stock reagent (200x), 20x TE buffer and λ DNA standard (100µg/ml).

### **2.1.3. Enzyme-linked immunosorbent assay (ELISA) materials**

#### **Sodium carbonate buffer**

4.2g NaHCO<sub>3</sub> and 1.78g Na<sub>2</sub>CO<sub>3</sub> was dissolved in 1L dH<sub>2</sub>O and brought to pH 9.5.

#### **PBS 10x**

800g NaCl, 116g Na<sub>2</sub>HPO<sub>4</sub>, 20g KH<sub>2</sub>PO<sub>4</sub> and 20g KCl was dissolved in 10L dH<sub>2</sub>O and brought to pH 7.2.

#### **Washing buffer**

0.05% (v/v) Tween 20 in PBS.

#### **Phosphate citrate buffer**

10.19g anhydrous citric acid and 14.6g Na<sub>2</sub>HPO<sub>4</sub> was dissolved in 1l dH<sub>2</sub>O and brought to pH 5.5.

## ELISA substrates

Two different substrates were used. A 20mg *o*-Phenylenediamine dihydrochloride (OPD) tablet was dissolved in 50ml of phosphate citrate buffer containing 20 $\mu$ l H<sub>2</sub>O<sub>2</sub>. OPD was used for all ELISAs, with the exception of IL-12p70 which was developed with Tetramethyl benzidine (TMB) (Millipore).

## Stop solution

1M H<sub>2</sub>SO<sub>4</sub>

**Table 2. 6. ELISA kits**

Kit	Supplier	Cytokines
Mouse Duoset	R&D	IFN- $\gamma$ , IL-1 $\beta$ , IL-12p70, IL-13, IL-23 IL-33, GM-CSF, MIP-1 $\alpha$ , MCP-1, TNF- $\alpha$
Mouse ELISA Max	Biologend	IL-1 $\alpha$ , IL-10, IL-17
Murine ELISA Development Kit	Peprotech	Eotaxin, KC, MIP-2
BD OptEIA	BD	IgE

**Table 2. 7. ELISA monoclonal antibodies obtained from BD Pharmingen**

Cytokine	Capture antibody	Detection antibody
IL-6	Purified Rat Anti-Mouse IL-6	Biotin Rat Anti-Mouse IL-6
IL-12p40	Purified Rat Anti-Mouse IL-12 (p40/p70)	Biotin Rat Anti-Mouse IL-12 (p40/p70)

**Table 2. 8. Detection antibodies for measuring murine immunoglobulins**

Antibody	Detection antibody	Supplier
IgG1	Biotin Anti-Mouse IgG1	BD Pharmingen
IgG2b	Biotin Rat anti-Mouse IgG2b	BD Pharmingen
IgG2c	Goat Anti-Mouse IgG2c:HRP	AbD Serotec
IgE	Detection antibody from IgE BD OptEIA kit	BD Pharmingen



## 2.1.4. Real-time PCR materials

### RNA isolation

High Pure RNA Isolation Kit (Roche).

**Table 2. 9. Reverse transcription reagents**

Reagent	Supplier
dNTPs	Promega
Reverse Transcriptase buffer	Promega
Random primers 5'-NNNNNN-3'	MWG Biotech
RNaseOUT	Invitrogen
M-MLV Reverse Transcriptase	Promega

### Real-time PCR reagents

GoTaq® qPCR Master Mix (Promega) – includes qPCR master mix, CXR dye and nuclease-free H<sub>2</sub>O.

### Primers

Primers (MWG Biotech) were directed against mouse genes and designed to be intron-spanning to avoid amplification of genomic DNA. Dissociation curve analysis was performed after a completed real-time PCR to exclude non-specific products.

**Table 2. 10. Real-time primers**

Primer	Forward	Reverse
<b>β-actin</b>	5'-TCCAGCCTTCTTGGGT-3'	5'-GCACTGTGTTGGCATAGAGGTC-3'
<b>IL-10</b>	5'-AGGCGCTGTCATCGATTCTC-3'	5'-GACACCTTGGTCTTGGAGCTTAT-3'
<b>IL-12p35</b>	5'-GGTGAAGACGGCCAGAGAAA-3'	5'-GGCAACTCTCGTTCTTGTGTAG-3'
<b>IL-12p40</b>	5'-GTGTAACCAGAAAGGTGCGTT-3'	5'-TCGGACCCTGCAGGGAAC-3'

## 2.1.5. Flow cytometry materials

### FACS buffer

2% (v/v) foetal bovine serum in PBS.

### Annexin V binding buffer 10x

28.3g HEPES, 87.7g NaCl, 3.7g KCl, 0.96 MgCl<sub>2</sub> and 2.66g CaCl<sub>2</sub> dissolved in 1L dH<sub>2</sub>O.

**Table 2. 11. Reagents for measurement of cell death**

Reagent	Target	Supplier
Annexin V FITC	Phosphatidylserine	Gift from Prof. Seamus Martin (Smurfit Institute of Genetics, TCD)
Propidium iodide	DNA	Sigma
LIVE/DEAD® Fixable Dead Cell Stain Kit	Free amines	Invitrogen

Fluorescent **DQ Ovalbumin** (Invitrogen)

### Fluorescent alum

20µg of DQ Ovalbumin was added to 100µg of alum in PBS (final volume of 500µl) and was incubated on a rotor overnight at 4°C. Fluorescent alum was pelleted by centrifugation (10 minutes, 3500g, 4°C) and resuspended in 500µl of PBS. The level of OVA DQ binding to alum was determined by measuring protein content in the supernatants from OVA DQ/alum.

### Intracellular staining

FoxP3/Transcription Factor Staining Buffer Set (eBioscience) containing Fixation/Permeabilization Concentrate (4x stock solution), Fixation/Permeabilization Diluent and Permeabilization Buffer (10x, to be diluted in dH<sub>2</sub>O).

### OVA-specific iTag MHC Tetramer

H-2 Kb conjugated with PE loaded with SIINFEKL peptide (Beckman Coulter).

**Table 2. 12. List of antibodies**

Antigen	Fluorochrome	Clone	Supplier
CD16/CD32	Unconjugated	2.4G2	BD Pharmingen
CD11b	PE-Cy7	M1/70	BD Pharmingen
c-kit	FITC	2B8	BD Pharmingen
SiglecF	PE	E50-2440	BD Pharmingen
F4/80	PerCP-Cy5.5	BM8	eBioscience
Gr-1	APC-Cy7	RB6-8C5	BD Pharmingen
CD3	V450	17A2	eBiosciences
CD8a	APC	53-6.7	BD Pharmingen
CD44	FITC	IM7	BD Pharmingen
CD4	Alexa Fluor 780	RM4-5	eBiosciences
FoxP3	FITC	FJK-16s	eBiosciences
B220	Alexa Fluor 700	RA3-6B2	BD Pharmingen

### 2.1.6. Confocal microscopy materials

**Table 2. 13. Reagents for monitoring alum uptake**

Reagent	Function	Supplier
DQ Ovalbumin	Labelling particles	Invitrogen
Cholera Toxin Subunit B Alexa Fluor 647 (CTB-Alexa647)	Binds to lipid membrane	Molecular Probes
Draq7	DNA stain	Invitrogen
Hoechst 33258	DNA stain	Sigma

#### Mounting medium

ProLong® Gold Antifade Reagent with DAPI (Invitrogen).

## 2.2.Methods

### 2.2.1. Mice

**Table 2. 14. Mice strains**

Mouse strain	Background	Original source
C57BL/6	n/a	Harlan Olac (Bicester, UK)
IL-1RI <sup>-/-</sup>	C57BL/6	Jackson Laboratories (Maine, USA)
IL-1α <sup>-/-</sup>	C57BL/6	Prof. Nancy Rothwell (Manchester, UK)
IL-1α/β <sup>-/-</sup>	C57BL/6	Prof. Nancy Rothwell (Manchester, UK)
IL-18 <sup>-/-</sup>	C57BL/6	Jackson Laboratories (Maine, USA)
NLRP3 <sup>-/-</sup>	C57BL/6	Prof. Jurg Tschopp (Lausanne, Switzerland)
ASC <sup>-/-</sup>	C57BL/6	Genetech (California, USA)
Caspase-1/11 <sup>-/-</sup>	NOD	Jackson Laboratories (Maine, USA)
IL-17R <sup>-/-</sup>	C57BL/6	Amgen Inc (California, USA)
IL-33 <sup>-/-</sup>	C57BL/6	Sankyo Laboratory Service (Japan)
ST2 <sup>-/-</sup>	C57BL/6	Prof. Andrew MacKenzie (Cambridge, UK)
4Get	C57BL/6	Jackson Laboratories (Maine, USA)
4Get ST2 <sup>-/-</sup>	C57BL/6	Prof. Padraic Fallon (TCD, Ireland)
IL-10 <sup>-/-</sup>	C57BL/6	Jackson Laboratories (Maine, USA)

Mice were used at 8-16 weeks of age. C57BL/6 mice were obtained from Harlan Olac (Bicester, UK). IL-1RI<sup>-/-</sup>, IL-10<sup>-/-</sup>, IL-17R<sup>-/-</sup>, NLRP3<sup>-/-</sup> ASC<sup>-/-</sup>, caspase-1/11<sup>-/-</sup>, IL-18<sup>-/-</sup>, IL-33<sup>-/-</sup> 4Get, 4get ST2<sup>-/-</sup> and ST2<sup>-/-</sup> mice were bred in the Bioresource Unit at TCD. 4Get, 4get ST2<sup>-/-</sup> and ST2<sup>-/-</sup> mice were provided by Prof. Padraic Fallon (Institute of Molecular Medicine, TCD) and experiments on these mice were performed by Dr Hendrik Nel. IL-1α<sup>-/-</sup> and IL-1α/β<sup>-/-</sup> were provided by Prof. Stuart Allan (University of Manchester) and experiments on these mice were performed by Dr Graham Tynan. IL-17R<sup>-/-</sup> mice were provided by Dr Rachel McLoughlin (School of Biochemistry and Immunology, TCD). IL-10<sup>-/-</sup> mice were provided by Prof. Kingston Mills (School of Biochemistry and Immunology, TCD). IL-18<sup>-/-</sup> and caspase-1/11<sup>-/-</sup> mice were provided by Dr Matthew Campbell (Smurfit Institute of Genetics, TCD).

Animals were maintained according to the regulations of the EU and the Irish Department of Health. All procedures performed were conducted under animal licence number B100/3321 and were approved by the Trinity College Dublin Animal Research Ethics Committee (Ethical Approval Number 091210).

### **2.2.2. Cell Culture**

#### **Isolation and culture of murine BMDCs**

Bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 mice and mice on a C57BL/6 background. Mice were euthanized using CO<sub>2</sub> and their femurs and tibiae were dissected from the muscle tissue. The bone marrow was flushed out with cRPMI medium using a 27G needle. The cell aggregates were broken up using a 19G needle and the cell suspension was pelleted by centrifugation (400g, 5 minutes, room temperature). The pellet was then resuspended in 1ml of 0.88% ammonium chloride red blood cell lysis buffer for 2 minutes. Cells were then washed in cRPMI, centrifuged (400g, 5 minutes, room temperature) and resuspended in 10ml of cRPMI. Cells were counted and seeded in T175 flasks at  $4 \times 10^5$  cells/ml in cRPMI medium supplemented with 20ng/ml of GM-CSF. After 3 days, 30ml of fresh medium containing 20ng/ml of GM-CSF was added to the flask. On day 6, the cell culture supernatant containing all non-adherent cells was removed and 30ml of cRPMI containing 20ng/ml GM-CSF was added. After 2 days, a further 30ml of complete RPMI with 20ng/ml GM-CSF was added. Finally, two days later, the loosely adherent cells were harvested and counted. Cells were plated in cRPMI supplemented with 10ng/ml GM-CSF in 96 well round bottom plates at a concentration of  $0.625 \times 10^6$  cells/ml (200 $\mu$ l/well) or in 24-well plates at a concentration of  $1 \times 10^6$  cells/ml (500 $\mu$ l/well); cells were used the following day for experiments. The specific treatments and conditions for restimulation of BMDCs are outlined in each experimental figure legend.

#### **Culture of immortalised bone marrow-derived macrophages**

Immortalised bone marrow-derived macrophages (iBMMs) were a gift from Prof. Katherine Fitzgerald (University of Massachusetts Medical School, USA). iBMMs

were cultured in cRPMI medium and every 2 days they were scraped, washed with cRPMI and passaged at a 1 in 5 dilution. For experiments, iBMMs were plated at a concentration of  $0.5 \times 10^6$  cells/ml and were used the following day, at which point they had reached approximately 80-90% confluency. The specific treatments and conditions for restimulation of iBMMs are outlined in each experimental figure legend.

### **Culture of C2C12 cell line**

The C2C12 cell line was obtained from Dr Richard Porter (School of Biochemistry and Immunology, TCD). C2C12 cells were grown in cDMEM medium and were passaged every 3 days before reaching 100% confluency; cells were washed with PBS before 2% trypsin was added for 5 minutes followed by 2 washes with cDMEM. For experiments, C2C12 cells were plated at a concentration of  $1 \times 10^6$  cells/ml on day 0 and were stimulated on day 1, when their confluency reached approximately 80-90%. The specific treatments and conditions for restimulation of C2C12 cells are outlined in each experimental figure legend.

### **Culture of J558 cell line**

To obtain granulocyte-monocyte colony stimulating factor (GM-CSF) for the culture of murine BMDCs, the murine gene for GM-CSF was cloned into a mammalian expression vector containing a geneticin resistance gene and transfected into the plasmacytoma line X63-AgS (J558 cells). J558 cells were provided by Dr Nathalie Winter (INRA, French University François-Rabelais in Tours).

J558 cells were firstly grown at a concentration of  $1 \times 10^6$  cells/ml in cRPMI medium containing geneticin (1mg/ml) for 2 passages. After the second passage, cells were washed twice in cRPMI medium and re-seeded in the absence of geneticin ( $1 \times 10^6$  cells/ml). For further passages, cells were seeded at a lower density ( $0.25 \times 10^6$  cells/ml). The supernatants were collected from J558 cells at passage numbers 4 through to 9. The concentration of GM-CSF in supernatants was quantified by ELISA.

### **Isolation and culture of peritoneal cells (PerC)**

PerC were collected by washing the peritoneal cavity of mouse with 5ml of PBS. PerC were pelleted by centrifugation (400g, 5 minutes, 4°C). The supernatant was

collected and the PerC were resuspended in 1ml of cRPMI and counted. PerC were plated at  $1 \times 10^6$  cells/ml in cRPMI in 96-well round bottom tissue culture plates (200 $\mu$ l/well). PerC were restimulated *ex vivo* with the appropriate treatments. The specific treatments and conditions for restimulation of PerC are outlined in each experimental figure legend.

### **Isolation and culture of splenocytes and mediastinal lymph nodes**

Spleens and lymph nodes were collected into cold cRPMI. To obtain a single cell suspension, spleens and lymph nodes were passed through 40 $\mu$ m cell strainers and rinsed with 2ml of cRPMI. Cells were pelleted by centrifugation (400g, 5 minutes, 4°C). Cells were resuspended in 1ml of cRPMI and counted. Splenocytes were plated at  $2 \times 10^6$  cells/ml and mediastinal lymph node cells at  $1 \times 10^6$  cells/ml in cRPMI in 96-well round bottom plates (200 $\mu$ l/well). Cells were restimulated *ex vivo* with the appropriate treatments. The specific treatments and conditions for restimulation of PerC are outlined in each experimental figure legend.

## **2.2.3. Animal Studies**

### **Role of various cytokines in the innate immune response to alum**

(i) Mice (C57BL/6, caspase-1/11<sup>-/-</sup>, IL-1 $\alpha$ <sup>-/-</sup>, IL-1 $\alpha/\beta$ <sup>-/-</sup>, IL-1RI<sup>-/-</sup>, IL-10<sup>-/-</sup>, IL-17R<sup>-/-</sup>, IL-33<sup>-/-</sup>, NLRP3<sup>-/-</sup> or ST2<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed at the time points indicated in each experimental figure legend and the peritoneal cavity was washed with 5ml of PBS. The wash volume was collected and the PerC were harvested. In certain experiments, the mediastinal lymph nodes were also isolated. Single cell suspensions of PerC were analysed by flow cytometry and where indicated, chemokine and cytokine levels in PerC supernatants were measured by ELISA. Moreover, PerC and mediastinal lymph node cells ( $1 \times 10^6$ /ml) were restimulated *ex vivo* with specific treatments outlined in each experimental figure legend.

**Table 2. 15. Restimulations *ex vivo***

Restimulation	Final concentration	Stimulation time
Medium	n/a	24h or 72h
LPS	1µg/ml	24h
heat-killed <i>E. coli</i>	MOI 1:10	24h
CpG	5µg/ml	24h
Anti-CD3	200ng/ml	72h
PMA	25ng/ml	72h

Collected supernatants were analysed for cytokine production by ELISA

(ii) Transgenic IL-4-IRES-eGFP mice (4get mice) have an eGFP gene incorporated into the IL-4 gene. Thus it is possible to monitor expression of IL-4 by detecting eGFP in cells. 4get and ST2<sup>-/-</sup> 4get mice were injected intraperitoneally with PBS or alum (1mg/mouse). After 24 hours, mice were sacrificed and PerC were collected with 5ml of PBS. Single cell suspensions of PerC were analysed by flow cytometry to assess IL-4-eGFP expression.

(iii) C57BL/6 mice were injected intraperitoneally with PBS, alum (1mg/mouse), alum with Uricase (50U/mouse), MSU (0.5mg/mouse) or MSU with Uricase. After 1 hour, mice were sacrificed and PerC were collected in 5ml of PBS. Single cell suspensions of PerC were analysed by flow cytometry and peritoneal lavage chemokines and cytokines were measured by ELISA.

#### Measurement of adaptive immune responses

Mice (C57BL/6, IL-1RI<sup>-/-</sup>, IL-10<sup>-/-</sup>, IL-18<sup>-/-</sup>, IL-33<sup>-/-</sup> and ST2<sup>-/-</sup>) mice were injected intraperitoneally with PBS, antigen (50µg/mouse) or alum (1mg/mouse) co-administered with antigen (50µg/mouse). Where indicated, the mice were sacrificed on day 7. Otherwise, on day 14, serum was collected and the mice were boosted with the same formulations. On day 21, the mice were sacrificed. Blood was collected and centrifuged (400g, 10 minutes, room temperature) before the serum was removed. Antigen-specific antibody titres in the serum were measured by ELISA. Peritoneal cells were collected in 5ml of PBS. Where indicated, spleens and



mediastinal lymph nodes (draining LNs) were removed and cells were isolated. Single cell suspensions (spleen,  $2 \times 10^6$ /ml, mediastinal lymph node cells and PerC,  $1 \times 10^6$ /ml) were stained and analysed by flow cytometry and/or restimulated *ex vivo* with specific treatments as outlined in each experimental figure legend.

### **Restimulations *ex vivo***

Single cell suspensions of splenocytes, mediastinal lymph node cells and peritoneal cells were stimulated with medium alone, various concentrations of antigen (10, 50 and 250  $\mu$ g/ml), anti-CD3 (0.250  $\mu$ g/ml) or anti-CD3 (0.2  $\mu$ g/ml) with PMA (25ng/ml). After 3 days supernatants were removed and analysed for cytokines by ELISA.

#### **2.2.4. dsDNA detection**

dsDNA was detected in the acellular phase of peritoneal fluid using a commercially available Quanti-iT PicoGreen dsDNA kit. 50 $\mu$ l of neat or diluted peritoneal fluid in TE buffer,  $\lambda$  DNA standard (32.75-2000ng/ml) and TE buffer alone were added in triplicate onto a 96-well black plate. 50 $\mu$ l of PicoGreen working solution (1/200 diluted in TE buffer) was added to each well 5 minutes before measuring fluorescence using a spectofluorometer. Samples were excited at 480nm and the fluorescent signal representing PicoGreen binding to dsDNA was measured at 530nm. dsDNA concentrations were determined from a standard curve prepared using  $\lambda$  DNA of known concentrations.

#### **2.2.5. Flow cytometry**

All compensations were set up using anti-rat and anti-hamster Ig  $\kappa$  chain negative control compensation particles (BD Pharmingen) or, if not applicable, cells were stained with a single antibody or dye. Moreover, to distinguish autofluorescent cells from cells expressing low levels of individual surface markers, thresholds for auto fluorescence were established by staining samples with fluorescence-minus-one

(FMO) control stain sets in which a fluorochrome for a channel of interest was omitted.

Single cell suspensions were analysed using a Fortessa flow cytometer (BD Biosciences). All prepared samples were analysed firstly by FSC-A vs. SSC-A plots and debris were excluded from further analysis. Furthermore, to exclude doublets, cells were analysed on a FSC-A vs. FSC-H plot, while dead cells were excluded using Aqua LIVE/DEAD stain. Samples were acquired using Diva software and data were analysed using FlowJo software (Treestar, Oregon).

### **Measurement of cell death**

Cell death was assessed using Annexin V and PI staining or PI staining alone. For Annexin V staining, all washes, incubations and measurements were performed in 1x Annexin V binding buffer. PI staining was performed in PBS.

Cells were washed and then incubated with 1µg/ml Annexin V-FITC (200µl). After 5 minutes, cells were washed and resuspended in 200µl of binding buffer. Furthermore, PI was added directly to FACS tubes (1µg/ml) immediately before the acquisition of samples on the flow cytometer.

### **Fluorescent alum uptake**

Cells were incubated with fluorescent alum for 1-6h as specified in figure legends. Cells were washed in PBS and resuspended in 200µl of PBS. Fluorescent alum uptake was detected in the FITC channel on flow cytometer.

### **Flow cytometric analysis of cells after immunization**

PerC, mediastinal lymph node cells and splenocytes ( $1 \times 10^6$  cells) were pelleted by centrifugation (400g, 5 minutes, 4°C). Cells were washed by adding 200µl of PBS, pelleted by centrifugation (400g, 5 minutes, 4°C) and the supernatants were discarded. All subsequent washing steps were performed in the same way. Cells were stained with Aqua LIVE/DEAD (1/500 dilution in PBS, 0.25µl per 50µl of sample) for 25 minutes in the dark and on ice. After washing with PBS, cells were incubated with 50µl of FACS buffer mixed with anti-CD16/CD32 monoclonal antibodies to block FcγRII/III (0.25µg per sample). Cells were then stained with fluorochrome-labelled antibodies

(Tables 2. 16 and/or 2.17) (in 50µl FACS buffer), for 15 minutes in the dark and on ice. After 15 minutes, cells were washed twice with 200µl of FACS buffer and when only extracellular staining was performed, the cells were resuspended in FACS buffer and analysed by flow cytometry.  $1 \times 10^5$  single and live cells were acquired.

**Table 2. 16. Flow cytometry antibodies for PerC staining**

Antibody	Fluorochrome	Dilution	Final concentration
CD11b	PE-Cy7	1/2000	0.1µg/ml
F4/80	PerCP-Cy5.5	1/200	1µg/ml
Gr-1	APC-Cy7	1/200	1µg/ml
CD117 (c-kit)	PE-Cy7	1/1000	0.5µg/ml
SiglecF	PE	1/1000	0.2µg/ml

**Table 2. 17. Flow cytometry antibodies for regulatory T cell staining**

Antibody	Fluorochrome	Dilution	Final concentration
CD3	V450	1/200	1µg/ml
CD4	Alexa Fluor 780	1/200	1µg/ml
FoxP3	FITC	1/200	2.5µg/ml

Alternatively, cells were stained for nuclear FoxP3 using a FoxP3/Transcription Factor Staining Buffer Set. After washing with FACS buffer, cells were incubated with 100µl of Fixation/Permeabilization buffer for 15 minutes in the dark at room temperature. Cells were washed twice with 200µl of permeabilisation buffer, followed by incubation with anti-FoxP3 antibody diluted in 50 µl permeabilisation buffer for 20 minutes in the dark on ice. To eliminate non-specific FoxP3 staining, cells were washed twice with 200µl of permeabilisation buffer. Cells were resuspended in 200µl of FACS buffer and analysed by flow cytometry.  $1 \times 10^5$  single and live cells were acquired.

#### **Flow cytometric analysis of OVA-specific CD8+ T cells**

Mediastinal lymph node cells and splenocytes ( $2 \times 10^6$  cells) were transferred into round bottom sterile FACS tubes with lids. Cells were washed twice with 2ml of sterile PBS and cell pellets were resuspended with 100µl of FACS buffer containing 1µl

of OVA-specific tetramer. Cells were incubated for 2h in a tissue culture incubator (37°C, 5% CO<sub>2</sub>), followed by washing with 2ml of sterile PBS. Cells were stained with Aqua LIVE/DEAD (1/500 dilution in PBS, 0.25µl per 50µl of sample) for 25 minutes in the dark on ice. Without washing, 50µl of mixed fluorochrome-labelled antibodies (Table 2. 18) diluted in FACS buffer were added to the cells following the addition of anti-CD16/CD32 monoclonal antibodies to block FcγRII/III (0.25ug per sample). After a 15 minute incubation period in the dark on ice, the cells were washed twice with 200µl of FACS buffer, resuspended in 150µl of FACS buffer and analysed by flow cytometry. A minimum of 1x10<sup>6</sup> single and live cells were acquired.

**Table 2. 18. Flow cytometry antibodies for CD8+ T cell staining**

Antibody	Fluorochrome	Dilution	Final concentration
B220	Alexa Fluor 700	1/200	1µg/ml
CD3	V450	1/200	1µg/ml
CD4	Alexa Fluor 780	1/200	1µg/ml
CD8	APC	1/200	1µg/ml
CD44	FITC	1/200	2.5µg/ml
F4/80	PerCP Cy5.5	1/200	1µg/ml

## 2.2.6. Confocal microscopy

### Live cell imaging

BMDCs were plated at 0.5x10<sup>6</sup> cells/ml in cRPMI in 35mm glass bottom tissue dishes. Non-adherent cells were removed on the following day and 0.5ml of fresh, pre-heated cRPMI mixed with the DNA stain, Hoechst 33258 (1µg/ml) was added to cells. After 30 minutes, fluorescent alum and DRAQ7 (0.3µM) were added and cells were viewed using a Point Scanning Confocal Microscope with a heated stage and CO<sub>2</sub> chamber (Olympus FV100 LSM Confocal Microscope).

### Preparation of cover slips

16mm cover slips were incubated in concentrated nitric acid. After 1h, the cover slips were rinsed 10 times with sterile water and were incubated overnight in

methanol. After removing the methanol, the cover slips were left to dry in a laminar flow hood. Cover slips were autoclaved before use.

### **Fixed cell imaging**

Sterile cover slips were placed in a 12-well plate and BMDCs were plated in cRPMI supplemented with 10ng/ml of GM-CSF at a concentration of  $0.625 \times 10^6$  cells/ml (1ml/well). On the following day, non-adherent cells were removed and 0.5ml of fresh, pre-heated cRPMI was added to wells. After 30 minutes, fluorescent alum (1-20 $\mu$ g/ml) was added and cells were incubated for 1-6 hours. The cover slips were washed once with 1ml of PBS and were fixed by incubating with 1ml of 4% paraformaldehyde (prepared in PBS) for 40 minutes. Following incubation, the cover slips were washed 3 times with PBS and were blocked with 1% BSA in PBS (500 $\mu$ l). After 30 minutes, the cover slips were washed 3 times with PBS. 100 $\mu$ l of 0.1 $\mu$ g/ml cholera toxin subunit B conjugated with Alexa Fluor 647 was added to each cover slip and incubated for 30 minutes at room temperature. Following incubation, the cover slips were washed 3 times with PBS and rinsed with sterile water. 15 $\mu$ l of mounting medium was added onto the glass microscope slides and cover slips were mounted cell-side down. Prepared slides were left to dry at room temperature in the dark overnight and were then stored at 4°C. Cells were viewed using Point Scanning Confocal Microscope (Olympus FV100 LSM Confocal Microscope).

## **2.2.7. Enzyme-linked immunosorbent assay**

### **Preparation of peritoneal lavage supernatants for cytokine detection**

Peritoneal cavity lavages were centrifuged (5 minutes, 400g) and the resulting supernatants were transferred into Amicon® Ultra-4 3kDa Centrifugal Filter Units (Millipore). These tubes were centrifuged at 3500g (4°C) for 20-40 minutes until approximately 200 $\mu$ l supernatants remained (the dilution factor reached approximately 20x). The concentrated supernatants were collected and analysed by ELISA.

## Measurement of chemokine and cytokine concentrations

Concentrations of cytokines and chemokines in supernatants were measured using commercially available ELISA kits from R&D Systems, Biolegend and Peprotech or antibody pairs from BD Pharmingen. All antibodies and recombinant cytokine standard concentrations, buffers and diluents as well as incubation times for individual ELISA kit components are listed in Tables 2.19-2.22.

High binding plates (Greiner Bio One) were coated with capture antibody in PBS (40µl/well) and incubated overnight at 4°C. After washing with PBS/Tween, the plates were incubated for 2 hours at room temperature with 100µl of the appropriate blocking solution. The plates were then washed with washing buffer, 40µl of supernatant or serially diluted standard was added to the plates and incubated overnight at 4°C. The plates were then washed with washing buffer before detection antibody (40µl/well) was added. The plates were incubated for either 1 or 2h at room temperature. After washing, HRP-conjugated streptavidin or avidin (40 µl/well) was added to the plates. Finally, the plates were washed and o-Phenylenediamine dihydrochloride (OPD) or 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added. The enzyme reaction was stopped by the addition of 1M H<sub>2</sub>SO<sub>4</sub> (20µl/well). The OD values were determined by measurement of absorbance at 492nm (OPD) or 450nm (TMB) using a microtitre plate reader. Cytokine concentrations were determined using a standard curve prepared from recombinant cytokines of known concentrations.

### Becton Dickenson

Blocking solution: 10% (w/v) Milk in PBS, except IL-6 ELISA: 3% (w/v) BSA in PBS

Diluent: PBS

Detection antibody: 1 hour incubation

Xtravidin-HRP: 1/750, 30 minutes incubation

**Table 2. 19. BD reagents**

Cytokine	Capture Antibody	Detection Antibody	Standard concentrations
IL-6	1µg/ml	1µg/ml	5-5000pg/ml
IL-12p40	0.5µg/ml	1µg/ml	5-5000pg/ml

## **Peprotech**

Blocking solution: 1% (w/v) BSA in PBS

Diluent: 0.1% BSA (w/v) in PBS

Detection antibody: 1 hour incubation

Avidin-HRP: 1/2000 30 minutes incubation

**Table 2. 20. Peprotech ELISA reagents**

<b>Cytokine</b>	<b>Capture Antibody</b>	<b>Detection Antibody</b>	<b>Standard concentration</b>
<b>Eotaxin</b>	0.5µg/ml	1µg/ml	2-2000pg/ml
<b>KC</b>	0.25µg/ml	0.5µg/ml	1-1000pg/ml
<b>MIP-2</b>	0.5µg/ml	0.25µg/ml	1-1000pg/ml

## **R&D kits**

Blocking solution: 1% (w/v) BSA in PBS

Diluent: 1% (w/v) BSA in PBS

Detection antibody: 2 hour incubation

Streptavidin-HRP: 1/200, 30 minutes incubation

**Table 2. 21. R&D kits**

<b>Cytokine</b>	<b>Capture Antibody</b>	<b>Detection Antibody</b>	<b>Standard concentrations</b>
<b>IFN-γ</b>	4µg/ml	0.4µg/ml	30-2000pg/ml
<b>IL-1β</b>	4µg/ml	1.5µg/ml	1-1000pg/ml
<b>IL-13</b>	4µg/ml	0.2µg/ml	4-4000pg/ml
<b>IL-12p70</b>	4µg/ml	0.4µg/ml	2.5-2500pg/ml
<b>IL-23</b>	4µg/ml	0.1µg/ml	2.5-2500pg/ml
<b>IL-33</b>	0.8µg/ml	0.4µg/ml	1-1000pg/ml
<b>GM-CSF</b>	2µg/ml	0.05µg/ml	8-500pg/ml
<b>MIP-1α</b>	0.4µg/ml	0.1µg/ml	8-500pg/ml
<b>MCP-1</b>	0.2µg/ml	0.05µg/ml	4-250pg/ml
<b>TNF-α</b>	0.8µg/ml	0.2µg/ml	31-2000pg/ml

## Biolegend

Blocking solution: 1% (w/v) BSA

Diluent: 1% (w/v) BSA

Detection antibody: 1 hour incubation

Streptavidin-HRP: 1/1000, 30 minutes incubation

**Table 2. 22. Biolegend ELISA reagents**

Cytokine	Capture Antibody	Detection Antibody	Standard concentration
IL-1 $\alpha$	1/200	1/200	0.5-500pg/ml
IL-10	1/200	1/200	1-1000pg/ml
IL-17	1/200	1/200	1-1000pg/ml

## Antibody ELISAs

### Detection of antigen-specific IgG1, IgG2b, IgG2c and IgE

Serum antigen-specific antibody titres were measured using commercially available antibodies (BD). Medium binding plates (Greiner Bio One) were coated (50 $\mu$ l/well) with OVA or HSA (50 $\mu$ g/ml) in carbonate buffer and were incubated overnight at 4°C. Plates were washed with washing buffer and non-specific binding sites were blocked by incubating with 10% (w/v) dried milk (or 1% (w/v) BSA in PBS for IgE ELISA) for 2 hours at room temperature. After washing, serum samples were added at a starting dilution of 1/100 (IgE 1/10) and were serially diluted one in two across the plate in PBS before being incubated overnight at 4°C. Plates were washed and biotinylated antibodies specific for IgG1 (0.125 $\mu$ g/ml), IgG2b (0.1125 $\mu$ g/ml) and IgE (1/500), or HRP-conjugated antibodies specific for IgG2c (0.5 $\mu$ g/ml), were added to the plates (50 $\mu$ l/well) for 2h at room temperature. Plates were washed; IgG1 and IgG2b antibodies were detected by incubating with HRP-conjugated streptavidin from Sigma (1/750) and IgE antibodies were detected using HRP-conjugated streptavidin from BD (1/200). Finally, plates were washed and OPD substrate dissolved in phosphate citrate buffer (50 $\mu$ l/well, 0.4mg/ml) was added. The enzyme reaction was stopped by the addition of 1M H<sub>2</sub>SO<sub>4</sub> (20 $\mu$ l/well). The OD values were obtained using a Versa Max Microplate Reader measuring absorbance at 492nm.



### **Detection of total IgE**

Total IgE concentrations were measured in serum using a commercially available ELISA kit (BD OptEIA™). High-binding plates (Greiner Bio One) were coated overnight (50µl/well) with IgE capture antibody (1/250) diluted in PBS. After washing with washing buffer, non-specific binding sites were blocked by incubating the plates with 10% (v/v) FCS in PBS for 2h at room temperature. Plates were washed. Serum samples at a starting dilution of 1/10 or an IgE standard (100ng/ml) were added (50µl/well) and serially diluted 1/2 across the plate in PBS. Plates were incubated overnight at 4°C. Afterwards, plates were washed and IgE detection antibodies (1/500, 50µl/well) were added for 2h at room temperature. After washing, cytokines were detected by incubating plates with HRP-conjugated streptavidin (1/250, 50µl/well). Finally, plates were washed and OPD was added. The enzyme reaction was stopped by addition of 20µl of 1M H<sub>2</sub>SO<sub>4</sub>. The OD values were determined by measurement of absorbance at 492nm. IgE concentrations were determined using a standard curve prepared from a recombinant IgE standard.

## **2.2.8. Relative Quantitation of Gene Expression**

### **RNA isolation**

The total RNA content of cells was isolated using a High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol. Briefly, cells were lysed, added to a column and incubated with DNase. After three washing steps, RNA was eluted and stored at -80°C.

### **Reverse transcription**

To produce cDNA, 200ng of RNA was reverse transcribed. The concentration of RNA was adjusted to 200ng by addition of H<sub>2</sub>O up to a volume of 5µl (Table 2. 23). To exclude genomic DNA contamination, one control sample (noRT) was prepared where the RT enzyme was replaced by H<sub>2</sub>O. After a PCR cycle (Table 2. 24), cDNA was diluted with 20µl H<sub>2</sub>O and was stored at -20°C.

**Table 2. 23. Reverse transcription mix**

Reagent	Volume ( $\mu$ l)	Concentration
dNTPs	2	2mM
RT buffer	2	1x
Random primers	0.5	50ng/ml
RNaseOUT	0.25	10U per sample
M-MLV enzyme	0.25	50U per sample
RNA+H <sub>2</sub> O/ H <sub>2</sub> O	5	200ng per sample

RNA was transcribed using the following PCR cycle:

**Table 2. 24. PCR cycle**

Temp	Time	Cycles
25°C	10min	1
42°C	30min	1
95°C	3min	1

### Real time PCR

Real-time PCR was performed in ABGene 96-well plates with the GoTaq kit (Promega) according to the protocol provided and using the 7500 Fast Real-Time PCR System (Applied Biosystems). Samples were prepared as outlined in Table 2.25. To exclude genomic DNA contamination in samples and in reagents, two control samples were prepared. One control sample was prepared from the “noRT” reverse transcription reaction, while the second control was prepared by replacing cDNA with H<sub>2</sub>O.

**Table 2.25. Real-time PCR mix**

Reagent	Volume per sample ( $\mu$ l)
Forward primer	0.5
Reverse primer	0.5
qPCR Mix	2.5
H <sub>2</sub> O	3.5
CXR	0.05
cDNA/noRT/H <sub>2</sub> O	3

Data from real-time PCR was obtained using the following PCR cycle:

**Table 2. 26. Real-time PCR cycle**

Temp	Time	Cycles
95°C	2min	1
95°C	3s	40
60°C	30s	

Data were analysed using Relative Quantification on Applied Biosystem 7500 Software, which provided cycle threshold (Ct) values. Ct is defined as the number of cycles required for the fluorescent signal to cross a threshold, exceeding background fluorescence. The change in gene expression was normalized to  $\beta$ -actin expression from the corresponding sample ( $Ct_{Gene} - Ct_{\beta-actin} = \Delta Ct$ ). Furthermore,  $\Delta Ct$  from control samples were averaged and were subtracted from the  $\Delta Ct$  of each sample ( $\Delta Ct_1 - \Delta Ct_{ctrl\ mean} = \Delta \Delta Ct_1$ ). Fold induction was calculated as  $2^{(-\Delta \Delta Ct)}$ .

### 2.2.9. Statistical analysis

Statistical analysis was performed using Graphpad Prism v5.02 software. The means for three or more groups were compared using one-way ANOVA. The Tukey multiple comparisons test was used to identify differences between individual groups. The means for two groups were compared by unpaired Student's t test.

## **CHAPTER 3**

# **THE ROLE OF INFLAMMASOMES AND IL-1 IN ALUM-INDUCED INNATE IMMUNE RESPONSES**



### 3.1.Introduction

IL-1 $\beta$ , a member of the IL-1 family, is a potent inducer of inflammation, which can be beneficial or detrimental for the host. At the site of damage or infection, IL-1 $\beta$  promotes the infiltration of inflammatory cells, due to induction of vasodilatation, upregulation of adhesion molecules and chemokine production (258). IL-1 $\alpha$  shares the same receptor complex with IL-1 $\beta$ , but it is expressed and released differently, which results in distinct biological functions. IL-1 $\alpha$  is an endogenous danger signal, stored in the nuclei of many cells, which can be released during necrosis (258). IL-1 $\beta$  expression and secretion is tightly regulated. It requires the first signal to become expressed in the form of pro-IL-1 $\beta$ , followed by a second signal, which is needed for processing the cytokine into its active form (99). It has been demonstrated that inflammasomes are commonly engaged in pro-IL-1 $\beta$  processing via caspase-1 activation (34). The NLRP3 inflammasome, composed of NLRP3, ASC adaptor protein and pro-caspase-1 is described as a common sensor of many compounds (33), including uric acid (259), cholesterol crystals (260), amyloid plaques (261) or viral DNA (36). Interestingly, it has also been shown that aluminium-containing adjuvants activate NLRP3 inflammasome assembly *in vitro*, which leads to caspase-1-dependent activation of IL-1 $\beta$  (236, 239). Moreover, release of IL-18, another member of IL-1 family, is induced by alum in a NLRP3-dependent manner (239). Furthermore, it was also demonstrated that alum promotes IL-1 $\alpha$  secretion *in vitro* (238). However, even though there are reports demonstrating that alum induces the secretion of IL-1 $\alpha$ , IL-1 $\beta$  and IL-18 *in vitro*, there is relatively little information on the role of these cytokines in alum-induced innate immune responses *in vivo* (239-241).

While alum is the most widely used vaccine adjuvant, until recently its mode of action was not well understood. It has been demonstrated that alum promotes an inflammatory response at the site of injection, including chemokine and cytokine release, followed by inflammatory cell recruitment, including neutrophils, eosinophils and inflammatory monocytes (237, 241). Some of these processes have been shown to be mediated by IL-1. For instance, in IL-1RI-deficient mice there is a reduction in neutrophil infiltration to the site of alum injection (244). Furthermore, MyD88, an adaptor protein for IL-1 and TLR signalling, is required for antigen-loaded

inflammatory monocyte recruitment into draining lymph nodes following alum injection (237). To investigate whether these effects were due to IL-1 $\beta$  secretion, similar experiments were done in NLRP3 or caspase-1-deficient mice. However, these reports show conflicting data. In NLRP3-deficient mice immunised with alum, there was a partial reduction in neutrophil, eosinophil and inflammatory monocyte influx into the site of injection, and inflammatory monocytes failed to migrate to draining lymph nodes (239). However, these effects at the site of injection were not observed in caspase-1-deficient mice (241), even though both reports showed decreased IL-1 $\beta$  secretion at the site of alum injection in the absence of NLRP3 or caspase-1.

Strikingly, there is also uncertainty regarding the role of IL-1 and inflammasomes in alum-induced adaptive immunity. It was shown that MyD88 and TRIF-double deficiency did not alter antigen-specific IgM, IgG or IgE production after alum immunisation, suggesting that IL-1R signalling is dispensable (235). In contrast, initial reports showed that NLRP3 played an essential role in induction of antigen-specific IgG1 antibodies by alum (236, 242). However, this has since been challenged by others, who found no defect in alum induced adaptive immune responses in NLRP3 or caspase-1-deficient mice (239, 241).

Overall, there is a great deal of confusion regarding the role of IL-1 and inflammasomes in alum induced immune responses. Importantly, there is a lack of a direct comparison of innate and adaptive immune responses in mice deficient in different members of the IL-1 family and inflammasome components.

### **3.2.Aim**

- To comprehensively dissect the role of IL-1, IL-18 and the inflammasome components, NLRP3, ASC and caspase-1, in alum-induced innate immune responses.

### 3.3. Results

#### 3.3.1. Optimising flow cytometry staining and analysis for the mouse peritonitis model

Intraperitoneal injection is widely used as a convenient method to determine local innate immune responses to compounds and vaccines due to easy isolation of peritoneal cells (PerC). In naïve mice, there are resident cells in the peritoneum, including macrophages and mast cells, however, after alum injection, there is massive infiltration of neutrophils, eosinophils and inflammatory monocytes (237, 241). In order to assess alum-induced innate immune responses in mice, a gating strategy was established. Firstly, acquired cells were gated using forward and side scatter parameters, to exclude any debris, followed by exclusion of dead cells using Aqua LIVE/DEAD stain. To make sure that only single cells were analysed, cells were gated using forward scatter area and height (Figure 3. 1. A). To gate resident macrophages in the live single cell suspension, two markers were used, CD11b and F4/80, both highly expressed by resident peritoneal macrophages (Figure 3. 1. B). Neutrophils and inflammatory monocytes are rare populations in naïve or PBS-injected mice, therefore, to find these two populations, PerC from alum-injected mice were stained and analysed. Both populations express CD11b and Gr-1, comprising two myeloid differentiation antigens, Ly6C and Ly6G. Importantly, their gating can be difficult if eosinophils are not excluded, due to eosinophil expression of F4/80 and low levels of Gr-1. Therefore, SiglecF<sup>+</sup> cells were gated out from CD11b<sup>+</sup> cells (Figure 3. 1. C and D). Neutrophils and inflammatory monocytes can be distinguished by expression of F4/80, inflammatory monocytes are CD11b<sup>+</sup>Gr-1<sup>+</sup>F4/80<sup>+</sup> (Figure 3. 1. C) while neutrophils are CD11b<sup>+</sup>Gr-1<sup>high</sup>F4/80<sup>-</sup> (Figure 3. 1. D). Resident mast cells and a small population of eosinophils can be detected in PBS-injected mice. These populations express no or low levels of Gr-1, but can be distinguished using c-kit and SiglecF, respectively. Mast cells can be gated as Gr-1<sup>-</sup>SiglecF<sup>-</sup>c-kit<sup>+</sup> and eosinophils as Gr-1<sup>low</sup>SiglecF<sup>+</sup>c-kit<sup>-</sup> cells.



### 3.3.2. Injection of alum induces innate immune responses

Having established a gating strategy to identify the following cell subsets: resident macrophages, mast cells, inflammatory monocytes, neutrophils and eosinophils in the peritoneal cavity, it was necessary to determine the kinetics of cell infiltration into the site of alum injection. Therefore, C57BL/6 mice were injected intraperitoneally with PBS or alum (1mg/mouse). After 24h, 3 and 7 days, PerC were collected. As expected, mice injected with PBS had a high percentage of resident macrophages, some mast cells and eosinophils, but few inflammatory monocytes or neutrophils (Figure 3. 2). 24h post alum injection, there was a significant infiltration of inflammatory monocytes, neutrophils and eosinophils, while resident macrophages and mast cells were not detected. Between 3 and 7 days following alum injection, the percentage of neutrophils and eosinophils decreased over time, while the percentage of inflammatory monocytes increased. Interestingly, mast cells and resident macrophages were not detected in peritoneal cavity even 7 days post alum injection (Figure 3. 2). Importantly, alum administration resulted in a significantly higher total cell number in the peritoneum (Figure 3. 3 A), which reached a peak 24h post injection, but quickly waned. Interestingly, 7 days post injection, the total number of peritoneal cells was reduced when compared to the PBS control group. Overall, alum mediated the depletion of resident macrophages and mast cells from the peritoneum (Figure 3. 3 B), while concomitantly promoting the transient influx of inflammatory monocytes, neutrophils and eosinophils (Figure 3. 3 C).

To determine which factors are engaged in recruitment of innate cells after alum administration, a number of key cytokines and chemokines were measured in peritoneal lavage fluid 1, 3, 6 and 24 hours post injection (Figure 3. 4). There was a marked increase in peritoneal concentrations of the pro-inflammatory cytokines, IL-1 $\beta$  and IL-6 in mice receiving alum. While alum induced a massive increase in levels of IL-6 as quickly as 1h post injection, IL-1 $\beta$  was detected later, after 3h. Interestingly, the concentrations of these cytokines were lower 24 post alum injection. In addition, assays were carried out for IL-1 $\alpha$ , but this cytokine was not detected at any time point tested (data not shown). Neutrophils and eosinophils can be recruited to the site of injury by different chemokines, either the granulocyte chemotaxins, MIP-1 $\alpha$  and MIP-

2, or/and by the neutrophil chemotaxin, KC and the eosinophil chemotaxin, eotaxin, respectively. Injection of alum induced secretion of all these chemokines. Interestingly, KC and MIP-2, were up regulated after only 1h and they were detected for up to 24h, while MIP-1 $\alpha$  was detected from 1h, but it only reached high levels 24h post injection. One of the chemokines which can promote monocyte infiltration into site of alum injection is monocyte chemoattractant protein-1 (MCP-1). Indeed, it was detected as early as 1 hour post injection and its high expression persisted up to 24 hours. Overall, alum induced a significant upregulation of pro-inflammatory cytokines and chemokines, which correlated with rapid and massive cell infiltration at the site of administration.

### **3.3.3. IL-1 is required for alum-induced neutrophil infiltration**

Considering that alum significantly augmented production of IL-1 $\beta$  at the site of injection, it was important to determine whether this cytokine plays a role in the recruitment of cells into the injection site. Wild-type and IL-1RI-deficient mice were injected intraperitoneally with PBS or alum and 24h later PerC were isolated. As expected, alum induced massive cell infiltration in wild-type mice at the site of injection, however, in IL-1RI<sup>-/-</sup> mice the total PerC number was significantly reduced (Figure 3. 5 A). To determine if this was a global or selective effect, different cell populations were analysed by flow cytometry. Alum induced complete mast cell and resident macrophage depletion in both wild-type and IL-1RI-deficient mice. Moreover, infiltration of inflammatory monocytes and eosinophils into the site of alum injection did not require IL-1. However, the number of infiltrating neutrophils was significantly reduced in IL-1RI<sup>-/-</sup> mice (Figure 3. 5 B and C).

IL-1RI is a receptor subunit which has three known ligands, IL-1 $\alpha$ , IL-1 $\beta$  and IL-1Ra. Both, IL-1 $\alpha$  and IL-1 $\beta$  have been implicated in mediating neutrophil infiltration after tissue injury. Therefore, in order to investigate which ligand of IL-1RI is responsible for alum-induced neutrophil infiltration, mice deficient in both IL-1 $\alpha$  and IL-1 $\beta$  and in IL-1 $\alpha$  alone were used. Wild-type, IL-1 $\alpha$ / $\beta$ <sup>-/-</sup> and IL-1 $\alpha$ <sup>-/-</sup> mice were injected intraperitoneally with PBS or alum and 24h later PerC were isolated. As expected, alum induced cell infiltration in wild-type mice at the site of injection. In IL-1 $\alpha$ / $\beta$ <sup>-/-</sup>

mice total PerC number was significantly reduced, while in IL-1 $\alpha$ <sup>-/-</sup> mice it was marginally decreased (Figure 3. 6 A). In wild-type mice, alum induced substantial neutrophil recruitment at the site of injection. Interestingly, neutrophil influx was almost undetectable in mice deficient in both IL-1 $\alpha$  and IL-1 $\beta$ , while it was significantly, but not fully, reduced in IL-1 $\alpha$ <sup>-/-</sup> mice. Inflammatory monocyte recruitment was lower in IL-1 $\alpha$ / $\beta$ <sup>-/-</sup> mice and slightly increased in IL-1 $\alpha$ <sup>-/-</sup> mice, but these changes were not significant (Figure 3. 6 B). Moreover, similarly to results with IL-1RI-deficient mice, infiltration of eosinophils into the site of alum injection did not require IL-1 $\alpha$  or IL-1 $\beta$  (Figure 3. 6 C).

Having established that IL-1 has a selective role in neutrophil infiltration to the alum injection site, it was next determined whether another member of the IL-1 family, IL-18, has any role in innate immune response to alum. Wild-type and IL-18-deficient mice were injected with PBS or alum and 24h later PerC were isolated. Injection of alum increased peritoneal cell number in both wild-type and IL-18-deficient mice, but there was no significant difference between these two mouse strains (Figure 3. 7 A). There was also no difference in depletion of resident macrophages and mast cells between wild-type and IL-18<sup>-/-</sup> mice. Furthermore, the recruitment of neutrophils, inflammatory monocytes and eosinophils was also IL-18-independent (Figure 3. 7 B and C).

### **3.3.4. The inflammasome is not required for neutrophil infiltration into the site of alum injection**

Both, IL-1 $\alpha$  and IL-1 $\beta$  contributed to alum-induced neutrophil infiltration to the site of injection. IL-1 $\beta$ , but not IL-1  $\alpha$ , has to be processed to become bioactive and it has been shown *in vitro* that NLRP3-dependent caspase-1 activation is crucial for alum-induced IL-1 $\beta$  processing. Therefore, to determine whether the IL-1 $\beta$ -dependent neutrophil infiltration into the site of alum injection is also NLRP3-dependent, wild-type and NLRP3-deficient mice were injected with PBS or alum for 24h. Interestingly, in contrast to IL-1RI<sup>-/-</sup> mice, alum induced comparable cell infiltration into the site of injection in wild-type and NLRP3-deficient mice (Figure 3. 8 A). Depletion of resident macrophages and mast cells was NLRP3-independent.

Furthermore, there was no difference in influx of neutrophils, inflammatory monocytes or eosinophils (Figure 3. 8 B and C).

The role of NLRP3 inflammasome is well described in the context of crystal-induced IL-1 $\beta$  secretion. However, there are also other inflammasome sensors, including NLRP1, NLRC4 and AIM2, which can mediate caspase-1-dependent IL-1 $\beta$  cleavage. All these inflammasomes, including NLRP3, contain the adaptor protein ASC, which mediates pro-caspase-1 cleavage. Thus, even though there was no role for NLRP3 in alum-driven neutrophil infiltration into the site of injection, it was still possible that other inflammasome might mediate these effects. In order to determine the role of ASC in infiltration of cells to the site of alum injection, wild-type and ASC-deficient mice were injected with PBS or alum for 24h. As with NLRP3-deficient mice, alum induced comparable cell infiltration into the site of injection in wild-type and ASC-deficient mice (Figure 3. 9 A). Furthermore, resident macrophages and mast cells were depleted, while inflammatory monocytes and eosinophils infiltrated the site of alum injection to a similar extent in wild-type and ASC<sup>-/-</sup> mice (Figure 3. 9 B and C). Importantly, in contrast to IL-1RI<sup>-/-</sup> mice, but similarly to NLRP3<sup>-/-</sup> mice, there was no difference in the influx of neutrophils into the site of alum injection between wild-type and ASC-deficient mice (Figure 3. 9 B).

Having shown that the inflammasome components, NLRP3 and ASC, do not play a role in neutrophil infiltration nor in the influx of other cells to the site of alum injection, it was crucial to determine whether there is a role for caspase-1 in alum-driven cell recruitment. Therefore, caspase-1-deficient mice were used. However, caspase-1<sup>-/-</sup> mice were generated using embryonic stem cells from 129 mice, which have intrinsic caspase-11-deficiency. It has been hypothesised that due to the close location of these two genes, backcrossing did not lead to a loss of the additional deficiency. Indeed, it has been shown that commercially available caspase-1-deficient mice also carry a deficiency in caspase-11 and they are in fact caspase-1 and caspase-11 double knockout mice (262). Thus, C57BL/6 and caspase1/11-deficient mice were injected with PBS or alum for 24h. Similarly to NLRP3-deficient and ASC-deficient mice, alum induced comparable cell infiltration to the injection site in wild-type and caspase-1/11-deficient mice (Figure 3. 10 A). Interestingly, caspase-1/11<sup>-/-</sup> mice had significantly more resident macrophages and mast cells in

the peritoneal cavity than control mice. However, the absence of caspase-1 and caspase-11 did not affect depletion of these cells after alum administration (Figure 3. 10 B and C). Importantly, similarly to NLRP3<sup>-/-</sup> and ASC<sup>-/-</sup> mice, there was no difference in the influx of neutrophils into the site of alum injection between wild-type and caspase-1/11-deficient mice (Figure 3. 10 B). Furthermore, eosinophils infiltrated the site of alum injection in both wild-type and caspase-1/11<sup>-/-</sup> mice to the same extent (Figure 3. 10 C). In contrast, there was a striking effect of caspase-1/11-deficiency on alum-induced recruitment of inflammatory monocytes. The ability of alum to recruit this population was greatly reduced in caspase-1/11-deficient mice (Figure 3. 10 B).

Interestingly, while neutrophil influx was abrogated only in the absence of IL-1 $\beta$  signalling, this effect was not mediated by inflammasomes. Therefore, to assess if specific inflammasome components were required to induce IL-1 $\beta$  secretion *in vivo*, acellular peritoneal cavity fluid isolated from NLRP3<sup>-/-</sup>, ASC<sup>-/-</sup>, caspase-1/11<sup>-/-</sup> and wild-type mice injected intraperitoneally with PBS or alum for 24h was tested for the presence of IL-1 $\beta$ . Strikingly, in contrast to *in vitro* results (Figure 4. 6), alum induced IL-1 $\beta$  secretion in an inflammasome-independent manner at the site of injection (Figure 3. 11 B, C and D). Furthermore, the presence of pro-inflammatory IL-6 and neutrophil chemokines KC and MIP-2 was tested in peritoneal fluid. Interestingly, alum-induced secretion of IL-6 was significantly decreased in IL-1RI<sup>-/-</sup> mice, while mice deficient in the inflammasome components, NLRP3 and caspase-1, had significantly increased levels of IL-6 (Figure 3. 11). Release of KC was also partly dependent on IL-1, while NLRP3 and caspase-1 or caspase-11 deficiency led to increased secretion of this chemokine. On the other hand, alum-induced MIP-2 production was not dependent on IL-1 or inflammasome components (Figure 3. 11).

### **3.3.5. IL-17 is not required for neutrophil influx at the site of alum injection**

IL-17 is a potent inducer of pro-inflammatory mediators, including the neutrophil chemoattractant, KC (263). Furthermore, IL-17 can be induced by IL-1 $\alpha$  and IL-1 $\beta$ , which were shown here to be crucial for neutrophil influx after alum administration. Therefore, to determine the role of IL-17 in infiltration of cells to the

site of alum injection, wild-type and IL-17R-deficient mice were injected with PBS or alum. In contrast to IL-1RI-deficient mice, alum induced comparable cell infiltration into the site of injection in wild-type and IL-17R-deficient mice (Figure 3. 12 A). Importantly, influx of neutrophils into the site of alum injection was not IL-17-dependent (Figure 3. 12 B). Furthermore, resident macrophages and mast cells were depleted, while inflammatory monocytes infiltrated the site of alum injection to a similar extent in wild-type and IL-17R<sup>-/-</sup> mice. Surprisingly, the lack of IL-17 signalling resulted in a significant increase in eosinophil recruitment to the site of alum injection (Figure 3. 12 C). Overall, these data demonstrate that the IL-1-dependent alum-induced neutrophil influx is not dependent on IL-17.

### **3.3.6. Alum induces cell death-independent DNA accumulation**

Host DNA is a recently described DAMP which is released early after alum administration and accumulates at the site of injection (250). It was also reported that DNA-containing extracellular traps are released by CD11b<sup>+</sup> cells in the peritoneal cavity after alum administration (229). In order to determine whether, IL-1 and inflammasomes contribute to alum-induced host DNA release, wild-type, IL-1RI<sup>-/-</sup>, NLRP3<sup>-/-</sup>, ASC<sup>-/-</sup> and caspase-1/11<sup>-/-</sup> mice were injected with alum or PBS and 24h later, the acellular phase of the peritoneal cavity fluid was analysed for the presence of dsDNA. Strikingly, DNA accumulation was partly dependent on caspase-1 or caspase-11 and IL-1 signalling, while NLRP3 and ASC did not affect DNA release (Figure 3. 13).

To determine whether host DNA release is dependent on cell death or rather on the accumulation of neutrophils and inflammatory monocytes at the site of alum injection, wild-type mice were injected intraperitoneally with alum or PBS. After 1, 3, 6 and 24h the peritoneal cavity was washed with PBS and peritoneal cells and the acellular peritoneal cavity fluid were isolated. Interestingly, even though alum induced significant cell recruitment 24h post injection, there was a decrease in total cell number early after injection (Figure 3. 14 A), which corresponds with massive cell death 1 hour post injection (Figure 3. 14 B and C). Furthermore, consistent with the slow increase in total cell number, the number of infiltrating neutrophils and

inflammatory monocytes was most marked 24h post alum injection (Figure 3. 14 D, E and F). Importantly, maximal DNA release was evident 24 hours post alum injection (Figure 3. 14 G) and not when alum induced death was most marked. Overall, these data suggest that host DNA release is more dependent on cell accumulation than cell death.

### **3.3.7. IL-1 and IL-18 are dispensable for alum-induced adaptive immune responses**

Having established that alum induced IL-1 $\alpha$  and inflammasome-independent IL-1 $\beta$  release at the site of injection, which led to neutrophils influx, it was important to determine the role of IL-1 in alum-driven adaptive immunity. Furthermore, while IL-18 does not have any apparent role in alum-driven innate responses, it is possible that it influences adaptive responses.

In order to determine the role of IL-1 and IL-18 in antigen-specific antibody responses after alum immunisation, wild-type, IL-18<sup>-/-</sup> and IL-1RI<sup>-/-</sup> mice were immunised with PBS, OVA alone or OVA co-administered with alum. After 14 days, mice were boosted with the same treatments. Sera collected on days 13 and 21 from immunised mice were analysed for the presence of OVA-specific IgG1, IgG2b, IgGc and IgE. The patterns of OVA-specific IgG1, IgG2b and IgGc after prime and boost immunisation were similar, thus only the data from day 21 are presented. Immunisation with OVA and alum in all mice resulted in an increase of OVA specific IgG1 in comparison to OVA alone. However, there are no significant differences in antigen-specific IgG1 between WT and IL-1RI<sup>-/-</sup> or IL-18<sup>-/-</sup> mice immunised with alum (Figure 3. 15). Furthermore, similar levels of OVA-specific IgG2b, IgG2c and IgE were detected in mice after alum immunisation (Figure 3.15).

To establish whether the ability of alum to promote antigen-specific CD8 T cell responses depend on IL-1 or IL-18, mediastinal lymph node cells and splenocytes isolated from vaccinated mice on day 21 were analysed by flow cytometry. Cells were gated to exclude debris, doublets and dead cells. B220<sup>+</sup> and F4/80<sup>+</sup> cells were excluded and CD3<sup>+</sup>CD8<sup>+</sup>CD4<sup>-</sup> T cells were gated. OVA-specific CD8 T cells were detected using K<sup>b</sup>/SIINFEKL tetramer and upregulation of CD44, a T cell activation

marker (Figure 3.16 A). As expected, alum promoted induction of OVA-specific CD8 T cells both in the mediastinal lymph nodes and spleens in wild-type mice, while OVA alone did not. The lack of IL-1 or IL-18 did not have a significant impact on the number of tet<sup>+</sup> CD8 T cells in mLN and spleens (Figure 3. 16 B and C).



### 3.4. Discussion

Innate immune responses evolved to recognise and react quickly to pathogenic challenge. Importantly, while pathogen-derived PAMPs are recognised by various well-described receptors, including TLRs and RLRs, which induce potent immune responses, endogenous particulates can drive sterile inflammation but the underlying mechanisms are less well understood. Cholesterol, uric acid, and amyloid- $\beta$  can form crystals, which although variable in chemical structure and origin, drive similar response, including NLRP3 inflammasome-dependent IL-1 $\beta$  secretion, at least *in vitro* (259-261). Furthermore, when administered to mice, they trigger common pathways to generate pro-inflammatory responses, including IL-1 $\beta$  secretion (240, 259-261). However, in many cases it is still controversial whether the IL-1 $\beta$  produced *in vivo* is a result of inflammasome-dependent or independent pathways.

The discovery that the most commonly used adjuvant alum drives IL-1 $\beta$  in a NLRP3-dependent manner caused great excitement (236, 242). At the time it seemed that this solved the mystery of alum adjuvanticity. However, many subsequent reports demonstrated that there is a limited role for inflammasomes in alum-driven adaptive immune responses (239, 241, 243). The reason for these discrepancies is not yet clear. Therefore, the current study investigated in detail the role of inflammasomes in alum-induced immune responses, including their capacity to induce IL-1 $\beta$  processing *in vivo* and subsequent inflammation.

Firstly, it has been confirmed that alum promotes inflammatory responses at the injection site. In agreement with other studies (237, 241), alum induced a rapid innate immune response, which was reflected in a peak cell influx 24h after injection. Neutrophils, eosinophils and inflammatory monocytes were recruited, while resident macrophages and mast cells disappeared. Interestingly, the alum-induced cellular influx decreased over time and 7 days post injection, there were barely any cells detected in the peritoneum. This acute inflammation was mediated by massive cytokine and chemokine release shortly after alum injection. The above findings demonstrate that alum is recognised rapidly and promotes an acute innate immune response at the site of injection. One of the key molecules engaged in triggering inflammation is IL-1. As established by others (239, 241) and here, alum drives IL-1 $\beta$

release *in vivo*. Alum promoted IL-1 $\beta$  release as early as 3h post injection and it was present in the peritoneum for at least 24h post injection.

Mice deficient in IL-1RI fail to generate neutrophilic inflammation in response to some particulates. For instance, administration of MSU, cholesterol crystals or asbestos lead to IL-1-dependent neutrophil recruitment (240, 259, 260). In contrast, neutrophil infiltration induced by the pathogen-associated molecular pattern, Zymosan is IL-1-independent (264). As demonstrated before (240), alum-induced neutrophil influx required IL-1RI, which was also reflected in a significant decrease in total PerC numbers recruited into the site of alum injection. The disappearance of resident macrophages and mast cells and the infiltration of inflammatory monocytes and eosinophils was IL-1-independent. Importantly, the lack of IL-1 signalling led to a significant decrease in concentrations of IL-6 and the neutrophil chemokine KC, which may explain the reduction in neutrophil recruitment.

IL-1RI has three ligands, IL-1Ra, IL-1 $\alpha$  and IL-1 $\beta$ , but there is limited information about the relative contribution of IL-1 $\alpha$  and IL-1 $\beta$  to sterile inflammation. Necrotic cells induce neutrophil infiltration, which is abrogated in IL-1RI-deficient mice (118) and furthermore, this can be inhibited by neutralisation of IL-1 $\alpha$  (118, 265). However, the specific roles of IL-1 $\alpha$  and IL-1 $\beta$  have not yet been studied in detail in the inflammatory responses to many particulates and crystalline materials, including alum. Similarly to IL-1RI<sup>-/-</sup> mice, IL-1 $\alpha$ / $\beta$ <sup>-/-</sup> mice completely failed to recruit neutrophils following the injection of alum. Interestingly, the influx of neutrophils was partly reduced in IL-1 $\alpha$ <sup>-/-</sup> mice, which suggests that both IL-1 $\alpha$  and IL-1 $\beta$  have a role in alum-induced neutrophil recruitment. While IL-1 $\alpha$  was not detected in the peritoneum, it may be released after alum-induced necrosis, but quickly sequestered by the IL-1R complex. To determine IL-1 $\alpha$  secretion *in vivo*, injecting IL-1RI-deficient mice (thus allowing IL-1 $\alpha$  to accumulate) with alum and assaying for cytokine after 1 hour, where massive cell death occurs, might be informative.

Alum-induced IL-1 $\beta$  processing is NLRP3, ASC and caspase-1-dependent *in vitro* (236), therefore it could be assumed that any effect of alum-induced IL-1 $\beta$  *in vivo* would be also dependent on the NLRP3 inflammasome. Indeed it has been reported that alum-induced cell recruitment at the site of injection is partly dependent on NLRP3 (239). However, the current study found that NLRP3 is fully dispensable for

alum-induced cell recruitment, including neutrophils. Furthermore, the adaptor protein ASC was also not required for cell infiltration following alum injection and caspase-1/11-deficient mice had intact neutrophil infiltration at the site of alum injection. Overall, these data suggest that mice deficient in NLRP3, ASC and caspase-1 can secrete IL-1 $\beta$  at the site of alum injection, which indicates caspase-1-independent processing.

Overall, the findings in the current thesis suggest that the ability of alum to drive IL-1 $\alpha$  and IL-1 $\beta$ -dependent neutrophil recruitment is an inflammasome-independent process. It remains to be established how IL-1 $\beta$  is processed *in vivo* following alum injection. Interestingly, it has been also demonstrated that while the injection of cholesterol crystals failed to recruit neutrophils in mice deficient in either IL-1 $\alpha$ , IL-1 $\beta$  or IL-1RI, this infiltration was only partly abrogated in NLRP3, ASC and caspase-1-deficient mice (260). This suggests caspase-1-independent processing of IL-1 $\beta$  following cholesterol crystal injection. There are alternative possible mechanisms of IL-1 $\beta$  processing, including serine proteases, such as elastase, proteinase-3 and cathepsin G (111, 266) or another caspase, caspase-8 (112, 113). Interestingly, it seems that IL-1 $\beta$  processing *in vivo* might be dependent on the type of inflammation. For instance, it has been reported that IL-1 $\beta$  can be processed by distinct enzymes in different murine models of arthritis. While various experimental models of arthritis share a dependency on IL-1, the IL-1 $\beta$  can be processed either by caspase-1, proteinase-3 or neutrophil elastase. The induction of acute inflammation in the K/BxN serum transfer mouse model of arthritis leads to caspase-1-independent neutrophil infiltration, where IL-1 $\beta$  processing is mediated by neutrophil elastase (267). In another experimental model, *Streptococcus pyogenes* cell wall (SCW) fragments-induced arthritis, IL-1 $\beta$  is cleaved by the neutrophil-derived serine protease, proteinase-3. In contrast, repeated injection of SCW fragments led to chronic inflammation, which is attenuated in caspase-1-deficient mice (268). Furthermore, while it has been demonstrated that silica crystals induce IL-1 $\beta$  secretion *in vitro* in a NLRP3 inflammasome-dependent manner, caspase-1 deficiency did not reduce silica-induced neutrophil infiltration to the same extent as in IL-1 $\beta$  or IL-1RI-deficient mice. However, neutrophil recruitment was decreased in cathepsin C-deficient mice. Cathepsin C processes various proforms of serine proteinase, and can therefore,

participate in alternative processing of IL-1 $\beta$ . Interestingly, in the same report it was demonstrated that the lack of cathepsin C did not have any impact on silica-induced IL-1 $\beta$  secretion *in vitro* (269).

Alum induces acute inflammation, which promotes a massive IL-1-dependent and caspase-1-independent neutrophil infiltration into the site of injection. Therefore it is possible in this context that IL-1 $\beta$  can be processed by neutrophil-derived serine proteases. However, to prove this it will be necessary to use serine proteinase inhibitors during alum injection and assess inflammation in cathepsin C-deficient mice.

IL-1 is a key player in promoting neutrophil infiltration into the site of damage or infection, but there are reports showing that subsequent IL-1-induced cytokines can be decisive in this process. For instance, IL-17 is crucial for neutrophil influx into the site of *Staphylococcus aureus* infection, because it stimulates production of chemokines, including the neutrophil chemokine, KC (263). However, the current study suggests that IL-17 is not required for alum-induced neutrophil recruitment. Surprisingly, eosinophil recruitment was increased in IL-17R-deficient mice, which suggests that one of the members of IL-17 family (IL-17A, IL-17F or IL-25) can block alum-induced eosinophil influx. Therefore, it will be interesting to determine how blocking IL-17 receptor signalling might increase alum-driven eosinophil influx.

Dendritic cells are antigen-presenting cells, which are capable of linking innate and adaptive immune responses. Alum induces DC infiltration into the site of injection, but also promotes the recruitment of inflammatory monocytes. These inflammatory monocytes were demonstrated to carry antigen into draining lymph nodes and to differentiate into antigen-presenting dendritic cells (237). Interestingly, while recruitment of inflammatory monocytes into the lymph nodes was not tested in the current study, the infiltration of these cells into the site of alum injection was measured. Interestingly, only caspase-1/11<sup>-/-</sup> mice failed to recruit inflammatory monocytes, while this effect was not observed in IL-1RI, NLRP3 or ASC-deficient mice. However, these mice were bred on NOD background, while C57BL/6 mice were used as controls. It cannot be excluded that alum would recruit different cell populations in NOD and C57BL/6 mice. However, alum induced infiltration of other cell populations, including neutrophils, in a comparable manner in C57BL/6 and caspase-1/11<sup>-/-</sup> mice. The inflammatory caspases, caspase-1 and caspase-11 shape host defence by

promoting pyroptosis and induction of pro-inflammatory IL-1 $\beta$ . While caspase-1 is directly involved in IL-1 $\beta$  cleavage, caspase-11 can promote caspase-1 activity (262). However, the inflammatory monocyte recruitment was intact in alum injected IL-1RI-deficient mice, which excludes the involvement of IL-1 $\beta$ . However, it is possible that both caspases might have additional protein targets, which can have an impact on immune responses beyond inflammasome activation. For instance caspase-11 has been implicated in intracellular TLR4-independent LPS sensing (270). Furthermore, there is a report suggesting that caspase-11 has an important role in cell migration. Caspase-11 has been shown to interact with Aip1, which is involved in actin depolymerisation during chemotaxis (271). However, it is unclear how this effect would be critical specifically for recruitment of monocytes and not other cell populations. Overall, it will be vital to test whether alum-induced inflammatory monocyte recruitment is impaired in mice selectively deficient only in caspase-11.

One of the danger signals induced after alum injection is host DNA, which has been suggested to be released by dying cells (250). However, the finding that DNA release at the site of alum injection is sustained at least up to 24h post alum administration (250) suggests that it might be an active process. Indeed, it is demonstrated in this thesis that alum-induced cell death and DNA release did not occur at the same time. While cell death was most marked after 1 hour, DNA accumulated in the peritoneum over time and reached a peak around 24h following alum injection. Interestingly, it was also reported that the alum depot contains DNA in structures which resemble extracellular traps (ETs) (229). ETs are a recently described facet of the innate immune response against pathogens. They consist of DNA nets, which bind antimicrobial peptides and proteases, where bacteria can be entrapped and killed (230). ET formation can be stimulated by various stimuli, including LPS, PMA, IL-8 and monosodium urate crystals and can be released by cells, including neutrophils, eosinophils, monocytes, macrophages, basophils and mast cells (230, 272, 273). Alum induced the infiltration of cells, including neutrophils and inflammatory monocytes, as quickly as 3h after injection and their accumulation reached a significant level at 24h. Importantly, extracellular DNA also accumulated slowly, reaching a peak level at 24h post alum injection, which suggests that it is released by infiltrating cells. Surprisingly, DNA levels were reduced in mice deficient in IL-1RI and

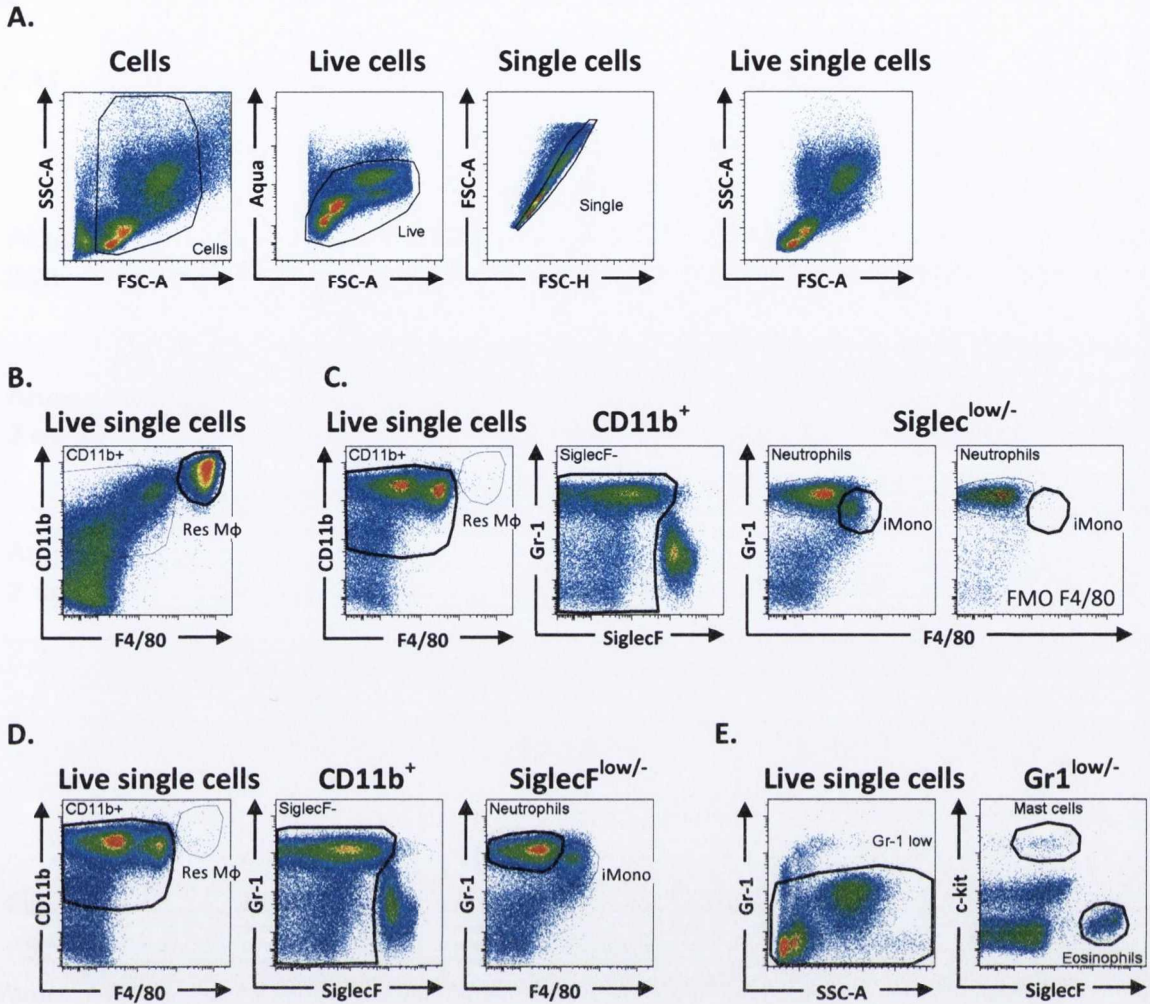
caspase-1/11. This reduction in concentrations of DNA correlated with the decrease in neutrophil and inflammatory monocyte recruitment, respectively.

Furthermore, the current results indicate that alum is not toxic to infiltrating cells at later time points (6 and 24h), because the level of cell death is comparable to mice injected with PBS. This is consistent with previous reports where it was noticed that alum forms a depot by 4h post injection (229), which may explain its loss of local toxicity. Overall, while the source of host DNA may be different to that suggested in the original report (250), it is still an important danger signal which can modulate adaptive immune responses.

Although IL-1 $\beta$  is released early after alum injection and together with IL-1 $\alpha$ , is critical for neutrophil infiltration, their role in driving alum-induced adaptive immune responses is not clear. In this report it is demonstrated that alum drives antibody production and promotes cytotoxic T cell expansion in an IL-1-independent manner. This is surprising especially, since it has been observed that IL-1RI-deficient mice had a decreased level of extracellular DNA at the site of injection. It has been demonstrated by others that the digestion of extracellular DNA by DNase I during alum immunisation led to a significant reduction in the level of antigen-specific IgG1 and IgE (250), due to impairment of DC-T cell interaction (252). However, IL-1RI-deficient mice immunised with alum had comparable levels of antigen-specific IgG1 and IgE, which suggests that the partial decrease in extracellular DNA after alum injection is not sufficient to block alum-induced immune responses. In general, these results support previous reports which demonstrated that MyD88 signalling (235) and IL-1 $\beta$  processing were dispensable in alum-induced antibody responses (239, 241, 243), but are in conflict with reports suggesting that these responses were inflammasome-dependent (236, 242).

Alum promotes not only NLRP3-dependent IL-1 $\beta$  secretion *in vitro*, but also IL-18. Surprisingly, it is demonstrated here that IL-18 does not play a role in innate or adaptive immune responses following alum immunisation. However, it is possible that IL-18 plays a substantial role in alum-driven immune responses beyond the scope of this study. In particular the role of IL-1 and IL-18 in the promotion of T and B cell memory should be addressed.

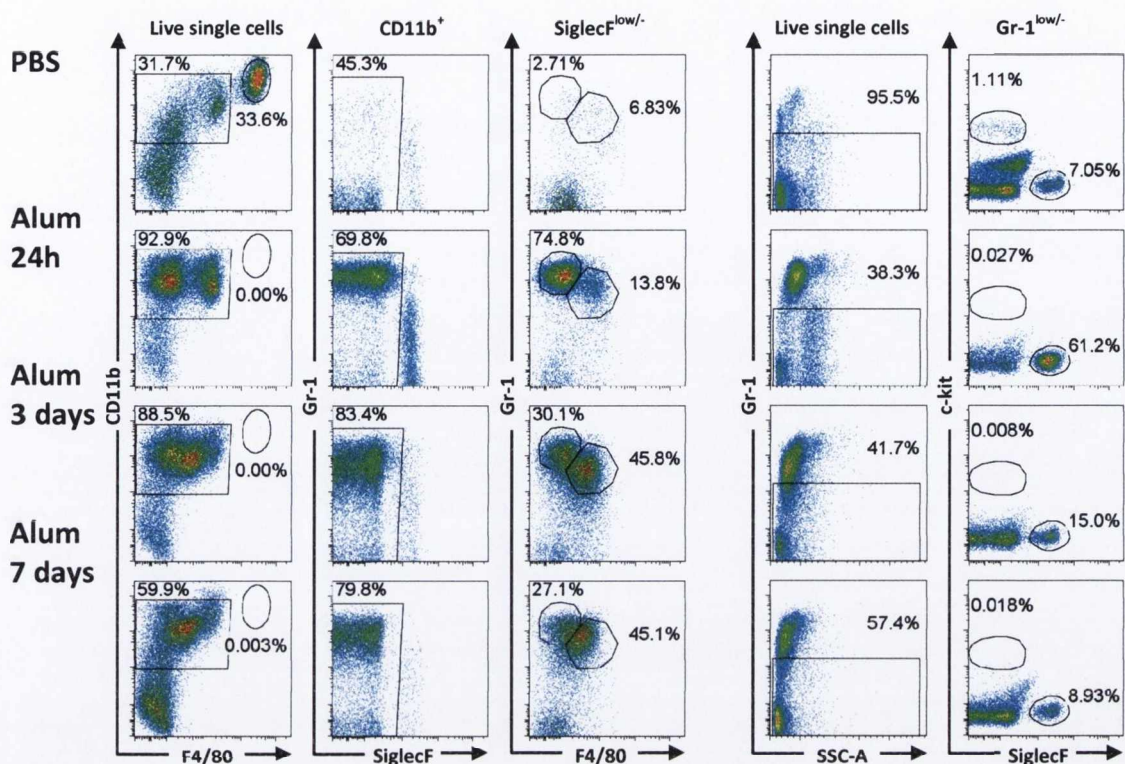
Overall, the current findings suggest that while alum activates the NLRP3 inflammasome by dendritic cells or other cells *in vitro*, the induction of innate and adaptive immunity *in vivo* is independent of inflammasomes. Furthermore, while IL-1 $\alpha$  and inflammasome-independent IL-1 $\beta$  production at the site of alum injection promotes innate responses, this is dispensable for antibody production or cytotoxic T cell expansion.



**Figure 3.1 Gating strategy for peritoneal cells.** Peritoneal cells from C57BL/6 mice were isolated 24h post PBS (A, B, E) or alum injection (C, D) and were stained with Aqua LIVE/DEAD and antibodies against CD11b, F4/80, Gr-1, c-kit and SiglecF. Cells were acquired using BDFortessa flow cytometry. (A) Acquired cells were gated to include only live and single cells for further analysis. (B) Resident peritoneal macrophages (Res Mφ) were gated as CD11b<sup>high</sup>F4/80<sup>high</sup>, (C) Inflammatory monocytes (iMono) as CD11b<sup>+</sup>Gr-1<sup>+</sup>F4/80<sup>+</sup> and (D) neutrophils as CD11b<sup>+</sup>Gr-1<sup>high</sup>F4/80<sup>-</sup>. (E) Mast cells were gated as Gr-1<sup>-</sup>SiglecF<sup>-</sup>c-kit<sup>+</sup> and eosinophils as Gr-1<sup>low</sup>SiglecF<sup>+</sup>c-kit<sup>-</sup>.

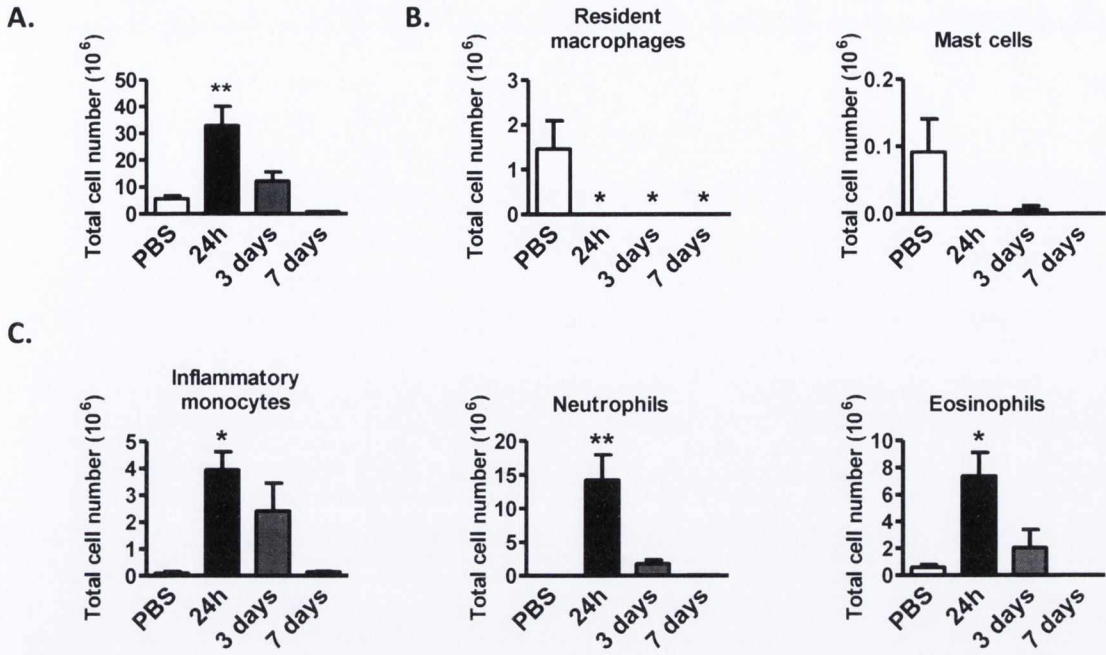






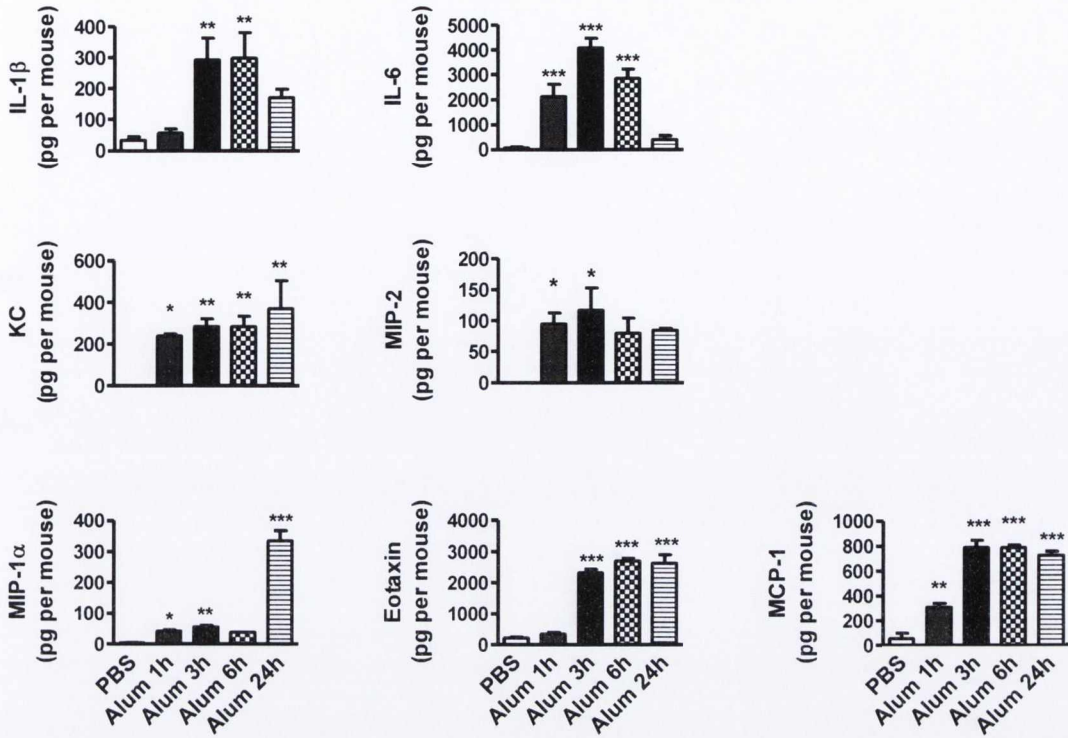
**Figure 3. 2. Characterization of the innate response after injection with alum.** C57BL/6 mice were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 h, 3 days and 7 days following injection and the peritoneal cavity was washed with PBS. Cells were counted, stained and analysed by flow cytometry for the presence of resident peritoneal macrophages (CD11b<sup>high</sup>F4/80<sup>high</sup>), mast cells (Gr-1<sup>-</sup>SiglecF<sup>-</sup>c-kit<sup>+</sup>), inflammatory monocytes (CD11b<sup>+</sup>Gr-1<sup>+</sup>F4/80<sup>+</sup>), neutrophils (CD11b<sup>+</sup>Gr-1<sup>high</sup>F4/80<sup>-</sup>) and eosinophils (Gr-1<sup>low</sup>SiglecF<sup>+</sup>c-kit<sup>-</sup>). Results are representative of two independent experiments.





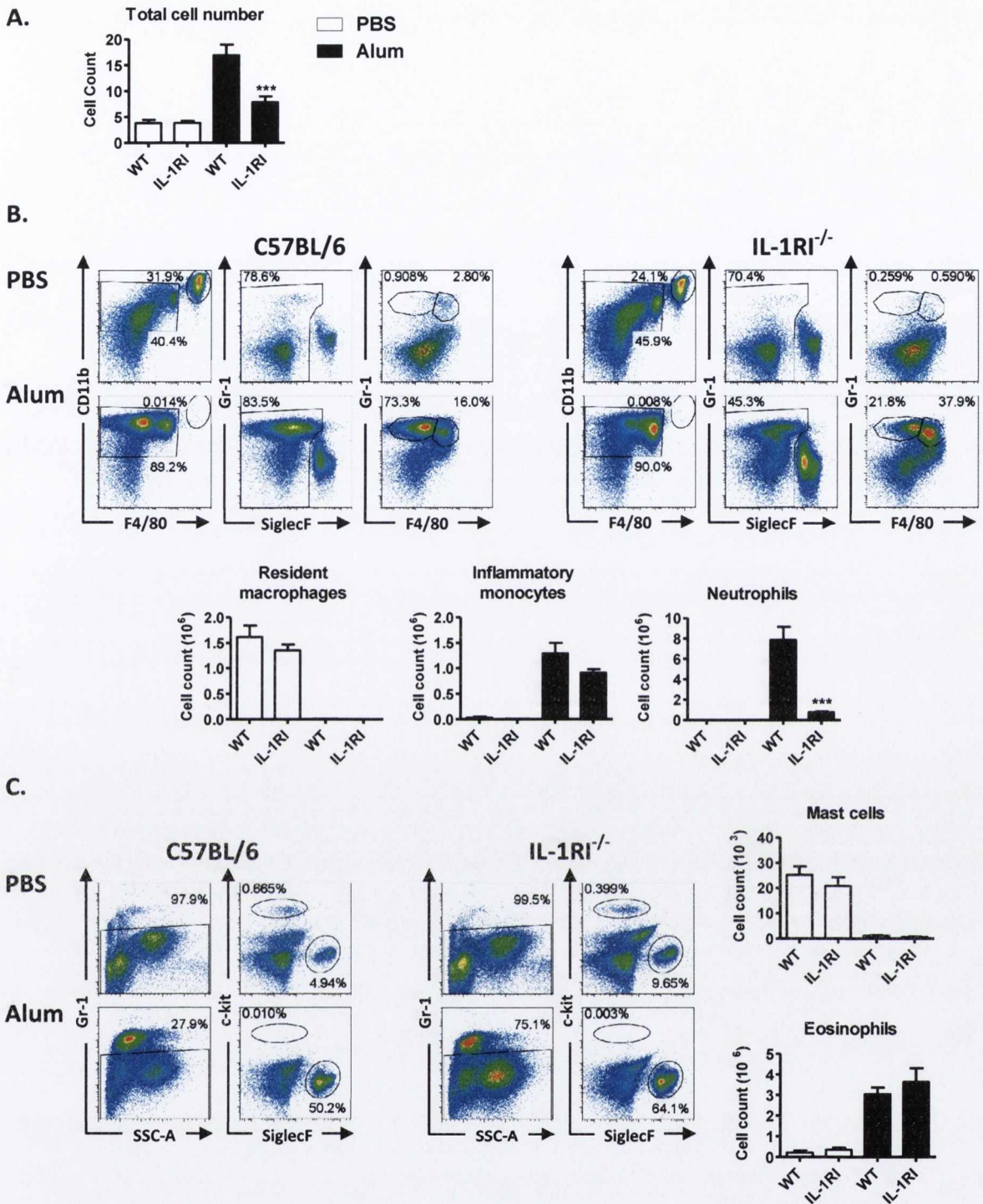
**Figure 3. 3. Alum induces a rapid innate immune response at the site of injection.** C57BL/6 mice were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 h, 3 days and 7 days following injection and the peritoneal cavity was washed with PBS. Cells were counted (A), stained and analysed by flow cytometry for the presence of (B) resident macrophages, mast cells (C) inflammatory monocytes, neutrophils and eosinophils. PBS v Alum \* $p < 0.05$ , \*\*\* $p < 0.001$ . Results are representative of two independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group.





**Figure 3. 4. Alum promotes cytokine and chemokine release at the site of injection** C57BL/6 mice were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 1, 3, 6 and 24 hours following injection and the peritoneal cavity was washed with PBS. The acellular phase of the peritoneal lavage fluid was analysed for the presence of IL-1 $\beta$ , IL-6, KC, MIP-2, MIP-1 $\alpha$ , eotaxin and MCP-1. PBS v Alum \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Results are pooled from two independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group.

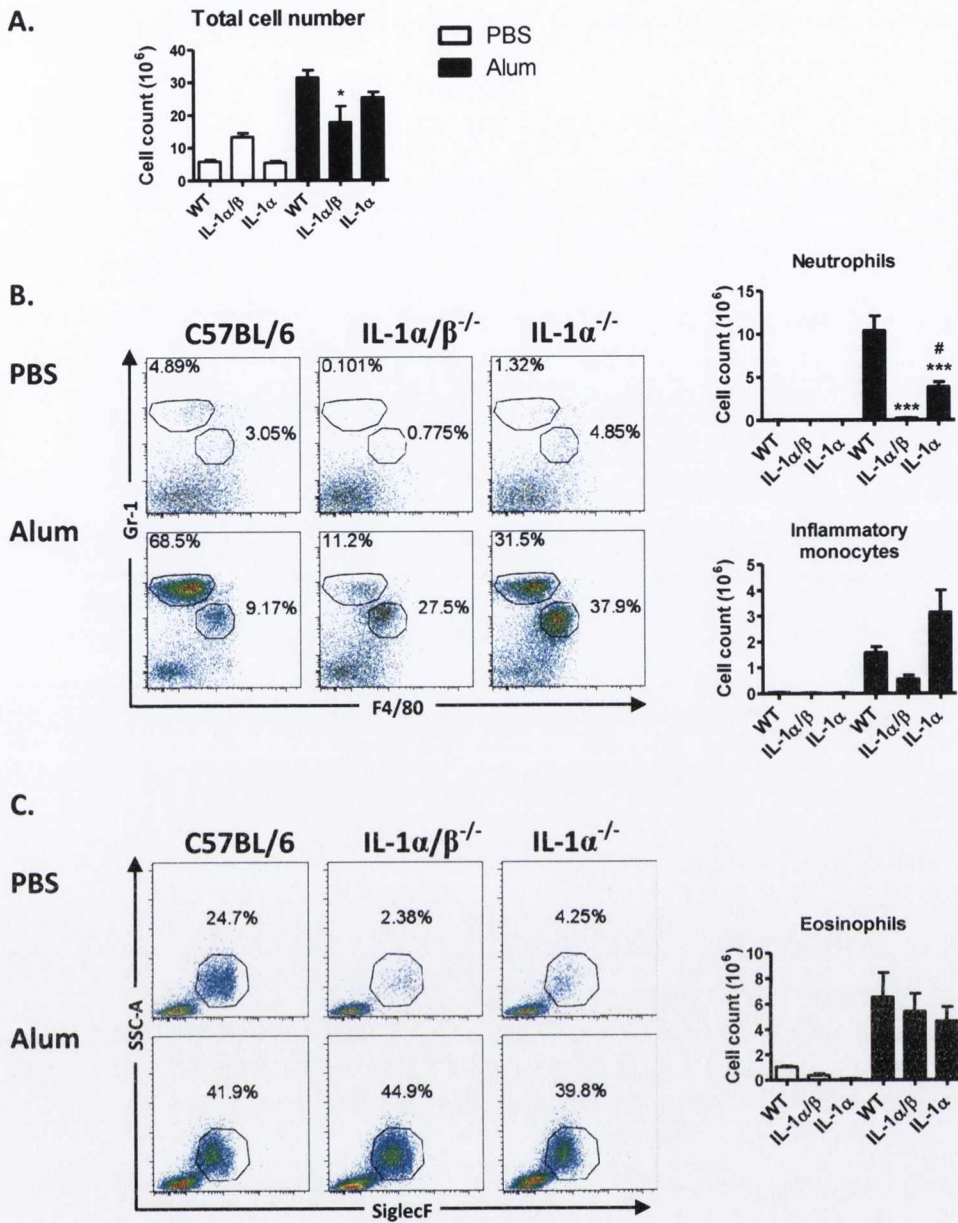




**Figure 3. 5. IL-1 controls neutrophil infiltration into the site of alum injection.** Mice (C57BL/6 and IL-1RI<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were counted (A), stained and analysed by flow cytometry for the presence of (B) resident macrophages, inflammatory monocytes, neutrophils, (C) eosinophils and mast cells. Wild-type Alum v IL-1RI<sup>-/-</sup> Alum \*\*\*p<0.001. Results are representative of three independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group.

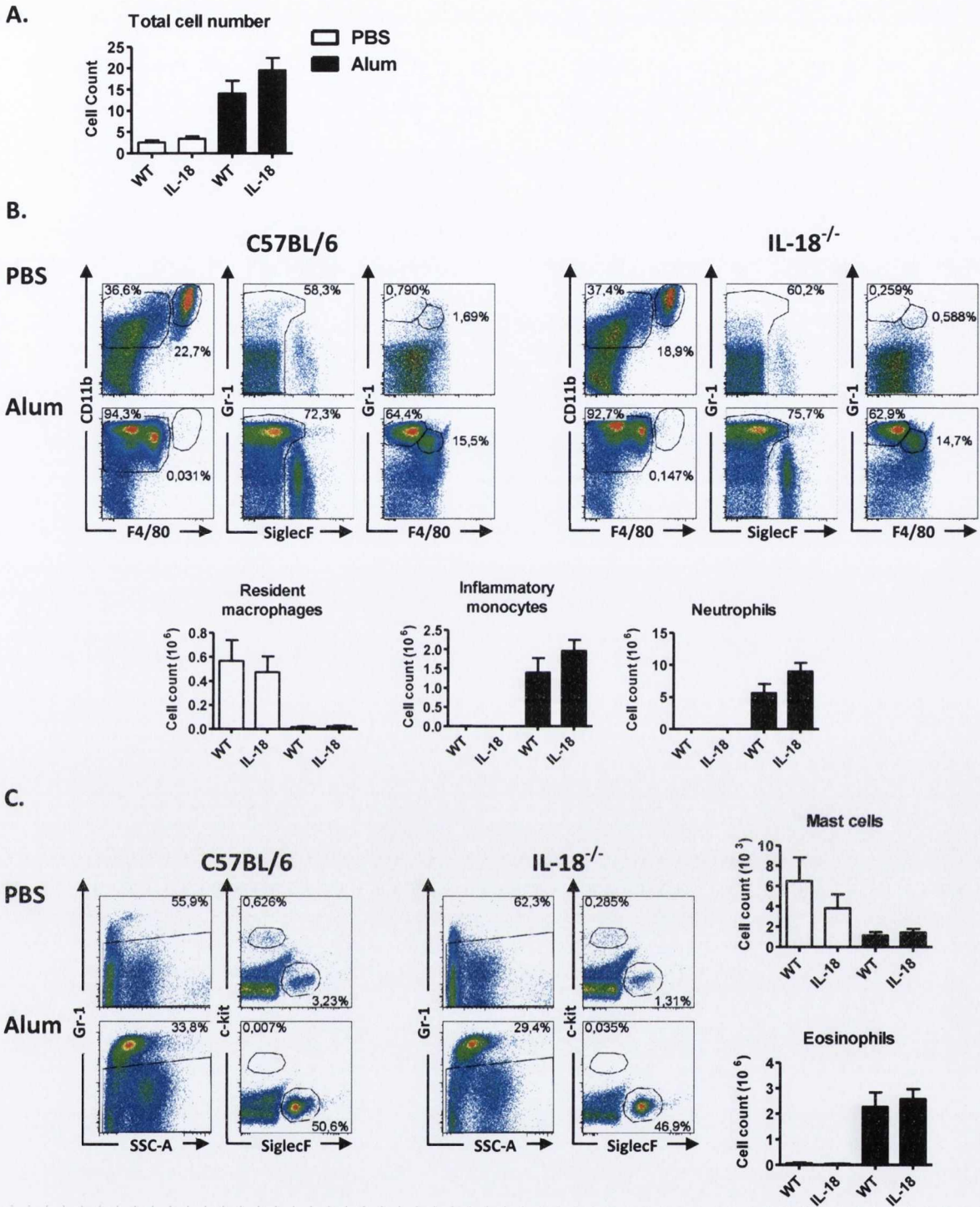






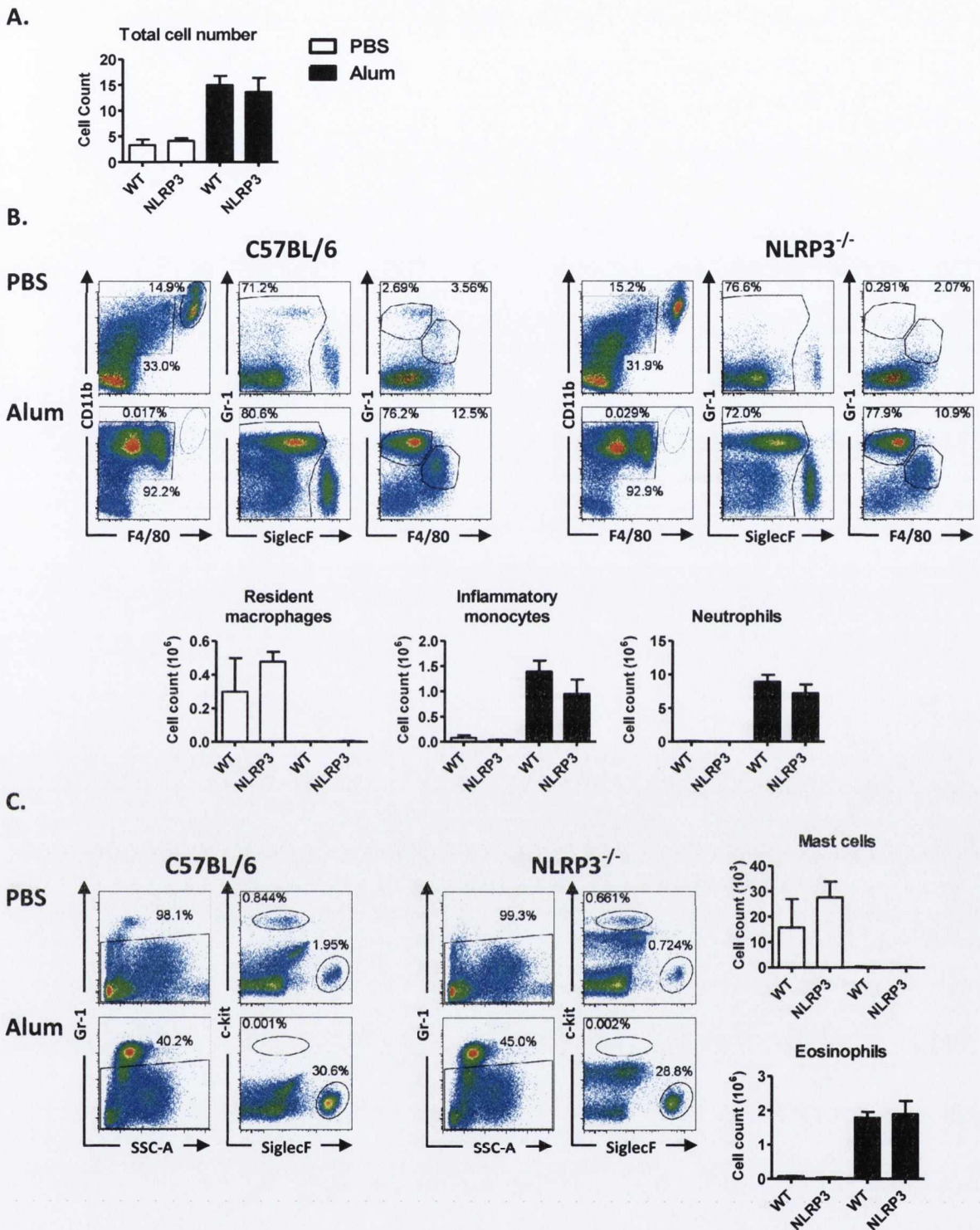
**Figure 3. 6. Both IL-1RI ligands, IL-1 $\alpha$  and IL-1 $\beta$ , control neutrophil recruitment into the site of alum injection.** Mice (C57BL/6, IL-1 $\alpha$ / $\beta$ <sup>-/-</sup> and IL-1 $\alpha$ <sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were counted (A), stained and analysed by flow cytometry for the presence of (B) neutrophils, inflammatory monocytes (gated on live single CD11b<sup>+</sup> cells) and (C) eosinophils (gated on live single Gr-1<sup>low/-</sup> cells). Wild-type Alum v IL-1 $\alpha$ / $\beta$ <sup>-/-</sup> Alum \*\*\*p<0.001. IL-1 $\alpha$ / $\beta$ <sup>-/-</sup> Alum v IL-1 $\alpha$ <sup>-/-</sup> Alum #p>0.05. Results represent one experiment. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group. Experiment performed by Dr Graham Tyan.





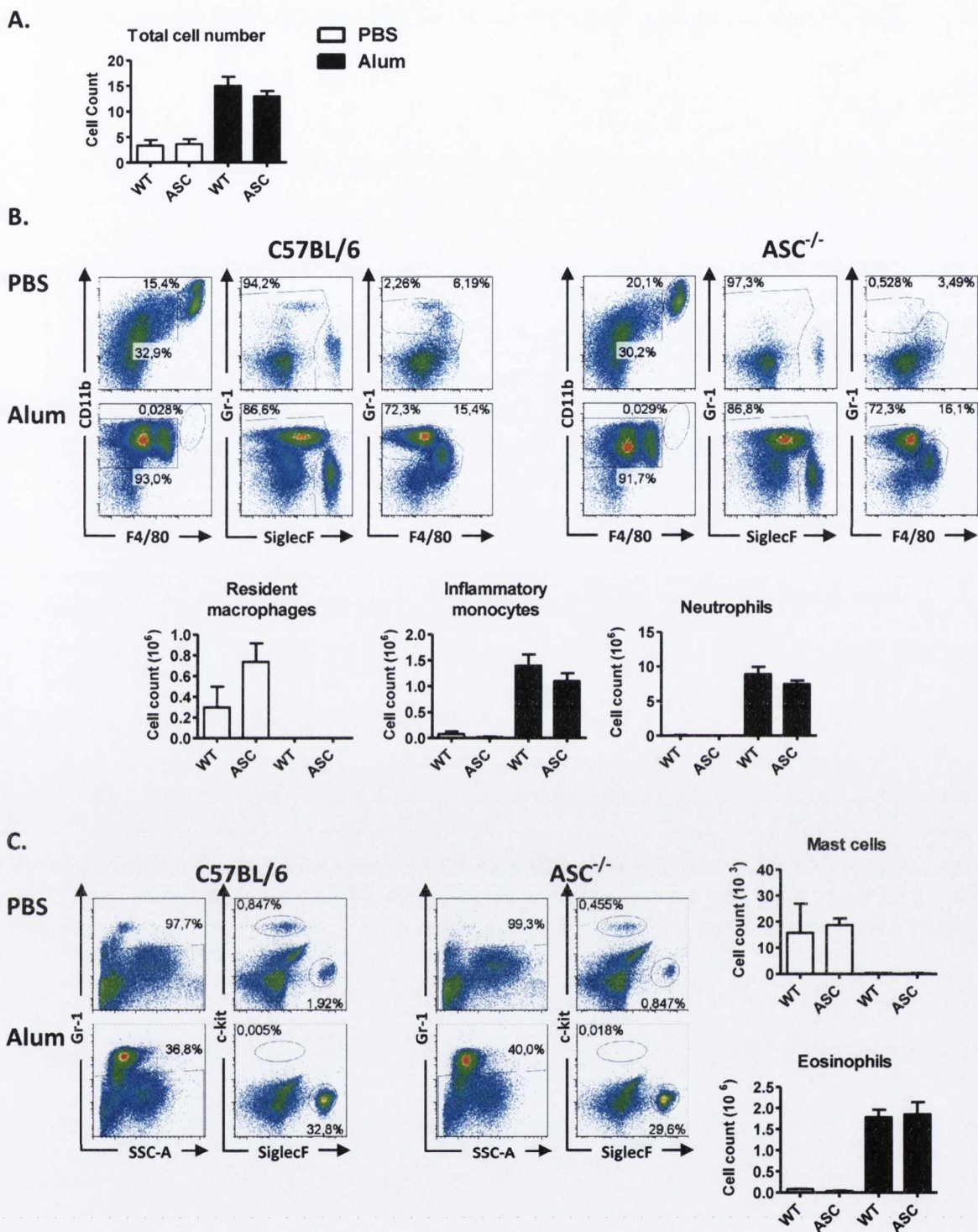
**Figure 3. 7. The recruitment of inflammatory cells following alum injection is not IL-18 dependent.** Mice (C57BL/6 and IL-18<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were counted (A), stained and analysed by flow cytometry for the presence of (B) resident macrophages, inflammatory monocytes, neutrophils, (C) eosinophils and mast cells. Results are representative of two independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group.





**Figure 3. 8. The recruitment of inflammatory cells following alum injection is not NLRP3 dependent.** Mice (C57BL/6 and NLRP3<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were counted (A), stained and analysed by flow cytometry for the presence of (B) resident macrophages, inflammatory monocytes, neutrophils, (C) eosinophils and mast cells. Results are representative of three independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group.

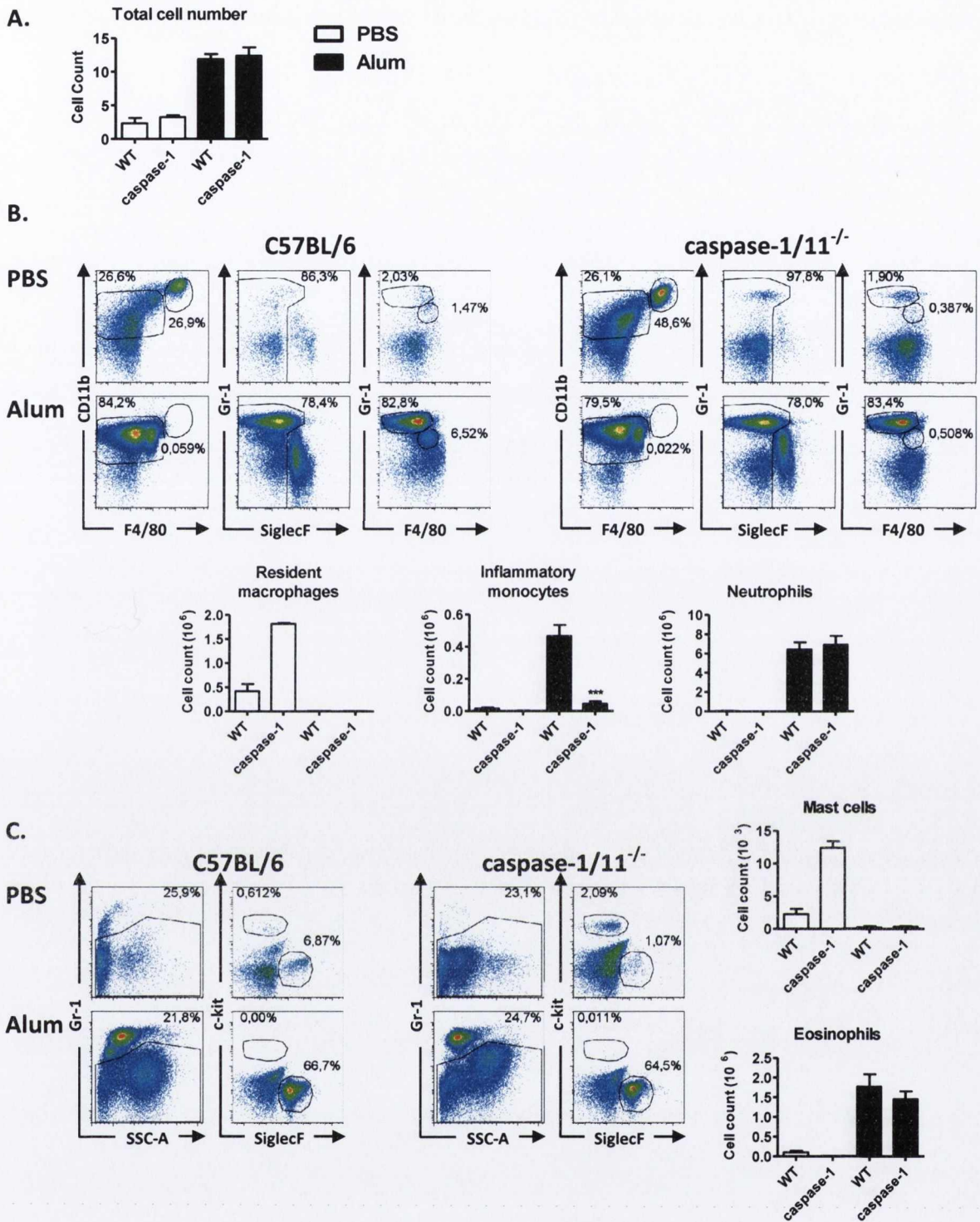




**Figure 3. 9. ASC is not required for the recruitment of inflammatory cells to the site of alum injection.** Mice (C57BL/6 and ASC<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were counted (A), stained and analysed by flow cytometry for the presence of (B) resident macrophages, inflammatory monocytes, neutrophils, (C) eosinophils and mast cells. Results are representative of two independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group.



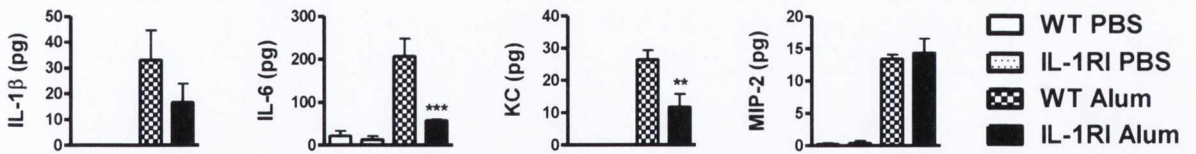




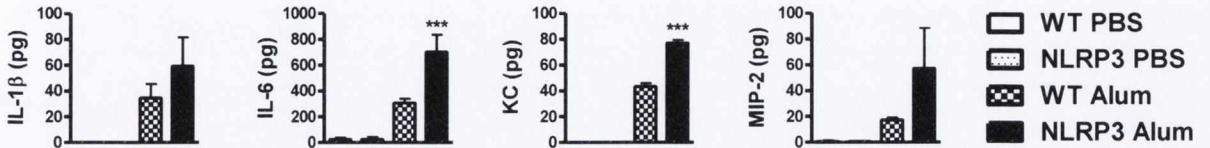
**Figure 3. 10. Caspase-1 or caspase-11 is involved in recruitment of inflammatory monocytes to the site of alum injection.** Mice (C57BL/6 and caspase-1/11<sup>-/-</sup>) were injected with PBS or alum (1mg/mouse) intraperitoneally. Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were counted (A), stained and analysed by flow cytometry for the presence of (B) macrophages, neutrophils, (C) eosinophils and mast cells. Wild-type Alum v caspase-1/11<sup>-/-</sup> Alum \*\*\*p<0.001. Results are representative of two independent experiments. Error bars show means ± SEM for 3-4 mice per experimental group.



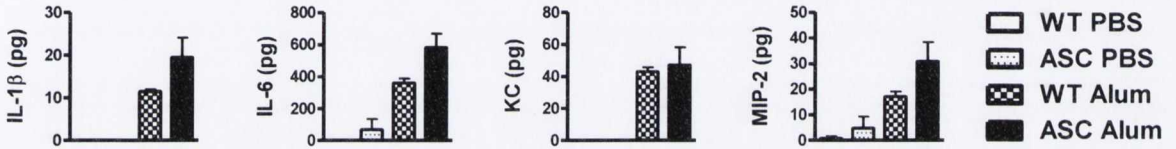
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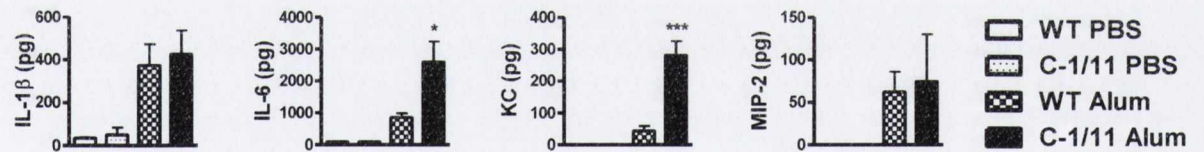
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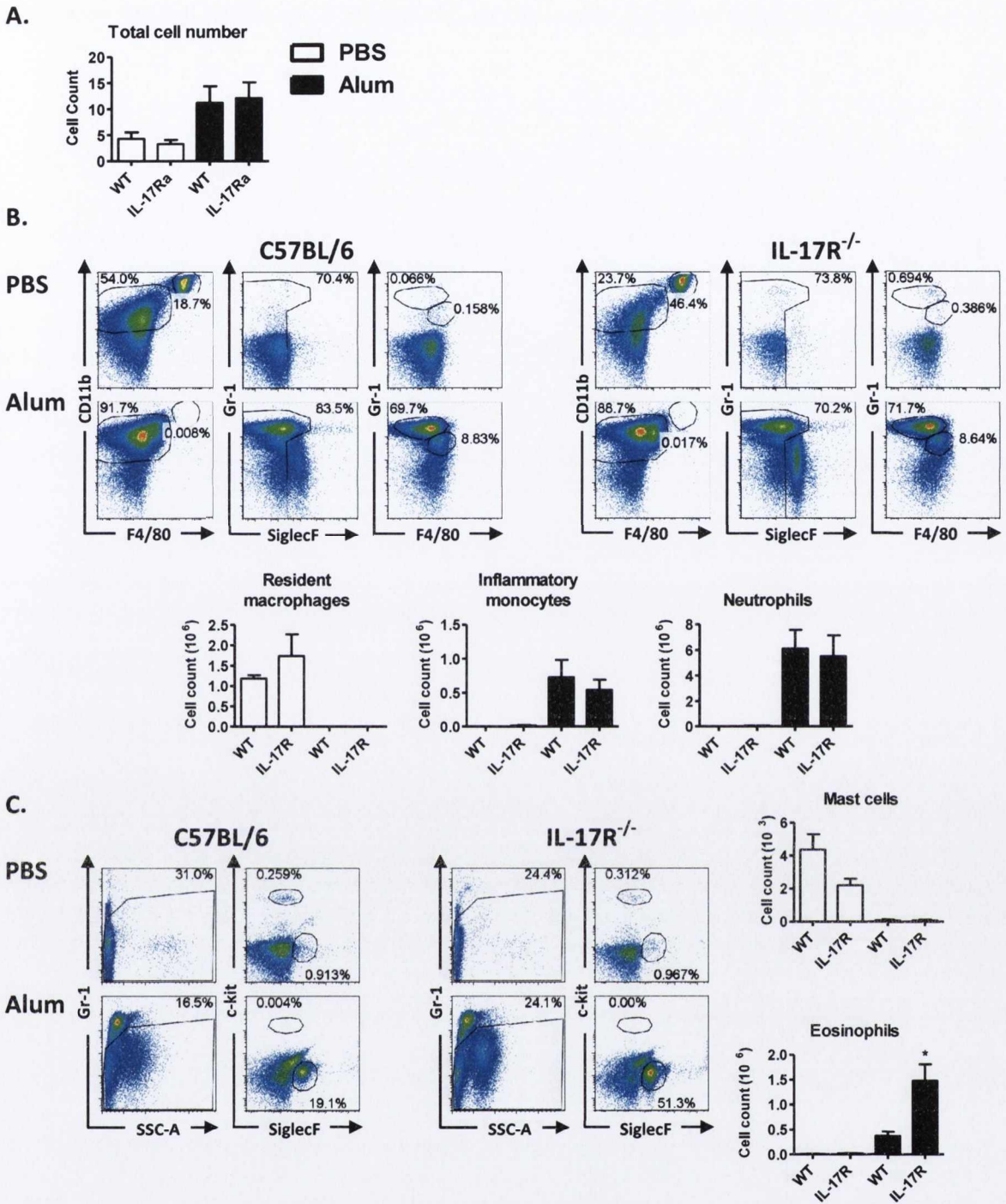


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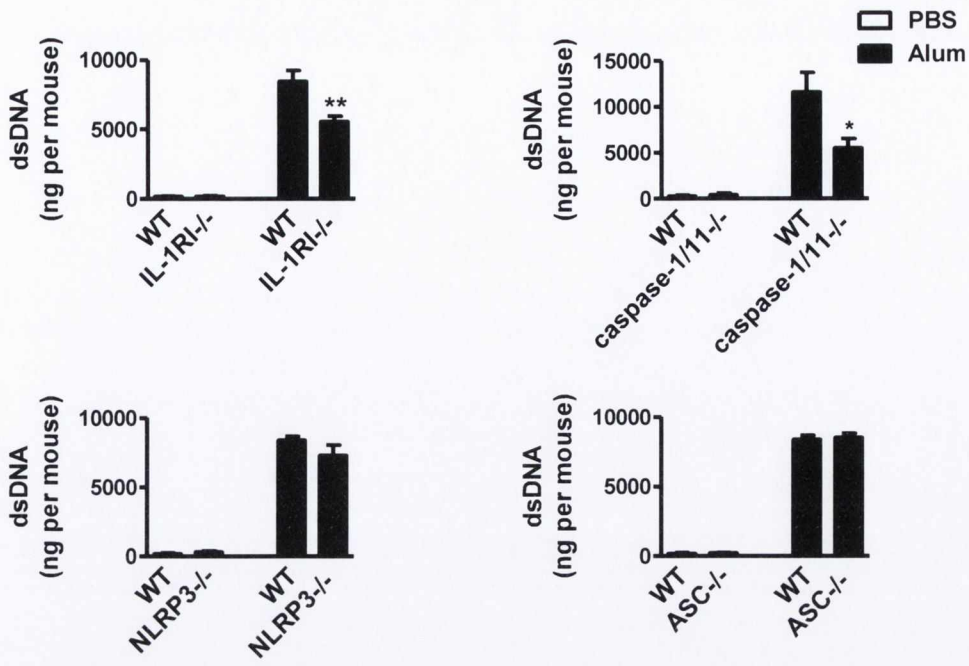
**Figure 3. 11. Alum-induced release of IL-6 and KC is IL-1-dependent.** C57BL/6, IL-1RI<sup>-/-</sup> (A), NLRP3<sup>-/-</sup> (B), ASC<sup>-/-</sup> (C) and caspase-1/11<sup>-/-</sup> (D) mice were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. The acellular phase of the peritoneal lavage fluid was analysed for the presence of IL-1α (undetected), IL-1β, IL-6, KC and MIP-2. Alum (C57BL/6) v Alum (IL-1RI<sup>-/-</sup>, NLRP3<sup>-/-</sup>, ASC<sup>-/-</sup>, caspase-1/11<sup>-/-</sup>) \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. Results are pooled from two independent experiments (IL-1RI<sup>-/-</sup>, NLRP3<sup>-/-</sup> and ASC<sup>-/-</sup>) or represent one experiment (caspase-1/11<sup>-/-</sup>). Error bars show means ± SEM for 3-4 mice per experimental group.





**Figure 3. 12. Lack of IL-17 boosts eosinophil infiltration into the site of alum injection.** Mice (C57BL/6 and IL-17R<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Peritoneal cells were counted (A), stained and analysed by flow cytometry for the presence of (B) resident macrophages, inflammatory monocytes, neutrophils, (C) eosinophils and mast cells. Wild-type Alum v IL-17R<sup>-/-</sup> Alum \*p<0.01. Results represent one independent experiment. Error bars show means ± SEM for 3 mice per experimental group.

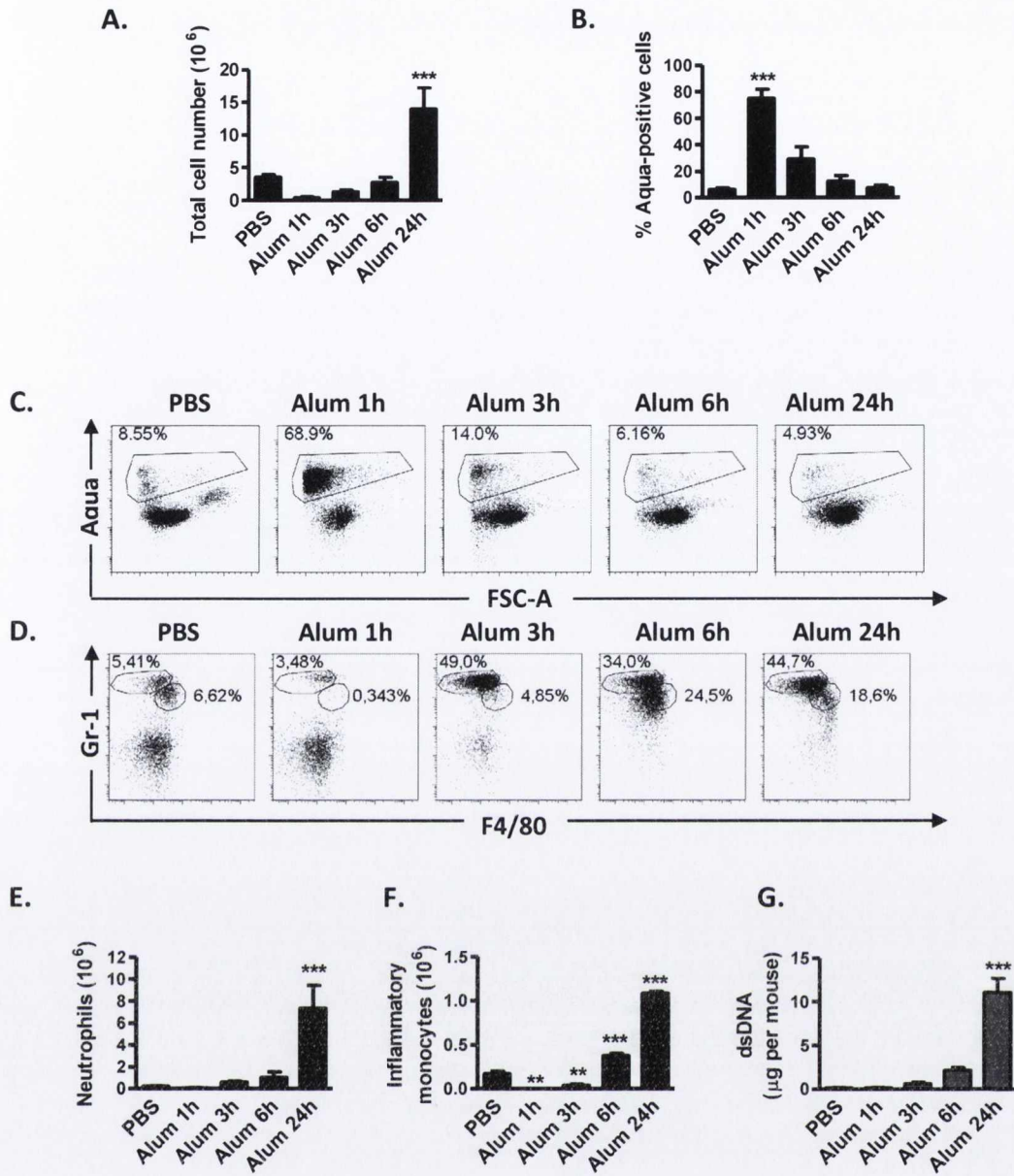




**Figure 3.13. Alum-induced DNA release at the site of injection is partly IL-1 and caspase-1 or caspase-11 dependent.** Mice (C57BL/6, IL-1RI<sup>-/-</sup>, NLRP3<sup>-/-</sup>, ASC<sup>-/-</sup> and caspase-1/11<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24h following injection and the peritoneal cavity was washed with PBS. The acellular phase of the peritoneal lavage fluid was analysed for the presence of dsDNA. Alum (C57BL/6) v Alum (IL-1RI<sup>-/-</sup>, NLRP3<sup>-/-</sup>, ASC<sup>-/-</sup> or caspase-1/11<sup>-/-</sup>) \*p<0.05, \*\*\*p<0.001. Results are representative of two independent experiments (C57BL/6, IL-1RI<sup>-/-</sup>, ASC<sup>-/-</sup> and NLRP3<sup>-/-</sup> mice) or represent one experiment (caspase-1/11<sup>-/-</sup> mice). Error bars show means ± SEM for 3-4 mice per experimental group.

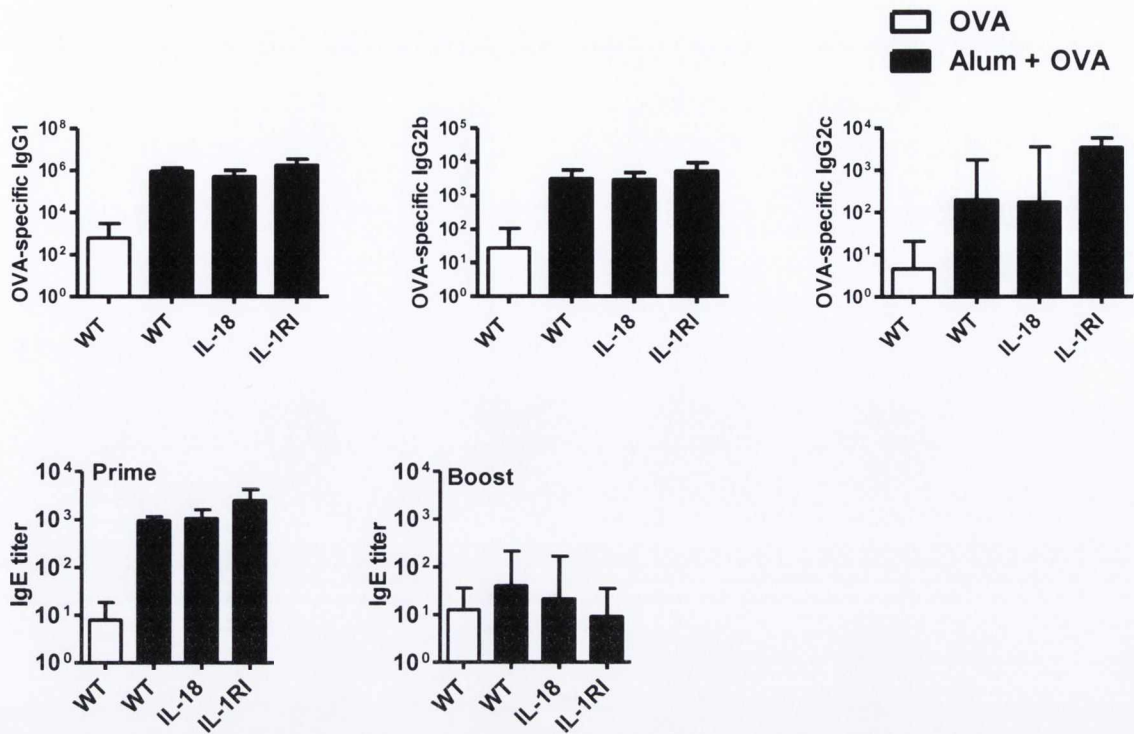






**Figure 3. 14. Alum-induced cell death and dsDNA release occur at different time points.** C57BL/6 mice were injected with PBS or alum (1mg/mouse) intraperitoneally. Mice were sacrificed 1h, 3h, 6h and 24h following injection and the peritoneal cavity was washed with PBS. (A) Peritoneal cells were counted. (B, C) Peritoneal cells were stained with Aqua LIVE/DEAD and cell death was analysed by flow cytometry. (D, E, F) Peritoneal cells were stained for the presence of neutrophils ( $CD11b^+Gr-1^{high}F4/80^-$ ) and inflammatory monocytes ( $CD11b^+Gr-1^+F4/80^+$ ) (G) The acellular phase of the peritoneal lavage fluid was analysed for the presence of dsDNA. PBS v Alum \*\*\* $p < 0.001$  (A, B, E, F) Results are combined from two independent experiments with 3-4 mice per experimental group. Error bars show means  $\pm$  SEM. (C, D) Results are representative of two independent experiments.





**Figure 3. 15. IL-1 and IL-18 are not required for antigen-specific antibody production after alum immunisation.** Mice (C57BL/6, IL-18<sup>-/-</sup> and IL-1RI<sup>-/-</sup>) were immunised intraperitoneally with PBS, EndoOVA alone or EndoOVA in combination with alum on days 0 and 14. On days 13 and 21, blood was collected. Blood samples were centrifuged and serum was removed. The serum samples were analysed for OVA-specific IgG1, IgG2b, IgG2c and IgE by ELISA. OVA-specific antibody responses are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal serum dilutions to a cut-off point of 2 standard deviations above the mean OD from sera of PBS injected mice. Results are from one experiment. Error bars show means  $\pm$  SEM for 4-5 mice per experimental group.



## **CHAPTER 4**

# **IL-33 MODULATES ALUM-INDUCED IMMUNE RESPONSES**



## 4.1. Introduction

Polly Matzinger proposed the “danger theory”, which suggests that the immune system responds principally to “dangerous” situations like stress, injury and cell death (274), events which are associated with release of endogenous danger signals, or damage-associated molecular patterns (DAMPs). DAMPs are released when cells lose plasma membrane integrity, which can trigger alarm and inflammation (275). Aluminium adjuvants are not recognised by any specific receptor, but promote lipid raft formation and receptor-independent cell activation (232). However, alum also promotes cell death inducing local necrosis in vaccinated muscle tissue (207). Furthermore, following intraperitoneal injection in mice, there is a substantial increase in cell death in the peritoneal cavity (250). The nature of alum-induced cell death was never fully characterised but it has been reported that *in vitro*, alum-induced macrophage death is cathepsin B/S-dependent (276). A number of forms of cell death have been associated with the induction of inflammation: pyroptosis, pyronecrosis and necrosis (277) which have different mechanisms of initiation but all result in a loss of membrane integrity. Necrosis was long regarded as an uncontrolled form of cell death, but recent data shows that it can be regulated e.g. through RIP kinases (278). While pyroptosis is associated with inflammasome activation and is caspase-1 and NLR protein-dependent (279), pyronecrosis, is independent of caspase-1, but employs NLRs, the adaptor protein ASC and cathepsin B (280). Furthermore, pyronecrosis is associated with release of the danger signal High Mobility Group Box 1 (HMGB-1) (280), while pyroptosis promotes IL-1 $\beta$  release (279). Interestingly, neither of these types of cell death were found to be involved in alum-induced macrophage death *in vitro* (276).

To date, two DAMPs have been shown to be released following alum administration: uric acid (237) and host DNA (250). Uric acid is generated during the catabolism of purines and is present in extracellular fluid under physiological conditions. When its concentrations are high it crystallises which leads to monosodium urate (MSU) crystal formation. Alum induces the release of high concentrations of uric acid (250) and depletion of alum-induced uric acid by uricase results in decreased antigen-specific T cell and humoral responses, which indicates



that uric acid plays an important role in alum adjuvanticity (237). Host DNA, is also released shortly after injection and is partly responsible for the induction of antigen-specific humoral immunity (250). Importantly, there might be additional signals which are involved in shaping the immune response after vaccination. One possible candidate is IL-33, a member of the IL-1 family (281), which is expressed in the nucleus of many cells, mainly by those exposed to the external environment (137). The mechanism of IL-33 release is not fully elucidated, but it has been suggested that it is primarily released by necrotic cells (138). Interestingly, IL-33 promotes humoral responses and upon exposure to IL-33, type 2 innate lymphoid helper cells and TH2 cells produce IL-5 and IL-13 and naïve T cells are polarised toward TH2 cells (142, 144, 145). In addition, IL-33 modulates functions of innate immune cells, including eosinophils and basophils. Furthermore, ST2 and IL-33 deficient mice have an impaired TH2 immune response toward parasites and in an established allergy model (150, 282-284).

Based on the toxicity of alum and the TH2-promoting properties of IL-33, it is hypothesised that alum induces the release of IL-33, which modulates innate and adaptive immune responses.

#### **4.2.Aims**

- To characterise the nature of cell death promoted by alum
- To investigate whether alum promotes IL-33 release
- To investigate the role of IL-33 in alum-mediated innate immune responses and its potential to influence the outcome of alum immunisation

## 4.3. Results

### 4.3.1. Alum induces cell death *in vitro*

Aluminium-containing adjuvants induce local inflammation and irritation at the site of injection (250, 276). In order to assess the cytotoxic effect of alum *in vitro*, BMDCs were incubated with the adjuvant at concentrations from 5-2000 $\mu\text{g}/\text{ml}$ . After 15 minutes, 1, 3 and 6 hours, cells were collected, stained with propidium iodine (PI) and analysed by flow cytometry. PI was used to distinguish viable from dead cells, because it only incorporates into DNA of cells where the structural integrity of the cell membrane is compromised. BMDCs incubated with high concentrations of alum for as little as 15 minutes were PI<sup>+</sup> (Figure 4. 1 A) and the cytotoxicity of alum increased with time (Figure 4. 1 B). Furthermore, a significant percentage of BMDCs were PI<sup>+</sup> after 3 and 6h of treatment when incubated with lower concentrations of alum (100-500 $\mu\text{g}/\text{ml}$ ) (Figure 4. 1. A).

### 4.3.2. Alum is phagocytosed by dendritic cells

It has been demonstrated that alum is recognised in a receptor-independent manner via its interactions with membrane lipids and it has been proposed that alum is not internalised by DCs, but rather a process of abortive phagocytosis leads to uptake of antigen alone (232). However, others showed that alum can be phagocytosed by macrophages within a few hours (240, 285). Low concentrations of fluorescent alum, up to 20 $\mu\text{g}/\text{ml}$ , were used to exclude its cytotoxic effect on cells. Therefore, to assess whether alum can be internalised by dendritic cells, BMDCs were incubated with fluorescent alum for 1, 3 and 6 hours and analysed by flow cytometry. Interestingly, most BMDCs were Alum OVA DQ-positive as early as 1 hour after incubation with particles and there was no marked increase in uptake after 3 or 6 hours (Figure 4. 2 A and B). Furthermore, to validate that fluorescent alum was phagocytosed and not only attached to the cell membrane, live imaging was performed. Fluorescent alum was added to BMDCs and cells were incubated in the confocal microscopy chamber (at 37°C, 5% CO<sub>2</sub>) for 100 minutes, and single images were taken every minute to monitor particle uptake. Alum was clearly phagocytosed

after a 1 hour incubation, while earlier, after 30 minutes, it was interacting with the surface of dendritic cells. Interestingly, DRAQ7, cell death marker, which stains DNA of cells with ruptured membranes, was added, but it did not stain cells at these early time points (Figure 4. 2. C). To make sure that DRAQ7 can be visualised by confocal microscopy, at the end of the experiment cells were treated with 10% H<sub>2</sub>O<sub>2</sub> and cell death was observed by DRAQ7 incorporation (data not shown). To further investigate whether alum is inside cells, BMDCs incubated with fluorescent alum for 6 hours were stained with cholera toxin B, which binds to the cell surface. Alum particles can clearly be identified within cells (Figure 4. 3). Since alum can be phagocytosed by DCs, the next question was whether the cytotoxic effect of alum was dependent on the uptake of particles. BMDCs were pre-treated with cytochalasin D, a fungal toxin which inhibits actin polymerisation and phagocytosis, prior to addition of a cytotoxic concentration of alum (400µg/ml). After 1, 3 and 6 hours, cells were collected, stained with PI and analysed by flow cytometry. As previously shown, alum enhanced the number of PI<sup>+</sup> cells compared to controls. However, cell death induced by alum was inhibited by cytochalasin D (Fig. 4. 4. A and B).

#### **4.3.3. Alum induces necrosis *in vitro***

Since phagocytosed alum promoted cell death in BMDCs the next question addressed was what kind of cell death is it? For a long time only two types of cell death were described: apoptosis and necrosis. One of the early changes in apoptotic cells is translocation of phosphatidylserine (PS) to the outer surface of the plasma membrane, which can be detected by flow cytometry with the use of fluorochrome-conjugated annexin V. In order to determine whether alum can induce apoptosis, FITC-conjugated annexin V (AnnV-FITC) was used. BMDCs were incubated with alum or actinomycin D, which was used as a positive control to induce apoptosis in BMDCs. After 1, 3 and 6 hours of treatment, cells were stained with annexin V and PI to distinguish apoptotic and necrotic cell death. As was expected, actinomycin D triggered apoptosis in BMDCs as early as 3h after exposure and actinomycin D-treated BMDCs were either PI<sup>-</sup>AnnV<sup>-</sup> or PI<sup>-</sup>AnnV<sup>+</sup> (Figure 4. 5. A). BMDCs incubated with alum did not stain selectively with annexin V at any time point. (Figure 4. 5. A). In order to

further confirm that alum does not induce apoptosis, BMDCs were pre-treated with the pan-caspase inhibitor ZVAD-fmk before addition of alum. After 3h cells were collected, stained with PI and analysed by flow cytometry. Inhibition of caspases did not affect the cytotoxicity of alum (Figure 4. 5. B).

Having established that alum induced cell death but did not promote apoptosis, it was necessary to determine whether alum can promote necrosis. However, necrosis is only a final destination of cells which can die due to different triggers and pathways. Recently, two new types of cell death were described: pyroptosis and pyronecrosis. Pyroptosis is inflammation-associated cell death, which relies on caspase-1 rather than apoptotic effector caspases (279). Alum is a known activator of the NLRP3 inflammasome and caspase-1 and it was determined whether alum-induced cell death is NLRP3, ASC or caspase-1-dependent. Firstly, BMDCs were pre-incubated with the caspase-1 inhibitor, YVAD-fmk. In addition, wild-type, ASC-deficient and NLRP3-deficient BMDCs were incubated with alum. After 3h, cells were collected and cell death was assessed by incorporation of PI. Alum promoted cell death and its capacity to induce cell death was not dependent on NLRP3 or caspase-1, which indicates that alum does not induce pyroptosis (Figure 4. 5. C). Next, it was evaluated whether alum promotes pyronecrosis. Pyronecrosis is caspase-1-independent, but NLRP3 and cathepsin B-dependent (280). Since alum-induced cell death is NLRP3-independent this would suggest it is not pyronecrosis. However, to confirm this, BMDCs were pre-treated with the cathepsin B inhibitor, CA-074-Me followed by incubation with alum. After 3h, cells were collected and cell death was assessed by staining with PI. Alum induced cell death and the presence of a cathepsin B inhibitor did not have an impact on its cytotoxicity (Figure 4. 5. D).

Particle uptake, followed by inflammasome assembly and lysosomal rupture are required for alum-induced IL-1 $\beta$  production by DCs (238). Since neither NLRP3, ASC, caspase-1 nor cathepsin B were required for cell death, their role in alum-driven IL-1 $\beta$  secretion was confirmed. BMDCs were primed with LPS for 3h, followed by 6h incubation with alum. As expected, a lack of inflammasome components, as well as inhibition of caspases and cathepsin B resulted in a significant decrease in IL-1 $\beta$

production. Furthermore, inhibition of actin polymerization by cytochalasin D also led to a decrease in IL-1 $\beta$  secretion (Figure 4. 6).

Having established that alum induced necrosis but not pyroptosis or pyronecrosis, the next question addressed was whether autophagy is involved in alum-induced cell death. Autophagy is a self-degradation process to maintain cell homeostasis (178). Interestingly, autophagy has been observed to accompany cell death (286). Two inhibitors were used to block autophagy, 3-methyladenine (3-MA) and chloroquine. 3-MA blocks class III PI3 kinases, which are involved in autophagosome formation, an initial event in autophagy, whereas chloroquine targets lysosomes and prevents their fusion with autophagosomes, which is a later event (Figure 1.13). BMDCs were pre-treated with autophagy inhibitors, prior to the addition of alum. After 6h, cells were collected, stained with PI and analysed by flow cytometry. Interestingly, pre-treatment with 3-MA, but not chloroquine, prevented alum-induced cell death (Figure 4. 7). Autophagy has also been implicated in regulation of IL-1 $\beta$  production. It has been demonstrated that autophagy inhibition increases IL-1 $\beta$  secretion by cells treated with LPS alone or LPS with the NLRP3 inducers, MSU crystals, nigericin or ATP (180, 287). In order to determine the role of autophagy in alum-induced IL-1 $\beta$  secretion, BMDCs were stimulated with LPS and then pre-incubated with 3-MA, before addition of alum at concentrations from 100-500 $\mu$ g/ml for 6 or 24h. Interestingly, inhibition of autophagy significantly decreased IL-1 $\beta$  secretion induced by LPS and alum at both time points, while 3-MA enhanced IL-1 $\beta$  secretion in LPS-primed BMDCs after 24h, but not 6h (Figure 4. 8). Overall, these results indicate that alum induces necrosis in dendritic cells, which can be blocked by an inhibitor of autophagy.

#### **4.3.4. Macrophages and muscle cells are susceptible to alum-driven cell death**

In order to demonstrate that alum-induced cell death is not restricted to dendritic cells, other innate immune cells, immortalised bone marrow-derived macrophages (iBMM), and non-hematopoietic cells, the muscle cell line C2C12, were used. iBMMs and C2C12 cell were incubated with a range of different concentrations

of alum (5-2000 $\mu$ g/ml). After 1 and 6 hours, cells were stained with PI and analysed by flow cytometry. Alum enhanced the number of PI<sup>+</sup> cells in both iBMMs and C2C12 cells (Figure 4. 9). However, the capacity of alum to induce cell death in iBMMs and C2C12 cells was different. iBMMs were more susceptible to cell death compared not only to C2C12 cells but also to BMDCs. iBMMs were killed even by concentrations of alum as low as 100 $\mu$ g/ml after only 1h of incubation. High concentrations of alum (500-2000 $\mu$ g/ml) killed almost 100% of iBMMs after 6h (Figure 4. 9 B). C2C12 cells were more resistant to alum-induced cell death. Alum did not significantly enhance C2C12 cell death after 1h and only higher concentrations of alum at the 6h time point were found to induce significant cytotoxicity (Figure 4. 9 A). Since alum-induced dendritic cell death was autophagy-dependent, the next question addressed was whether, inhibition of autophagy can block alum-induced cell death in immortalised bone-marrow derived macrophages. Thus, iBMMs were pre-treated with the inhibitors, 3-MA and chloroquine, followed by incubation with alum. After 6h cells were stained with PI and analysed by flow cytometry. As before, alum induced massive cell death. However, only pre-incubation with 3-MA, but not with chloroquine, prevented alum-induced cell death (Figure 4. 10).

#### **4.3.5. Alum induces cell death *in vivo***

Even though it was proven that alum could induce cell death *in vitro*, the most important and relevant question was whether alum can promote cell death *in vivo*. As shown before, alum induced massive cell death after intraperitoneal injection (Figure 3. 17. B), which was significant 1 hour post injection. To confirm this finding, C57BL/6 mice were injected intraperitoneally with PBS or alum (1mg/mouse). After 1h, the peritoneal cavity was washed and PerC were stained with Aqua Live/Dead Stain or PI to assess the percentage of cell death. As was expected, only a small percentage of PerC isolated from PBS-injected mice were PI<sup>+</sup> or Aqua<sup>+</sup>. However, alum injection induced massive cell death in the peritoneal cavity (Figure 4. 11). More than 50% of alum-treated PerC were PI<sup>+</sup>. Moreover, using the Aqua Live/Dead stain more than 60% of PerC from alum injected mice were dead.

#### 4.3.6. Alum induces early IL-33 release at the site of injection

Necrotic cells can release and induce secretion of pro-inflammatory cytokines into the surrounding tissue, leading to activation of the innate immune system. Since alum induced cell death *in vivo* the ability of alum to promote the release of pro-inflammatory chemokines and cytokines was investigated. The acellular peritoneal fluid isolated from mice injected intraperitoneally with PBS alone or alum for 1 hour was analysed for the presence of the cytokines IL-1 $\beta$ , IL-6, IL-33 and the chemokines KC, MIP-2, MIP-1 $\alpha$ , MCP-1 and eotaxin. Interestingly, IL-33 was released 1 hour post alum injection. Furthermore, there was a massive induction of pro-inflammatory IL-6, the neutrophil chemoattractants, KC, MIP-2, and the monocyte and macrophage chemoattractants, MIP-1 $\alpha$  and MCP-1. However, significant levels of IL-1 $\beta$ , IL-1 $\alpha$  and eotaxin were not detected (Figure 4. 12 A). To confirm that IL-33 is released shortly following alum-induced cell death, peritoneal acellular fluid isolated from mice injected intraperitoneally with PBS or alum for 1, 3, 6 and 24 hours was tested for the presence of IL-33. Alum only induced significant IL-33 release 1 hour post injection.

The release of IL-33 very quickly after alum administration was never described before. However, other damage-associated molecular patterns, including uric acid, have been implicated in alum-driven immune response (151). Therefore, in order to determine whether IL-33 release is uric acid-dependent, uricase was used to deplete uric acid levels during immunisation. Mice were immunised with PBS, alum, monosodium urate (MSU) crystals and with alum or MSU crystals in the presence of uricase. After 1 hour, mice were sacrificed, the peritoneal cavity was washed and PerC and the acellular peritoneal supernatants were collected. Firstly, supernatants from the peritoneal fluid were analysed for the presence of IL-33. At 1 hour post injection, alum induced upregulation of IL-33, whereas MSU crystals did not. Importantly, addition of uricase did not have any significant impact on alum-induced IL-33 (Figure 4. 13 A). To confirm that injected uricase was active, the effect of uric acid-depletion on alum and MSU crystal-induced cell infiltration was investigated. Isolated PerC were analysed for the presence of neutrophils and resident macrophages (Figure 4. 13 B). At early time points alum and MSU injection decreased the total cell number in the peritoneum, probably due to cell death, while uricase did not block this effect (Figure

4. 13 C). As was expected, alum and MSU crystals promoted the disappearance of resident macrophages and the recruitment of neutrophils to the site of injection. Importantly, even though the addition of uricase did not have any impact on cytokine secretion, it inhibited the recruitment of neutrophils after alum and MSU crystal injection (Figure 4. 13 B and C). The disappearance of macrophages was also partly reversed only when uricase was added to the MSU crystals, but not when uricase was injected with alum (Figure 4. 13 B and C).

#### **4.3.7. The IL-33/ST2 axis is required for alum-induced infiltration of eosinophils**

Due to the fact that IL-33 was released after alum injection, it was crucial to determine whether the lack of the IL-33 would alter innate immune responses induced by alum. Therefore, wild-type and IL-33<sup>-/-</sup> mice were injected intraperitoneally with PBS or alum and sacrificed after 24 hours. The peritoneal cavity was washed and cells were isolated and analysed by flow cytometry to determine the numbers of resident macrophages, mast cells, neutrophils, inflammatory monocytes, and eosinophils. Alum induced comparable infiltration of cells at the site of injection in wild-type and IL-33-deficient mice (Figure 4. 14 A). Moreover, the lack of IL-33 did not alter the depletion of resident macrophages or mast cells and the infiltration of inflammatory monocytes and neutrophils (Figure 4. 14 B and C). However, the number of eosinophils was decreased significantly in IL-33-deficient mice (Figure 4. 14 C).

In order to confirm that alum-induced recruitment of eosinophils was dependent on IL-33 signalling, mice lacking the IL-33 receptor, ST2, were used. Wild-type and ST2<sup>-/-</sup> mice were injected with PBS or alum. After 24h, PerC were isolated and analysed by flow cytometry to monitor cell influx following injection. Similarly to IL-33-deficient mice, the absence of ST2 did not have a significant impact on total cell infiltration (Figure 4. 15 A). Furthermore, inflammatory monocyte and neutrophil influx was slightly increased in ST2-deficient mice (Figure 4. 15 B and C), while eosinophil recruitment was significantly decreased (Figure 4. 15 D).

Previous experiments using 4Get mice showed that IL-4-expressing cells, probably eosinophils, were recruited to the peritoneal cavity in response to alum



administration (241, 249). Thus, the next question addressed was whether IL-33 has any impact on the recruitment of IL-4 producing cells. 4Get and ST2<sup>-/-</sup> 4Get mice were injected intraperitoneally with PBS or alum. After 24 hours, PerC were isolated, and analysed by flow cytometry. Moreover, cytopins of peritoneal cells were prepared. Firstly, it was confirmed that infiltration of eosinophils was decreased in 4Get ST2-deficient mice (Figure 4. 16 A and C) Moreover, in accordance with results from ST2-deficient mice, 4Get ST2<sup>-/-</sup> mice did not have impaired infiltration of neutrophils and the alum-induced depletion of resident macrophages was not altered (Figure 4. 16 C). Next it was determined whether eosinophils and Gr-1<sup>int</sup> cells are the source of IL-4 after immunisation with alum. As expected, 4Get mice immunised with alum upregulated IL-4 production. However, when ST2-deficient 4Get mice were injected with alum there were fewer IL-4 producing cells in the peritoneal cavity (Figure 4. 17 A). Overall, ST2-deficiency led to a reduction in the numbers of IL-4<sup>+</sup> Gr-1<sup>+</sup> cells and IL-4<sup>+</sup> eosinophils recruited to the peritoneal cavity by alum (Figure 4. 17 B).

#### **4.3.8. ST2 blocks alum-induced IgG isotype switching**

Having established that alum induced necrosis at the site of injection, which led to the release of the IL-33 and ST2-dependent IL-4<sup>+</sup> eosinophil influx, it was important to address the role of IL-33 in alum-driven humoral immune responses.

In order to determine the requirement of the IL-33 receptor, ST2, in antibody responses after alum immunisation, wild-type and ST2<sup>-/-</sup> mice were immunised with PBS, OVA alone or OVA co-administered with alum. After 14 days, mice were boosted with the same treatments. Sera collected on days 13 and 21 from immunised mice were analysed for the presence of OVA-specific IgG1, IgG2b, IgGc and IgE. Moreover total IgE levels were measured in sera as a indicator of TH2 responses. The levels of OVA specific antibodies after prime and boost immunisation were similar, thus only the data from day 21 are presented. Immunisation with OVA and alum in wild-type and ST2-deficient mice resulted in an increase of OVA specific IgG1 in comparison to OVA alone. However, there are no significant differences in antigen-specific IgG1 between WT and ST2<sup>-/-</sup> mice immunised with alum (Figure 4. 18 A). Interestingly, significant OVA-specific IgG2b and IgG2c titres were only detected in ST2-deficient

mice after alum immunisation (Figure 4. 18 A). Immunisation with alum resulted in an increase in OVA-specific IgE and total IgE levels in wild-type mice. However, ST2-deficiency led to a decrease in both OVA-specific and total IgE levels (Figure 4. 18 B and C). The decrease in total IgE in ST2<sup>-/-</sup> mice compared to wild-type mice was significant only for prime immunisation (sera from day 13), while boosting led to a reduction in the difference between wild-type and ST2<sup>-/-</sup> mice.

In order to confirm that this striking effect of ST2-deficiency was due to IL-33 release at the site of alum-induced necrosis, wild-type and IL-33<sup>-/-</sup> mice were immunised with PBS, OVA alone or OVA co-administered with alum. After 14 days, mice were boosted with the same treatments. Sera collected on days 13 and 21 from immunised mice were analysed for the presence of OVA-specific antibodies. Surprisingly, in contrast to ST2-deficient mice, IL-33<sup>-/-</sup> mice had similar levels of OVA-specific IgG1, IgG2b and IgE after alum immunisation when compared to wild-type mice. However, similarly to ST2<sup>-/-</sup> mice, OVA-specific IgG2c titres were higher in IL-33<sup>-/-</sup> mice (Figure 4. 19 A). Additionally, the lack of IL-33 led to a decrease in total IgE levels, which was significant only for prime immunisation (sera from day 13), while boosting led to a reduction in the difference between wild-type and IL-33<sup>-/-</sup> mice (Figure 4. 19 B).

#### **4.3.9. IL-33 is not required for induction of antigen-specific CD8 T cells following alum immunisation**

It has been demonstrated that IL-33 released during necrosis can act on CD8 T cells directly and this process is necessary for induction of potent cytotoxic CD8 T cell responses to viruses (143). To establish whether alum-induced antigen-specific CD8 T cell responses depend on IL-33 release, wild-type and IL-33<sup>-/-</sup> mice were immunised with PBS, OVA alone or OVA co-administered with alum. After 14 days, mice were boosted with the same treatments. After 7 days mediastinal lymph nodes and spleens were collected and single cell suspensions were analysed by flow cytometry. Cells were gated to exclude debris, doublets and dead cells. B220<sup>+</sup> and F4/80<sup>+</sup> cells were excluded and CD3<sup>+</sup>CD8<sup>+</sup>CD4<sup>-</sup> T cells were gated. OVA-specific CD8 T cells were detected using K<sup>b</sup>/SIINFEKL tetramer and upregulation of CD44, a T cell activation

marker (Figure 4. 19 A). As expected, alum promoted induction of OVA-specific CD8 T cells both in mediastinal lymph nodes and spleens in wild-type mice, while OVA alone did not. Lack of IL-33 did not decrease the number of tet<sup>+</sup> CD8 T cells in mLN and spleens (Figure 4. 19 C). Surprisingly in fact even, some IL-33-deficient mice immunised with alum had even more OVA-specific CD8 T cells in spleen than wild-type mice although the effect did not reach significance.

#### 4.4. Discussion

In developed countries there is a schedule of vaccinations, beginning early in life to confer protection against common childhood diseases. However, there are still diseases which we cannot prevent by vaccination using traditional approaches. Therefore, there are attempts to develop new vaccines, many of them with novel adjuvants, which are designed to be safe and effective. However, according to the predictions of the “danger model”, without any danger signals the consequence of immunisation will be tolerance rather than protective immunity. Therefore, it is important to ask whether adjuvants and vaccines can work without some degree of toxicity? It has been reported that alum, which is regarded as a safe adjuvant, can kill cells *in vitro*, induce local cell death and release endogenous danger signals at the site of injection (237, 250, 276).

Findings presented in this chapter confirm that alum can induce cell death *in vitro*. Cell death was assessed in the muscle cell line, C2C12, immortalised bone marrow-derived macrophages and bone marrow derived-dendritic cells. These cells were used since DCs and macrophages are alum sensors and muscle cells are predominant at the site of human intramuscular vaccine administration. Interestingly, the kinetics of cytotoxicity vary between different types of cells. Macrophages are the most susceptible to alum-induced cell death while C2C12 muscle cells were least susceptible. These differences may reflect a differential capacity of the cells to interact with alum. DCs were shown to interact intimately with alum within seconds of initial contact via lipid rafts (232), which can delay phagocytosis and subsequent cell death. On the other hand, macrophages do not seem to sense alum through lipid rafts (232), but they have been shown to internalise and accumulate alum efficiently (207, 234). Furthermore, Flach and colleagues questioned whether DCs can phagocytose alum and suggested that after rare events of alum phagocytosis DCs always die. Therefore, a detailed analysis of the ability of DCs to phagocytose alum was carried out. Surprisingly, within 1 hour almost all BMDCs were fluorescent alum<sup>+</sup> when measured by flow cytometry. It was also confirmed by confocal microscopy that alum interacted first with membranes, but within 30 minutes it was internalised by cells. Furthermore,

pre-treatment with cytochalasin D led to inhibition of alum-driven cell death. Cytochalasin D inhibits actin polymerisation, which is crucial in phagocytosis.

Having confirmed that DCs are capable of taking up alum and the inhibition of phagocytosis by cytochalasin D blocks their cell death, next the nature of alum-induced cell death was studied in BMDCs. The type of cell death is very important in the context of immune responses. Apoptosis is regarded as non-immunogenic, due to the fact that membrane integrity is maintained and the cell cannot release DAMPs. In contrast, necrotic cell death is associated with cell swelling, a loss of membrane integrity and the release of endogenous danger signals, which can induce inflammation (277). Even though necrosis was described as an uncontrolled process, recent data shows that it might be regulated (278). Recently, new types of cell death were reported, pyroptosis (279) and pyronecrosis (280). Both are characterised by rapid plasma membrane rupture and the release of pro-inflammatory intracellular contents. However, pyroptosis and pyronecrosis require different cellular machinery. Pyroptosis depends on inflammasome formation, caspase-1 and NLRP3, while pyronecrosis is caspase-1-independent, but NLRP3 and cathepsin B-dependent.

Firstly, it has been confirmed that alum does not induce apoptosis, as alum-treated BMDCs do not express Annexin V, an apoptotic marker and the cell death cannot be blocked by the pan caspase inhibitor, ZVAD. However, DCs incorporated PI, a dye which accumulates in cells with ruptured membranes, suggesting necrotic cell death. Recently, it has been suggested by Jacobson and colleagues that alum induces a novel necrotic type of cell death *in vitro*, which results from lysosomal destabilisation (276). They demonstrated that alum induced macrophage death which is not pyroptosis (caspase-1-dependent necrosis) or necroptosis (RIP-1-mediated necrosis). Likewise, it has been established here that alum-induced dendritic cell death was not mediated by pyroptosis or pyronecrosis, as alum-induced cell death cannot be prevented by a lack of the inflammasome components, NLRP3 and ASC, or by inhibiting caspase-1 and cathepsin B. Jacobson *et al.* demonstrated that alum-induced bone marrow-derived macrophage death can be blocked by a high concentration of the cathepsin B inhibitor (CA-074-Me) and that BMMs deficient in cathepsin B or cathepsin S are resistant to alum-induced cell death. However, no role for cathepsin B was seen in the current study. Importantly, alum

adjuvant, alhydrogel composed of aluminium hydroxide alone, which is applied for human vaccination, was used for all experiments in this thesis, while Jacobson and colleagues used Imject Alum, a combination of aluminium hydroxide and magnesium hydroxide. The formulations differ in crystalline composition and also their immunostimulatory properties. Furthermore, different cells were used in these experiments. The dendritic cells used in this thesis were not primed with any PRR agonist, while in the Jacobson did study macrophages were primed with LPS, which might also change the vulnerability to cell death. Overall, from the current study it can be concluded that the alum adjuvant, Alhydrogel, induces necrosis, but not apoptosis, pyronecrosis or pyroptosis in dendritic cells.

In recent years other types of cell death were described, including autophagic cell death. This has led to some controversy as autophagy is regarded as a process to maintain cell homeostasis by self degradation of cell components during starvation, recycling damaged organelles and misfolded proteins, but also targeting toxic components and intracellular pathogens (288). However, cytotoxic effects can also be attributed to massive autophagy, which results in self-destruction. Autophagic cell death involves an increased accumulation of autophagic vacuoles and subsequent cell death, which can be avoided by specific inhibition of autophagy (289). Interestingly, it has been demonstrated that alum is an autophagy inducer (180). Furthermore, it is demonstrated here for the first time that blocking autophagy prevents alum-induced cell death *in vitro*. Pre-treatment with 3-MA led to inhibition of alum-driven cell death in the case of both bone marrow derived dendritic cells and immortalised macrophages. 3-MA can suppress autophagy by targeting class III PI3 kinases thus interfering with the formation of autophagosomes, an early event in autophagy. Another autophagy inhibitor, chloroquine targets later events during autophagy, namely lysosomal hydrolases and the fusion of autophagosomes with lysosomes. Pre-treatment of BMDCs and iBMMs with chloroquine did not lead to suppression of alum-induced cell death. This discrepancy suggests that only the inhibition of early events in autophagy can prevent alum-induced cell death while once autophagosomes are formed cell death cannot be prevented. It is possible that overwhelming phagocytosis of alum by dendritic cells induces autophagy which cause irreversible cellular damage and consequently cell death by necrosis. However, to prove that

alum-driven cell death is indeed an autophagic event, additional experiments will have to be conducted, as the observation that 3-MA modulates alum-induced cell death is only a starting point for further experiments. For instance, experiments with dendritic cells in which proteins involved in autophagosome formation and elongation (e.g. the PI3 kinase III complex protein Beclin-1 or the elongation conjugate proteins Atg 5 or Atg 16) are silenced by siRNA should be conducted.

Recently, it has been demonstrated that autophagy can modulate immune responses by targeting cytokine production. One of the most interesting examples is IL-1 $\beta$ . Autophagy inhibits IL-1 $\beta$  secretion by different means, including sequestering pro-IL-1 $\beta$  and inflammasomes into autophagosomes and targeting them for degradation (180, 181). Thus, inhibition of autophagy by 3-MA or Wortmannin or using cells deficient in autophagy induction led to an increase in IL-1 $\beta$  secretion when cells are stimulated with LPS alone or with LPS and various inflammasome inducers (180, 181, 287). Indeed, it is also reported in this thesis that 3-MA increased LPS-induced IL-1 $\beta$  secretion. Surprisingly, suppression of autophagy by 3-MA, blocked alum-induced IL-1 $\beta$  secretion. This result is in contrast to previous experiments where it has been shown that 3-MA in the presence other NLRP3 inflammasome inducers, MSU crystals and nigericin, resulted in upregulation of IL-1 $\beta$  (180, 287). There is only one report, which suggests that autophagy might facilitate secretion of proteins, including IL-1 $\beta$ , rather than their degradation. Dupont and colleagues demonstrated that basal autophagy inhibits IL-1 $\beta$  secretion, but once autophagy is induced there is enhanced IL-1 $\beta$  secretion (290). Overall, the role of autophagy in IL-1 $\beta$  secretion may depend on timing and the specific type of stimuli. Based on this report and some studies on unconventional protein secretion in yeasts it has been proposed that there could be two types of autophagy: secretory and degradative (291). However, the difference at a molecular level between the two types of autophagosomes and how their fate is decided remains unclear. Thus, it remains to be determined whether alum can induce secretory autophagy which facilitates IL-1 $\beta$  release, while at the same time inducing degradative autophagy to deal with the massive burden of phagocytosed alum.

Crucially, alum not only induces cell death *in vitro*, but also *in vivo*. In accordance with a previous report (250), intraperitoneal injection of alum induced

a significant increase in the percentage of dead cells in the peritoneal cavity. However, Marichal and colleagues analysed peritoneal exudate cell death in mice injected with alum after 3 to 24 hours, when substantial cell recruitment can be observed. Therefore, the reported percentages of cell death may be lower than expected due to dilution by infiltrating cells. Analysis of cell death 1 hour post alum injection shows that alum potently kills resident cells; at least 50% of cells were PI and Aqua LIVE/DEAD positive. Both markers only stain cells in which membrane integrity is lost, which further confirms that alum induces necrosis.

The “danger theory” suggests that immune responses are induced by “danger signals” released by stressed and injured tissues. One potential danger signal, IL-33, a novel cytokine in the IL-1 family (281) is expressed by multiple tissues, but it is thought to be released only during necrosis (138). Therefore, IL-33 levels were analysed in the peritoneal lavage fluid at different time points following injection. Interestingly, IL-33 is released *in vivo* as early as 1 hour post alum injection, which correlates with massive cell death. IL-33 cannot be detected at later time points. This suggests that alum-induced cell death is rapid and very shortly after injection “danger signals” are released. Indeed, other endogenous danger molecules, uric acid and host DNA were detected as early as 2 and 3 hours post alum administration, respectively (237, 250). To confirm that IL-33 is released independently of uric acid, mice were depleted of uric acid by the addition of uricase, the enzyme which inactivates uric acid. It has been reported that intraperitoneal injection of alum or MSU crystals results in uric acid-dependent neutrophil infiltration, which can be reversed by the addition of uricase (237, 251). In experiments presented in this report uricase inhibited neutrophil infiltration induced by both alum and MSU injection. Importantly, depletion of uric acid did not significantly reduce IL-33 release. This suggests that MSU crystals are not required for IL-33 release.

Intraperitoneal injection of alum induces immune response in different immunological compartments. Firstly, at the site of injection, alum can induce necrosis and release of cytokines, including IL-33, IL-1 $\beta$ , IL-6 and chemokines, including KC, MIP-1 $\alpha$ , MIP-2, MCP-1 and eotaxin, which drive innate responses. In accordance with previous reports (237, 241), in wild-type mice alum injection induced the infiltration of inflammatory monocytes and neutrophils and the disappearance of resident



macrophages and mast cells. This alum-driven innate response was intact in both IL-33-deficient and ST2-deficient mice. However, the lack of IL-33/ST2 signalling impaired eosinophil recruitment to the site of injection. Indeed, it was reported previously, that IL-33 induces eosinophil migration (138, 292) and also activation (138, 281). Moreover, over-expression of IL-33 leads also to eosinophil influx. For instance, in a mouse model of atopic dermatitis, skin-specific stable expression of IL-33 induced eosinophil infiltration into the skin and systemic eosinophilia (293). Furthermore, as with alum injection, it has been demonstrated that eosinophils are recruited in an IL-33/ST2-dependent manner during parasite infections and allergy (150, 283, 294). Importantly, it has yet to be established which cells release IL-33 after alum administration and what kind of cells sense it. In the intraperitoneal model used here, resident macrophages and mast cells are sensors of alum. It has been demonstrated that eosinophils are recruited only in mice which have mast cells present in the peritoneum and that this is IL-5-dependent. It is probable that alum-induced IL-33 drives IL-5 secretion by mast cells, which were observed to express ST2 (146).

The role of eosinophils in alum-driven immune responses is somewhat controversial. It has been demonstrated that Gr-1<sup>+</sup> granulocytes, which resemble eosinophils, play a crucial role in modulating alum-induced immune responses. Following alum administration Gr-1<sup>+</sup> cells accumulate and produce IL-4 at the site of injection and later also in the spleen. This IL-4 production plays a pivotal role in suppressing TH1 responses, because a lack of IL-4 signalling or depletion of Gr-1<sup>+</sup> cells lead to decreased production of IgE and increased levels of antigen-specific (TH1-related) IgG2a/c. However, there is conflicting data regarding whether these Gr-1<sup>+</sup> IL-4-producing cells are indeed eosinophils, because mice lacking eosinophils do not exhibit a similar phenotype to IL-4 deficient mice after alum immunisation (241). Furthermore, it has been demonstrated that mast cells and IL-5, which are crucial for eosinophil influx after alum injection, are dispensable for alum adjuvanticity (241). Therefore, it was determined using 4Get and ST2-deficient 4Get mice whether the lack of IL-33/ST2 correlates with the reduction of IL-4 production by eosinophils and/or Gr-1<sup>+</sup> cells. Indeed, IL-4 production was reduced not only in eosinophils, but also in Gr1<sup>int</sup> cells, which indicates that alum-driven infiltration and/or stimulation of IL-4<sup>+</sup> cells is ST2 signalling dependent.

As mentioned before, IL-4 producing cells have been shown to potently modulate alum-driven immunity, by blocking cellular immune responses. Therefore, in order to determine if IL-33/ST2 is able to modulate alum-induced humoral and cellular immune responses, wild-type, ST2-deficient and IL-33-deficient mice were immunised and later boosted with OVA in alum. As was expected, immunisation of wild-type mice with OVA co-administered with alum resulted in elevated total IgE, detectable levels of OVA-specific IgE and high titres of OVA-specific IgG1, but low titres of IgG2b and IgG2c, an indication of a TH2 type response. ST2-deficiency and IL-33-deficiency did not reduce the capacity of alum to promote IgG1 responses, as mice immunised with alum had the same level of OVA-specific IgG1 antibodies compared to alum immunised wild-type mice. However, when other OVA-specific antibody titres were compared, there was a significant difference between wild-type and ST2<sup>-/-</sup> or IL-33<sup>-/-</sup> mice. While titres of OVA-specific IgG2b and IgG2c were significantly elevated in ST2-deficient mice, there was only slightly increased OVA-specific IgG2c in IL-33<sup>-/-</sup> mice. Furthermore, while both ST2 and IL-33 deficiency led to a reduction in total IgE concentrations, only in ST2<sup>-/-</sup> mice was there a decrease in antigen-specific IgE responses. Interestingly, the change in alum-driven antigen-specific antibodies in ST2<sup>-/-</sup> mice is in line with previous reports which demonstrated that deficiency in the TH2 associated cytokines IL-4, IL-6 and IL-13 did not alter antigen specific IgG1, but elevated IgG2a (equivalent of IgG2c) titres and decreased IgE levels after alum immunization (247, 248, 295). However, it is very surprising that this effect is not fully mediated by IL-33, which has been shown by many to induce strong innate type 2 responses. Clearly, it is possible that while alum injection induces local release of IL-33, which binds to ST2 and promotes eosinophil influx, there is an additional role for ST2, which is not dependent on the release of this alarmin.

Indeed, it has been suggested before that the simple relationship between IL-33 and ST2 as a ligand and a receptor is not exclusive. It was questioned whether IL-33 can have its own function as a nuclear factor, and that ST2 may exhibit unique ligand-independent inhibitory properties (296). In fact, ST2 is evolutionary older than its ligand. Sequences homologous to human ST2 have been identified in mouse (67%), chicken (43%) and zebra fish (34%), so the receptor is present in all jawed vertebrates. In contrast, IL-33 protein homology has been reported between human and mouse

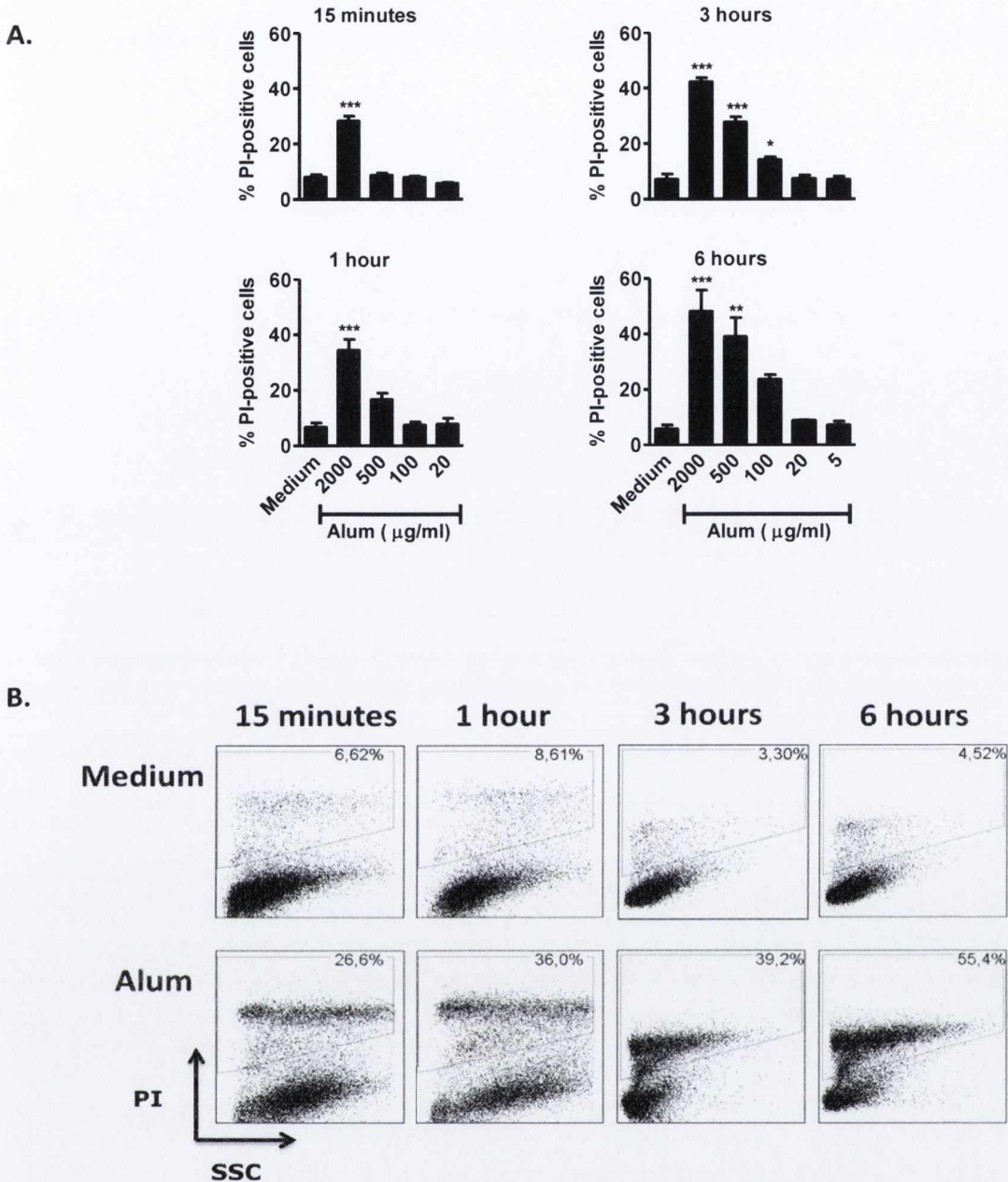
(54%), but the cytokine has not been found in species which evolved before rodents (296). Is it therefore possible that ST2 evolved in placental mammals to recognise extracellular IL-33, which would be released during necrosis as an alarmin, while at the same time fulfilling other IL-33-independent functions?

Indeed, there are reports which suggest a role for the ST2 receptor during inflammation which seems to be independent of IL-33 recognition. For instance, the induction of tolerance by low doses of LPS make wild-type and IL-33-deficient mice resistant to lethal dose of LPS, while ST2-deficient mice succumb to the LPS challenge (150, 297). Furthermore, induction of pathology in the animal model of multiple sclerosis, EAE, is independent of IL-33 (150), while ST2-deficiency was found to worsen disease outcome (298). On the other hand, airway inflammation, induced by two sensitizations with alum and OVA, develops normally in ST2<sup>-/-</sup> mice (299), while IL-33<sup>-/-</sup> mice exhibit attenuated inflammation (150). Overall, these data suggest that there are conditions whose outcome differentially depends on IL-33 and ST2, ST2 alone or IL-33 alone. Therefore, to elucidate the role of IL-33 and ST2 signalling it is important to use both mouse strains.

The ST2 receptor belongs to the Toll-like/IL-1-receptor superfamily, which share an intracellular TIR domain and can bind adaptor proteins e.g. MyD88 and Mal. The IL-33/ST2/IL-1RaP complex recruits MyD88, IRAK1 and IRAK4 and induces the activation of NF- $\kappa$ B and MAP kinases (281). However, it has also been demonstrated that ST2 can inhibit the functions of MyD88 and Mal, which are necessary for TLR and IL-1R1 receptors to be functional, by sequestering these adaptor proteins (297). Therefore, it is possible that upon IL-33 binding, ST2 activates downstream signals, whereas during stimulation of IL-1R1 or TLRs ST2 acts as an inhibitory receptor, similarly to SIGIRR. Interestingly, the absence of MyD88, the ST2 adaptor protein, does not seem to have a similar effect to ST2-deficiency following alum immunisation. MyD88 and Trif-deficient mice immunised with alum have similar antigen-specific IgG1, IgG2c and IgE antibody titres when compared to wild-type mice (235). ST2-IL-33 signalling might not be exclusively dependent on MyD88, therefore, other adaptor proteins may be involved in ST2 signal transduction. For instance, the role of Mal during alum immunisation has not been studied and would be an interesting avenue to pursue.

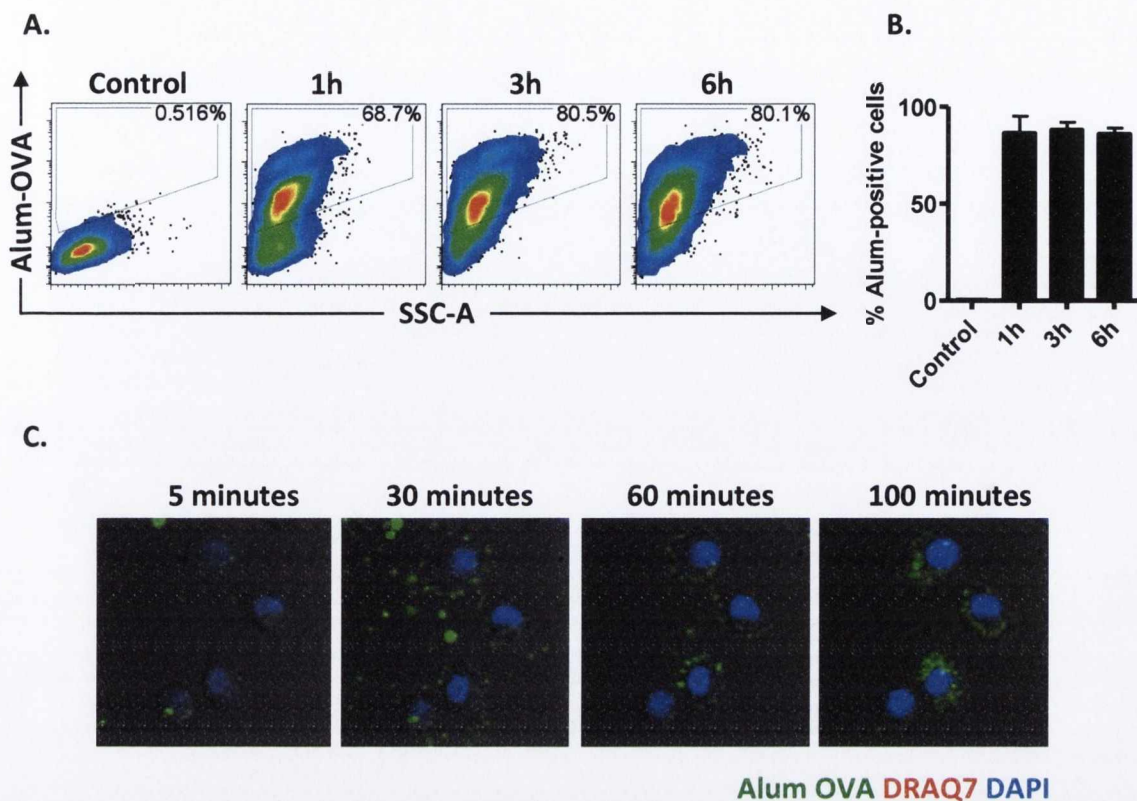
Overall, there is a clear role of IL-33 in modulation of the innate immune response to alum, whereas ST2 impacts on innate and also on adaptive immune responses.





**Figure 4. 1. Alum induces cell death in BMDCs.** DC from C57BL/6 mice were stimulated with alum at concentrations from 5-2000µg/ml. After 15 minutes, 1, 3 and 6h, cell death was assessed by incorporation of PI. Cells were analysed by flow cytometry. Medium v Adjuvant \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. (A) Results are expressed as the mean ± SEM of three independent experiments. (B) Representative results for 2000µg/ml alum.

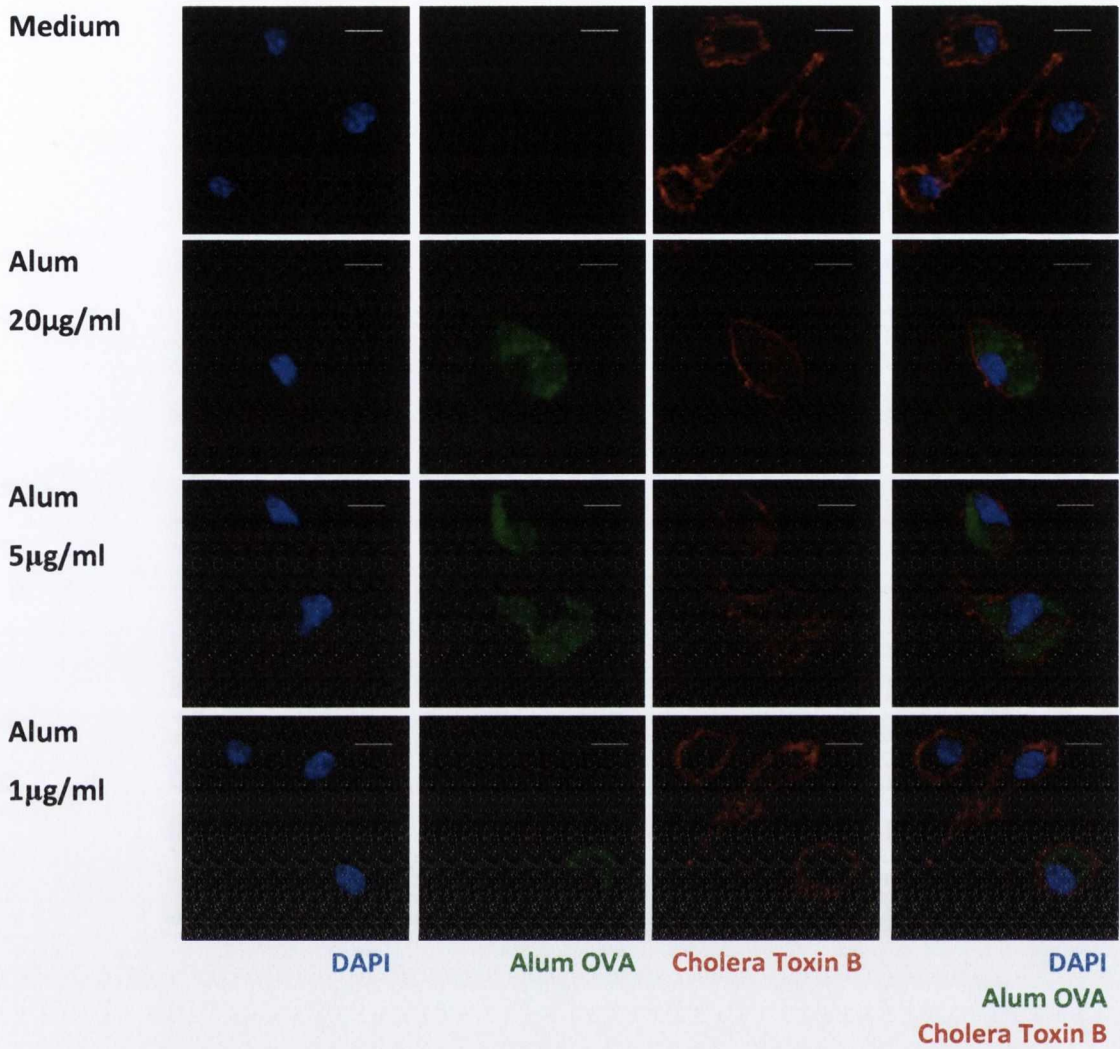




**Figure 4. 2. Alum is phagocytosed by dendritic cell within one hour.** (A, B) DC from C57BL/6 mice were incubated with alum and fluorescent OVA DQ at a concentration of 20 $\mu$ g/ml. After 1, 3 and 6h, alum OVA DQ uptake was assessed by flow cytometry. (C) 20 $\mu$ g/ml of Alum OVA DQ and two DNA stains, Hoechst 33258 and DRAQ7, were added to DC and cells were placed in confocal microscopy incubator for live cell imaging. Individual images were taken every minute up to 100 minutes. (A, C) Representative results from 3 independent experiments. (B) Results are expressed as the mean  $\pm$  SEM of three independent experiments.



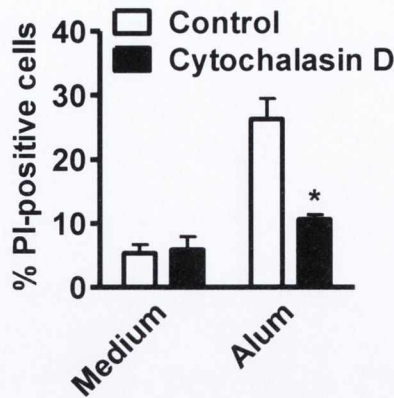




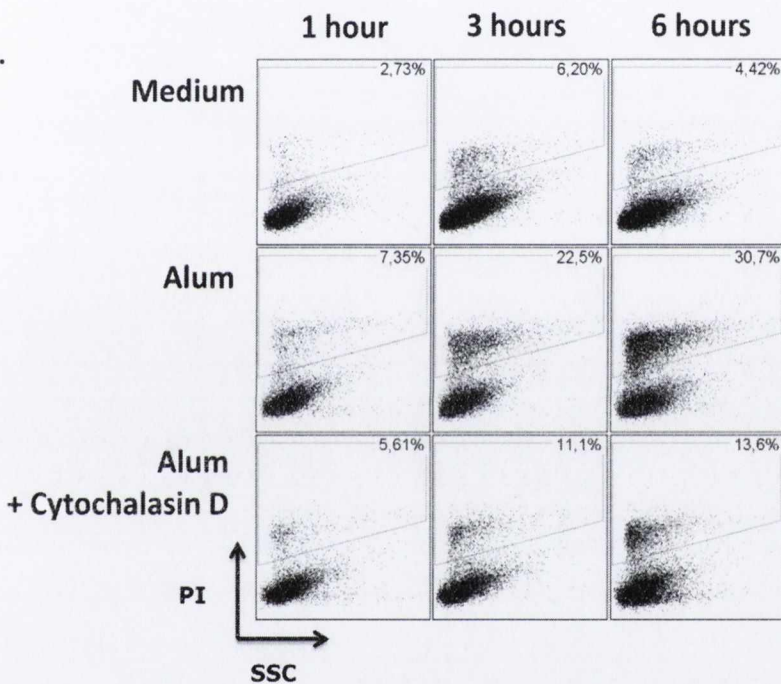
**Figure 4. 3. Alum is phagocytosed by dendritic cells.** DC from C57BL/6 mice were incubated with medium or 1, 5 or 20µg/ml fluorescent alum (green). After 6h cells were washed and cell membranes were stained with fluorescent cholera toxin B (red) and nuclei were stained with DAPI (blue), followed by analysis by confocal microscopy. Scale bars, 10 µm. Representative data from two independent experiments.



A.

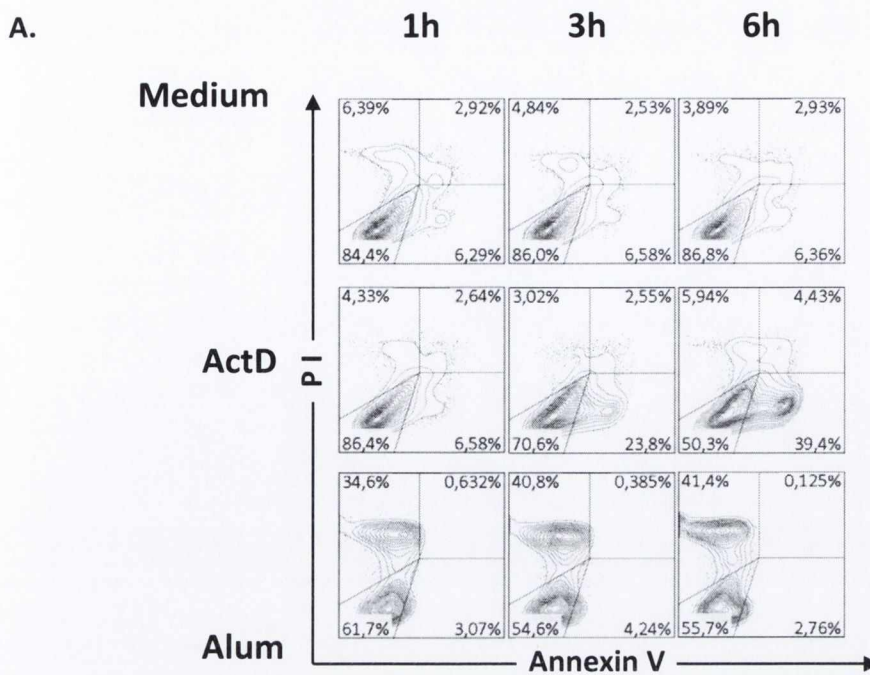


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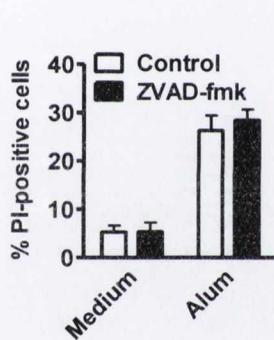


**Figure 4. 4. Alum-induced cell death depends on actin polymerisation.** DC from C57BL/6 mice were incubated with medium or alum (400µg/ml). In addition DC were treated as above in the presence of Cytochalasin D (5µM). Cells were collected after (A) 3h or (B) 1, 3 or 6h and stained with PI. Cell death was assessed by flow cytometry. Alum v Alum + Cytochalasin D \*p<0.05. (A) Results are expressed as the mean ± SEM of two independent experiments. (B) Representative data from two independent experiments.

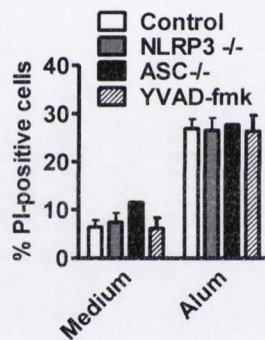




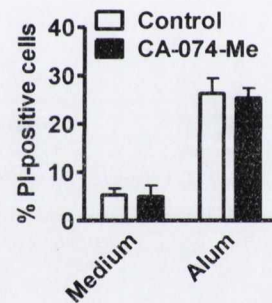
**B.**



**C.**



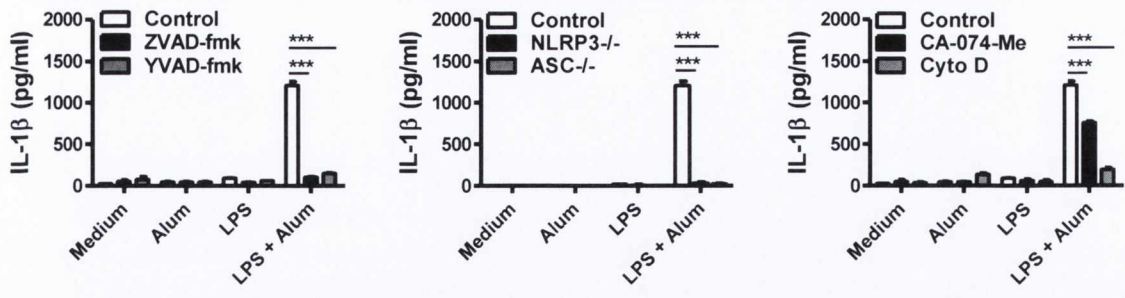
**D.**



**Figure 4. 5. Alum does not induce apoptosis, pyroptosis or pyronecrosis in BMDCs.**

(A) DC from C57BL/6 mice were incubated with 2000 $\mu$ g/ml alum or 5 $\mu$ M actinomycin D. After 1, 3 and 6 hours, cells were stained with annexin V and PI. Cell death was assessed by flow cytometry. Results are representative of three independent experiments. (B, C, D) DC from C57BL/6, NLRP3<sup>-/-</sup> and ASC<sup>-/-</sup> mice were incubated with medium or alum (400 $\mu$ g/ml). In addition, wild-type DC were treated as above in the presence of (B) Caspase inhibitor, ZVAD-fmk (10 $\mu$ M), (C) Caspase-1 inhibitor YVAD-fmk (10 $\mu$ M) or (D) Cathepsin B inhibitor, CA-074-Me (10 $\mu$ M). Cells were collected after 3h and stained with PI. Cell death was assessed by flow cytometry. Results are expressed as the mean  $\pm$  SEM of two or three independent experiments.



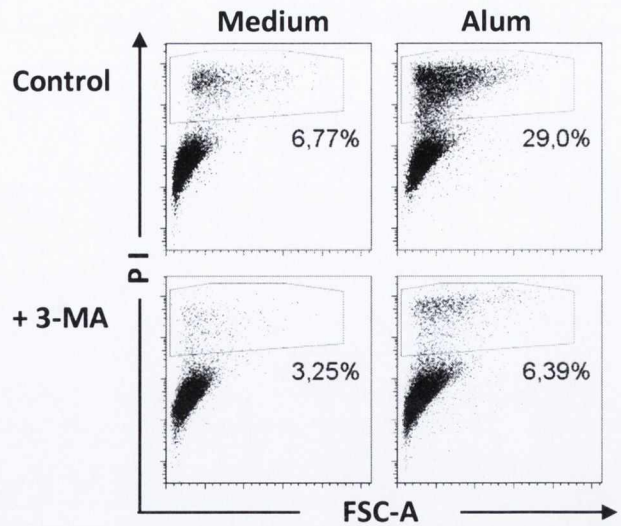
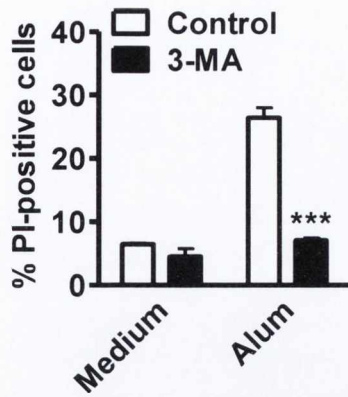


**Figure 4. 6. Alum-induced IL-1 $\beta$  production is dependent on phagocytosis, cathepsin B and inflammasome assembly.** DC from C57BL/6, NLRP3<sup>-/-</sup> and ASC<sup>-/-</sup> mice were primed with LPS (5ng/ml) for 3 hours, followed by incubation with medium or alum (400 $\mu$ g/ml). In addition wild-type DC were treated as above in the presence of ZVAD-fmk (10 $\mu$ M), YVAD-fmk (10 $\mu$ M), CA-074-Me (10 $\mu$ M) or Cytochalasin D (5 $\mu$ M). Supernatants were collected after 24h and tested for the presence of IL-1 $\beta$  by ELISA. Results are representative of two independent experiments. Error bars show means  $\pm$  SD for treatments tested in triplicate. LPS + Alum (C57BL/6) v LPS+ Alum + Inhibitor or LPS + Alum (NLRP3<sup>-/-</sup>, ASC<sup>-/-</sup>) \*\*\* p<0.001.

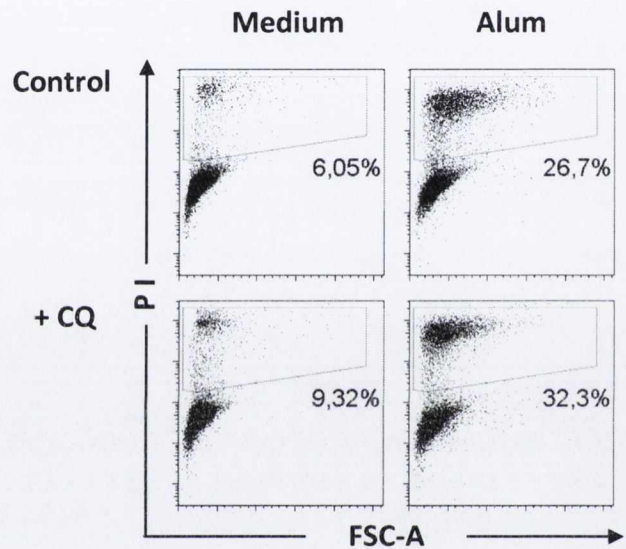
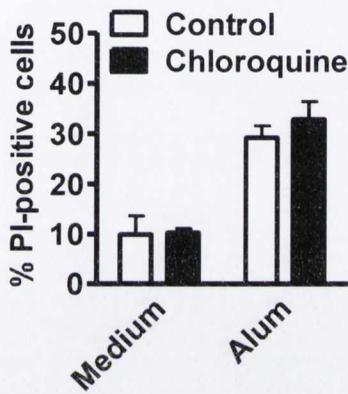




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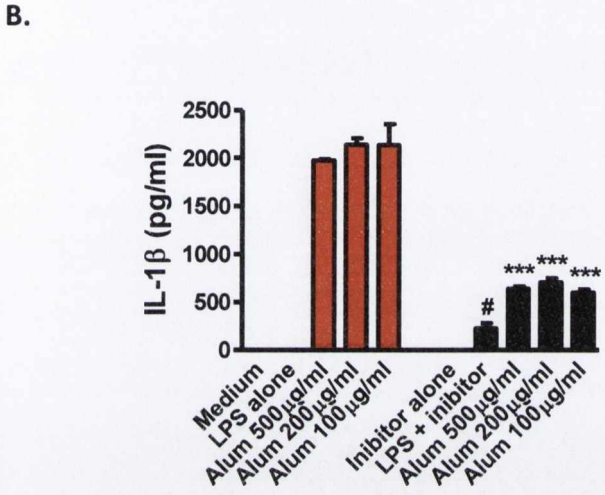
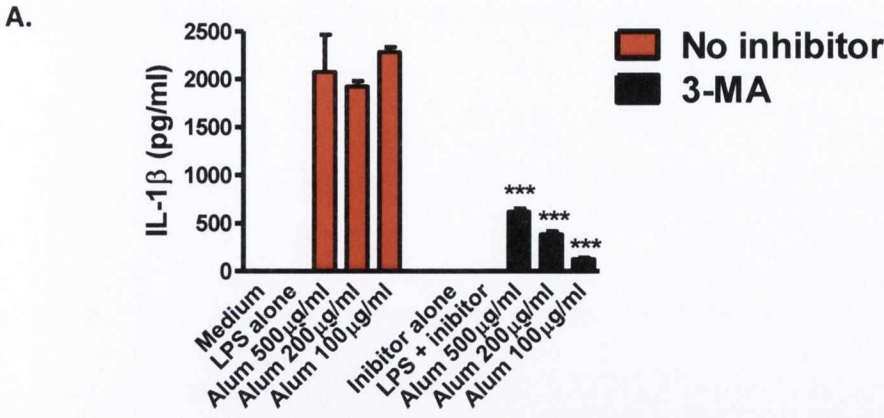


B.



**Figure 4. 7. Alum-induced dendritic cell death can be inhibited by blocking an early stage in autophagy.** DC from C57BL/6 mice were incubated with medium or alum (500µg/ml). In addition DC were treated as above in the presence of (A) 3-MA (5mM) or (B) chloroquine (10µM). Cells were collected after 6h and stained with PI. Cell death was assessed by flow cytometry. Alum v Alum + Inhibitor \*\*\* $p < 0.001$ . (A, B) Results are expressed as the mean  $\pm$  SEM of three independent experiments.

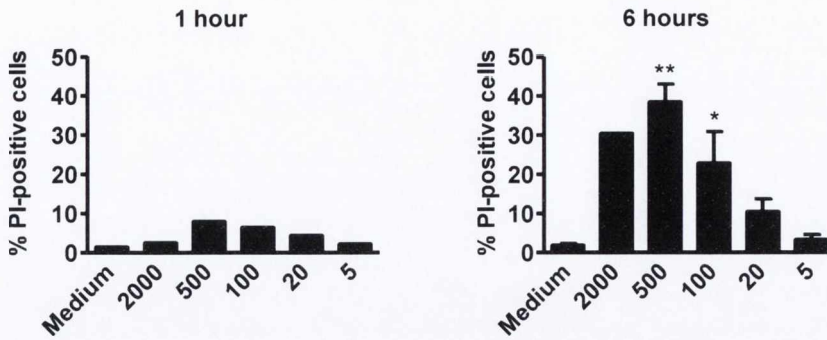




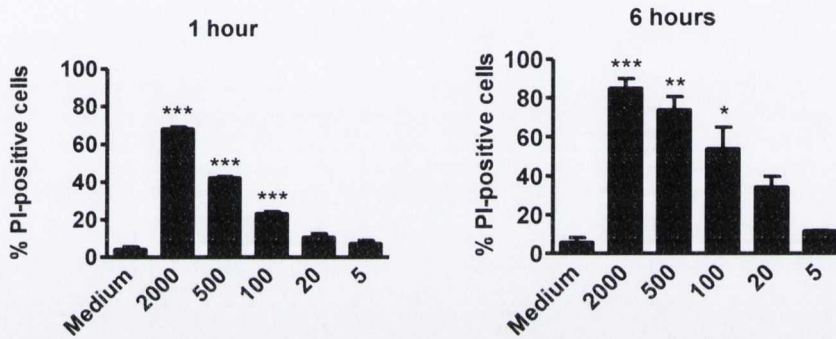
**Figure 4. 8. Blocking autophagy results in a decrease in alum-induced IL-1 $\beta$  secretion.** DC from C57BL/6 mice were incubated with medium, LPS (5ng/ml) alone or LPS with alum at concentrations from 100-500 $\mu$ g/ml. In addition, cells were treated as above in the presence of the autophagy inhibitor, 3-MA (5mM). Supernatants were collected after (A) 6h and (B) 24h and IL-1 $\beta$  concentrations were determined by ELISA. 3-MA + Alum + LPS v Alum + LPS, \*\*\*  $p < 0.001$ . 3-MA + LPS v LPS alone #  $p < 0.05$ . Results are representative of two independent experiments. Error bars show means  $\pm$  SD for each experimental group tested individually in triplicate.



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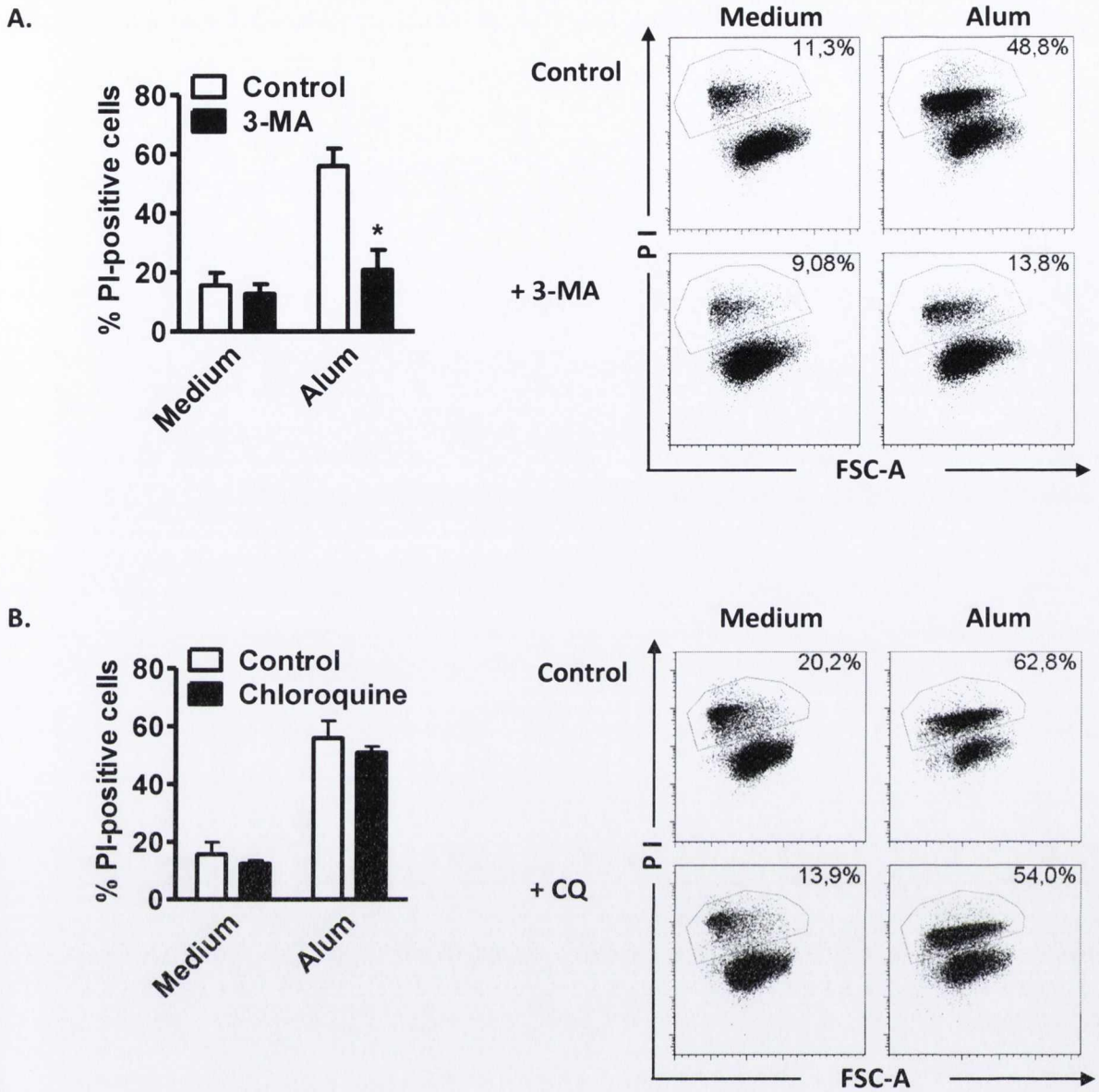


B.



**Figure 4. 9. Macrophages and muscle cells are susceptible to alum-induced cell death.** (A) C2C12 muscle cells and (B) immortalised bone-marrow derived macrophages were incubated with alum at concentrations from 5-2000 $\mu$ g/ml. After 1 and 6h, cell death was assessed by PI incorporation. Cells were analysed by flow cytometry. Medium v Adjuvant \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Results are expressed as the mean  $\pm$  SEM of three independent experiments.

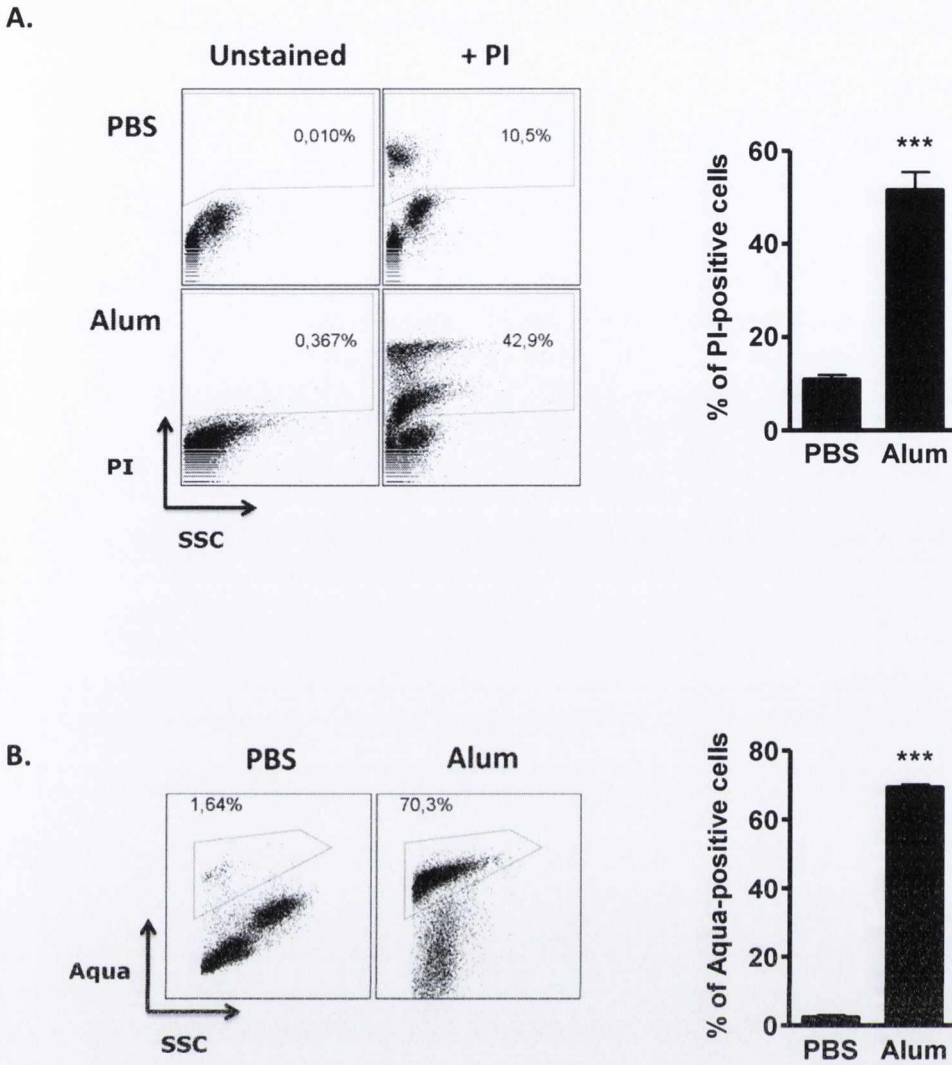




**Figure 4. 10. The autophagy inhibitor, 3-MA, blocks alum-induced cell death in macrophages.** Immortalised BMMs were incubated with medium or alum (500µg/ml). In addition DC were treated as above in the presence of (A) 3-MA (5mM) or (B) chloroquine (10µM). Cells were collected after 6h and stained with PI. Cell death was assessed by flow cytometry. Alum v Alum + Inhibitor \*p<0.01. (A, B) Results are expressed as the mean ± SEM of two independent experiments.

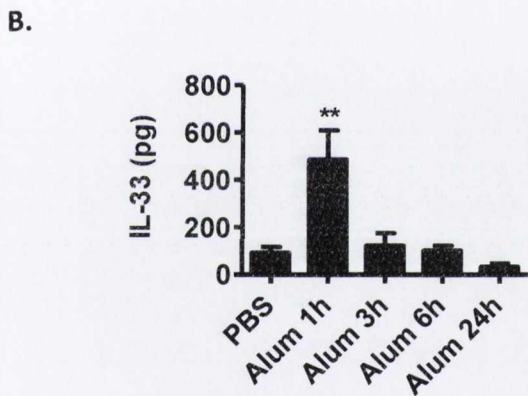
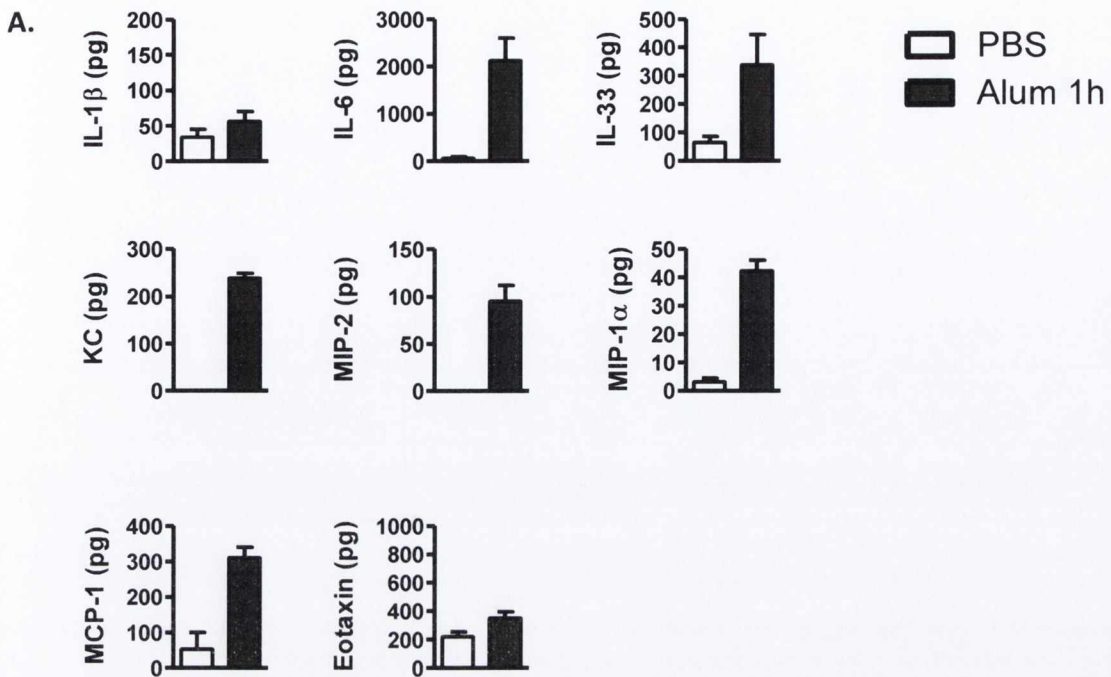






**Figure 4. 11. Alum induces cell death *in vivo*.** C57BL/6 mice were injected with PBS or alum (1mg/mouse) intraperitoneally. Mice were sacrificed 1 hour following injection and peritoneal exudate cells were isolated. PerC were stained with (A) PI or (B) Aqua LIVE/DEAD stain and cell death was assessed by flow cytometry. PBS v Alum \*\*\* $p < 0.001$ . Results are representative of three independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group.

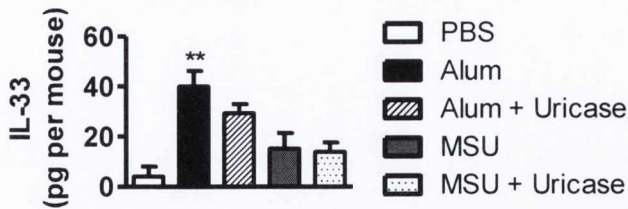




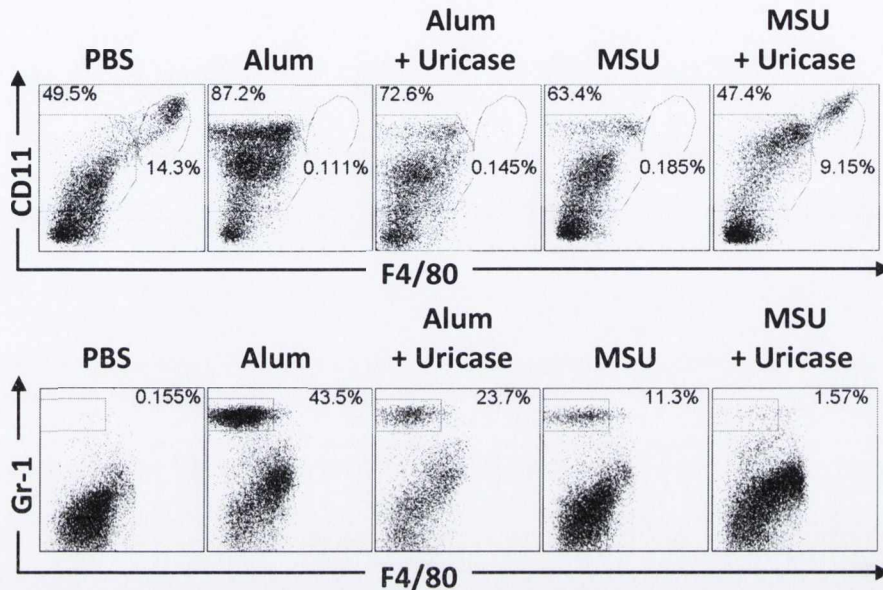
**Figure 4. 12. Alum promotes early cytokine and chemokine release at the site of injection.** C57BL/6 mice were injected with PBS or alum (1mg/mouse) intraperitoneally. Mice were sacrificed 1, 3, 6 and 24 hours following injection and the peritoneal cavity was washed with PBS. The acellular phase of the peritoneal lavage fluid was analysed for the presence of IL-1 $\alpha$  (undetected), IL-1 $\beta$ , IL-6, IL-33, KC, MIP-2, MIP-1 $\alpha$ , MCP-1 and eotaxin (pg per mouse). PBS v Alum \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Results are representative of two independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group.



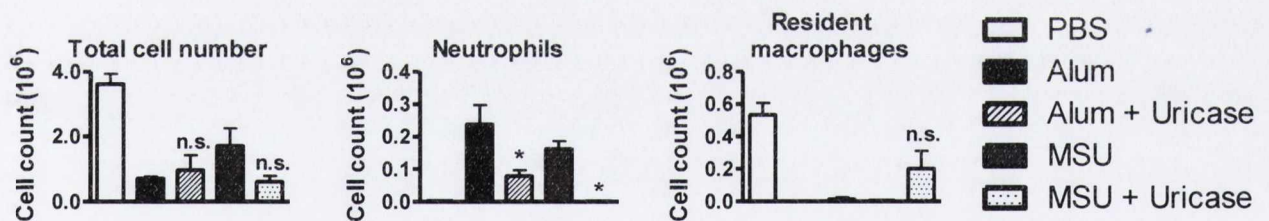
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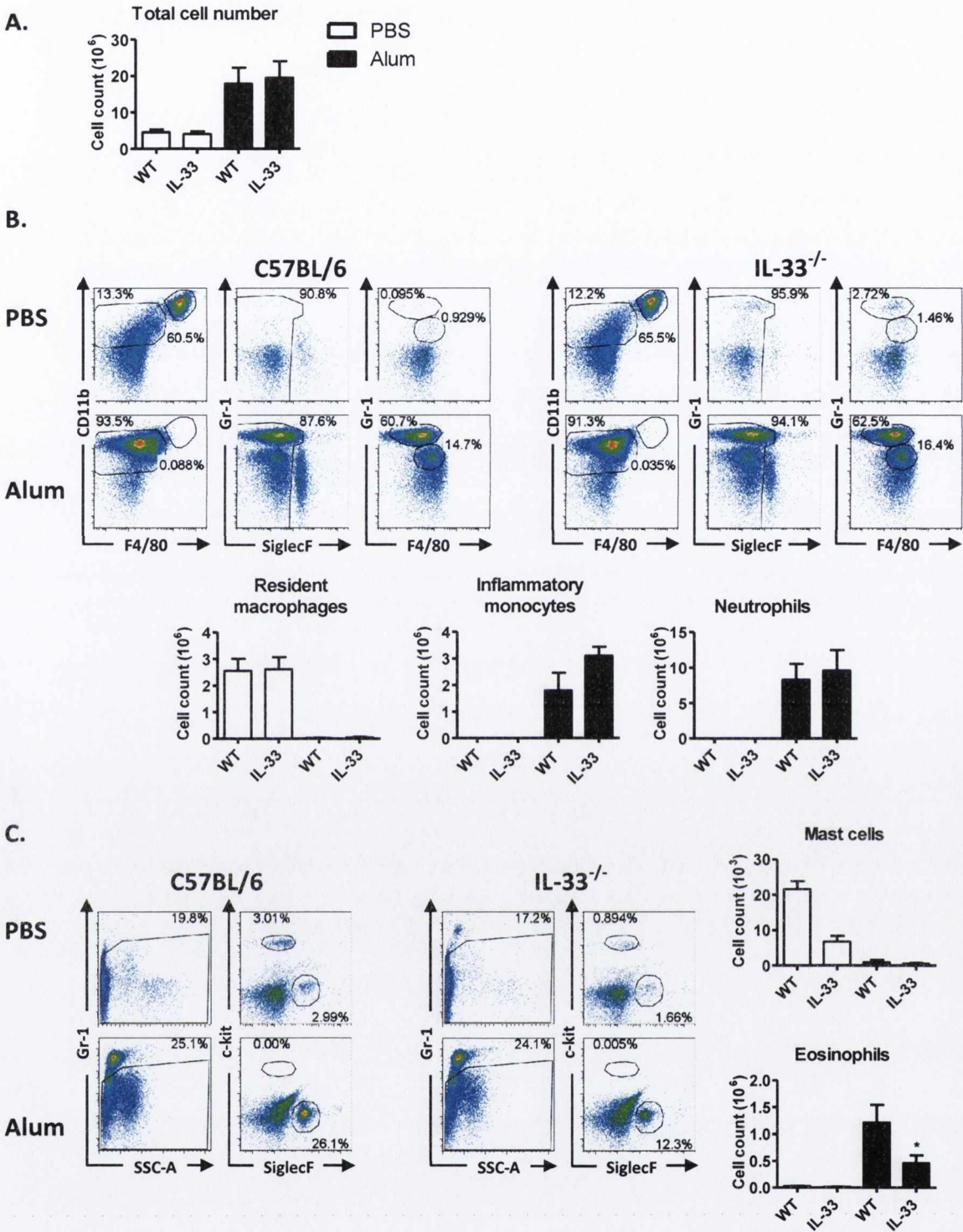


C.



**Figure 4. 13. Alum-driven IL-33 release is not uric acid-dependent.** C57BL/6 mice were injected intraperitoneally with PBS, alum (1mg/mouse) and MSU crystals (0.5mg/mouse) alone or in the presence of Uricase (50U/mouse). Mice were sacrificed 1 hour following injection and the peritoneal cavity was washed with PBS. (A) The acellular phase of the peritoneal lavage fluid was analysed for the presence of IL-33. PBS v Alum or MSU crystals \* $p < 0.05$ , \*\* $p < 0.01$ . (B, C) PerC were analysed for the presence of large peritoneal macrophages (B, higher panel) and neutrophils (B, lower panel). Alum or MSU crystals v Alum or MSU crystals + Uricase \* $p < 0.05$ . Results are representative of two independent experiments. Error bars show means  $\pm$  SEM for 3 mice per experimental group.

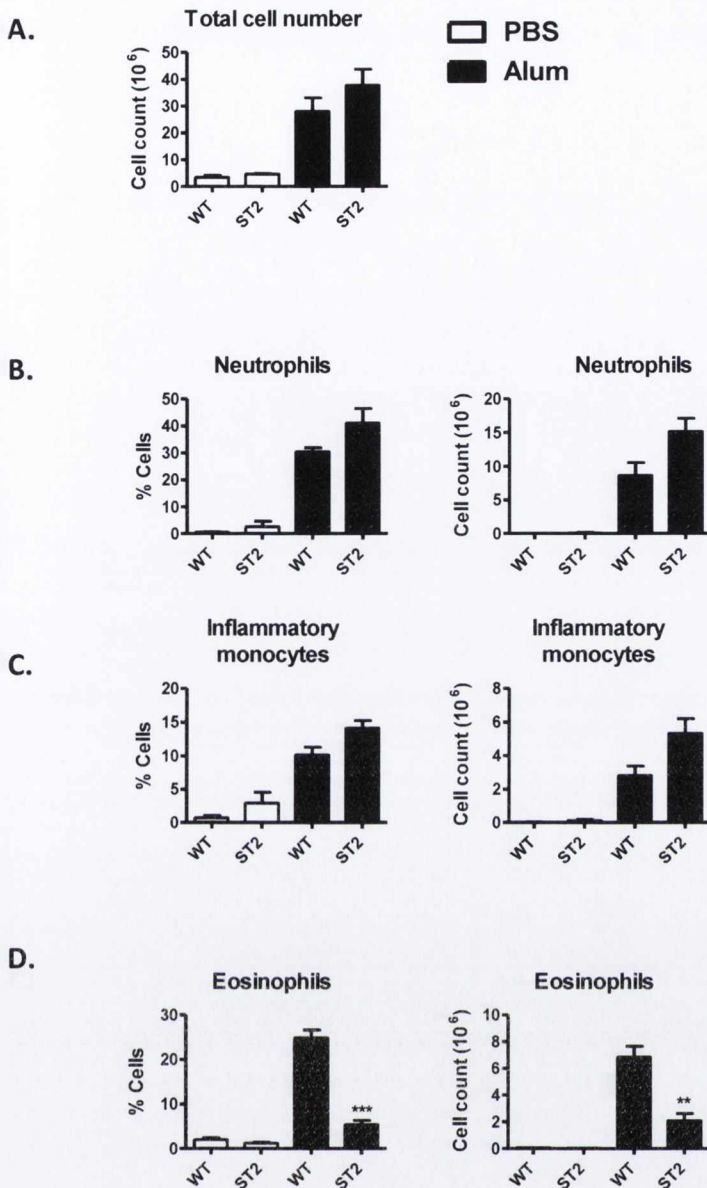




**Figure 4. 14. IL-33 is required for eosinophil infiltration into the site of alum injection.** Mice (C57BL/6 and IL-33<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were counted (A), stained and analysed by flow cytometry for the presence of (B) resident macrophages, inflammatory monocytes, neutrophils, (C) eosinophils and mast cells. Wild-type Alum v IL-33<sup>-/-</sup> Alum \*p<0.05. Results represent one experiment. Error bars show means ± SEM for 4 mice per experimental group.

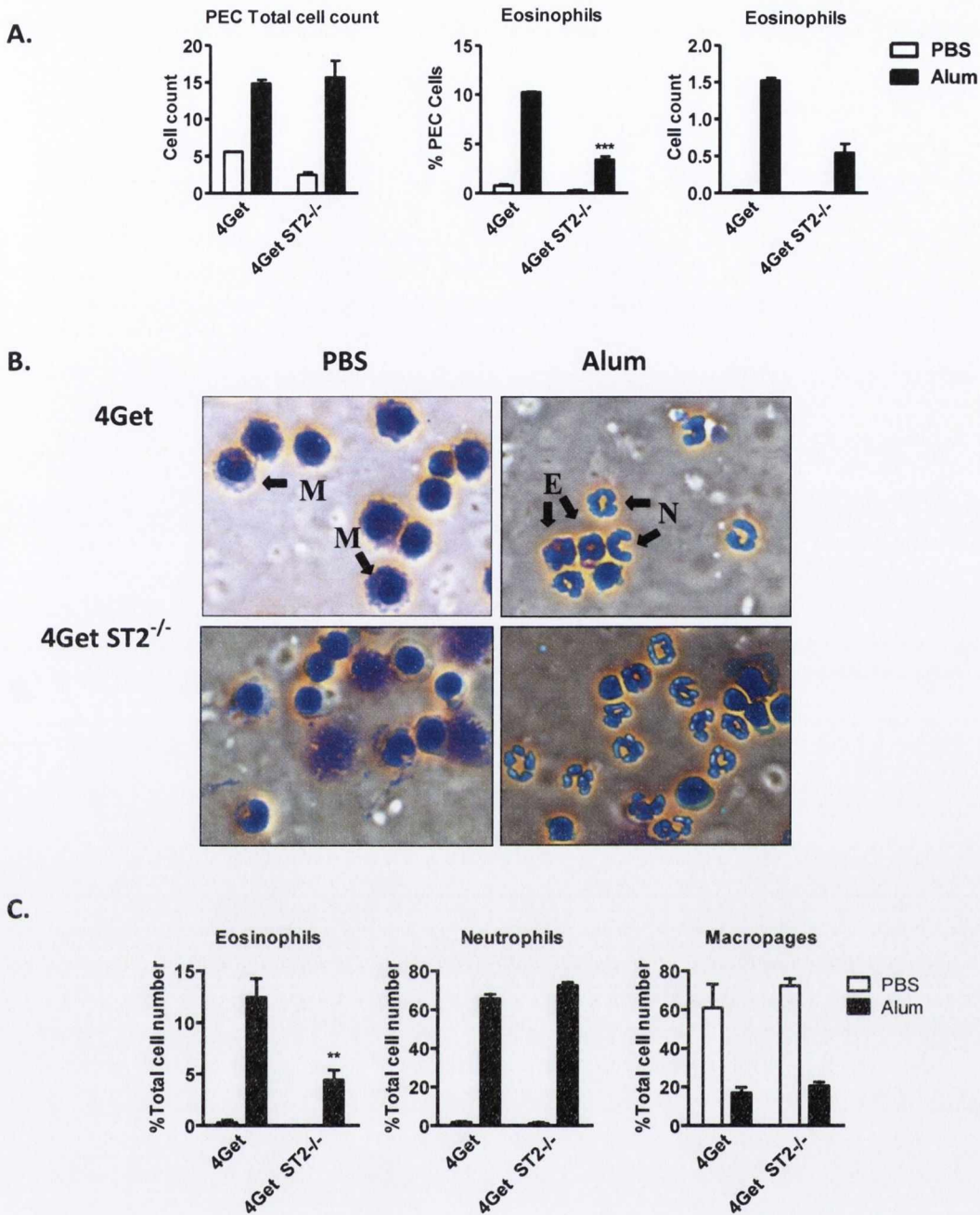






**Figure 4. 15. IL-33 controls eosinophil infiltration into the site of alum injection via ST2 signalling.** Mice (C57BL/6 and ST2<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were counted (A), stained and analysed by flow cytometry for the presence of (B) neutrophils, (C) macrophages and (D) eosinophils. Wild-type Alum v ST2<sup>-/-</sup> Alum \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. Results are from one experiment. Error bars show means ± SEM for 3 mice per experimental group. Experiment performed by Dr Hendrik Nel.

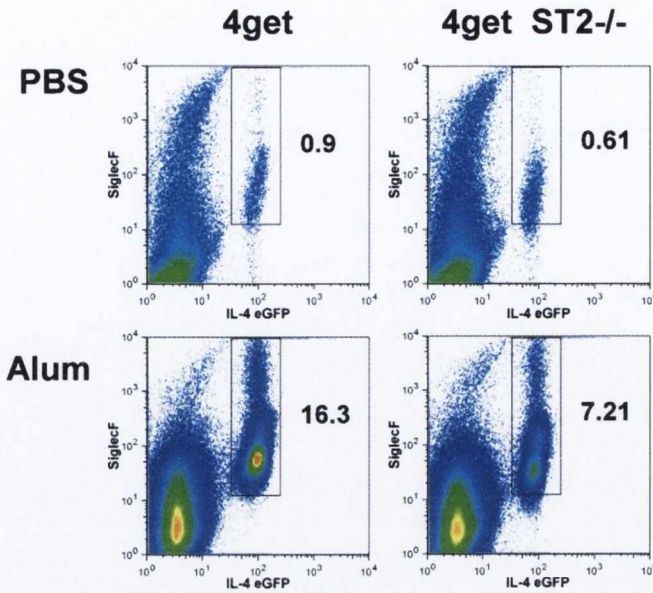




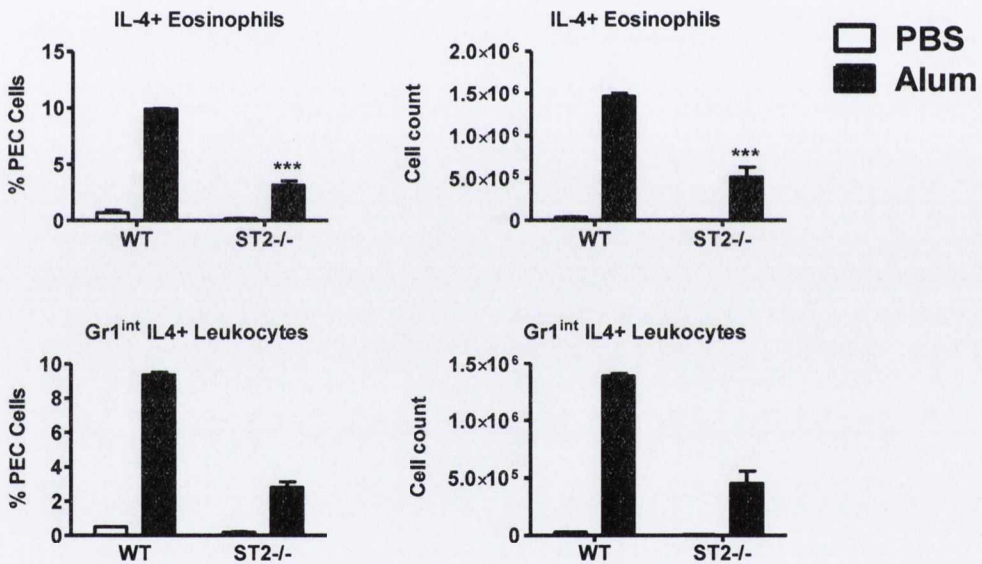
**Figure 4. 16. Lack of ST2 receptor in 4Get mice selectively impairs eosinophil recruitment after alum administration.** Mice (4Get and 4Get ST2<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. (A) Isolated PerC were counted, stained and analysed by flow cytometry for the presence of eosinophils. (B, C) Differential staining on the cytopins (50000 PerC) was done with Thermo Scientific Shandon Kwik-Diff. Arrows: N – neutrophils, E – eosinophils, M – Macrophages. 4Get Alum v 4Get ST2<sup>-/-</sup> Alum \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Results are representative of two independent experiments. Error bars show means  $\pm$  SEM for 3 mice per experimental group. Experiment performed by Dr Hendrik Nel.



A.

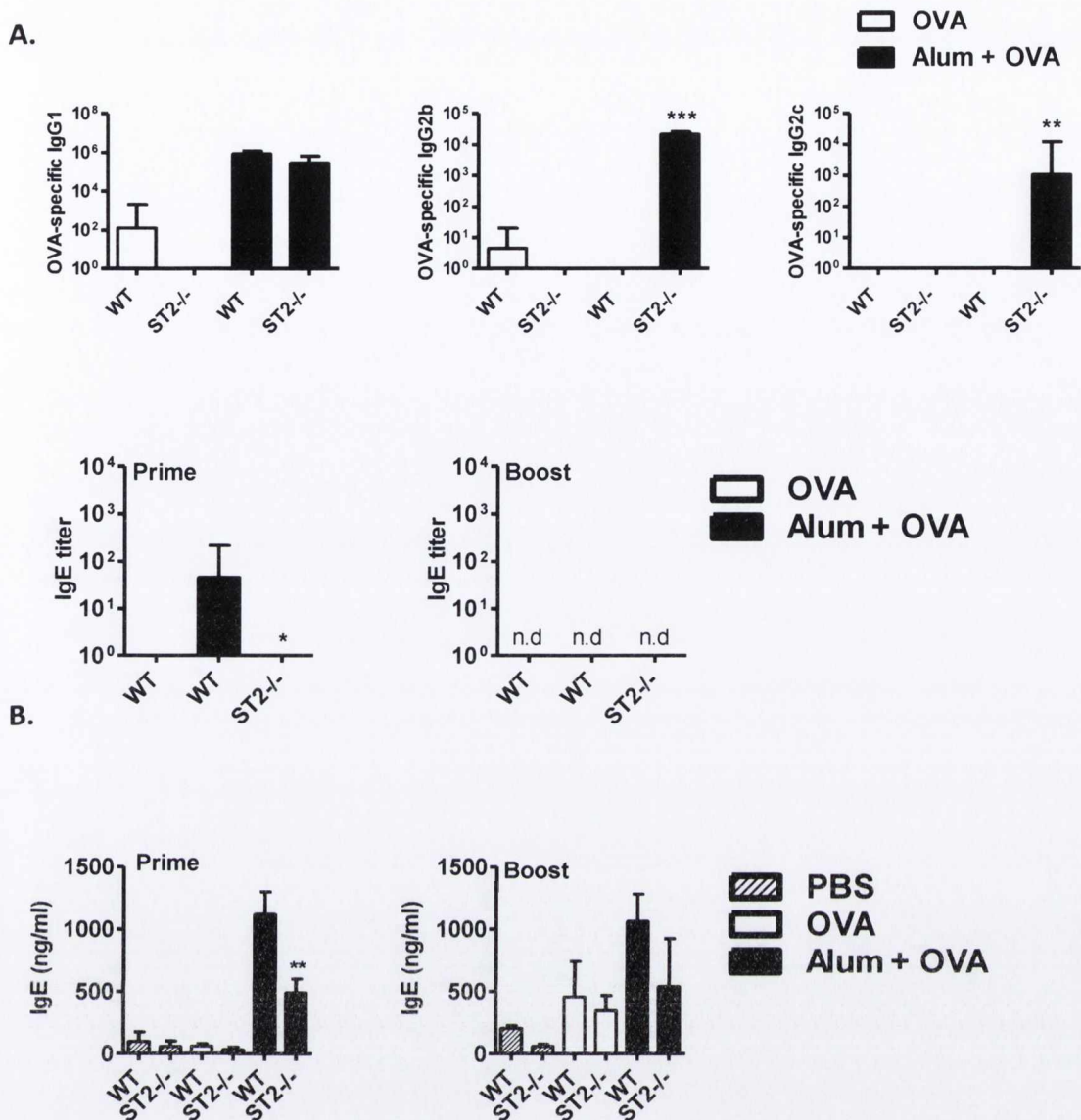


B.



**Figure 4. 17.** IL-33 induces IL-4 production by eosinophils and Gr-1<sup>+</sup> cells. Mice (4Get and 4Get ST2<sup>-/-</sup>) were injected with PBS or alum (1mg/mouse) intraperitoneally. Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were stained and analysed by flow cytometry for the presence of IL-4 expressing eosinophils and Gr-1<sup>int</sup> cells. (A) Representative data. (B) Results are pooled from two independent experiments. 4Get Alum v 4Get ST2<sup>-/-</sup> Alum \*\*\*p<0.001. Experiments performed by Dr Hendrik Nel.

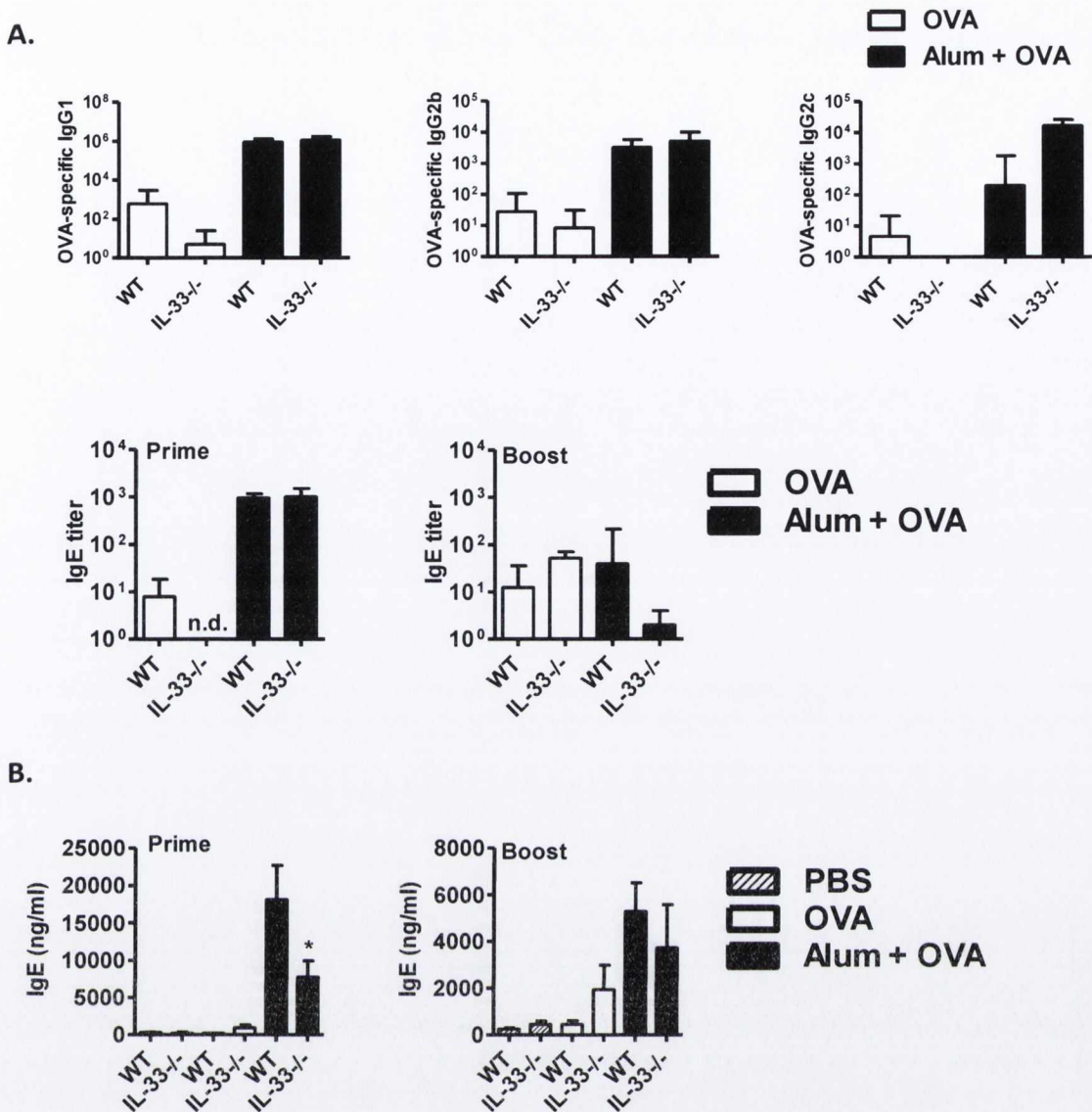




**Figure 4. 18. ST2 blocks TH1-biased IgG isotype switching after alum immunisation.** Mice (C57BL/6 and  $ST2^{-/-}$ ) were immunised intraperitoneally with PBS, OVA alone or OVA in combination with alum on days 0 and 14. On days 13 and 21 blood was collected. Blood samples were centrifuged and serum was removed. (A) The serum samples were analysed for OVA-specific IgG1, IgG2b, IgG2c and IgE by ELISA. OVA-specific antibody responses are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal serum dilutions to a cut-off point of 2 standard deviations above the mean OD from sera of PBS injected mice. (B) Total levels of IgE were measured by specific ELISA. Wild-type Alum + OVA v  $ST2^{-/-}$  Alum + OVA \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Results are representative of two independent experiments. Error bars show means  $\pm$  SEM for 4-5 mice per experimental group.

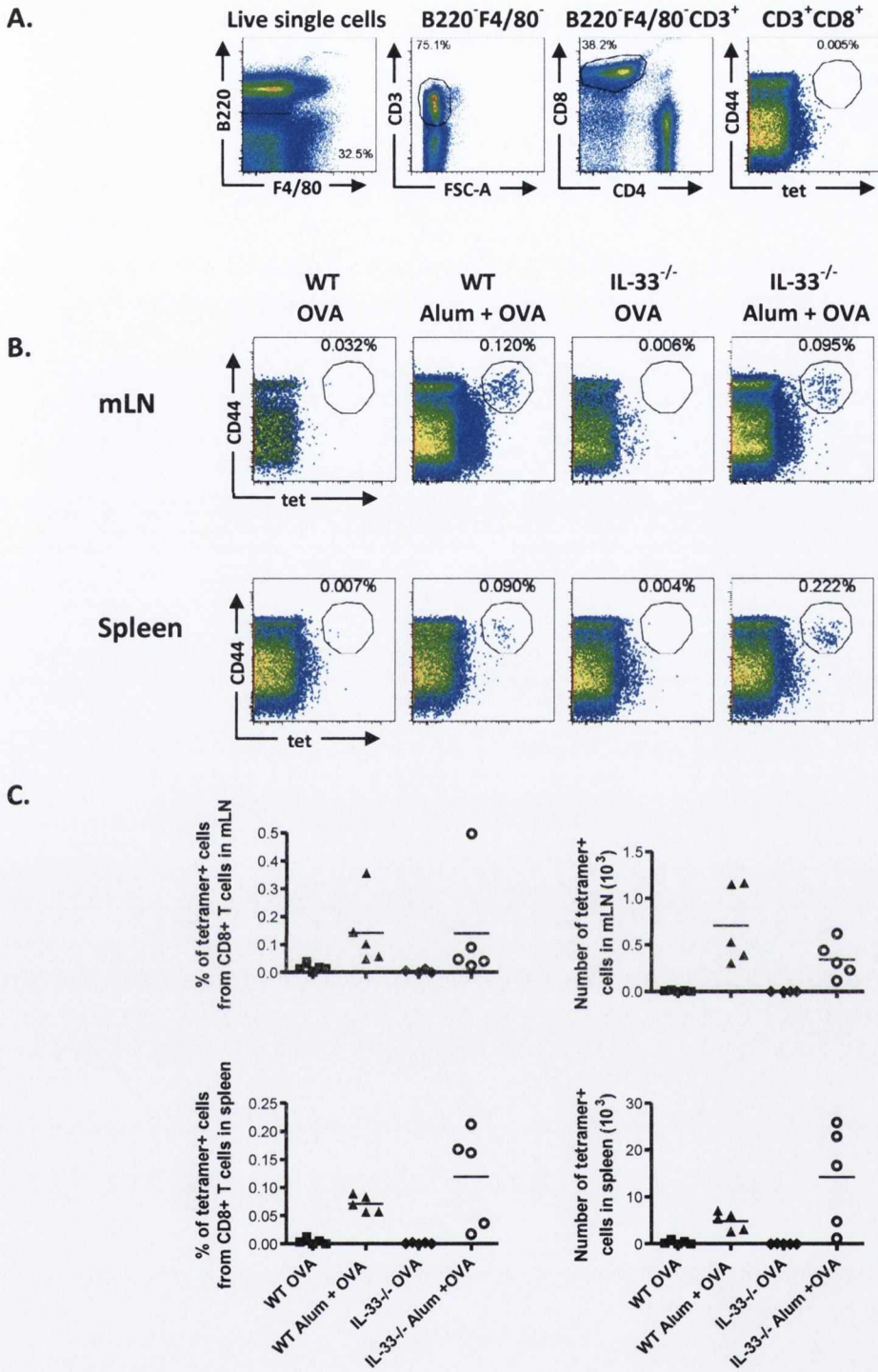






**Figure 4. 19. IL-33 has limited effect on antigen-specific IgG2c and total IgE responses after alum immunisation.** Mice (C57BL/6 and IL-33<sup>-/-</sup>) were immunised intraperitoneally with PBS, EndoOVA alone or EndoOVA in combination with alum on days 0 and 14. On days 13 and 21, blood was collected. Blood samples were centrifuged and serum was removed. (A) The serum samples were analysed for OVA-specific IgG1, IgG2b, IgG2c and IgE by ELISA. OVA-specific antibody responses are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal serum dilutions to a cut-off point of 2 standard deviations above the mean OD from sera of PBS injected mice. (B) Total levels of IgE were measured by specific ELISA. Wild-type Alum + OVA v IL-33<sup>-/-</sup> Alum + OVA \*p<0.05. Results are from one experiment. Error bars show means ± SEM for 4-5 mice per experimental group.





**Figure 4. 20. IL-33 is not required for the induction of antigen-specific CD8 T cells after alum immunization.** Mice (C57BL/6 and IL-33<sup>-/-</sup>) were immunised intraperitoneally with PBS, EndoOVA alone or EndoOVA in combination with alum on days 0 and 14. Mice were sacrificed on day 21 and spleens and mediastinal lymph nodes were collected. The number of tetramer<sup>+</sup> cells in lymph nodes and spleen were examined by flow cytometry. (A) Gating strategy. (B) tet<sup>+</sup>CD44<sup>hi</sup> CD8 T cells gated on live single CD3<sup>+</sup>CD8<sup>+</sup>CD4<sup>-</sup> B220<sup>-</sup>F4/80<sup>-</sup> cells. (C) The percentage and numbers of tet<sup>+</sup> CD8<sup>+</sup> T cells in mediastinal lymph nodes and spleens. Results are from one experiment with 4-5 mice per experimental group.



## **CHAPTER 5**

### **MODULATION OF IL-10 AND IL-12**

### **PRODUCTION BY ALUM**



## 5.1. Introduction

Regulatory mechanisms are required to maintain immunological homeostasis. However, for vaccination any adjuvant-induced suppressive mechanism can be undesirable as this can compromise the induction of protection against pathogens. Furthermore, strong TH1 and TH17 immune responses are needed to develop efficient immunity toward pathogens including HIV virus (300) and *Mycobacterium tuberculosis* (301). Currently, the most widely used adjuvant for human vaccination, alum, is inefficient in the activation of TH1 and TH17 immune responses which makes it unsuitable for developing vaccines against the above pathogens. Therefore, novel strategies are under investigation to enhance cell-mediated immune response induction by particulate adjuvant formulations. For example, combinations of alum with PAMPs, particularly the TLR4 ligand, MPLA, are used clinically and other formulations are under evaluation (204).

While there is a range of cytokines which can polarise immune responses, the IL-12 family of cytokines can potently shape cell-mediated immunity (158). TH1 responses and IFN- $\gamma$  production are induced by bioactive IL-12 (IL-12p70), a heterodimeric cytokine composed of two subunits, p35 and p40. p35 is a unique subunit of IL-12p70, while p40 is shared by IL-12 and IL-23 (158). IL-12p70 is preferentially produced by antigen-presenting cells, including dendritic cells. Due to the role of IL-12 family cytokines in polarisation of adaptive immune responses, their expression is tightly regulated. Secretion of these cytokines can be inhibited by various mechanisms, which are best characterized for cells activated by TLR ligands. TLR ligands, not only induce production of pro-inflammatory cytokines by DCs, but also activate additional inhibitory signalling pathways to prevent excessive inflammation. One of the earliest identified mechanisms which negatively regulates secretion of cytokines, including IL-12p70, is activation of PI3 kinase (164). PI3 kinase activation leads to inhibition of IL-12 and subsequently TH1 responses, which can lead to TH2 polarisation. In addition to TLR ligands, endogenous stimuli can also induce PI3 kinase activation. Recent reports suggest that monosodium urate crystals and alum induce Syk-PI3 kinase signalling in dendritic cells following crystal interactions with membrane lipids (232, 302). Moreover, *in vivo* experiments with a Syk inhibitor and in mice deficient in PI3 kinase  $\delta$  show that



this pathway is important for early recruitment of inflammatory dendritic cells to the site of MSU crystal injection and development of TH2 responses (251).

Immune responses can be also limited by IL-10, a cytokine which can be produced by cells from both the innate and adaptive immune system and is involved in suppressing functions of many immune cells. Importantly, IL-10 is potent regulator of TH1 responses. Abrogation of IL-10 signalling in mice immunised with OVA in the presence of low concentrations of LPS leads to development of an OVA-specific TH1 response. However, the same concentrations of LPS are not sufficient to prime for TH1 responses in the presence of IL-10 (175). Moreover, IL-10 can influence the magnitude of TH1 responses and protection induced by some vaccines. For instance, immunisation with *Leishmania* antigens together with CpG induces not only TH1 cells, but also IL-10-producing T cells. To overcome the suppressive effect of IL-10, three immunisations are required for protection against *Leishmania* infection. However, addition of anti-IL-10R blocking antibodies at the time of immunisation enhanced the magnitude of TH1 responses to mediate protection after a single immunization (303).

It is known that alum is not an effective adjuvant for promoting TH1 and TH17 responses, but the reasons for this are not well understood. It is hypothesised here that alum can induce immunoregulatory mechanisms, which actively block induction of cellular immune responses.

## 5.2.Aims

- To investigate the effects of alum on TLR-induced cytokines produced by dendritic cells.
- To assess the ability of alum to promote IL-10 production, and thereby, compromise cellular immune responses in vivo

## 5.3. Results

### 5.3.1. Alum inhibits IL-12 production by dendritic cells

In order to determine the effect of alum on cytokine production by dendritic cells, LPS or CpG primed BMDCs were incubated with alum (0.5-200 $\mu$ g/ml). After 24h supernatants were analysed by ELISA for the TH1 and TH17 cell polarising cytokines; IL-12p70, IL-12p40, IL-1 $\beta$ , IL-23 and IL-6. As reported previously, alum alone did not induce cytokine secretion in non-primed DCs. Moreover, in agreement with previous studies, TLR ligand-primed DCs treated with increasing doses of alum secreted IL-1 $\beta$ . Both TLR agonists, LPS and CpG, induced significant production of IL-12p70 by dendritic cells. However, when LPS or CpG-primed DCs were treated with alum, IL-12p70 secretion was inhibited even by low concentrations of the adjuvant (2 $\mu$ g/ml). Similarly, alum inhibited IL-12p40, IL-6 and IL-23 secretion, but not to the same extent as IL-12p70 (Figure 5. 1).

Having demonstrated that alum selectively inhibits IL-12p70 secretion, the effect of alum on IL-12p35 and IL-12p40 expression was investigated. DCs were stimulated with LPS alone or in the presence of alum for 3, 6 and 12h. Expression of IL-12p35, IL-12p40 and  $\beta$ -actin was determined by real-time PCR. As was expected, LPS-induced IL-12p35 and IL-12p40 mRNA expression, as early as 3h after stimulation. However, only IL-12p35 expression was down-regulated by alum at each time point. IL-12p40 expression was not significantly affected by addition of alum, which indicates that the inhibition of IL-12p35 is not a nonspecific effect (Figure 5. 2 A). Furthermore, to determine the optimal concentration of alum which decreases IL-12p35 expression, DCs were stimulated with LPS alone or in the presence of a range of alum concentrations. Cells were stimulated for 6 hours, which is an optimal time point to assess IL-12p35 expression in LPS-stimulated BMDCs and IL-12p35 and  $\beta$ -actin mRNA levels were determined by real-time PCR. In accordance with results obtained by ELISA, alum inhibits IL-12p35 expression at a concentration as low as 20 $\mu$ g/ml (Figure 5. 2 B).

In order to evaluate whether IL-12p70 inhibition is restricted to alum (Alhydrogel), the particulate adjuvants, aluminium phosphate (Adju-Phos), Calcium Phosphate, 1 $\mu$ m Poly-D,L-lactide-co-glycolide (PLG), 430 nm Polystyrene particles (PS) and Chitosan and the alum-induced danger signal Monosodium Urate crystals (MSU) were analysed (Figure 5. 3 and 5. 4). DCs were stimulated with LPS alone or LPS with

different concentrations of adjuvants or MSU. After 24h, supernatants were collected and analysed for cytokine production. Firstly, to ensure that the adjuvants and MSU crystals were acting as expected, their effect on IL-1 $\beta$  secretion was determined. Previous reports demonstrated that particulate adjuvants and MSU activate the NLRP3 inflammasome and induce IL-1 $\beta$  secretion (238). As was expected, IL-1 $\beta$  was detected in all treatment groups. However, there were considerable differences in potency between adjuvants. Adju-Phos, Alhydrogel and PLG were more potent inducers of IL-1 $\beta$  compared to Calcium Phosphate or PS particles. The inhibition of IL-12p70 secretion was detected in the case of all adjuvants, except chitosan. The most potent inhibitors of IL-12p70 were Adju-Phos, Alhydrogel and PLG, and the weakest Calcium Phosphate or PS particles. Remarkably, chitosan was the only adjuvant which induced IL-1 $\beta$  secretion, but did not inhibit IL-12p70.

### **5.3.2. Alum inhibits IL-12p70 secretion via PI3 kinase induction**

IL-12p70 secretion by DCs can be inhibited by several mechanisms including activation of PI3 kinase. TLR ligands including CpG can induce DCs to produce IL-12p70, however the engagement can simultaneously promote activation of PI3 kinase, which inhibits pro-inflammatory responses and acts as a negative regulator of TLR signalling (164). In order to determine the role of PI3 kinase in the inhibition of IL-12p70 by alum, DCs were pre-incubated with Wortmannin, a broad spectrum inhibitor of PI3 kinases, and then stimulated with LPS and alum (0.5-200 $\mu$ g/ml). Inhibition of PI3 kinase promoted a significant reversal of IL-12p70 inhibition by alum, while LPS induced IL-12p70 was not increased by Wortmannin. Moreover, IL-12p40 inhibition was reversed by inhibition of PI3 kinase while IL-1 $\beta$  levels were decreased (Figure 5. 5 A). In addition, IL-12p35 and IL-12p40 expression was determined by real-time PCR. LPS-induced IL-12p35 was inhibited by alum and this effect was reversed by inhibiting PI3 kinases. LPS-induced IL-12p40 was not inhibited by alum. Moreover, Wortmannin enhanced LPS-induced IL-12p40 expression (Figure 5. 5 B).

PI3 kinases negatively regulate IL-12p70 production in DCs via two distinct pathways, by activation of mTOR, which induces IL-10 production leading to autocrine inhibition of IL-12p70 or by blocking the activity of GSK, which is an IL-12 inducer. Therefore, in order to assess whether alum-induced IL-12p70 inhibition is

IL-10-dependent, wild-type and IL-10-deficient DCs were stimulated with LPS alone or in the presence of alum (2-100 $\mu$ g/ml) or monosodium urate crystals (2-100 $\mu$ g/ml) and IL-12p70 production was determined in supernatants. As shown previously, when wild-type primed DCs were treated with alum, IL-12p70 secretion was inhibited. Interestingly, IL-12p70 inhibition was also observed in IL-10 deficient DCs (Figure 5. 6 A). Furthermore, monosodium urate crystals, which are not as potent inhibitor as alum, were also able to inhibit IL-12p70 in IL-10-deficient DCs (Figure 5. 6 B). Overall, these results suggest an IL-10 independent mechanism of particle-induced IL-12p70 inhibition.

### 5.3.3. Alum induces IL-10 production *in vitro* and *in vivo*

IL-10 is the most potent known immunoregulatory cytokine and can regulate functions of many cells, including DCs. For instance, IL-10 is capable of inhibiting cytokine production and DC maturation. IL-12p70 inhibition induced by alum is IL-10-independent; however, it remains unknown whether IL-10 production *in vitro* or *in vivo* would impact alum-induced immune responses.

The first question addressed was whether alum can induce or enhance IL-10 production *in vitro*. DCs were stimulated with alum at concentrations from 0.5-200 $\mu$ g/ml in the presence of the TLR ligands, LPS and Zymosan and IL-10 secretion was measured. Alum alone did not promote IL-10 production by DCs. However, at a concentration of 50 $\mu$ g/ml alum enhanced LPS-induced IL-10 production, while Zymosan-induced IL-10 production was further increased by lower concentrations of alum (0.5-10 $\mu$ g/ml), (Figure 5. 7 A). Moreover, MSU crystals and Calcium Phosphate, promoted IL-10 secretion by primed DCs. Similarly to alum, the optimal concentrations of MSU and Calcium Phosphate, for promoting IL-10 secretion, were 50 $\mu$ g/ml (Figure 5. 7 B).

Having demonstrated that alum can enhance IL-10 secretion, the effect of alum on IL-10 mRNA expression was investigated. DCs were stimulated with LPS alone or in the presence of alum for 3, 6 and 12h. Expression of IL-10 and  $\beta$ -actin was determined by real-time PCR. As was expected, LPS enhanced IL-10 mRNA expression, which was most marked at 6h after stimulation. IL-10 expression was further upregulated by alum at later time points. (Figure 5. 7 C). To determine the optimal concentration of alum to increase IL-10 mRNA expression, DCs were stimulated with LPS alone or in the presence

of a range of alum concentrations. Cells were stimulated for 6 hours and IL-10 and  $\beta$ -actin mRNA levels were determined by real-time PCR. In accordance with results obtained by ELISA, alum significantly enhanced IL-10 expression at a concentration of 50 $\mu$ g/ml (Figure 5. 7 D)

Having established that alum can enhance IL-10 production in primed DCs, the next question addressed was whether alum can also augment IL-10 secretion *in vivo*. Wild-type mice were immunised intraperitoneally with PBS or alum. 24h, 3 and 7 days post injection, PerC were isolated and mediastinal lymph nodes and splenocytes were removed. Isolated cells were restimulated *ex vivo* with LPS, heat-killed *E. coli* or CpG or with anti-CD3 alone or together with PMA and IL-10 levels were measured in supernatants. Interestingly, peritoneal cells isolated from mice injected with PBS secreted high levels of IL-10 in response to all PRR agonists tested, whereas alum injection led to a reduced response. This effect was also observed in PerC restimulated with anti-CD3 alone or anti-CD3 with PMA, but it was less profound (Figure 5. 8 A). In contrast, injection of alum enhanced IL-10 secretion by mediastinal lymph node cells in response to HK *E. coli* and anti-CD3 with PMA. However, while restimulation with HK *E. coli* revealed significant IL-10 secretion 7 days post alum administration by mediastinal lymph nodes, restimulation with anti-CD3 and PMA showed that alum promoted IL-10 as early as 24h post injection. Furthermore, this effect persisted for at least 7 days (Figure 5. 8 B). Strikingly, the enhancement of IL-10 secretion in response to anti-CD3 and PMA was only observed in mediastinal lymph node cells, as splenocytes from alum-injected mice did not produce higher levels of IL-10 (Figure 5. 8 C).

Since one of the main producers of IL-10 are regulatory T cells, it was important to determine whether alum can promote regulatory T cell expansion. Wild-type mice were immunised intraperitoneally with PBS or alum. After 24h, 3 and 7 days, PerC were isolated and mediastinal lymph nodes and spleens were removed. Isolated cells were stained and analysed by flow cytometry. Cells were gated to exclude debris, doublet and dead cells, followed by gating for CD3<sup>+</sup>CD4<sup>+</sup> T cells. These cells were analysed for the presence of the nuclear transcription factor, FoxP3, which is the signature of regulatory T cells (Figure 5. 9 A). When the percentage of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells was compared between PBS and alum injected mice in the peritoneal cavity (Figure 5. 9 B), mediastinal lymph nodes (Figure 5. 9 C) and spleens (Figure 5. 9 D), there was no apparent expansion of regulatory T cells.

#### **5.3.4. Lack of IL-10 signalling does not compromise the innate immune response at the site of alum injection**

As showed above, peritoneal cells have a great capacity to produce IL-10 when restimulated with LPS, HK *E. coli* or CpG and this property is lost after alum injection. On the other hand, alum promotes a rapid innate immune response at the site of injection, inducing the influx of inflammatory monocytes, eosinophils and neutrophils. In order to determine whether IL-10 plays a role in restricting innate response at the site of alum injection, wild-type and IL-10-deficient mice were injected with PBS or alum and 24h later PerC were isolated and mediastinal lymph nodes removed. As expected, alum induced significant cell infiltration in wild-type mice at the site of injection. However, the lack of IL-10 only slightly increased the total PerC number (Figure 5. 10 A). To find out whether infiltration of different cell populations was skewed by IL-10 deficiency, PerC were analysed by flow cytometry. The injection of alum led to the complete depletion of mast cells and resident macrophages in both wild-type and IL-10-deficient mice. Moreover, there was comparable infiltration of inflammatory monocytes, neutrophils and eosinophils into the site of alum injection (Figure 5. 10 B and C).

The effect of IL-10 deficiency on cytokine production by PerC or mediastinal lymph node cells was also investigated. Isolated cells were restimulated with anti-CD3 to determine whether the lack of IL-10 would increase IFN- $\gamma$  or IL-17 production after alum injection. Alum administration increased IL-17 secretion in anti-CD3 restimulated PerC when compared to PBS injected mice, but this effect was not enhanced by IL-10-deficiency. Interestingly, the lack of IL-10 led to increased IFN- $\gamma$  production by PerC in PBS-injected mice, but there was no further enhancement in IFN- $\gamma$  secretion following alum administration. Additionally, there was no change in IL-5 production (Figure 5. 11 A). Similar results were seen when mediastinal lymph node cells were restimulated with anti-CD3; there was no increase in cytokine production observed in IL-10<sup>-/-</sup> mice (Figure 5. 11 B).

#### **5.3.5. IL-10 restricts the ability of alum to promote TH1 and TH17 responses**

In order to determine the effect of IL-10 on the capacity of alum to promote pro-inflammatory T cell responses, wild-type and IL-10-deficient mice were immunised with

PBS, OVA alone or alum co-administered with OVA and sacrificed 7 days post immunisation. Spleens were removed and cells were isolated from the peritoneal cavity. Cells were restimulated with OVA *ex vivo* and supernatants were analysed for IFN- $\gamma$  and IL-17, which are characteristic of TH1 and TH17 cells, respectively. As expected, alum did not significantly enhance antigen-specific IFN- $\gamma$  and IL-17 responses in splenocytes or PerC from wild-type mice. However, in IL-10-deficient mice immunised with alum there was a strong enhancement in antigen-specific IFN- $\gamma$  and IL-17 production by splenocytes compared to antigen alone (Figure 5. 12 B). Furthermore, in contrast to splenocytes, PerC isolated from IL-10<sup>-/-</sup> mice immunised with OVA alone produced IL-17 and IFN- $\gamma$ , but IFN- $\gamma$  secretion was significantly enhanced in IL-10<sup>-/-</sup> mice immunised with alum co-administered with OVA when compared to wild-type mice immunised with the same treatment although this was not antigen-specific (Figure 5. 12 A).

To determine the responsiveness of leukocytes from immunised wild-type and IL-10-deficient mice to a non-specific T cell stimulus, cells were stimulated with anti-CD3. Firstly, it was observed that there was an enhancement of IFN- $\gamma$  and IL-17 production by splenocytes and PerC from IL-10<sup>-/-</sup> mice immunised with OVA alone compared to wild-type cells when restimulated with anti-CD3 (Figure 5. 13 A and B). Furthermore, following anti-CD3 stimulation there was no significant difference in pro-inflammatory cytokine levels between splenocytes or PerC from IL-10-deficient mice immunised with OVA alone and alum with OVA (Figure 5. 13 A and B).

To evaluate the effect of IL-10 on antibody responses after alum immunisation, sera were isolated from wild-type and IL-10<sup>-/-</sup> mice immunised with PBS, OVA alone or alum co-administered with OVA 7 days post immunisation. Firstly, antigen-specific IgG1 titres were measured in serum. Immunisation of wild-type and IL10<sup>-/-</sup> mice with OVA and alum resulted in an increase of OVA-specific IgG1 in comparison to OVA alone. However, there were no significant differences in antigen-specific IgG1 between WT and IL-10<sup>-/-</sup> mice immunised with OVA and alum. Assays for antigen-specific IgG2b and IgG2c antibodies titres were also carried out, but these subclasses were barely detectable (Figure 5. 14).

Results from this adjuvant experiment performed on IL-10<sup>-/-</sup> mice suggested that in the absence of IL-10, alum can promote the generation of TH1 and TH17 responses. However, antigen-specific antibody responses were low as mice were immunised for only 7 days. Therefore, another *in vivo* study, which included a booster immunisation,

was performed. Additionally, to minimise the effect of any possible endotoxin contamination, instead of OVA, which was cleaned from potential LPS contamination in our laboratory, commercially available clinical grade LPS-free human serum albumin (HSA) was used as the antigen.

Wild-type and IL-10-deficient mice were immunised with PBS, HSA alone or alum co-administered with HSA and after 14 days mice were boosted in an identical manner. After 7 days, spleens were removed and cells were isolated from the peritoneal cavity. Cells were restimulated *ex vivo* with HSA and supernatants were analysed for the presence of IFN- $\gamma$  and IL-17. As expected, alum did not induce antigen-specific TH1 and TH17 cells in wild-type mice. However, in agreement with the *in vivo* study described above, vaccination with HSA and alum enhanced IFN- $\gamma$  production by peritoneal cells and splenocytes. However, there was no detectable antigen-specific IL-17 secretion (Figure 5. 15 A and B). Furthermore, to determine the responsiveness of splenocytes to nonspecific stimuli, cells were stimulated with anti-CD3. Firstly, it was observed that there was an enhancement of IFN- $\gamma$  production by splenocytes and PerC from IL-10<sup>-/-</sup> mice immunised with PBS alone compared to wild-type cells when restimulated with anti-CD3. This effect was not observed for IL-17 secretion (Figure 5. 16 A and B). Furthermore, following anti-CD3 stimulation there was no significant difference in pro-inflammatory cytokine levels between splenocytes and PerC from IL-10-deficient mice immunised with HSA alone or alum with HSA (Figure 5. 16 A and B).

To evaluate the effect of IL-10 on antibody responses after alum immunisation, sera were isolated from wild-type and IL-10<sup>-/-</sup> mice immunised with PBS, HSA alone or alum co-administered with HSA 7 days post booster immunisation. As expected, immunisation of wild-type and IL-10<sup>-/-</sup> mice with HSA and alum resulted in an increase of HSA-specific IgG1 in comparison to HSA alone. However, there were no significant differences between wild-type and IL-10<sup>-/-</sup> mice. Alum induced low titres of antigen-specific IgG2b and IgG2c in wild-type mice and their levels were not enhanced by the lack of IL-10 signalling (Figure 5. 17).



## 5.4. Discussion

The principal adjuvant used in human vaccination is alum, which promotes strong humoral responses, but is a poor inducer of cell-mediated immunity (304). The mechanisms underlying this selective immunomodulatory effect of alum are not fully understood. One of the possible explanations is the activation of immune regulatory pathways by the adjuvant. However, to date, the induction of anti-inflammatory effects by alum have not been widely investigated. Furthermore, to overcome the limitations of alum, combinational adjuvants have been developed, which aim to elicit stronger cellular immune responses than alum alone. For instance, combinations of alum with the TLR ligands, MPLA (203, 257) or CpG (231) were studied. However, there are studies which show some limitations of this approach. For instance, there is conflicting data regarding whether alum co-administered with MPL can provide better protection against influenza when compared to mice immunised with alum or MPL alone. The same research group demonstrated firstly that alum with MPL induced better protection against influenza than alum alone (257), but their second study showed that alum alone can provide better protection against influenza than alum co-administrated with MPL (256). Furthermore, it has been demonstrated that the addition of CpG did not improve alum induced adaptive immune responses measured in terms of antigen-specific antibodies. Interestingly, CpG alone induced higher levels of antigen-specific IgG2c than alum alone or alum with CpG, which might suggest that alum is somehow inhibiting CpG-mediated TH1 responses (231). Overall, there is considerable doubt that the addition of TLR ligands to alum result in enhanced protective immune responses. Therefore, it will be interesting to monitor the efficacy of HPV vaccine, which can be composed of alum alone (Gardasil) or alum with MPL (Cervarix) and see whether there is a difference in protection against cervical cancer in differently vaccinated women.

This study aimed to investigate the immunosuppressive effect of alum on immune responses. Firstly, the ability of alum to modulate TLR agonist-induced cytokine production *in vitro* was determined. In agreement with previous reports (236, 238), alum enhanced IL-1 $\beta$  production in LPS or CpG primed DCs. However, in contrast to IL-1 $\beta$ , alum inhibited secretion of the pro-inflammatory cytokines, IL-12, IL-23 and IL-6 with a particularly striking effect on IL-12p70 inhibition. Furthermore, IL-12p70 inhibition was not restricted to alum, as other particulate adjuvants and the danger

signal MSU also down-regulated TLR ligand-induced IL-12p70 secretion. IL-12p70, which is the bioactive form of IL-12, is composed of two subunits, IL-12p40 and IL-12p35. Expression of these subunits is differentially regulated; while IL-12p40 is expressed abundantly by immune cells, expression of IL-12p35 is tightly regulated, which prevents excessive TH1 responses (156). Interestingly, alum only inhibited IL-12p35, but not IL-12p40 expression, suggesting that alum may have a selective inhibitory effect on TH1 responses. IL-12 secretion can be down-regulated by phosphoinositide 3-kinases (PI3Ks) as PI3K-deficiency leads to excessive production of IL-12 by dendritic cells stimulated with TLR ligands (164). Moreover, it has been demonstrated that alum stimulates PI3 kinase activation in dendritic cells (232). Thus, whether alum inhibits IL-12p70 production via PI3 kinases was investigated by using the PI3K inhibitor, Wortmannin. DCs pre-incubated with Wortmannin and stimulated with LPS in the presence of alum expressed more IL-12p35 and produced more IL-12p70 than DCs which were not treated with the inhibitor. It has been reported that PI3 kinases can inhibit IL-12p70 production by upregulation of IL-10 secretion (165). However, alum inhibited IL-12 production in LPS-primed IL-10-deficient DCs, which suggests another pathway. It is possible that treatment of cells with alum promotes PI3 kinase-mediated GSK3 inhibition which leads to IL-10-independent IL-12 suppression. Furthermore, a recent paper demonstrated that alum-induced antigen-specific CD8<sup>+</sup> T cells express the inhibitory receptor PD-1. Interestingly, PI3 kinase activation in antigen-presenting cells leads to PD-L1 expression in HIV-infected DCs (305). It has been also demonstrated that PD-L1–PD-1 interaction is crucial in T cell exhaustion driven by HIV virus and that HIV-specific T cells upregulate PD-1 (306). Therefore, there may be a link between alum-induced PI3 kinase induction and expression of PD-1 on the surface of antigen-specific CD8<sup>+</sup> T cells in alum-immunised mice.

PI3 kinase activation does not only regulate IL-12 expression, but is also involved in the induction of IL-10. Together, PI3 kinase-mediated down-regulation of IL-12 and upregulation of IL-10 secretion can inhibit cellular immune responses. Interestingly, alum not only inhibited IL-12p70 production, but also increased the level of IL-10 secretion in DCs. Strikingly, MSU crystals and another adjuvant Calcium Phosphate significantly enhanced IL-10 secretion, which suggests a general mechanism of IL-10 induction by particulates. Interestingly, the ability of alum to enhance IL-10 secretion *in vitro* has been reported before (307), but the mechanism underlying this effect is still

unknown. It will be interesting to determine whether alum-driven IL-10 production is dependent on PI3 kinase signalling or other pathways.

Interestingly, alum-induced IL-10 secretion was also observed *ex vivo*. However, at the injection site alum did not enhance IL-10 secretion. Resident peritoneal cells were able to secrete high levels of IL-10 in unimmunised (data not shown) and PBS-injected mice but this was not seen in mice injected with alum. However, in these mice injection of alum primed mediastinal lymph node cells for enhanced IL-10 secretion. This was a selective effect, because there was no significant change in IL-10 production by splenocytes. Overall, these *in vitro* and *ex vivo* experiments suggest that alum enhances IL-10 production, which may in turn mediate regulatory effects during vaccination. Interestingly, one of the most significant producers of IL-10 are regulatory T cells, which are involved in restricting inflammation. However, regulatory T cells do not seem to play a role in limiting alum-induced immune response, as alum does not promote their expansion at the site of injection, in the draining lymph nodes or spleens. IL-10 can be produced by various cells from both the innate and adaptive immune system and it will be crucial to determine which cell type is responsible for IL-10 secretion in response to the injection of alum.

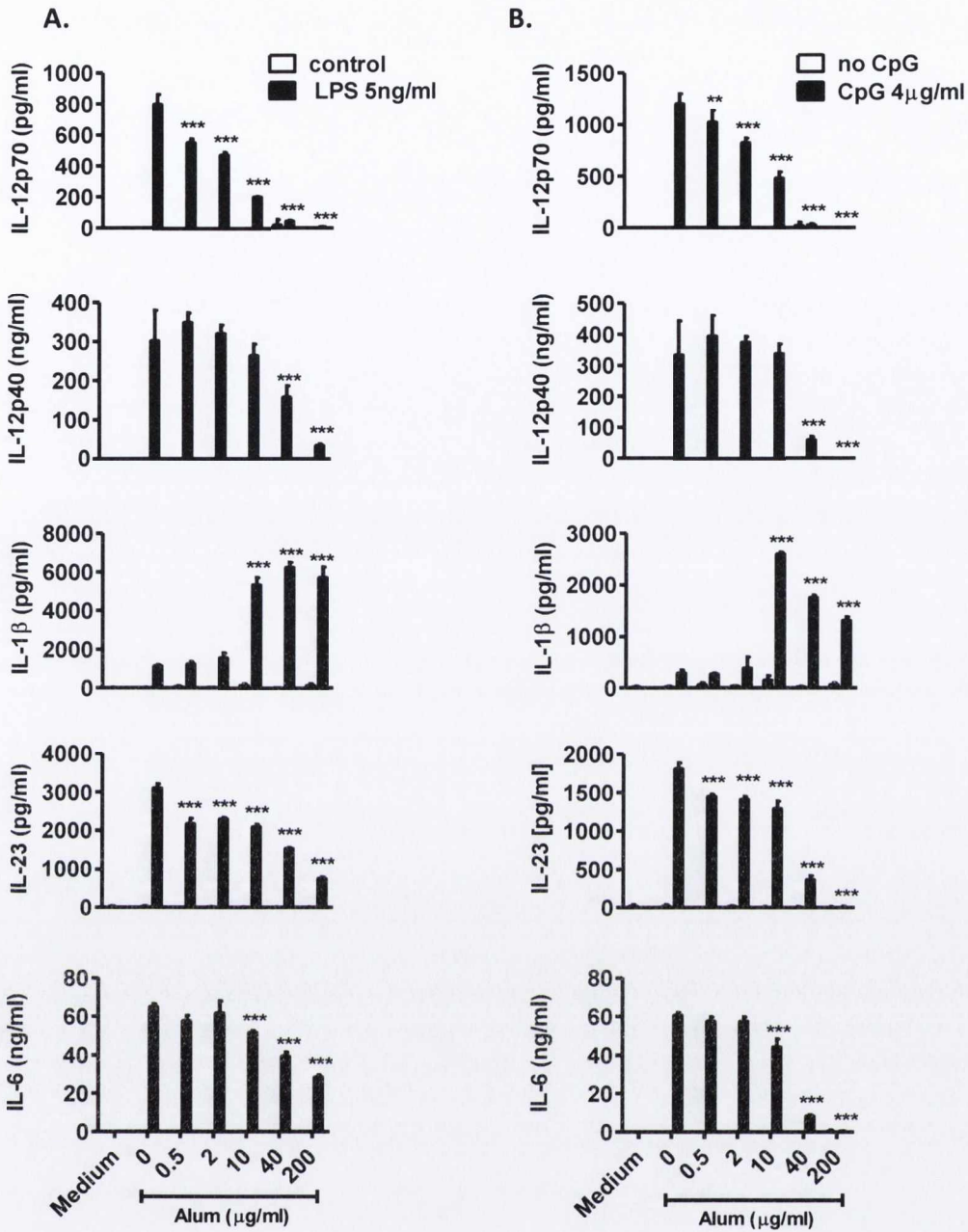
IL-10 can maintain the balance between tolerance and immunity and can limit both innate and adaptive immune responses. However, alum-induced inflammation at the site of injection is not restricted by IL-10. There is comparable infiltration of inflammatory cells into the peritoneal cavity, which suggests that IL-10-producing resident peritoneal cells do not have an impact on the innate immune response at the site of alum injection. However, it is possible that the lack of IL-10 might have an impact on DC-T cell interaction and polarisation of immune responses.

To investigate whether IL-10 is responsible for the limited ability of alum to promote TH1 and TH17 responses, both wild type and IL-10<sup>-/-</sup> mice were immunised with alum. As was expected, immunisation with OVA and alum did not result in significant antigen-specific IFN- $\gamma$  or IL-17 production in wild type mice. However, deficiency in IL-10 led to spontaneous and antigen-specific secretion of IFN- $\gamma$  by PerC and splenocytes in mice immunised with alum and OVA. Furthermore, following a booster immunisation with antigen and alum, the lack of IL-10 resulted in antigen-specific production of IFN- $\gamma$  but not IL-17. Interestingly, despite the fact that IL-

IL-10 compromises alum-induced TH1 responses there was no significant change in antigen-specific IgG1 antibody production in IL-10<sup>-/-</sup> mice.

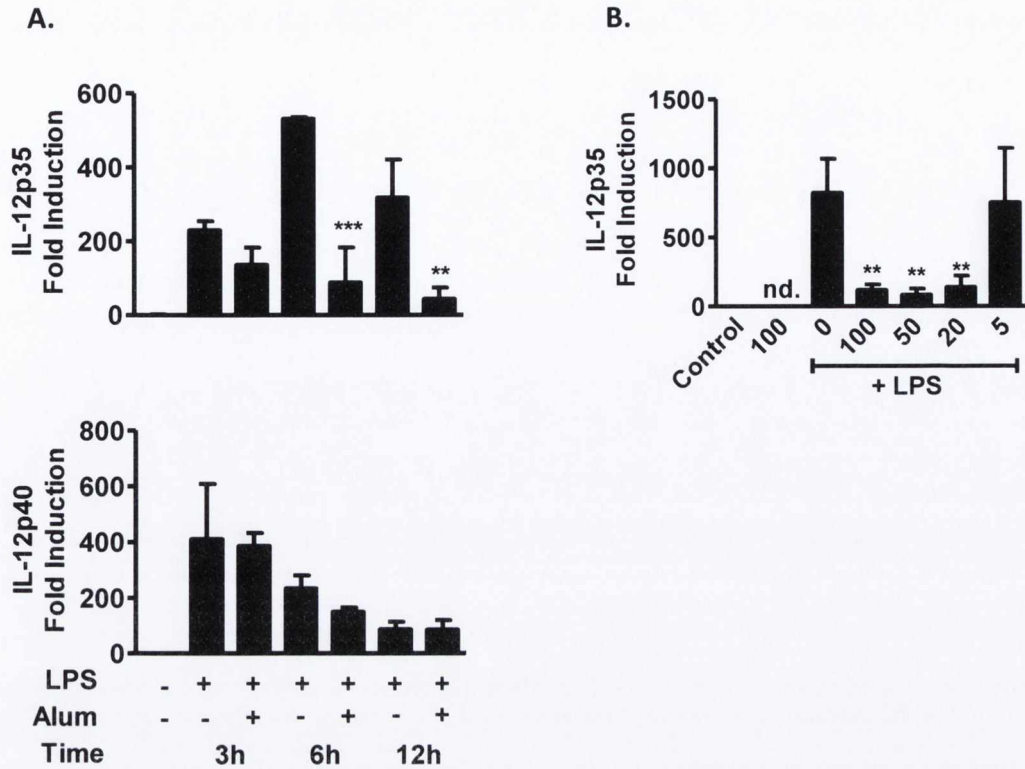
Overall, the data presented indicate that IL-10 exerts immunosuppressive effects during alum immunisation. In particular, the results suggest that alum-driven IL-10 can block TH1 responses.





**Figure 5. 1. Alum modulates pro-inflammatory cytokine production by dendritic cells.** DC from C57BL/6 mice were stimulated with (A) LPS (5ng/ml) or (B) CpG (4μg/ml) alone or with Alhydrogel (Alum) at concentrations from 0.5-200μg/ml. Concentrations of IL-12p70, IL-12p40, IL-1β, IL-23 and IL-6 were determined in 24h supernatants by ELISA. Adjuvant + LPS v LPS, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Results are representative of three independent experiments. Error bars show means  $\pm$  SD for each experimental group tested individually in triplicate.

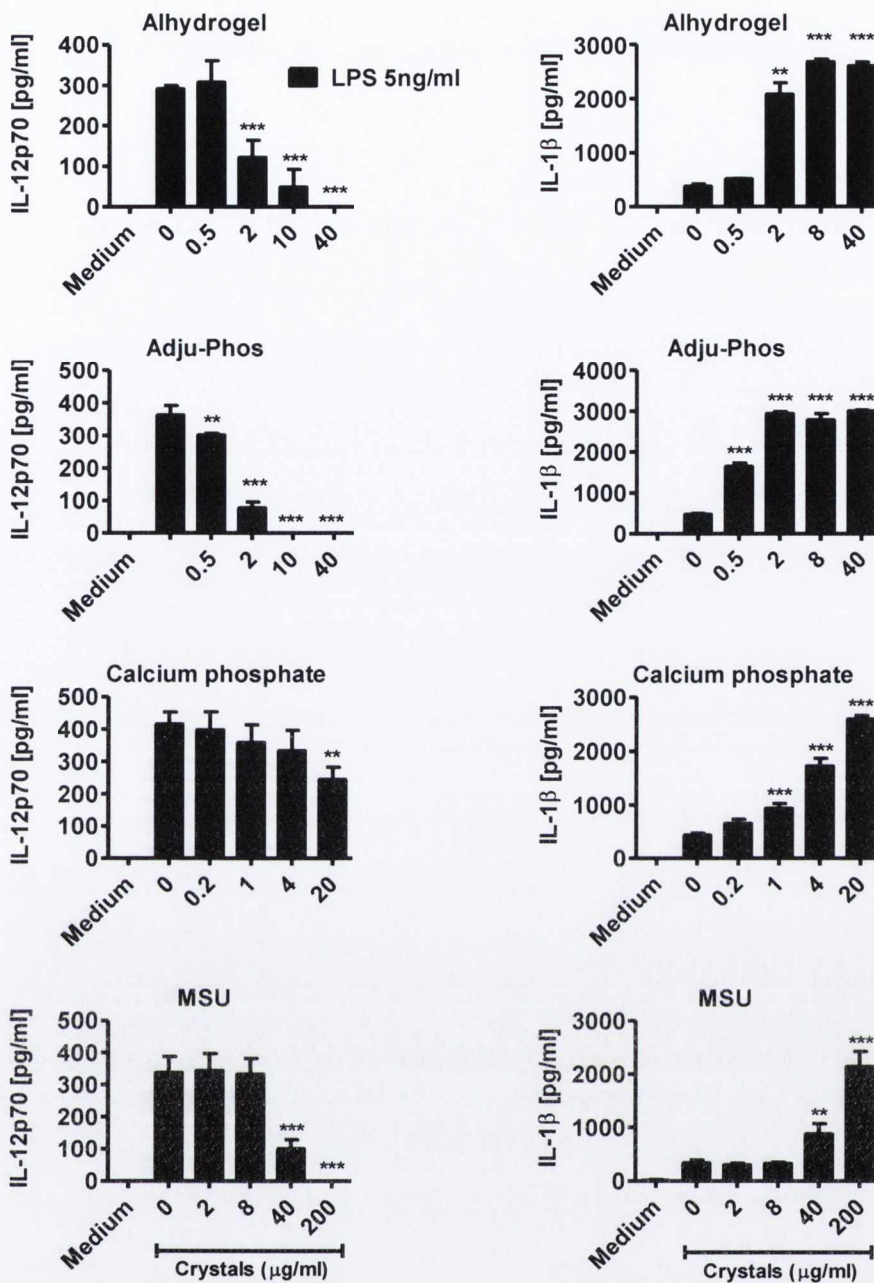




**Figure 5. 2. Alum selectively inhibits IL-12p35 expression.** (A) DC from C57BL/6 mice were incubated with LPS (5ng/ml) alone or together with alum (40μg/ml). The expression of IL-12p35 and IL-12p40 was determined in cells after 3, 6 and 12h by real time PCR. Alum + LPS v LPS only, \*\* p<0.01 \*\*\* p<0.001. (B) DC were incubated with LPS (5ng/ml) alone or LPS with Alhydrogel (Alum) at concentrations from 5-100μg/ml. The expression of IL-12p35 was determined in cells after 6h by real time PCR. Alum + LPS v LPS only \*\*p<0.01 All results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.

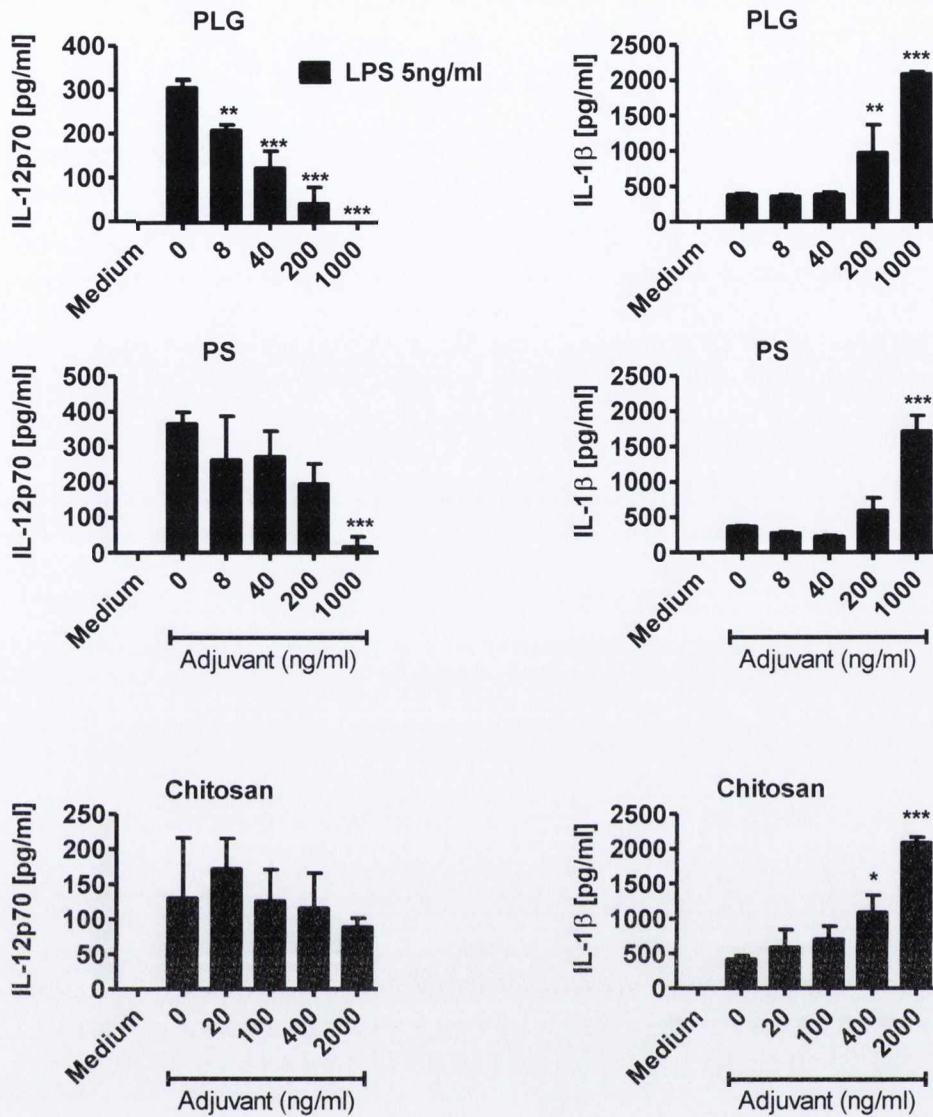






**Figure 5. 3. Aluminium-related adjuvants and the endogenous danger signal uric acid inhibit IL-12 production by dendritic cells.** DC from C57BL/6 mice were stimulated with LPS (5ng/ml) alone or together with Alhydrogel (0.5-40μg/ml), Adju-Phos (0.5-40μg/ml), Calcium Phosphate adjuvant (0.2-20μg/ml) or MSU crystals (2-200μg/ml). Concentrations of IL-12p70 and IL-1β were determined in 24h supernatants by ELISA. Adjuvant + LPS v LPS, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.

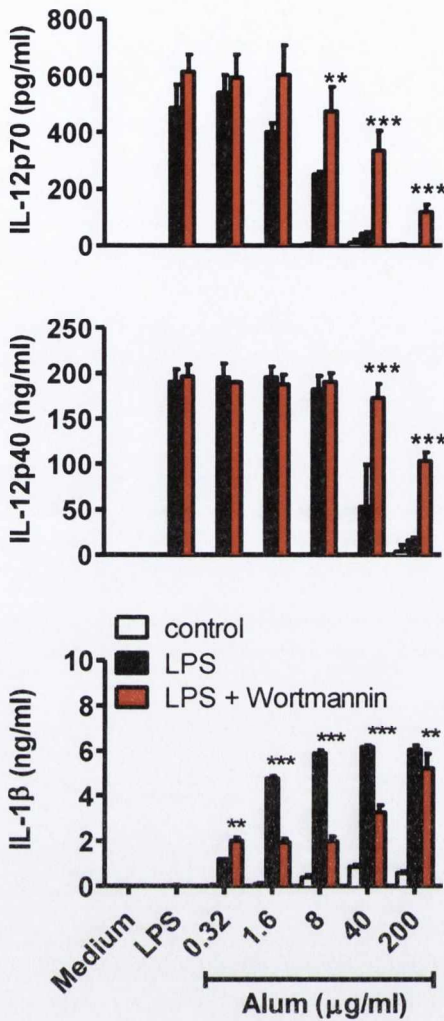




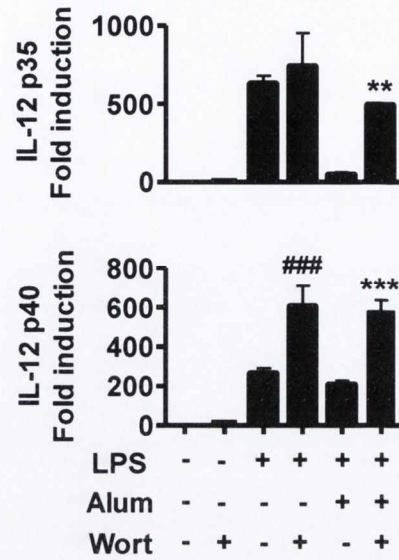
**Figure 5. 4. Particulate adjuvants with the exception of chitosan inhibit IL-12 production by dendritic cells.** DC from C57BL/6 mice were stimulated with LPS (5ng/ml) alone or together with PLG (8-1000μg/ml), PS (8-1000μg/ml) or chitosan (20-2000ng/ml). Concentrations of IL-12p70 and IL-1β were determined in 24h supernatants by ELISA. Adjuvant + LPS v LPS, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.



A.



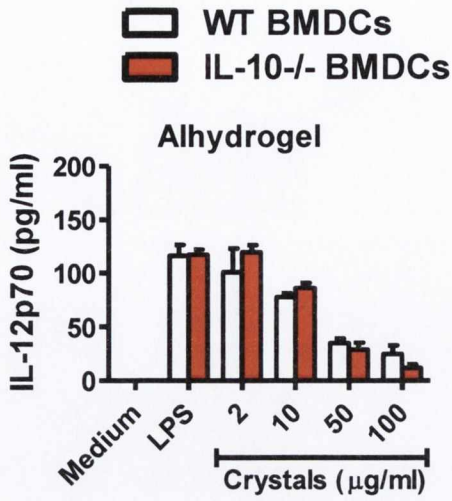
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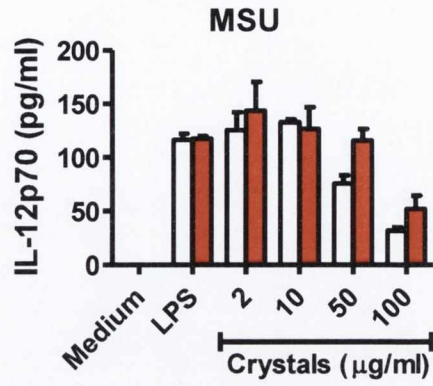
**Figure 5. 5. The inhibition of IL-12 by alum is reversed by inhibiting PI3 kinase.** (A) DC from C57BL/6 mice were incubated with medium, LPS (5ng/ml) alone or LPS with Alhydrogel at concentrations from 0.32-200μg/ml. In addition, cells were treated as above in the presence of the PI3 kinase inhibitor, Wortmannin (5μM). Supernatants were collected after 24h and IL-12p70, IL-12p40 and IL-1β concentrations were determined by ELISA. (B) DC were incubated with alum (8μg/ml), LPS (10ng/ml) or alum with LPS in the presence or absence of Wortmannin. IL-12p35 and IL-12p40 mRNA expression was determined 6h after treatment by real time PCR. Wortmannin + Alum + LPS v Alum + LPS, \*\*p<0.01, \*\*\*p<0.001. Wortmannin + LPS v LPS alone ###p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.



A.



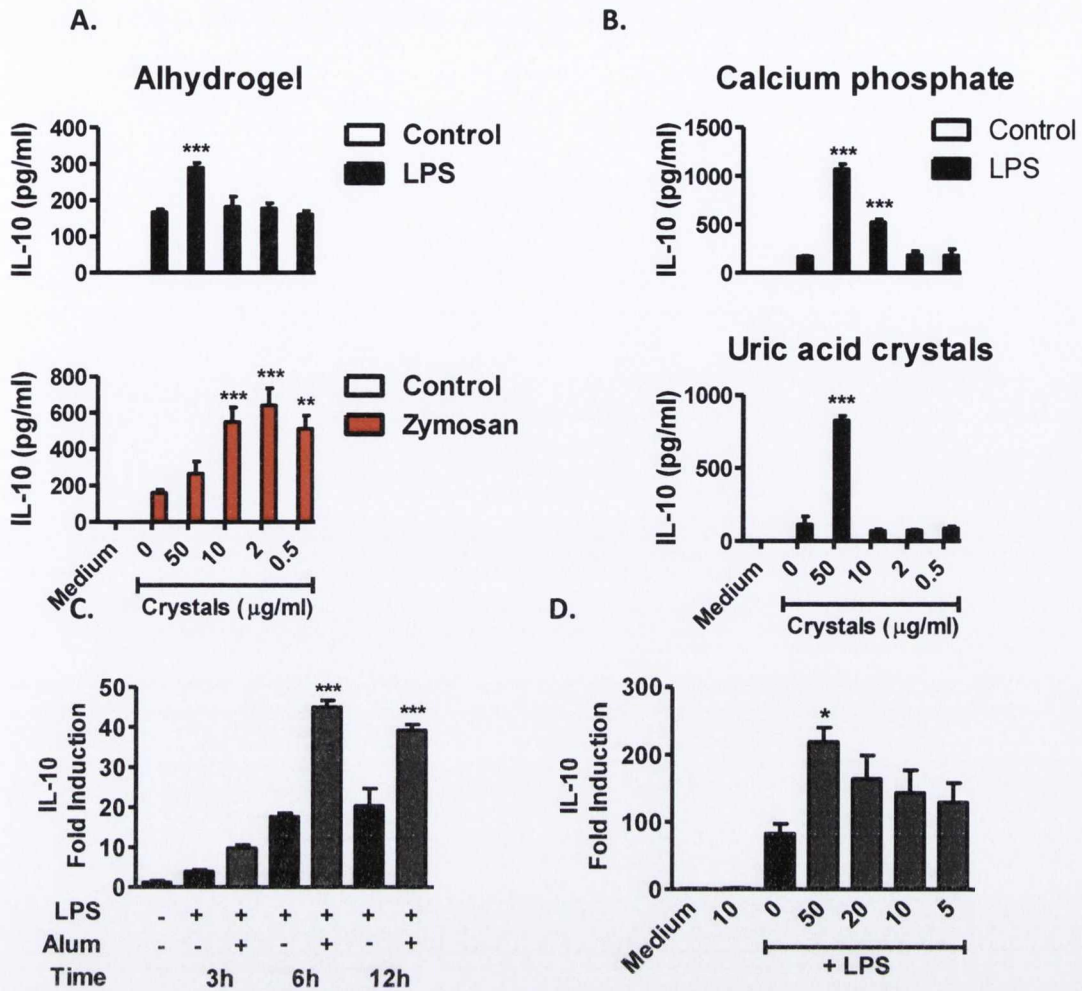
B.



**Figure 5. 6. The inhibition of IL-12p70 by alum and monosodium urate crystals is not IL-10-dependent.** DC from C57BL/6 and IL-10-deficient mice were incubated with medium, LPS (5ng/ml) alone or LPS with Alhydrogel or MSU crystals at concentrations from 2-100µg/ml. Supernatants were collected after 24h and IL-12p70 concentrations were determined by ELISA. Results are representative of two independent experiments. Error bars show means  $\pm$  SD for each experimental group tested individually in triplicate.

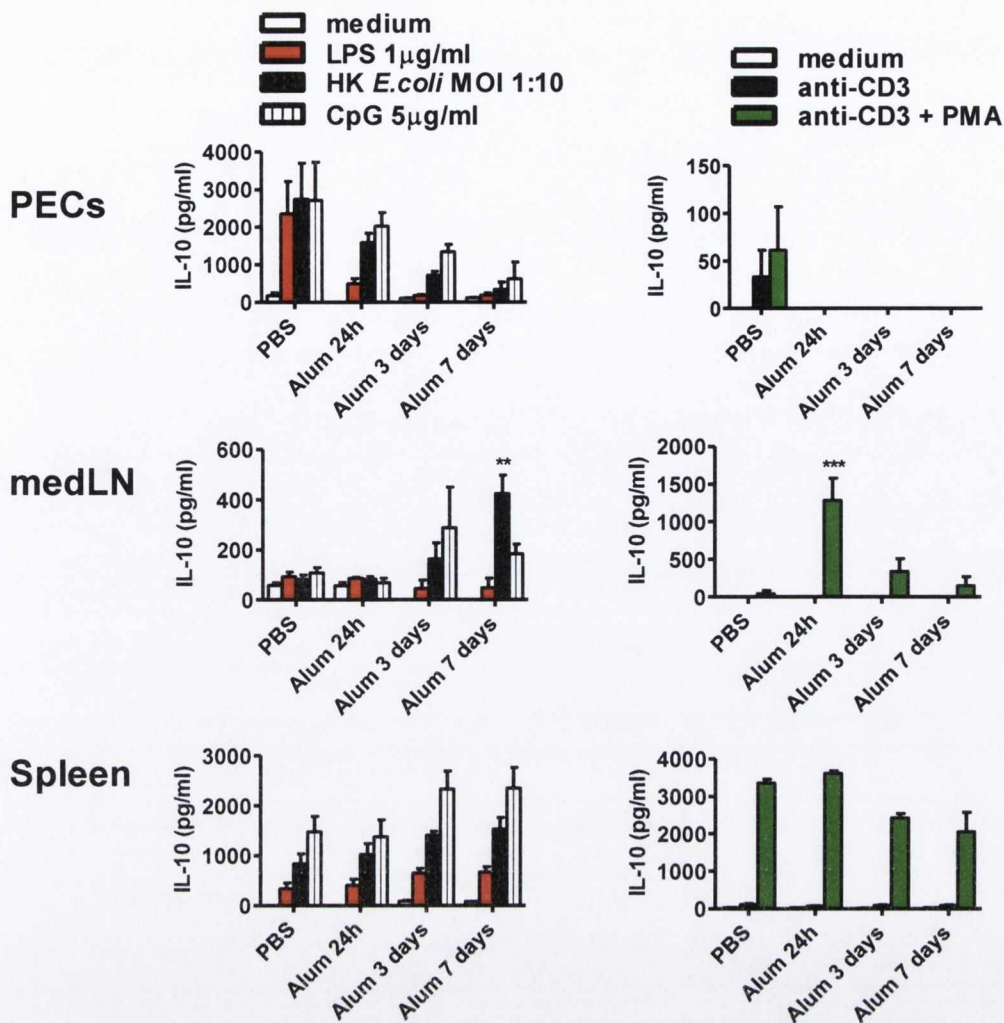






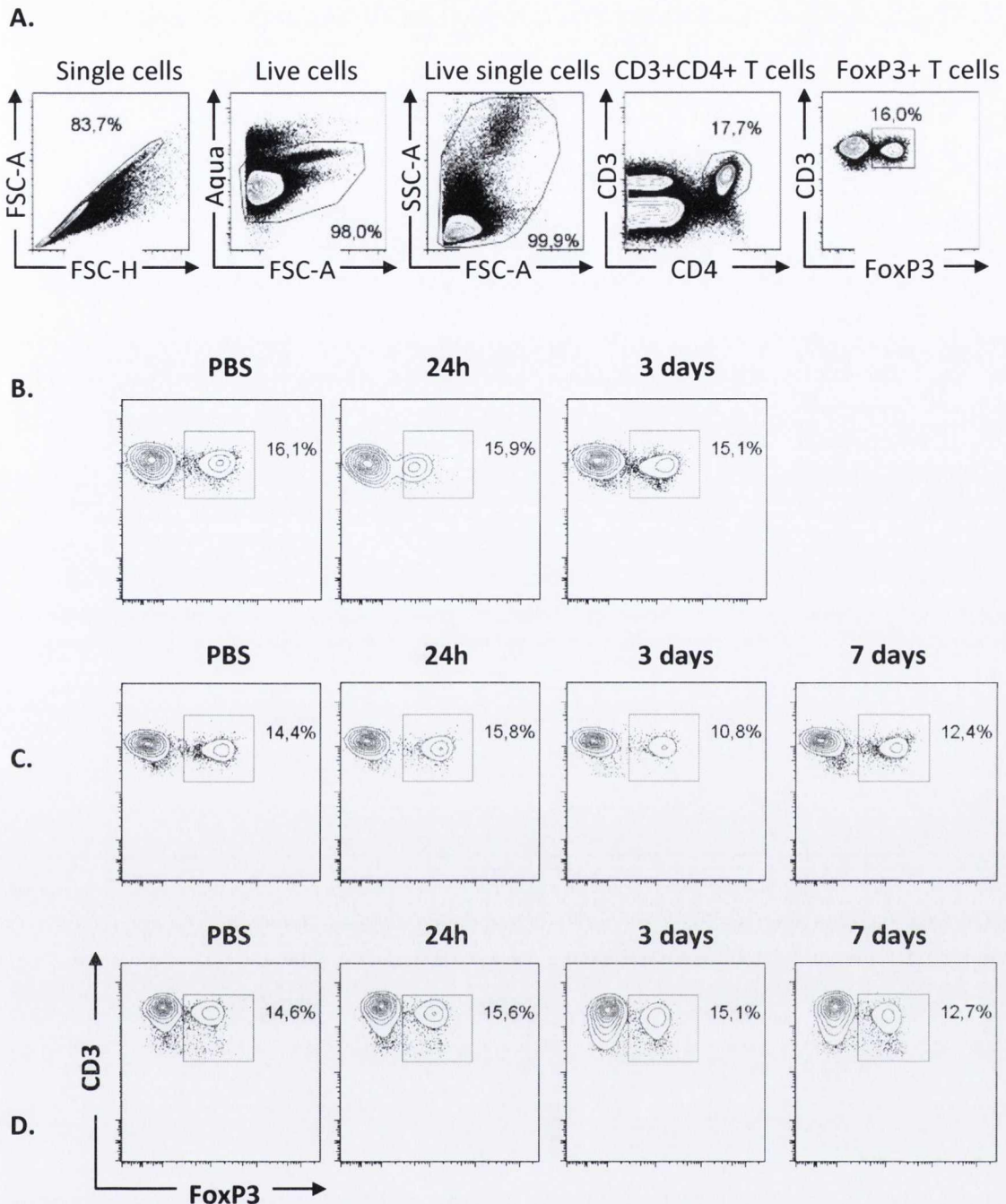
**Figure 5. 7. Alum, Calcium Phosphate and monosodium urate crystals promote IL-10 production by dendritic cells.** (A) DC from C57BL/6 mice were stimulated with LPS (5ng/ml) or Zymosan (1 $\mu$ g/ml) alone or together with Alhydrogel (0.5-50 $\mu$ g/ml) or (B) they were stimulated with LPS (5ng/ml) alone or together with Calcium Phosphate adjuvant (0.5-50 $\mu$ g/ml) or monosodium urate crystals (0.5-50 $\mu$ g/ml). Concentrations of IL-10 were determined in 24h supernatants by ELISA. (C) DC were incubated with LPS (5ng/ml) alone or together with alum (20 $\mu$ g/ml). The expression of IL-10 mRNA was determined in cells after 3, 6 and 12h by real time PCR. (D) DC were incubated with LPS (10ng/ml) alone or with alum at concentrations from 5-50 $\mu$ g/ml. The expression of IL-10 mRNA was determined in cells after 6h by real time PCR. Alum + LPS v LPS only \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001. All results are representative of two independent experiments. Error bars show means  $\pm$  SD for each experimental group tested individually in triplicate.





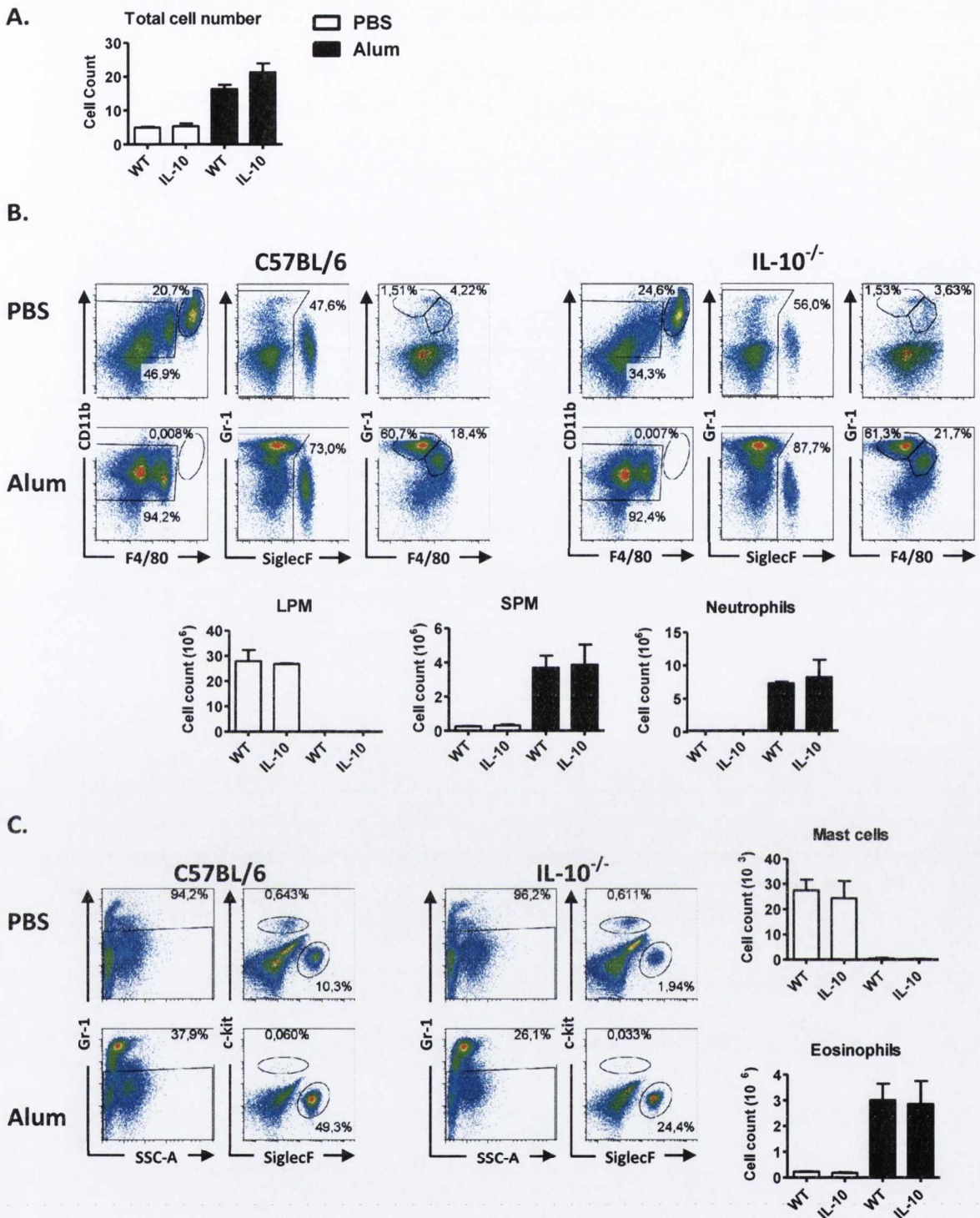
**Figure 5. 8. Injection of alum primes draining lymph node cells to secrete IL-10.** C57BL/6 mice were injected with PBS only or alum (1mg/mouse) intraperitoneally. Mice were sacrificed at 24 hours, 1, 3 and 7 days following injection. PerC, mediastinal lymph nodes and spleens were isolated. Isolated cells were stimulated with medium, LPS (1 $\mu$ g/ml), heat-killed *E. coli* (MOI 1:10) or CpG (5 $\mu$ g/ml) for 24h or anti-CD3 (200ng/ml) alone or anti-CD3 with PMA (25ng/ml) for 3 days. Supernatants were collected and analysed for IL-10 by ELISA. PBS v alum (24h, 1 day, 3 days or 7 days) \*\*  $p < 0.01$  \*\*\* $p < 0.001$ . Results are representative of two independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group tested in triplicate.





**Figure 5. 9. Alum does not promote expansion of FoxP3<sup>+</sup> regulatory T cells.** C57BL/6 mice were injected with PBS or alum (1mg/mouse) intraperitoneally. Mice were sacrificed 24 hours, 3 days or 7 days following injection. The peritoneal cavity was washed with PBS and mediastinal lymph nodes and splenocytes were removed. Isolated PerC, mediastinal lymph node cells and splenocytes were stained and analysed by flow cytometry for the presence of FoxP3<sup>+</sup> T cells. (A) Gating strategy. FoxP3<sup>+</sup>CD4<sup>+</sup> T cells in the peritoneum (B), mediastinal lymph nodes (C) and spleen (D). Results are representative of two independent experiments with for 3-4 mice per experimental group.

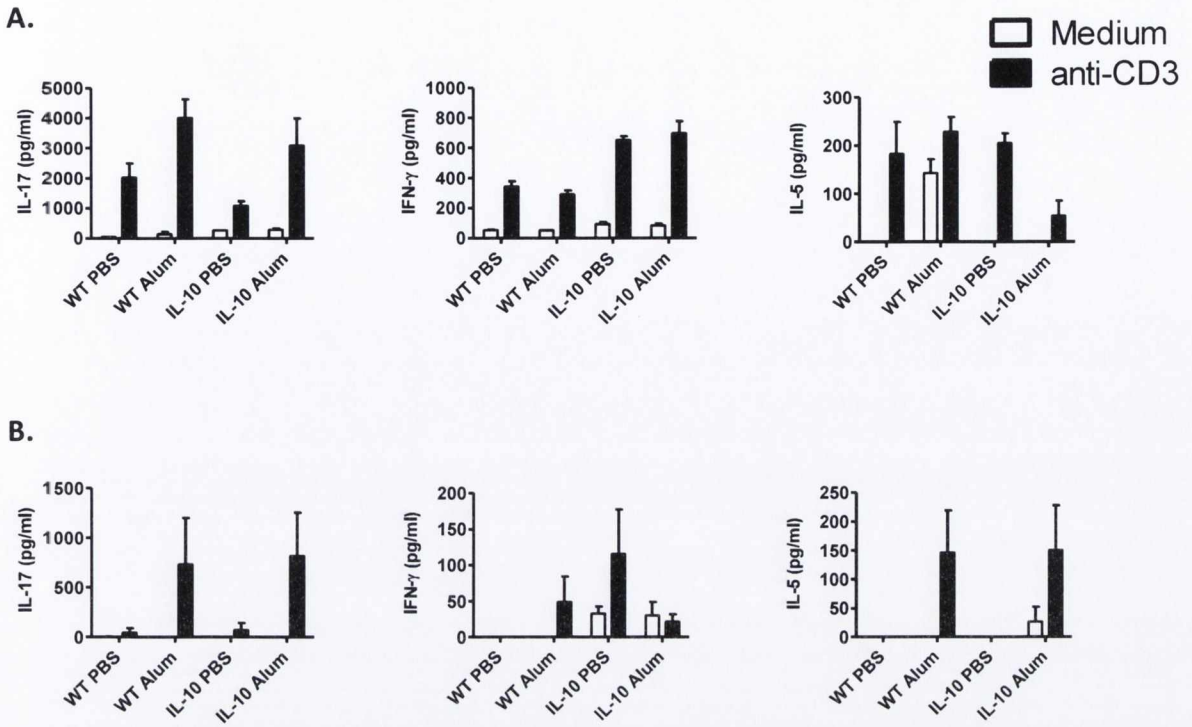




**Figure 5. 10. IL-10 does not compromise induction of innate immune response at the site of alum injection.** Mice (C57BL/6 and IL-10<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse) intraperitoneally. Mice were sacrificed 24 hours following injection and the peritoneal cavity was washed with PBS. Isolated PerC were counted (A) stained and analysed by flow cytometry for the presence of (B) macrophages, neutrophils, (C) eosinophils and mast cells. Results are representative of two independent experiments. Error bars show means  $\pm$  SEM for 3-4 mice per experimental group.

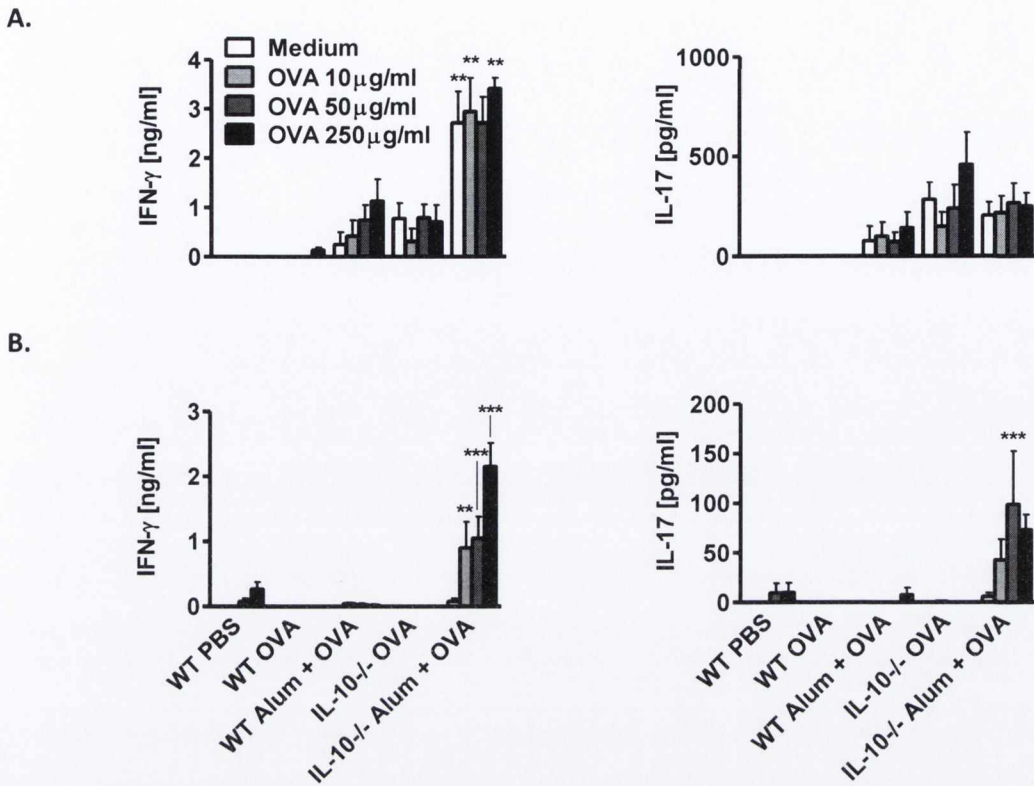






**Figure 5. 11. IL-10 deficiency does not modulate alum-induced cytokine production at the site of injection or in the draining lymph nodes.** Mice (C57BL/6 and IL-10<sup>-/-</sup>) were injected intraperitoneally with PBS or alum (1mg/mouse). Mice were sacrificed 24 hours following injection, the peritoneal cavity was washed with PBS and mediastinal lymph nodes were removed. Isolated cells were stimulated with medium or anti-CD3 (200μg/ml) for 72h. Collected supernatants were analysed for cytokine production by ELISA. (A) PerC. (B) Mediastinal lymph nodes. Results are representative of two experiments. Error bars show means ± SEM for 3-4 mice per experimental group tested in triplicate.

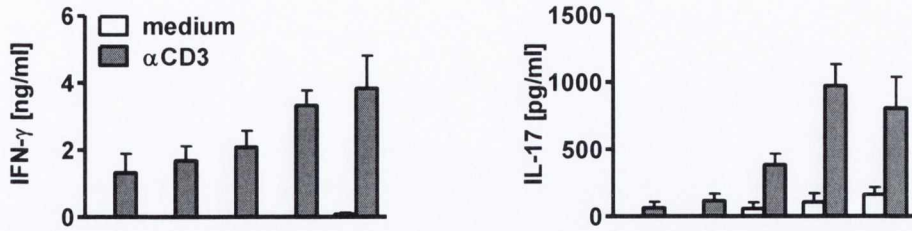




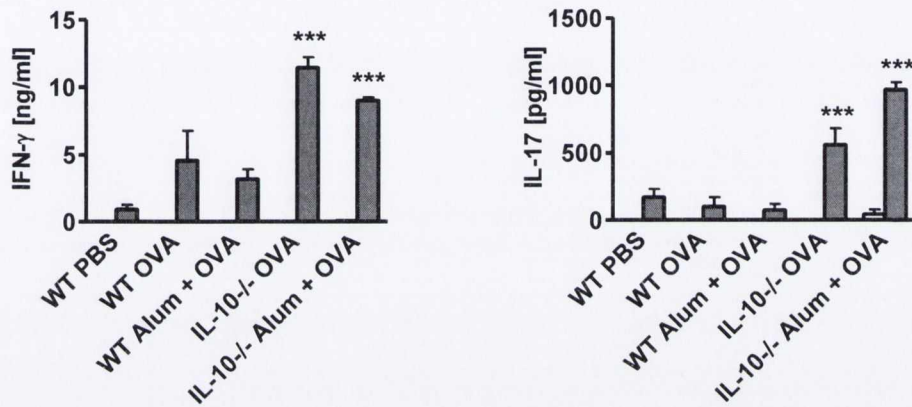
**Figure 5. 12. In the absence of IL-10, alum promotes OVA-specific splenic TH1 and TH17 responses.** Mice (C57BL/6 and IL-10<sup>-/-</sup>) were immunised intraperitoneally with PBS, OVA alone or OVA in combination with alum. After 7 days, mice were sacrificed and spleens and PerC were removed. Isolated cells were stimulated with OVA (10, 50 and 250 µg/ml). After 3 days, supernatants were collected and analysed for IFN-γ and IL-17 by ELISA. (A) PerC. (B) Spleens, Wild-type OVA or Alum + OVA (Medium, OVA 10, 50, 250µg/ml) v IL-10<sup>-/-</sup> OVA or Alum + OVA (Medium, OVA 10, 50, 250µg/ml) \*\*p<0.01, \*\*\*p<0.001 Results represent one experiment. Error bars show means ± SEM for 5 mice per experimental group.



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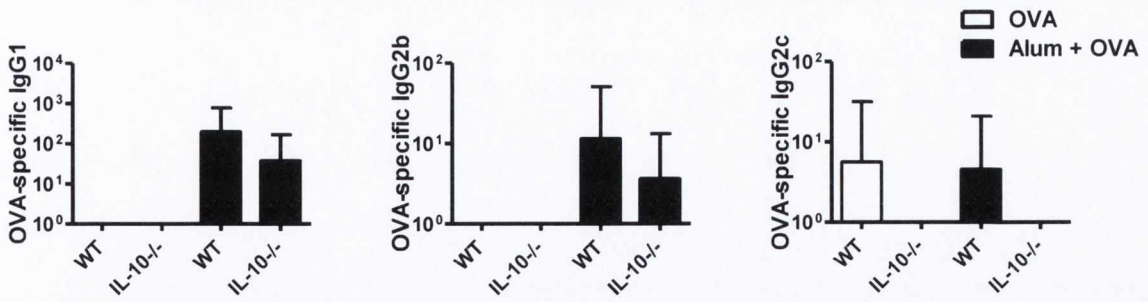


B.



**Figure 5. 13.** In the absence of IL-10, cells from peritoneal cavity and spleens are primed to secrete higher levels of IFN- $\gamma$  and IL-17 in response to anti-CD3. Mice (C57BL/6 and IL-10<sup>-/-</sup>) were immunised intraperitoneally with PBS, OVA alone or OVA in combination with alum. After 7 days, mice were sacrificed and PerC and spleens were removed. Isolated cells were stimulated with medium or anti-CD3 (0.2  $\mu$ g/ml). After 3 days, supernatants were removed and analysed for IFN- $\gamma$  and IL-17 by ELISA. (A) PerC. (B) Spleens, Wild-type OVA or Alum + OVA (Medium, anti-CD3) v IL-10<sup>-/-</sup> OVA or Alum + OVA (Medium, anti-CD3) \*\*\*p<0.001 Results represent one experiment. Error bars show means  $\pm$  SEM for 5 mice per experimental group.



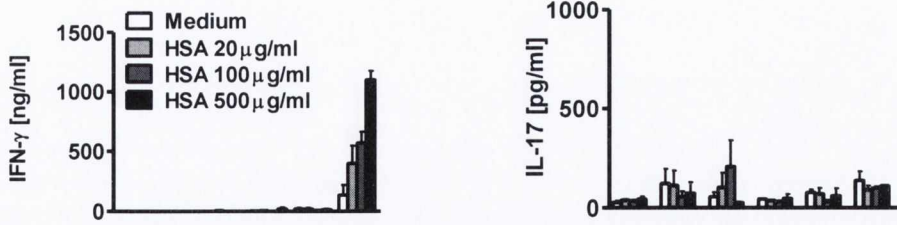


**Figure 5. 14. IL-10 does not inhibit antibody production induced during alum immunisation.** Mice (C57BL/6 and IL-10<sup>-/-</sup>) were immunised intraperitoneally with PBS, OVA alone or OVA in combination with alum. After 7 days blood was collected. Blood samples were centrifuged and serum was removed. The serum samples were analysed for IgG1, IgG2b and IgG2c by ELISA. Antibody responses are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal serum levels to a cut-off point of 2 standard deviations above sera from PBS injected mice. Results represent one experiment. Error bars show means  $\pm$  SEM for 5 mice per experimental group.

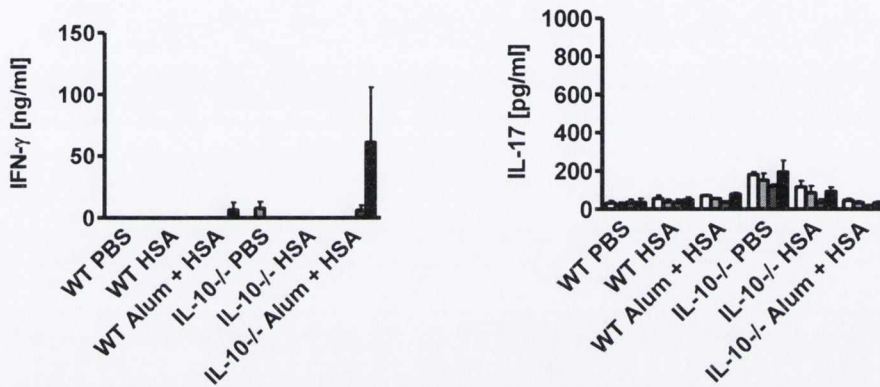




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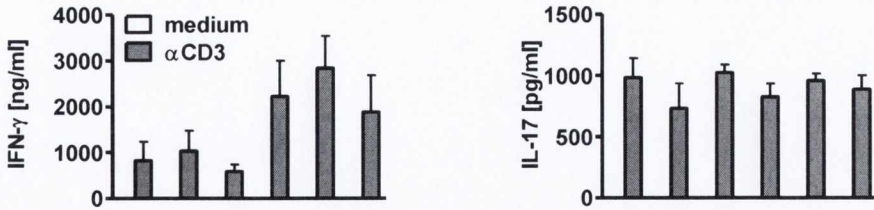
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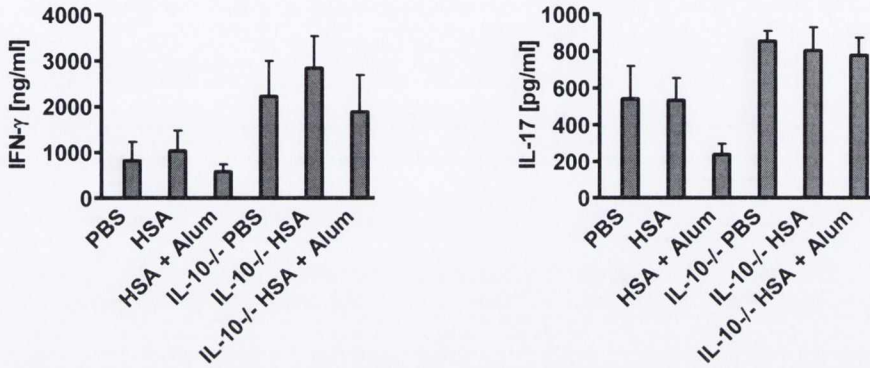
**Fig 5. 15. In the absence of IL-10, alum promotes antigen-specific splenic TH1 responses.** Mice (C57BL/6 and IL-10<sup>-/-</sup>) were immunised intraperitoneally with PBS, HSA alone or HSA in combination with alum. After 14 days mice were boosted with the same treatments. Mice were sacrificed 7 days post boost and spleens and PerC were removed. Isolated cells were stimulated with HSA (20, 100 and 500 μg/ml). After 3 days, supernatants were collected and analysed for IFN-γ and IL-17 by ELISA. (A) PerC. (B) Spleens. Results are representative of two experiments. Error bars show means ± SEM for 5 mice per experimental group.



A.

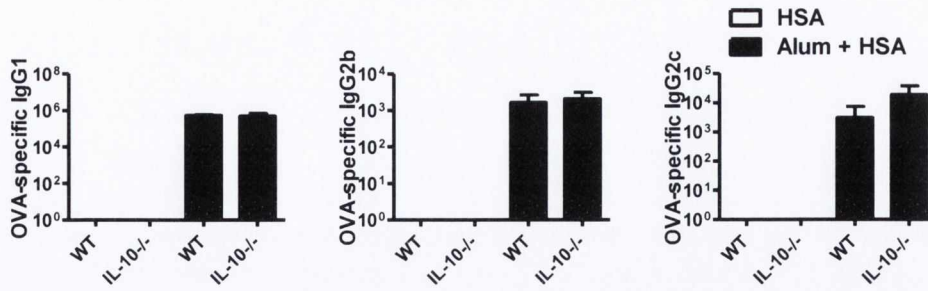


B.



**Figure 5. 16. In the absence of IL-10, PerC and splenocytes from alum-injected mice are not primed to secrete increased levels of IFN- $\gamma$  and IL-17 in response to anti-CD3.** Mice (C57BL/6 and IL-10<sup>-/-</sup>) were immunised intraperitoneally with PBS, HSA alone or HSA in combination with alum. After 14 days, mice were boosted with the same treatments. Mice were sacrificed 7 days post boost and spleens and PerC were removed. Isolated cells were stimulated with medium or anti-CD3 (0.2  $\mu$ g/ml). After 3 days, supernatants were removed and analysed for IFN- $\gamma$  and IL-17 by ELISA. (A) PerC. (B) Spleens. Results are representative of two experiments. Error bars show means  $\pm$  SEM for 5 mice per experimental group.





**Figure 5. 17. IL-10 does not regulate alum-driven IgG responses.** Mice (C57BL/6 and IL-10<sup>-/-</sup>) were immunised intraperitoneally with PBS, HSA alone or HSA in combination with alum. After 14 days, mice were boosted with the same treatments and 7 days post boost mice were sacrificed and blood was collected. Blood samples were centrifuged and serum was removed. The serum samples were analysed for IgG1, IgG2b and IgG2c by ELISA. Antibody responses are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal serum levels to a cut-off point of 2 standard deviations above sera from PBS injected mice. Results are representative of two experiments. Error bars show means  $\pm$  SEM for 5 mice per experimental group.



## **CHAPTER 6**

### **GENERAL DISCUSSION**





## 6. General discussion

The history of adjuvants started in the 1920s when it was demonstrated that the co-administration of various foreign materials could enhance antitoxin responses to tetanus or diphtheria toxoids. For instance, in 1925, Ramon used some quite bizarre materials, such as agar, saponins, starch oil or breadcrumbs and observed improved antitoxin responses (196). In 1926, Glenny demonstrated that tetanus toxoid precipitated with potassium aluminium sulfate induced stronger antibody responses than toxoid alone (219). This empiric discovery led to the development of aluminium adjuvants which have been applied in many vaccine formulations where neutralising antibodies are required for protection (198). However at that time and for many subsequent years, there was little knowledge available about vaccine immunology and the mechanisms which underlie protective immunity against infections. Therefore, it is not surprising that in 1989 Charles Janeway called aluminium adjuvants “immunologist’s dirty little secret” (308), as alum was successfully used in human vaccination, but the mechanism of its action was poorly understood. Currently, there is increasing interest and knowledge about alum-induced immunity, but it is still not fully understood how it provides protection. Aluminium adjuvants drive a potent humoral immune response and are widely used in vaccines including those against tetanus, pertussis, diphtheria, HBV and *Haemophilus influenzae* type b. However, alum is a poor inducer of cellular immune responses and therefore, it is unsuitable for vaccines against intracellular pathogens, such as *Mycobacterium tuberculosis* or HIV.

Alum forms a depot at the site of injection (219, 229) and it was thought that this depot was essential to drive sustained adaptive immune responses by facilitating slow release of the antigen. However, it is acknowledged now that the adjuvanticity of alum is more complex. The induction of adaptive immunity depends qualitatively and quantitatively on the early innate immune responses triggered by vaccines. In recent years there have been many interesting reports describing early events following alum injection. It has been demonstrated that alum induces cell death at the site of injection and this leads to the release of the damage-associated molecular patterns, uric acid and host DNA (237, 250). Furthermore, alum recognition promotes chemokine and cytokine release at the site of injection, which induces cell

recruitment, including neutrophils, inflammatory monocytes, eosinophils and Gr-1<sup>+</sup> IL-4-producing cells (237, 241, 249). However, it still remains unknown which cells are the “sensors” of alum. It has been demonstrated that resident macrophages and mast cells can sense alum after intraperitoneal injection and promote innate immune responses, but their depletion does not abrogate alum adjuvanticity (241). As alum is recognised by dendritic cells in a receptor-independent manner (232), it is therefore possible that any cell can sense alum by this mechanism and drive immune responses.

The major part of this thesis was dedicated to understanding the mechanism of alum-induced cell death and how it can drive innate responses. It has also been hypothesised that alum can potentially induce immunoregulatory mechanisms, which might inhibit cellular immunity during vaccination.

Importantly, this study confirmed that alum is a potent inducer of cell death. Alum promotes cell death in all types of cells tested, dendritic cells, macrophages and the muscle cell line C2C12. The mechanism of alum-induced cell death was studied in detail in dendritic cells, as these cells are responsible for inducing adaptive immune responses. Interestingly, alum does not promote either non-inflammatory, apoptosis or inflammatory pyroptosis or pyronecrosis. However, it potentially induces a form of necrosis, which can be inhibited by the autophagy inhibitor, 3-methyladenine. While this is not sufficient proof that alum promotes autophagic cell death (289), it is an interesting concept worth further detailed studies. It has been demonstrated before that alum induces lysosome disruption (240, 285), thus, it seems likely that dendritic cells overloaded with alum will try to eliminate it from the cytoplasm by autophagy. The excessive amount of alum particles inside the cell might result in massive autophagosome formation and subsequent cell death. However, this has to be confirmed experimentally. Furthermore, alum is not only toxic *in vitro*, but also induces quick and massive cell death in the peritoneal cavity following injection. The mechanism of alum-induced cell death *in vivo* has not been studied, but the incorporation of PI and Aqua LIVE/DEAD stain by peritoneal cells demonstrate that they lose their cell integrity, suggesting necrosis. It remains unknown whether it can be inhibited by autophagy inhibitor 3-MA similarly to the *in vitro* findings.

Interestingly, host DNA release has been attributed to alum-driven cell death at the site of injection (250). However, this study demonstrates that cell death occurs

shortly after alum injection, while DNA accumulates later and reaches significant levels around 24h post injection. This DNA accumulation correlates with neutrophil and inflammatory monocyte recruitment rather than cell death. It seems likely that it is an active process, for instance, the result of the release of extracellular traps (ETs) by recruited cells. Indeed it has been demonstrated that after alum injection, CD11b<sup>+</sup> cells, release ETs (229). Furthermore, this study established that mice deficient in IL-1RI or caspase-1/11 exhibit lower extracellular DNA content in the peritoneal cavity after alum injection, which might correlate with lower numbers of infiltrating neutrophils and inflammatory monocytes in these mice. Interestingly, the DNA concentrations are significantly reduced, but not abrogated, which suggests that cells other than neutrophils and monocytes can also release DNA.

A major finding from this work is that alum induces IL-33 release at the site of injection. IL-33 is nuclear protein which has been suggested to be released during necrosis (138). Indeed, this study shows that IL-33 release coincides closely with alum-induced cell death, suggesting passive release rather than secretion. Interestingly, IL-33 drives eosinophil recruitment and the accumulation of IL-4-producing cells in the peritoneal cavity. Importantly, it has been proposed that these cells are responsible for suppressing TH1-related immune responses during alum immunisation, including antigen-specific IgG2c antibody production (249). Therefore, this study establishes a new alum-driven DAMP, IL-33, which is crucial in polarising immune responses.

Apart from IL-33, alum promotes the release of many other chemokines and cytokines, including IL-1 $\beta$ . It has been demonstrated that IL-1 is responsible for the neutrophil influx after alum injection, but the specific contributions of IL-1 $\alpha$  and IL-1 $\beta$  has not been explored (240). Here, it is demonstrated that both IL-1 $\alpha$  and IL-1 $\beta$  are responsible for accumulation of neutrophils after alum injection. Interestingly, IL-1 $\alpha$  cannot be detected at the site of alum injection. It might be the case that it is quickly sequestered by IL-1RI-expressing cells following its release. Furthermore, IL-1 $\beta$  processing seems to be differently regulated *in vitro* and *in vivo*. *In vitro*, IL-1 $\beta$  secretion is dependent on NLRP3 inflammasome formation, whereas its release is intact following alum injection in mice deficient in the inflammasome components NLRP3, ASC and caspase-1. Importantly, the current results suggest that the IL-1 $\beta$

secreted *in vivo* is fully bioactive, as alum-induced IL-1 $\beta$  contributes to neutrophil infiltration. Overall, this suggests a role for alternative pathways for IL-1 $\beta$  processing and release. Interestingly, the data in Chapter 3 indicates that caspase-1 or caspase-11 independently of IL-1 is responsible for inflammatory monocyte recruitment into the site of alum injection.

Overall, this report proposes three distinct pathways which control cell recruitment to the injection site following alum injection. Importantly, it will be crucial to study whether IL-1, IL-33 or caspase-1/11 also mediate cell influx into the draining lymph nodes, especially inflammatory monocytes and dendritic cells, which have been shown to have a crucial impact on shaping adaptive immune responses to alum (237).

Dendritic cells are the key player in the adaptive immune responses, because they polarise naïve T cells into specific effector T subsets. It has been demonstrated before that dendritic cells recognise alum by receptor-independent sensing and alum promotes lipid raft formation, which induces PI3 kinase-Syk signalling pathways (232). Interestingly, while it has been suggested that alum can be sensed but is not phagocytosed by DCs (232), here it has been demonstrated that dendritic cells can in fact efficiently phagocytose alum. However, it seems likely that PI3 kinase activation occurs during alum recognition, as it is demonstrated in this study that alum inhibits IL-12p70 production by dendritic cells via PI3 kinase. Furthermore, the current study shows that in parallel with a profound acute inflammatory responses, alum exerts anti-inflammatory properties, promoting IL-10 secretion by dendritic cells and also by unknown cellular sources after immunisation. These results indicate that alum can potentially inhibit pro-inflammatory responses during dendritic cell priming and induce anti-inflammatory responses after immunisation.

The ultimate question asked during this study was whether components of the alum-driven innate immune responses described in this thesis would have any impact on adaptive immunity. It seems likely that some effects at the site of injection might be dispensable for alum-driven adaptive immune responses. For instance, the lack of IL-1 does not impair antigen-specific IgG1 or IgE antibody production, but it decreased neutrophil infiltration and DNA concentration at the site of alum injection. On the other hand, this study demonstrates that lack of the IL-33 receptor ST2 had a profound effect on the IgG antibody switch. While the level of antigen-specific IgG1

was not changed, there was an increase in the titres of antigen-specific IgG2b and IgG2c. This effect correlates with the reduced number of IL-4 producing cells, including eosinophils, at the alum injection site. Furthermore, it has been established that peritoneal cells and splenocytes isolated from alum-injected, IL-10-deficient mice secrete increased antigen-specific IFN- $\gamma$ . This indicates that alum-induced IL-10 *in vivo* might block pro-inflammatory adaptive immune responses. While the data obtained with model antigens, either human or chicken albumin, is very informative, it would be interesting to study adaptive immunity using alum co-administered with relevant pathogen-derived candidate vaccine antigens and assess protective immunity in a model when cellular responses are essential.

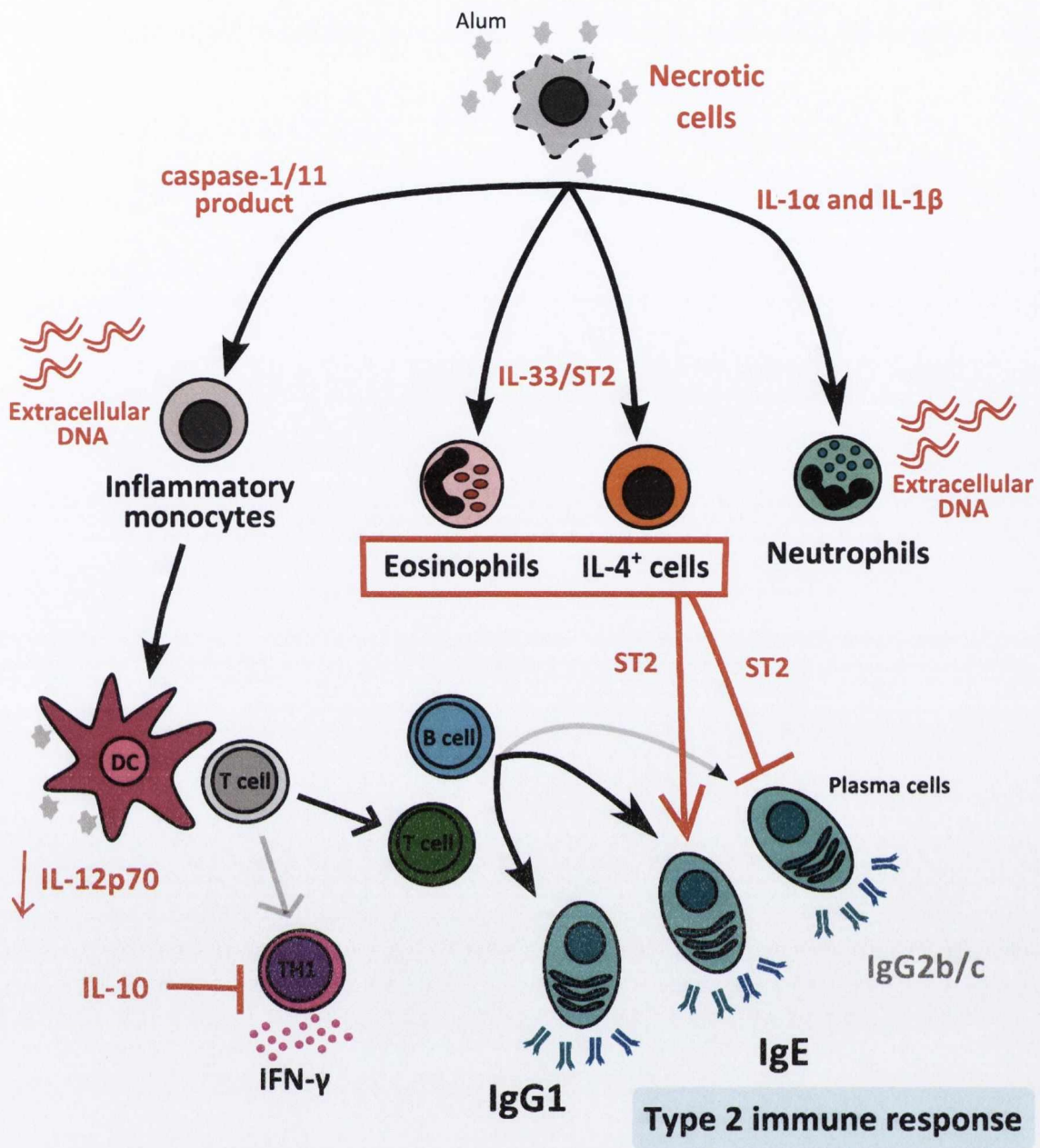
Alum induces humoral, type 2 immune responses, which partly resemble immune responses towards helminth parasite infection. Is it therefore possible that alum is sensed in a comparable manner to these parasites? It has been proposed that infection with helminth parasites and other metazoan parasites led to the evolution of type 2 immune responses (309) and specifically that they evolved from existing wound healing mechanisms to deal with parasite infections (310). It is fascinating to consider that alum might also trigger mechanisms similar to wound repair.

Alum and helminth parasite recognition and subsequent innate responses have common features. Parasites induce physical damage during entrance and migration through the body of the host (309). Moreover, it has been demonstrated that some helminths induce IL-33 release which is important for worm expulsion (144, 283, 284, 294). It has been also observed that *Schistosoma mansoni* eggs also induce the appearance of IL-4 producing cells (249). While alum is an inert material, it induces massive cell death at the site of injection, which leads to IL-33 release. Alum also strongly promotes eosinophil and IL-4-producing cell infiltration into the site of injection. Furthermore, wound healing also depends on IL-4 and eosinophils. Eosinophils can mediate tissue remodelling, myofibroblast differentiation and overall wound healing (311). Furthermore, it has been demonstrated that IL-4 producing eosinophils are crucial in tissue healing. For instance, in toxin-induced muscle or liver damage, IL-4 produced by eosinophils is critical for tissue regeneration (312, 313). Overall, it seems likely that while parasites and alum can be recognised differently,

they may induce similar type 2 immune responses which are crucial for maintaining tissue homeostasis.

Furthermore, wound healing requires reconstruction of the injured tissue and also suppression of pro-inflammatory responses (314). While suppression of pro-inflammatory responses is well documented for helminth parasites (310), there is little knowledge of alum-induced immunoregulatory properties. However, in this study it is demonstrated that alum can suppress IL-12p70 production and also promote IL-10 secretion. While alum can directly inhibit IL-12p70 secretion in DCs, the cellular source of IL-10 is unknown. Therefore it is possible that IL-10 production after alum injection might be an indirect result of massive damage at the site of injection and the induction of wound healing mechanisms.

Overall, this thesis explored the immunomodulatory properties of alum and their role in shaping innate and adaptive immunity during alum immunisation (Figure 6. 1). It has been demonstrated that early events following alum injection can promote polarisation of immune responses, which have an impact on the poor efficacy of alum in inducing cellular immunity.



**Figure 6. 1. A proposed model for how alum promotes innate and adaptive immune responses.** Alum induces cell death locally which promotes the release of chemokines and cytokines, including IL-1 $\alpha$ , IL-1 $\beta$  and IL-33. These cytokines recruit neutrophils, eosinophils and IL-4-producing cells. Additionally, an unknown product of caspase-1/11 cleavage promotes accumulation of inflammatory monocytes into the site of injection. Activated inflammatory monocytes give rise to DCs. In the draining lymph nodes DCs present antigen and activate antigen-specific CD4<sup>+</sup> T cells. Primed T cells activate antigen-specific B cells which give rise to plasma cells secreting IgG1, IgE and low titres of IgG2b and IgG2c. IL-4-producing cells, including eosinophils, suppress TH-1 related IgG2b and IgG2c antibody production and enhance IgE antibody production. Alum inhibits IL-12p70 production, but promotes IL-10 which also inhibits cellular responses after vaccination. Neutrophils and extracellular DNA accumulate at the site of alum injection, but their role in adaptive immune responses is not fully understood.





## **CHAPTER 7**

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## **APPENDIX**