The role of natural killer cells in resolution of antigen-induced inflammation

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Thesis for the degree of Master of Science
University of Iceland
School of Health Sciences
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Ágrip

Hjöðnun bólgu er mikilvæg til að koma í veg fyrir að bólgan verði krónísk. Náttúrulegar drápsfrumur (NK frumur) gegna hlutverki í fyrstu vörnum gegn sýkingu. Markmið þessarar rannsóknar var að ákvarða hlutverk NK frumna í hjöðnun mótefnavaka miðlaðrar bólgu. Mýs voru sprautaðar í æð með mótefni gegn NK frumum (NK frumu euddur hópur) eða með viðmiðunarmótefni (viðmiðunarhópur). Áður en NK frumum var eytt, voru mýsnar bólusettar í tvígang undir húð með metýleruðu BSA (mBSA) og 24 klst eftir eyðingu NK frumna var lífhimnubólga framkölluð með því að sprauta mBSA í kviðarhol þeirra. Fyrir og á nokkrum tímapunktum eftir að lífhimnubólgu var komið af stað var kviðarholsvökva og frumum safnað, frumur taldar og tjáning yfirborðssameinda ákvörðuð með frumuflæðisjá. Styrkur frumuboða og leysanlegra viðtaka var ákvarðaður með ELISA aðferð. Mótefni gegn NK frumum fækkaði fjölda þeirra í kviðarholi um 50% 36 klst eftir að því var sprautað í æð (12 klst eftir framköllun bólgu). Flestar NK frumurnar voru þroskaðar, $CD11b^{+}CD27^{+}$ frumur. Fjöldi óþroskaðra $CD11b^{-}CD27^{+}$ NK frumna jókst eftir framköllun bólgu í viðmiðunarhópnum en ekki í NK frumu eydda hópnum. Enginn munur var á tjáningu þeirra NK frumu viðtaka sem mældir voru í hópunum tveimur. Í viðmiðunarhópnum hafði daufkyrningum fjölgað 6 tímum eftir framköllun bólgu en fækkað aftur og náð upphaflegum fjölda 48 klst eftir framköllun bólgu. Í NK frumu eydda hópnum náði fjöldi daufkyrninga hámarki 12 klst eftir framköllun bólgu og voru þeim þá tvöfalt fleiri en í vipmiðunarhópnum. Fjöldi daufkyrninga hélst hár í NK frumu eydda hópum meðan á rannsókninni stóð. Eyðing NK frumna hafði lítil áhrif á aðrar frumur en daufkyrninga en jók styrk og/eða framlengdi hækkuðum styrk IL-6, IL-12p40, G-CSF og IL-1ra. Þessar niðurstöður benda til þess að NK frumur séu mikilvægar fyrir bólguferlið og nauðsynlegar fyrir hjöðnun bólgu.

Abstract

Resolution of inflammation is important in preventing chronic inflammation. Natural killer (NK) cells are known to act as the first line of defence during an infection. This study examined the role of NK cells in the resolution of antigen-induced inflammation. Mice were injected intravenously with the NK cell depleting antibody, anti-asialo GM1, or a control antibody. Prior to NK cells depletion, the mice were immunized twice subcutaneously with methylated BSA (mBSA) and 24 hours after depletion, peritonitis was induced by injecting mBSA into their peritoneum. Prior to and at several time-points following peritonitis induction, peritoneal exudates were collected, cells counted and expression of surface molecules determined by flow cytometry. Concentrations of cytokines, soluble cytokine receptors and growth factors were determined by ELISA. The NK cell depletion antibody decreased the number of NK cells in the peritoneum by 50% 36 hours after injection of the antibody (12 h after induction of inflammation). Most of the NK cells were mature, $CD11b^{+}CD27^{+}$ cells. Immature $CD11b^{-}CD27^{+}$ NK cells increased after induction of inflammation in the control group but not in the depleted group. The NK cell depletion did not affect the expression of NK cell receptors. In the control group, neutrophils increased in numbers at 6 h and reached basal levels 48 h after induction of inflammation, whereas in the depleted group the neutrophils peaked at 12 h, and were at that time-point three time higher in number than that in the control group. In addition, in the depleted group the neutrophil numbers remained high throughout the study. NK cell depletion had little effect on the number or receptor expression of other cell types. The NK cell depletion increased the concentrations and /or prolonged higher levels of IL-6, IL-12p40, G-CSF and IL-1ra. These results demonstrate that NK cells affect the inflammation process and are necessary for resolution of antigen-induced inflammation.

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List of abbreviations

BCR – B cell receptor

cDC – Convention/classical dendritic cell

CLR – C-type lectin receptor

DAMP – Damage-associated molecular pattern

DC – Dendritic cell

DHA – Docosahexaenoic acid

EPA – Eicosapentaenoic acid

FSS – Forward scatter

G-CSF – Granulocyte-colony stimulating factor

GM-CSF – Granulocyte macrophage-colony stimulating factor

h – Hour

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

i.p. – Intraperitoneal

i.v. – Intravenous

KIR – Killer cell Ig-like receptor

LOX – Lipoxygenase

LT – Leukotriene

LX – Lipoxin

mBSA – methylated bovine serum albumin

MFI – Mean fluorescence intensity

NCR – Natural cytotoxicity receptor

NK – Natural killer

NLR – NOD-like receptor

NOD – Nucleotide-binding oligomerization domain

OVA – Ovalbumin

PAMP – Pathogen-associated molecular pattern

PD – Protectin

pDC – Plasmacytoid dendritic cell

PG – Prostaglandins

PMN – Polymorphonuclear

PRR – Pattern recognition receptor

RIG – Retinoic acid-inducible gene

RLR – RIG-I-like receptor

rM – Resolution macrophage

Rv – Resolvin

SEM – Standard error of mean

SSC – Side scatter

TCR – T cell receptor

TGF – Transforming growth factor

Th – T helper

TLR – Toll-like receptor

TNF – Tumour necrosis factor

TM – Thioglycolate medium

VEGF – Vascular endothelial growth factor

1 Introduction

1.1 The immune system

The immune system is made up of many cells and molecules that work together to protect the body from infections and harmful substances. The way the immune system responds depends on the type of infection or substance the body is being exposed to. The immune system is divided into two parts; the innate immune response and the adaptive immune response.

The first line of defence against infections is the physical and chemical barriers that prevent microbes from entering the body. When these barriers are evaded or breached the immune system comes into play. The innate immune system acts rapidly once it encounters the infection. Overlapping with the innate immune system is the adaptive immune system which takes days to develop and is capable of eliminating infections more efficiently than the innate immune system.

For the immune system to protect an individual it has to fulfil four main tasks. It has to recognise the presence of the infection, contain or eliminate the infection, regulate the immune response and finally protect the individual against recurring disease from the same pathogen or infection. Immunological recognition is carried out by both white blood cells of the innate immune system and lymphocytes of the adaptive immune system. For containing or elimination of infection, immune effector functions are carried out by the complement system of blood proteins, antibodies and lymphocytes and other white blood cells. Immune regulation is one of the most important tasks of the immune system and failure to self-regulate can lead to conditions such as allergies and autoimmune diseases. Immunological memory is the main task of the adaptive immune system that serves to protect against recurring diseases. It brings about an immediate and strong response to any recurring pathogens, infections or diseases (1).

1.2 The innate immune system

The innate immune system is the first to respond to pathogens and infection. However, it does not cause lasting immunity and is not specific to any specific pathogens.

The cells of the innate immune system include macrophages, granulocytes, mast cells, dendritic cells (DCs) and natural killer (NK) cells. They are derived from hematopoietic stem cells of the bone marrow. The hematopoietic stem cells give rise to the two main categories of the white blood cells, i.e. the lymphoid and the myeloid lineages. Cells of the myeloid lineages are precursors of the cells of the innate immune system, while cells of the lymphoid lineages are precursors of the cells of the adaptive system and will be discussed later.

Pathogens are first met by macrophages residing in the tissues. Receptors on macrophages, neutrophils and DCs recognise pathogen-associated molecular patterns (PAMPs) that are present on microorganisms. These receptors are known as pattern recognition receptors (PRRs) and they recognise structures like mannose-rich oligosaccharides, peptidoglycans and lipopolysaccharides in bacterial cell wall, and unmethylated CpG DNA. PAMPs are common to pathogens and as such the innate immune system is able to distinguish self from non-self. Binding of PPRs to PAMPs triggers the macrophages to engulf the bacterium and degrade it internally. It also triggers secretion of cytokines

and chemokines that in turn signal other innate cells such as the neutrophils and monocytes, out of the blood stream and into the infected area. This initiates the process of the inflammatory response (1).

1.2.1 Macrophages

Macrophages are resident phagocytic cells and are the mature form of monocytes, which circulate in the blood and migrate into tissues where they differentiate into macrophages. Macrophages are dynamic and heterogeneous cells due to the different mechanisms governing their differentiation, tissue distribution and responsiveness to stimuli (2). Macrophages have numerous functions in the innate immune system and play a major role in inflammation, host defence and tissue remodelling. They also play an essential role in triggering, instructing and terminating the adaptive immune response by collaborating with T and B cells (3).

In tissues, macrophages undergo differentiation directed by the tissue which makes them acquire specific morphological and functional properties. They become polarized/activated that classifies them into two different groups. They undergo the classical M1 activation stimulated by toll-like receptor (TLR) and interferon (IFN)-γ or the alternate M2 activation stimulated by interleukin (IL)-4/IL-13. M1 macrophages have an IL-12^{high}, IL-23^{high}, IL-10^{low} phenotype. They have high production of reactive nitrogen and oxygen intermediates, promote T helper type 1 (Th1) response and have a strong microbicidal and tumoricidal activity. M2 macrophages have an IL-12^{low}, IL-23^{low}, IL-10^{high} phenotype. They function mostly as scavengers in containment of parasites, tissue remodelling and immunoregulation (4, 5). These characteristics of the M1 and M2 macrophages have been obtained from monocytes differentiated *in vitro* in response to stimuli. However, little is understood about the phenotypes of macrophages in the various phases of the inflammatory response *in vivo*, and there has been deviations from these characteristics in some studies (6).

In addition to the M1 and the M2 phenotypes, macrophages can also be polarized into an M2 like state from signals like IL-10, glucocorticoid hormones, molecules released from apoptotic cells and immune complexes. The phenotype induced from these signals have shared properties with the M2 macrophages in having high mannose and scavenger receptor expression but are different in their secretion of chemokines (4). These macrophages also display pro-inflammatory characteristics like the M1 macrophages. These macrophages have been isolated from the resolving phase of the immune response and are therefore called resolution macrophages (rM) (6, 7)

1.2.2 Neutrophils

Neutrophils are the most abundant cells of the innate immune system. They play a major role in defence against infection. Neutrophils are one of three types of granulocytes produced in the bone marrow. They are called polymorphonuclear (PMN) leukocytes because of the segmented lobular nucleus or granulocytes because of the small granular like packages of proteins contained in their cytoplasm. These proteins consist of antimicrobial molecules like cationic peptides, proteases, lactoferrin and myeloperoxidase. These proteins help facilitate the functional properties of the neutrophils. Neutrophils are the first cells to be recruited to the site of infection or inflammation for

containment and clearance of the infection. They also send chemotactic signals that attract monocytes that differentiate into macrophages and DCs to be recruited to the site of infection (8).

Neutrophils are short-lived cells that circulate in the blood for a few hours and only enter tissues during infections. The bone marrow produces a steady number of mature neutrophils. Upon infection the number of neutrophils that are matured in the bone marrow can increase by about 10 fold. Once neutrophils receive inflammatory signals from microbes and resident macrophages in the infected area, they enter the infected site from the blood and release toxic products to kill and clear the invading pathogens by phagocytosis, degranulation and generation of reactive oxygen metabolites (9).

After successfully eliminating the infection or inflammation, neutrophils can change their phenotype from pro-inflammatory to anti-inflammatory and therefore have a role in resolution of the inflammation. Once the infection is cleared the neutrophils undergo death by apoptosis that preserves the neutrophil membrane integrity and prevents the uncontrolled release of its toxic cell contents. The apoptotic cells send signals called “find me” and “eat me” that recruit macrophages to clear them from the site of infection thereby preventing further tissue damage (9, 10).

1.2.3 Eosinophils

Eosinophils are another type of granulocytes also produced in the bone marrow from pluripotent stem cells. Eosinophils can be found in the circulating blood in low numbers while a majority of their number can be found within the mucosal tissues interfacing with the environment and within primary and secondary lymphoid tissues (11).

The eosinophil granules contain cytotoxic cationic proteins, cytokines, chemokines and growth factors, which are released upon activation. Eosinophils have numerous functions in the immune system, including regulation of the immune system and inflammation, maintenance of the epithelial barrier function, tissue remodelling, and bridging the innate and the adaptive immune systems (11, 12). Eosinophils have both inflammatory and anti-inflammatory functions. They are recruited to the inflammatory site by engaging the receptors for cytokines, immunoglobulins (Igs), or complement which can lead to the secretion of cytokines, such as IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, and transforming growth factors (TGF) β, chemokines, lipid mediators and cytotoxic granule cationic proteins (13). Yamada *et al.* showed the first evidence that eosinophils may act as anti-inflammatory and proresolving cells that are recruited and switched on during the resolution phase of acute peritonitis through the release of beneficial mediators such as protectin (PD) 1 (14).

1.2.4 Basophils

Basophils are the third type of granulocyte produced in the bone marrow and the least common, accounting for less than 1% of granulocytes in circulating blood and spleen. However, they can rapidly increase in number in response to inflammatory signals and move into the blood, spleen, lung and liver (15). Their lifespan is estimated to be 1–2 days (15).

Basophils can be activated by inflammatory mediators like antibodies, cytokines, proteases, TLR ligands and complement factors. Upon activation they can produce cytokines such as IL-4, IL-6, IL-13, and tumour necrosis factor (TNF)-α, effector molecules such as histamine, leukotriene (LT) C4 and

antimicrobial peptides, and chemotactic factors (16, 17). Basophils also influence Th2 cell responses and function as antigen presenting cells to promote Th2 cell differentiation (16).

1.2.5 Mast cells

Mast cells help to induce inflammation. They have large granules in their cytoplasm that are released when they are activated. Masts cells and basophils have often been mistaken as one another given their similar appearance and phenotypic properties. They both express the high affinity receptor for IgE and release a similar spectrum of mediators upon IgE cross-linking. However, markers that can define mast cells from basophils have been identified to distinguish between these cell types and the list continues to expand (15). Unlike basophils, mast cells differentiate in the tissues but both are produced in the bone marrow from common granulocyte-monocyte precursor. The granulocyte-monocyte precursor develops into mast cell precursor that is released into the circulation and matures once it migrates and localizes in tissues. Unlike basophils mast cells can survive for months (16, 17). Mast cells are located throughout the body, common at muscosal surfaces, and near blood and lymphatic vessels.

Mast cells can be activated by diverse stimuli and can release a spectrum of mediators including histamine, proteases and other enzymes, cytokines, chemokines, growth factors, arachidonic acid metabolites, and reactive oxygen and nitrogen species (18). Mast cells play a role in orchestrating allergic responses and in homeostasis. They can detect and initiate responses against microbes through receptors like TLRs, complement receptors and Fc receptors and release mediators, which recruit effector cells including neutrophils. Mast cells also play a role in the development of acquired immune responses by activating DCs and T cells, and can act as antigen presenting cells. They play a part in protecting the internal surfaces of the body against pathogens and are involved in responses to parasitic worms, bacterial, fungal and viral infections (18).

1.2.6 Dendritic cells

DCs are mononuclear phagocytes produced from the macrophage/DC progenitors (MDPs) in the bone marrow and within tissues they differentiate from monocytes under inflammatory conditions (19). The function of the DCs depends on the DC subset as well as their location and activation status. There are two main subsets of DCs, the conventional/classical DCs (cDCs), normally referred to as DCs and the plasmacytoid DCs (pDCs) (19, 20). The pDCs differ from the cDCs in that they live longer, carry characteristic immunoglobulin rearrangements and express TLR-7 and TLR-9. pDCs can be found in the bone marrow and peripheral organs. They specialize in antiviral immunity as their receptors can detect single stranded RNA and DNA viruses producing type I IFNs. On the other hand, cDCs are short lived and often replaced by blood-born precursors. They reside as immature DCs in the peripheral non-lymphoid tissues like the skin and mucosa and they sense, sample and process incoming antigens and have a high phagocytic activity (20). They become mature by activation from microbial products or inflammatory cytokines. Mature DCs are non-phagocytic and migrate to draining lymph nodes where they function as antigen presenting cells to naïve T cells inducing the activation and differentiation of the T cells. Thus mature DCs have a key role in bridging the innate and the adaptive immune systems (20, 21).

1.2.7 Natural killer cells

Natural killer (NK) cells are large granular lymphoid cells that respond to the presence of an infection without being antigen specific. NK cells are therefore regarded as part of the innate immune system and they act in the first line of defence during an infection.

In humans, NK cells are divided into two subsets, the $CD56^{dim}CD16^{+}$ NK cells (90% of NK cells) and the $CD56^{bright}CD16^{-}$ NK cells (10%). These two subsets differ in location, cytotoxicity and function. The $CD56^{dim}CD16^{+}$ NK cells are present in peripheral blood, secrete low levels of cytokines, express perforin and efficiently kill targeted cells. The $CD56^{bright}CD16^{-}$ NK cells are present in secondary lymphoid tissues like the lymph nodes and tonsils, exert low cytotoxicity, secrete high levels of cytokines, do not express perforin and may be involved in autoimmune diseases through their potential autoreactivity or through their interaction with DCs, macrophages or T cells (22, 23).

NK cell subsets, based on phenotype, function and anatomic features, are also found in mice and they have some similarities with NK cell subsets in humans. In mice, NK cells are divided into three subsets depending on their expression of CD11b and CD27: $CD11b^{dull}CD27^{+}$ NK cells, $CD11b^{+}CD27^{+}$ (double-positive) NK cells and $CD11b^{+}CD27^{dull}$ NK cells. The $CD11b^{dull}CD27^{+}$ NK cells are found in bone marrow and lymph nodes, the double-positive NK cells are in blood and spleen and the $CD11b^{+}CD27^{dull}$ NK cells in blood, spleen, lungs, liver and inflamed tissues. These different subsets depict the NK cell differentiation process from immature $CD11b^{dull}CD27^{+}$ NK cells, to the double positive NK cells to the most mature $CD11b^{+}CD27^{dull}$ NK cells (24).

NK cells destroy target cells using perforins and granzymes, cytolytic mediators stored in their cytoplasmic granules, which are released upon activation (25, 26). NK cells also produce cytokines like IFN-γ, TNF-α, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-5, IL-10, IL-13 and TGF-β, which are regulated by activation and inhibitory receptors (25).

NK cells express many receptors that either mediate activation or inhibition, with many of these belonging to the same families of molecules. The families of NK cell receptors in mammals include the Ig superfamily receptors which include the killer cell Ig-like receptors (KIRs), natural cytotoxicity receptors (NCRs), the structurally unrelated NKG2D receptor families (Table 1) (25). Whether the binding of an NK cell to a target cell results in inhibition or activation has been suggested to be controlled by the net sum of all these signals it receives.

The activating receptors on NK cells recognise self-ligands, like human ULBP and MIC molecules and the mouse RAE1, H60 and MULT1 molecules (24, 27). These self-ligands are rare on normal cells but appear during infection or malignant transformation. When the activating receptors come in contact with a target cell expressing these self-ligands, it results in activation of cell signalling and lysis mediated by the NK cells (28).

The inhibitory receptors on NK cells recognise unhealthy cells as described by the “missing self” hypothesis. These receptors recognise the self-MHC class I molecules on the cell surfaces and those without the molecules due to infection are destroyed (23, 24, 27). The KLRs on the other hand bind to non-classical MHC class I molecules (23, 24).

Table 1. A summary of NK receptor families and receptors in humans and mice. NK cell receptors can be structurally divided into two groups as Ig superfamily or killer cell lectin-like receptors. Table adapted from Jadidi-Niaragh et al. (25)

NK Receptors	Activating or Inhibitory	Structure	Ligand	Spices
KIR family	Both	Ig-like	MHC-I molecules	Humans
Ly49 family	Both	C-type lectin like	MHC-I molecules and nonself MH-I like molecules	Mice
NKG2/CD94 family	Both	C-type lectin like	Non classical MHC-I molecules HLA-E (humans) and Qa1 (mice)	Humans and mice
NKG2D family	Activating	C-type lectin like	MHC-I like molecules	Humans and mice
NCR family	Activating	Ig-like	Not related MHC-I molecules	Humans and mice
NKR-P1 family	Both	C-type lectin like	C-type lectin-like related Clr (Ocil) molecules	Humans and mice
KLRG1	Inhibitory	C-type lectin like	Cadherins	Humans and mice
2B4 receptor	Both	Ig-like	CD48	Humans and mice
DNAM-1	Activating	Ig-like	CD155 and CD112	Humans and mice

Cytokine and chemokine production by NK cells also make them regulators of both innate and adaptive immune responses (29). They affect other cell types like DCs, T cells, B cells and endothelial cells to maintain immune homeostasis and regulate autoimmunity. NK cells promote DC maturation and kill over-stimulated DCs and immature DCs. They also enhance the function of $CD4^+$ T cells, promote priming of $CD4^+$ Th1 cells by secreting IFN-γ, and kill activated T cells that do not express enough classical or non-classical MHC class I molecules. NK cells can also kill over-stimulated macrophages and B cells (23, 24).

By depleting NK cells in mice, it has been shown that they play a role in the inflammation course. They have been shown to be required for early host defence during infection by production of pro-inflammatory mediators like IL-22, IFN-γ and other pro-inflammatory cytokines (30). They have also been shown to interact with macrophages and participate in early eradication of bacteria and regulation of IL-12 during sepsis (31).

NK cells that are recruited early in the inflammatory response may also function in its resolution by triggering the apoptosis of neutrophils at the inflammatory site (32). In a study using co-culture experiments it was discovered that *in vitro*, the human NK cells triggered a caspase-dependent neutrophil apoptosis which was mediated by the NKp46 receptor and the Fas pathway. The data showed that the induction of neutrophil apoptosis was specific to the NK cells and also accelerated neutrophil apoptosis contributing to the resolution of the inflammation (32).

NK cells also function in the clearing of eosinophils and leukocytes by the expression of the RvE1 receptor CMKLR1, which is a pro-resolving mediator for inflammation (33). In this recent study on the effects of NK cells during the resolution of an allergic airway inflammation, it was observed that in the resolution phase the disappearance of eosinophils and T cells corresponded with the increased appearance of NK cells. In addition, depletion of the NK cells delayed the clearance of both the

eosinophils and T cells and decreased the expression of the resolvin (Rv) E1 receptor CMKLR1 on NK cells (33).

1.3 The adaptive immune system

The adaptive immune response is, in contrast to the innate immune response, specific to distinct pathogens and infection. As was stated above, the lymphoid lineage gives rise to the antigen-specific lymphocytes of the adaptive immune system. They are called antigen-specific as they have to be activated by an antigen before they can function. Lymphocytes that are not yet activated are called naïve lymphocytes and can be found circulating in the body. Once a naïve lymphocyte has met its specific antigen it becomes activated and differentiates into a fully functional lymphocyte called effector lymphocyte. Lymphocytes are of two types, the B lymphocytes (B cells) and the T lymphocytes (T cells). They have different roles in the immune system and different antigen receptors.

Upon binding of an antigen with the B cell receptor (BCR) on the B cell surface, the B cell proliferates and differentiates into a plasma cell. Plasma cells are the effector form of B cells and produce antibodies. The antibodies are a secreted form of the BCR and have identical antigen specificity. The antibody molecule is called Ig and it targets the antigen that first activated the original B cell.

T cells are activated by binding of a peptide fragment of an antigen displayed by MHC molecules with its T cell receptor (TCR). Upon activation T cells differentiate and proliferate into one of several different types of effector T cells depending on whether their function is to kill, activate or regulate. Cytotoxic T cells kill infected cells, Th cells provide signals that for example activate antigen-stimulated B cells and macrophages, and regulatory T cells suppress the activity of other lymphocytes and control immune responses.

Some antigen activated B cells and T cells differentiate into memory cells. Memory cells are the lymphocytes that are responsible for the long-lasting immunity that follows an exposure to a specific pathogen or vaccination. During the primary immune response, specific immunological memory is established. This guarantees that there is a rapid reinduction of the antigen-specific antibodies and effector T cells upon exposure to the same pathogen (1).

1.4 Inflammation

Inflammation is the body‘s normal defence mechanism to protect it from infections and other pathogenic insults and restore tissue homeostasis. The innate immune system is a major contributor to inflammation and is characterised by a complex sequence of events which involves an inductive phase followed by a resolution phase. Inflammation has three main roles in fighting against infection. The first is to deliver effector molecules to the site of infection and augment killing of the microorganisms. Second, to induce blood clotting to prevent spread of the infection to the blood stream. Third, to promote the repair of injured tissue (1). The changes in the blood vessels surrounding the infected area result in the four different characteristics of inflammation – pain, redness, heat and swelling.

Although inflammation is important in fighting against infection, it is equally important that after the infection has been cleared, the inflammatory cells exit the site and the inflammation is resolved. Failure of inflammation to resolve is believed to be a major cause of many diseases, such as rheumatoid arthritis, atherosclerosis, chronic obstructive pulmonary disease, periodontal disease, cancer, cardiovascular disease and Alzheimer‘s disease. In their review, Soehnlein and Lindbom (34) describe four phases of the inflammatory response , two phases involved in induction of inflammation and two in resolution as discussed below.

1.4.1 Induction of inflammation

The induction phase of the inflammatory response is comprised of the inflammatory inducers, the sensors that detect them, the inflammatory mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators (35). The inflammatory inducers, such as bacteria, viral and other microbial pathogens, have PAMPs and are recognised and sensed by the germline-encoded PRRs. PRRs also recognise endogenous molecules from damaged cells called damage-associated molecular patterns (DAMPs). The PRRs include the TLRs and C-type lectin receptors (CLRs), the cytoplasmic proteins, such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (36). These receptors are expressed by cells of the innate immune system, like macrophages, neutrophils and DCs. Sensing of pathogens by these receptors upregulates transcription of genes involved in inflammatory responses and induces the production of inflammatory mediators, such as cytokines (e.g. TNF-α, IL-1 and IL-6), chemokines (e.g. CCL2 and CXCL8), and prostaglandins (PGs). These mediators act on the target tissues and local blood vessels inducing vasodilation, extravasation of neutrophils and leakage of plasma to the infected site. The neutrophils recruited or already located in the tissues find and destroy the pathogens. The PRRs that become activated, the mediators induced and the targeted tissues involved in the process all depends on the type of infection and this decides the type of inflammatory response induced (35).

Phase I of the inflammatory response involves resident macrophages recognizing pathogens or tissue damage and recruitment of neutrophils to the area (Figure 1). The PAMPs or DAMPs are recognised by the PRRs expressed on resident macrophages which then promote the production of pro-inflammatory cytokines and chemokines that induce the recruitment of neutrophils to the area. Phase II involves neutrophil-mediated monocyte recruitment. After the extravasation of neutrophils to the infected area, they release preformed granule proteins, including reactive oxygen and nitrogen species, proteinase and elastase. They eliminate or neutralise the invading pathogen/damaged tissue and attract monocytes to the inflammatory site (34, 37).

1.4.2 Resolution of inflammation

The resolution phase of the inflammatory response, which was once thought to be a passive process, is now known to be an active process in which the inflammatory cells are depleted, receptors activated, and chemokines and cytokines depleted to bring the inflammatory process to a halt and clearing out the pro-inflammatory cells (38). In the absence of DAMPs and PAMPs at the inflammatory site, the pro-inflammatory mediators are turned off and anti-inflammatory mediators come into play to

clear out the cells involved. The resolution phase involves signals, which turn off the infiltration of neutrophils and induce uptake and clearance of apoptotic cells. This occurs in phase III of the inflammatory response (Figure 1) (34). A process called lipid mediator class-switching occurs which promotes the synthesis of mediators (e.g. lipoxin (LX)-A_4, resolvins, etc.) with anti-inflammatory and pro-resolving activity and promotes the clearance of chemokines, such as CCL3 and CCL5 and cytokines. This reduces neutrophil activity and their recruitment to the inflamed site and also promotes macrophage uptake of apoptotic and dead neutrophils. Phase IV involves termination of the inflammatory response by macrophages that adopt an anti-inflammatory signature following ingestion of apoptotic neutrophils. Here, macrophages help to restore homeostasis by clearing out dead cells and inducing TGF-β production. The macrophages clear the apoptotic cells by phagocytosis which prevents the activated macrophages from killing tissue-resident cells and also triggers the release of vascular endothelial growth factor (VEGF) and other growth factors that are crucial for tissue repair (34).

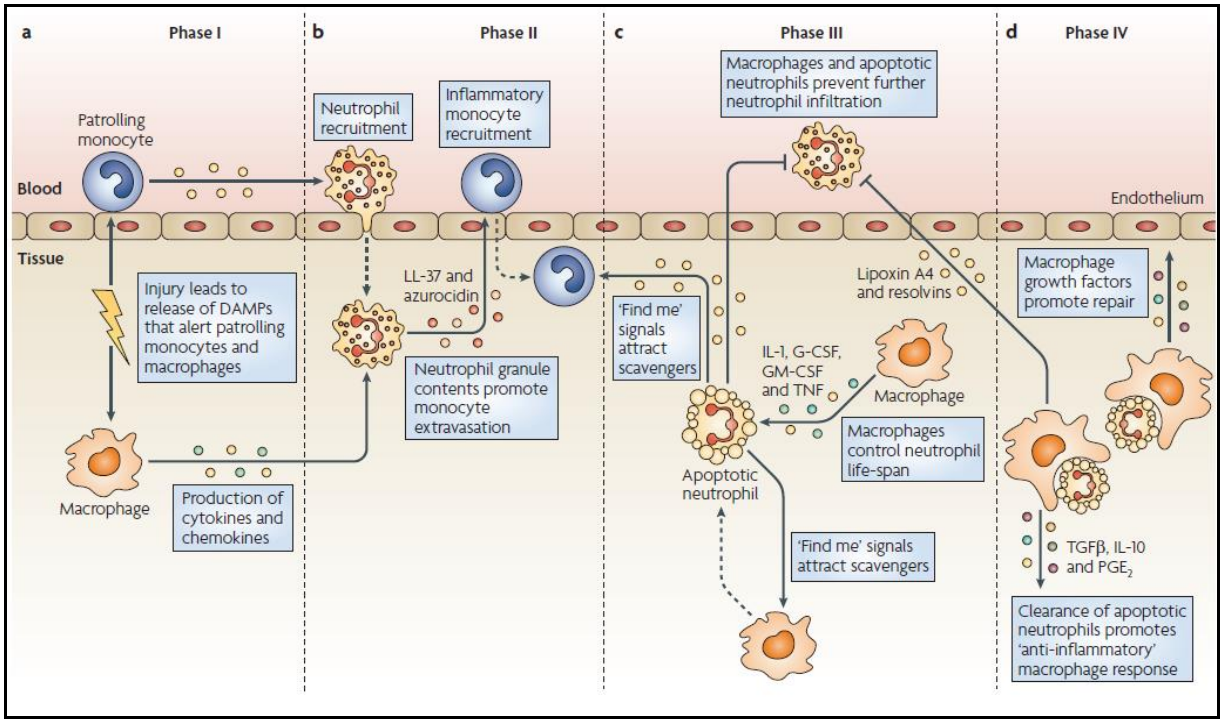

Figure 1. The induction and resolution phases of inflammation. (a) Phase I: macrophages fuel neutrophil recruitment. (b) Phase II: neutrophils recruit monocytes. (c) Phase III: phagocyte signals stop neutrophil influx. (d) Phase IV: macrophages restore homeostasis (34).

The inflammatory response is one continuous course combining both the induction and resolution phases. Therefore, there has to be a point at which induction stops and resolution begins. This occurs during lipid mediator class-switching (Figure 2). Prostaglandins and leukotrienes, generated by endothelial cells, neutrophils, monocytes and macrophages, initially promote inflammation in the induction phase. However, in the resolution phase, PGE_2 and PGD_2 now promote synthesis of mediators with anti-inflammatory and pro-resolving activity, such as LXs (34). LXs are produced by PMNs, by cell to cell interaction with platelets and epithelial cells, and other resident cells in response

to specific cytokines or growth factors. Macrophages can also promote LX production by the uptake of apoptotic neutrophils. LXA_4 decreases activity and inhibits neutrophil entry to the site of inflammation and promotes monocyte migration into the site of inflammation. In neutrophils, LXA_4 induces changes in the phosphorylation of cytoskeletal proteins that results in cell arrest. In macrophages, it induces mobilization of intracellular Ca^{2+} that promotes chemotaxis, stimulates migration and promotes non-phlogistic phagocytosis of apoptotic neutrophils. LXA_4 also decreases the synthesis of pro-inflammatory chemokines and cytokines (34). Other pro-resolving lipid mediators include resolvins and protectins. Resolvins are derived from the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Resolvins derived from EPA are termed E-resolvins and those derived from DHA, D-resolvin. E-resolvins are produced during endothelial cell-neutrophil interactions by neutrophil derived-5-lipoxygenase (5-LOX) after conversion of lipid precursors by endothelial cells or bacteria (34, 39). They bind ChemR23 on monocytes, macrophages and DCs and decrease TNF-α mediated NF-κB activation, forming an anti-inflammatory signalling pathway. D-resolvins act as inhibitors of neutrophil transendothelial migration. Protectins are derived from DHA and they have a similar role as D-resolvins. Both resolvins and protectins also increase surface expression of CCR5 on apoptotic neutrophils which leads to the clearance of pro-inflammatory chemoattractants, like CCL3 and CCL5, from the site of inflammation and termination of inflammatory cell influx (34).

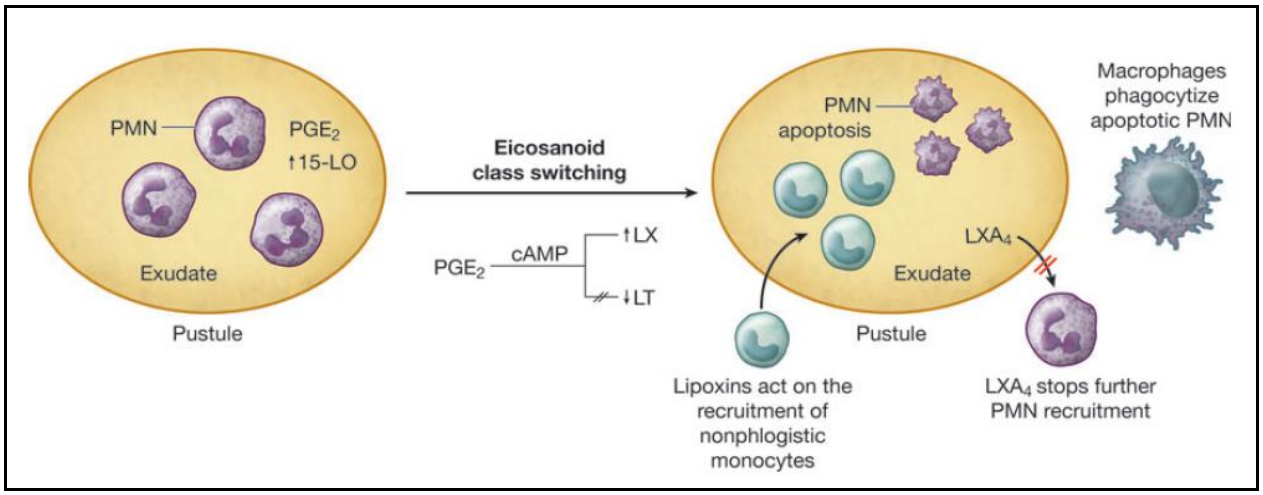

Figure 2. Lipid mediator class-switching. PMNs at the site of inflammation "switch" their phenotype to produce lipoxins and stop leukotriene biosynthesis thereby stopping further PMN entry (40).

Eosinophils have been shown to play a role in resolution of zymosan-induced acute inflammation and to produce specific pro-resolving and anti-inflammatory lipid mediators (14). The eosinophils were recruited to the inflammatory site, where they produced the pro-resolving lipid mediator PD1 via 12/15-LOX-initiated biosynthesis. PD1 is a DHA-derived lipid mediator that counter regulates PMN influx and stimulates macrophage ingestion of apoptotic PMNs (14).

Macrophages have one of the most important jobs in the resolution of inflammation. On polarization into M2 macrophages, not only do they phagocytose apoptotic cells and their secreted products by efferocytosis, they also trigger apoptosis of neutrophils by expressing death molecules like the Fas

ligand. The efferocytosis stimulates the macrophages to release TGF-β, which blocks TLR-induced pro-inflammatory signalling. TGF-β suppresses inflammation and stimulates tissue repair by inducing secretion of VEGF and collagen. After efferocytosis, the macrophages then leave the site of inflammation via the nearest lymphatics and the tissue is returned to homeostasis (37). Apoptotic neutrophils also promote resolution by producing signals to recruit macrophages and induce phagocytosis. One such signal is lactoferrin, which is released by the apoptotic cells and is a negative regulator of neutrophil recruitment and a chemoattractant for phagocytosing cells (34).

NK cells are active during the induction phase of inflammation but may also play a role in resolution of inflammation (33). In the induction phase NK cells produce IFN-γ and GM-CSF to enhance neutrophil survival (32). In the resolution phase they may trigger neutrophil apoptosis as was shown in a study where neutrophil apoptosis could be eliminated by an antibody to the NKp46 receptor on NK cells (32). Further indication of the role of NK cells in the resolution of inflammation comes from a study by Haworth *et al.* showing that NK cell depletion delays resolution of an allergic airway inflammation in mice (33). In that study the NK cell depletion impaired RvE's protective role as NK cells have the RvE receptor CMKLR1 (33). Further indication of the role of NK cells in resolution of inflammation comes from a study by Tomasdottir *et al.* (41). In the study, using murine antigen-induced inflammation, mice that were fed a fish oil diet had an early increase in the number of peritoneal NK cells that was not seen in mice fed the control diet. In addition mice fed the fish oil diet also had enhanced resolution of inflammation compared with that in mice fed the control diet (41). In the present study, the aim is to confirm the relationship between NK cells and resolution of inflammation.

1.5 Murine models of peritonitis

Several different murine models of peritonitis have been used to investigate inflammatory responses. They differ in the irritant or antigen used to induce the inflammation as well as the type of responses that are induced. Ragweed extract seemed to be what was used to induce peritonitis in mice in early experiments. The ragweed extract was known to induce selective eosinophilia within the peritoneal cavity. In a research of inflammation studying the production of IL-5 by NK cells, mice were immunized and challenged with a short ragweed extract resulting in infiltration of eosinophils during the allergic reaction (42).

Thioglycolate medium (TM) has frequently been used to study inflammation in the peritoneum. TM is a sterile irritant that induces inflammatory mediator production and leukocyte accumulation but the elicited macrophages in this model are highly phagocytic and may have properties different from those induced by other means. This model has primarily been used to study inflammatory macrophages and determining the role of particular mediators in inflammation.

Zymosan-induced peritonitis is also a commonly used method to study acute inflammation. Zymosan is a polysaccharide cell wall component from *Saccharomyces cerevisiae*. It induces an intense self-limited acute inflammation resulting from TLR activation, thus mimicking the initial inflammatory response to bacteria. Resolution of bacteria (40) and the effects of inflammation and

resolution on metabolomic profiles (43) have mostly been studied using this model. However, because of its short duration the inflammation entails little or no activation of adaptive immunity.

A murine model of antigen-induced peritonitis was first described by Cook *et al.* (44). They induced peritonitis by injecting an antigen, methylated bovine serum albumin (mBSA), into the peritoneum and compared the inflammatory response with that obtained using TM. They were able to induce an inflammatory response of equivalent magnitude, in terms of cell number, to that induced with TM. However, the inflammatory macrophages differed in terms of size, cell surface expression, and functional capabilities. This model of murine peritonitis involves an adaptive inflammatory response and was used by Tomasdottir *et al.* studying the role of dietary fish oil on the induction, resolution and adaptive phases of inflammation, where the hypothesis of this project was adapted from (41).

Another antigen-induced peritonitis model uses ovalbumin (OVA) to induce inflammation. This model has been used to study the effects of oral tolerance on allergic inflammation showing a milder peritonitis, affecting mostly neutrophils and eosinophils but no change in lymphocyte count in the OVA-tolerant mice. There was also a decrease in T cells and an increase in B cells compared with the immune OVA-challenged mice (45).

2 Aim

2.1 Hypothesis

NK cells that are recruited to the inflammatory site early in inflammation play a critical role in shaping the resolution process of the immune response.

2.2 Aims

To determine whether NK cells are important for resolution of antigen-induced inflammation.

Specific aims:

1. To determine the effect of NK cell depletion on the induction and resolution phase of the inflammation.
2. To determine the phenotype of NK cells in antigen-induced inflammation.

3 Materials and methods

3.1 Mice and diet

Female C57BL/6J mice weighing 18-20 g were purchased from Taconic Europe (Ejby, Denmark). All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland and complied with NRC´s Guide for the Care and Use of Laboratory Animals. Mice were housed five or eight per cage in humidity (45-55%) and temperature (23-25°C) controlled environment with a 12 h light and dark cycle. Mice were acclimated for one week prior to initiation of the experiments. All mice consumed water and food ad libitum.

3.2 Induction of peritonitis

Antigen-induced peritonitis was induced by immunizing the mice subcutaneously at the base of the tail with mBSA (Sigma) emulsified in an equal volume of complete Freund's adjuvant and with mBSA in incomplete Freund's adjuvant two weeks later. One week later, mice were challenged intraperitoneally (i.p.) with mBSA in saline. The mice were anesthetized with a mixture of hypnorm (VetaPharma), dormicum (Hoffman-La Roche) and sterile water (1:1:2) and killed by cervical dislocation. Peritoneal exudates, parathymic lymph nodes and spleen were collected before (0 h), 6, 12 and 48 h after peritonitis induction. Three to four mice were in each group and the experiment repeated twice.

3.3 Depletion of NK cells

To deplete NK cells, 70 µL anti-asialo GM1 antibodies (0.07 mg) (abs) (Wako Chemicals and eBioscience) or control rabbit IgG (Wako Chemicals) was injected intravenously (i.v.) into the mice 24 h before induction of inflammation (33). The effectiveness of the depletion was monitored by examining the number of NK cells at different time-points by flow cytometry.

3.4 Collection of peritoneal exudates

To collect peritoneal exudates (cells and fluid), after killing mice by cervical dislocation, 1.5 ml of cold sterile saline was injected into the peritoneum. The peritoneal fluid was collected and cells and supernatants separated by centrifugation. The cells were counted using Cell countess (Invitrogen), which gives the total cell number, and studied immediately. The supernatants were aliquoted and stored at -80^{o}C until they were used. Part of the fluid and cells was stored under nitrogen before being stored at -80°C.

3.5 Flow cytometry analysis

Peritoneal cells were suspended in a flow buffer and stained with fluorochrome-labeled antibodies. The cells were washed and fixed in a paraformaldehyde buffer. Prior to staining, unspecific binding was blocked by incubating the cells with 2% normal mouse, rat and Armenian hamster sera. The cells were collected using Navios flow cytometer (Beckman Coulter), where a typical forward and side

scatter gate was set to select live cells. The Kaluza program (Beckman Coulter) was used for analysis of the cells. The percentage of each cell type was determined and their total number calculated. In addition, the expression level was evaluated by measuring the mean fluorescence intensity (MFI).

3.5.1 Phenotype analysis of the innate cells and lymphocytes

Peritoneal cells were stained with antibodies against CCR3, Ly6G, CXCR2, F4/80, CD11b, CD90.2 and CD19 to identify neutrophils, eosinophils, macrophages and lymphocytes. Figure 3 describes the sequential gating procedure used to identify the different cell populations. Neutrophils were identified as $CXCR2^{+}Ly6G^{+}$ cells, eosinophils as $CCR3^{+}F4/80^{+}$ cells, macrophages as $F4/80^{+}CD11b^{+}$ cells and lymphocytes as $F4/80^{-}$ cells expressing either CD90.2 (T cells) or CD19 (B cells).

3.5.2 Phenotype analysis of the NK cells

The peritoneal cells were stained with antibodies against NK1.1, NKp46, CD27, CD43, CD69, CD107a, NKG2D, and CMKLR1. Figure 4 describes the sequential gating procedure used to identify the NK cells and their sub-populations. NK cells were identified as the cells expressing NK1.1. The $NK1.1^{+}$ NK cells were further gated to determine the number of NK cells present. The $NK1.1^{+}$ NK cells was further gated into three populations depending on their expression of CD11b and CD27, markers that determine the NK cell maturity (immature $CD11b^{-}CD27^{+}$, mature $CD11b^{+}CD27^{+}$ and most mature $CD11b^{+}CD27^{-}$). Finally, the percentage of each of these subpopulations of NK cells expressing NKp46, CD43, CD69, CD107a, NKG2D and CMKLR1 was determined.

3.6 Concentrations of cytokines and soluble cytokines receptors

Concentrations of the cytokines CXCL1, IL-6, IL-10, IL-12p40, G-CSF, INF-γ and TGF-β and the soluble cytokine receptors IL-1ra and sIL-6R in peritoneal exudates at all time-points were determined using DuoSet ELISAs (R&D Systems).

3.7 Statistical analysis

Results were expressed as mean values ± standard error of the mean. Two-way ANOVA was used to evaluate the overall difference between the two groups. A t-test was used to compare the two groups at a single time-point. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software) and P value of <0.05 considered significant. When the P value was between 0.05 and 0.1 it was indicated with (*).

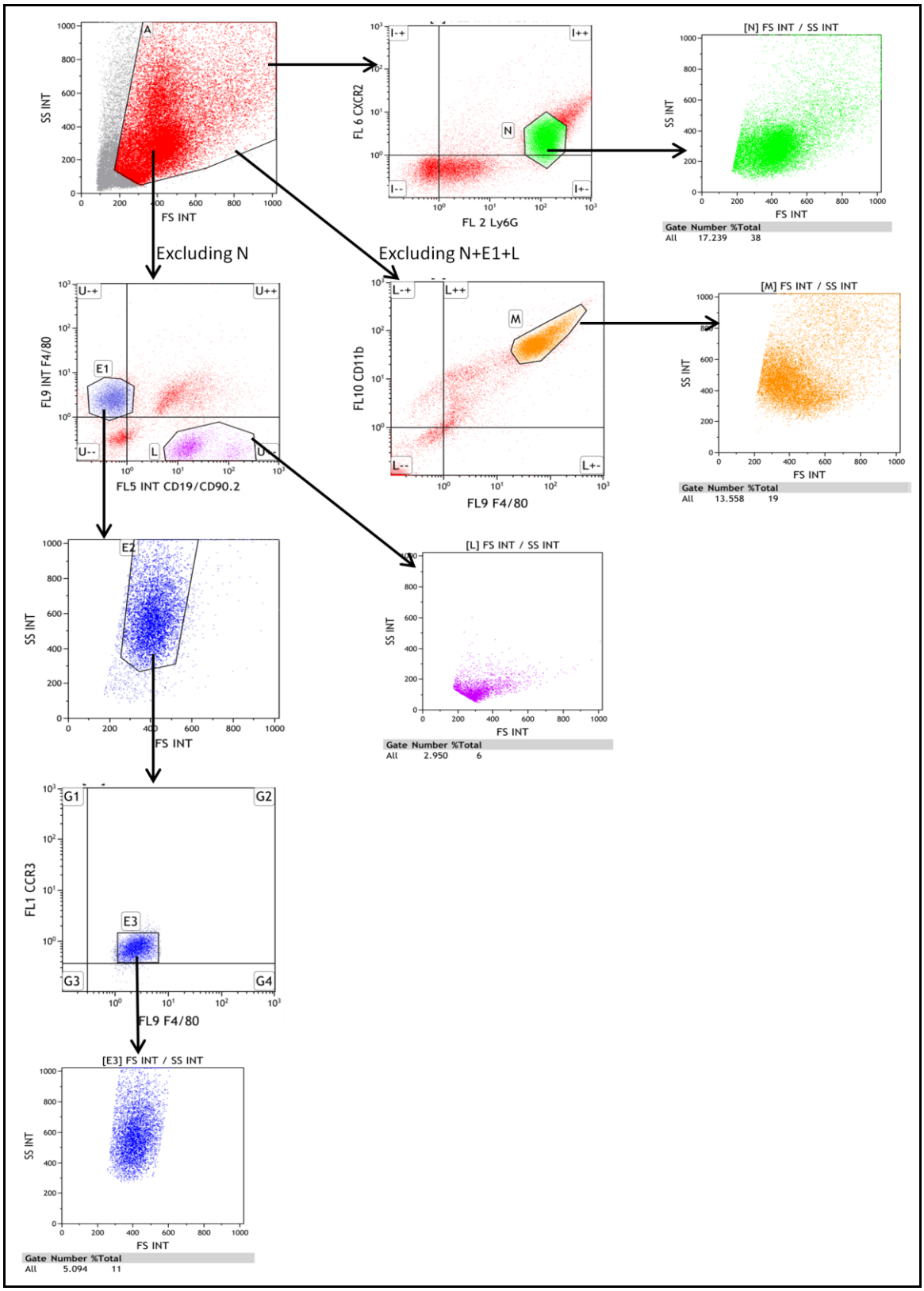

Figure 3. Gating procedure used to identify lymphocytes and innate cells in peritoneal fluid. First all known live cells are gated excluding dead cells (A). Neutrophils (N) are identified as CXCR3+Ly6G+ cells. On exclusion of neutrophils, eosinophils (E) are first determined as F4/80+CD90.2-CD19- cells, then selected for in the FSC/SSC plot and then confirmed as CCR3+F4/80+ cells. On exclusion of neutrophils, lymphocytes (L) are identified as CD90.2+CD19+F4/80- cells. Macrophages (M) are identified as F4/80+CD11b+ cells after exclusion of N, E and L.

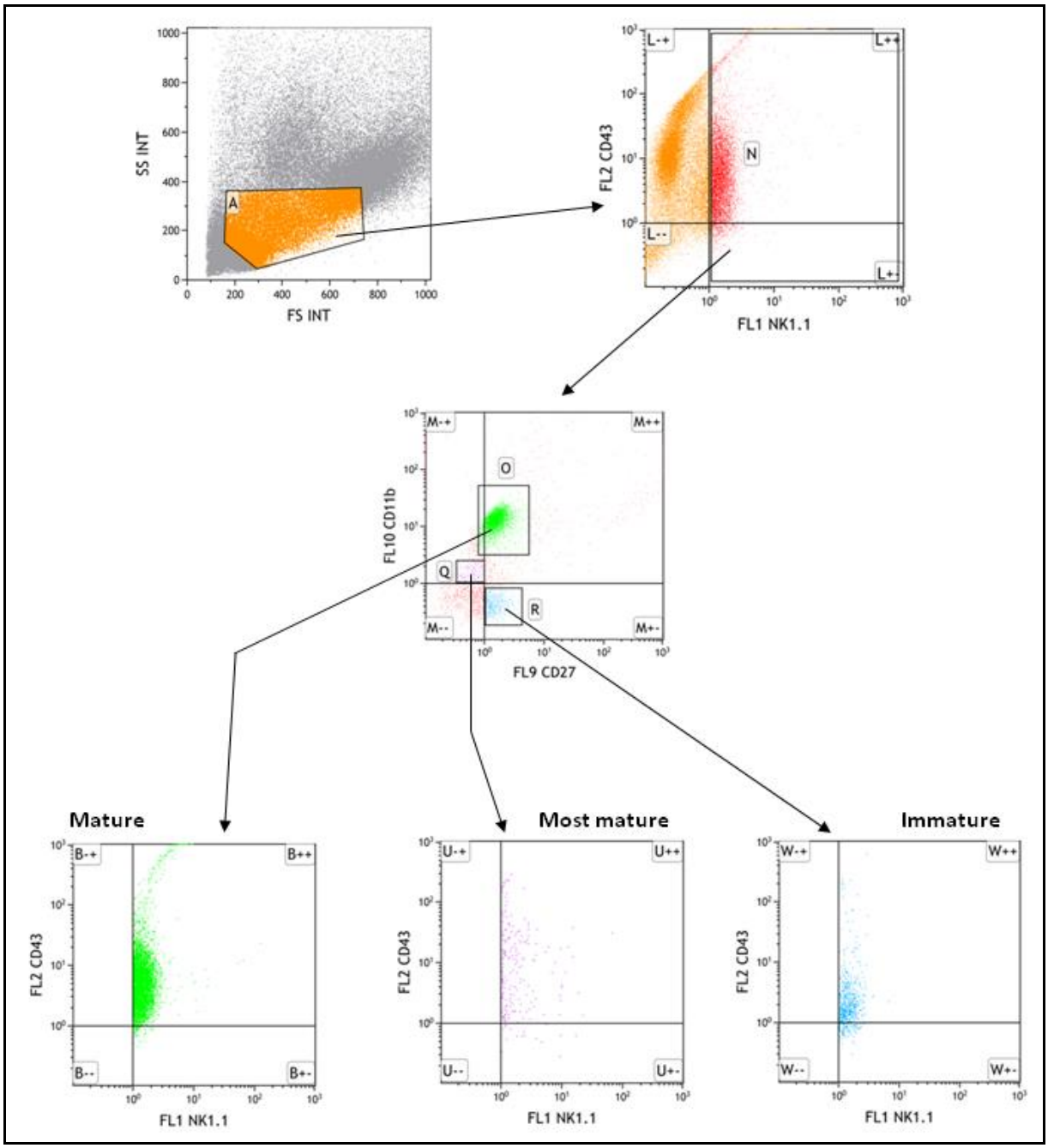

Figure 4. Gating procedure used to identify NK cells in peritoneal fluid. First all known NK cells are gated excluding other known cell types and dead cells (A). All NK1.1 NK cells (N) are identified as NK1.1+ cells. On selection of only NK1.1+ NK cells, three sub-populations of NK cells are gated and identified as immature CD11b-CD27+, mature CD11b+CD27+ and most mature CD11b+CD27- NK cells. They are selected for to determine the percentage of each of these subpopulations of NK cells expressing the other receptors.

4 Results

4.1 Effects of NK cell depletion on the number of peritoneal NK cells

Prior to induction of inflammation (0h; 24 h after administration of the depleting and the control antibodies) there was no difference in the number of peritoneal NK cells between the control and the depleted groups (Figure 5). However, at 6 h and 12 h after induction of inflammation (30 and 36 h after administrating the depleting and the control antibodies) there were less NK cells in the peritoneum of the mice in the depleted group (Figure 5) mounting to 50% decrease 36 h after administration of the depletion antibody. In addition, there was an overall effect of the treatment with the depleting antibody (P=0.011) between the depleted and the control groups within the first 36 h (Figure 5A). At 48 h the level of NK cell had increased in both groups and there was no longer a difference between the depleted and the control groups.

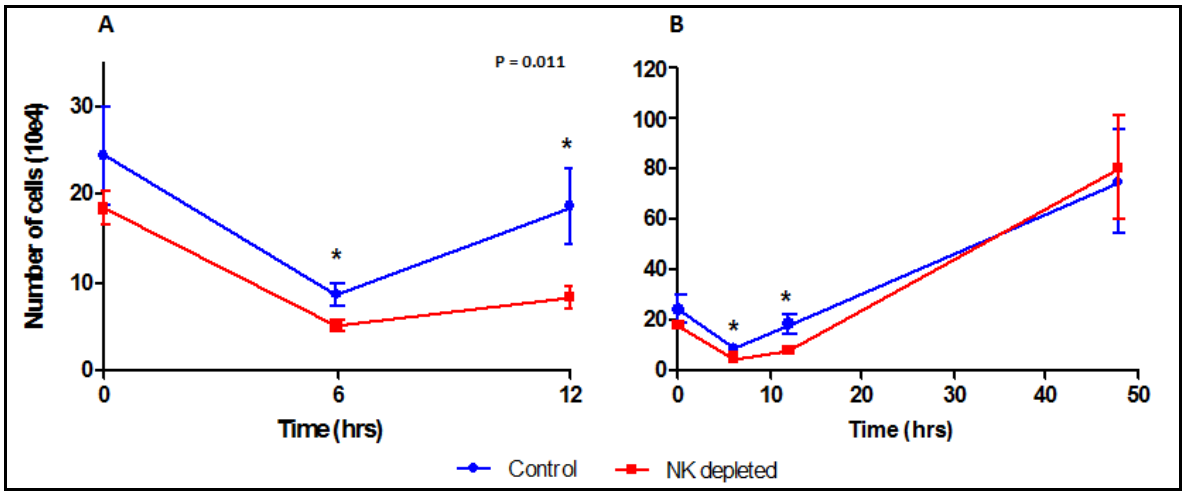

Figure 5. Effects of NK cell depletion on the number of peritoneal NK cells (A) up to 12 h (P of treatment on both groups = 0.011) and (B) up to 48 h. Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and peritoneal cells counted using Countess automated cell counter and stained with monoclonal antibodies and analysed by flow cytometer. P value on right hand corner represent the overall P value for the effect of treatment with the depleting antibody in both groups. Values are means ± SEM, n = 6-8. * Different from control at the same time-point, P<0.05.

4.2 Effects of NK cell depletion on subpopulations of NK cells

Three phenotypes of NK cells were identified. The immature $CD11b^{-}CD27^{+}$ NK cells, the mature $CD11b^{+}CD27^{+}$ NK cells and the most mature $CD11b^{+}CD27^{-}$ NK cells (Figure 6).

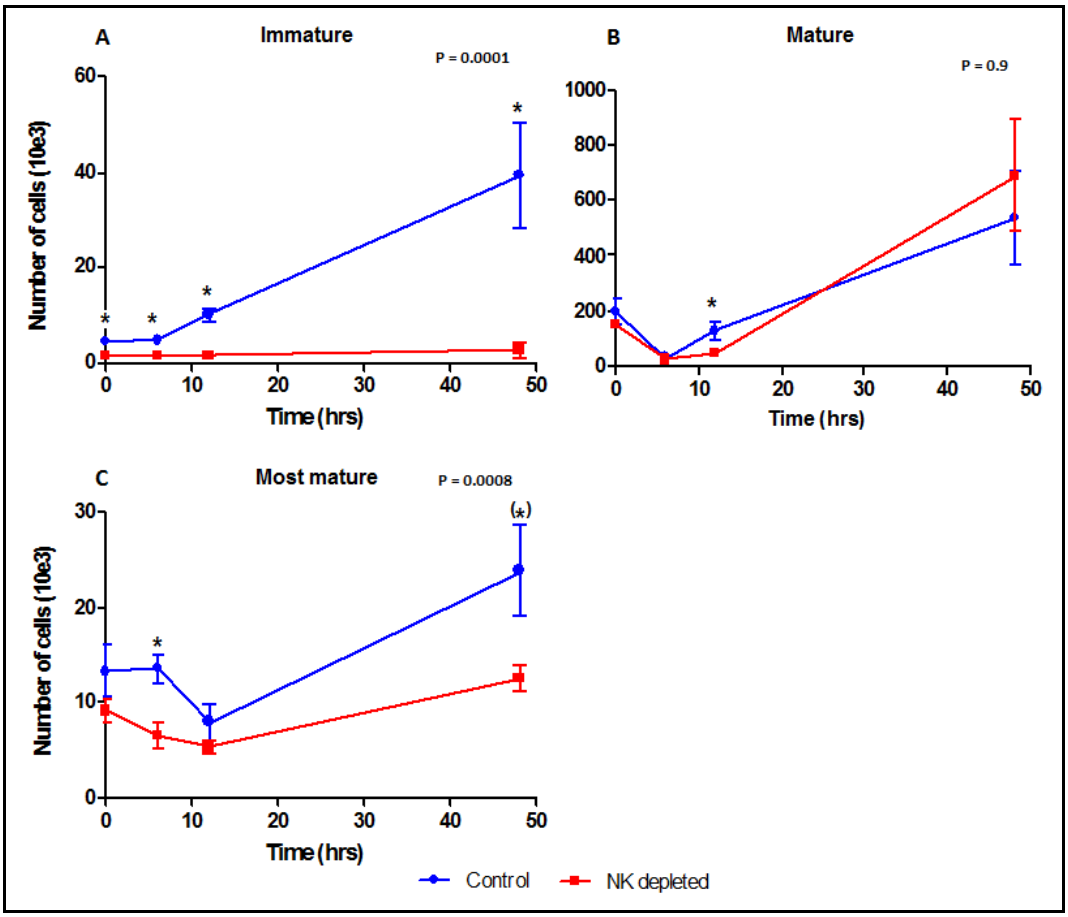

Figure 6. Effects of NK cell depletion on immature CD11b$^-$CD27$^+$ (A), mature CD11b$^+$CD27$^+$ (B) and most mature CD11b$^+$CD27$^-$ (C) NK cells. Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and peritoneal cells counted using Countess automated cell counter and stained with monoclonal antibodies and analysed by flow cytometer. P value on right hand corner represents the overall P value for the effect of treatment with the depleting antibody in both groups. Values are means ± SEM, n = 6-8. * Different from control at the same time-point, P<0.05. (*) Tendency towards being different from control at the same time-point, P<0.1.

4.2.1 CD11b$^-$CD27$^+$ NK cells

There were fewer immature NK cells in the peritoneum of mice in the depleted group than the control group prior to induction of inflammation and at all time-points during the inflammation (Figure 6A). At 48 h the number of immature NK cells had increased significantly in the control group, whereas there was no increase in the number of immature NK cell from what it was prior to induction of inflammation. The difference in the number of immature NK cells in the peritoneum of mice in the control and the depleted groups was 18 fold 48 h after induction of inflammation. There was an overall effect of the treatment with the depleting antibody (P=0.0001) between the depleted and the control groups.

4.2.2 CD11b^{+}CD27^{+} NK cells

The CD11b^{+}CD27^{+} NK cells were the majority of the total NK cells in both the control and the depleted groups at all time-points (Figure 6B). As with the total NK cells, the number of these mature NK cells had significantly increased at 48 h. At the 12 h time-point there were 50% fewer mature NK cells in the depleted group than in the control group but there was no difference in the number of mature NK cell between the two groups at other time-points. There was no overall effect of the treatment with the depleting antibody between the two groups.

4.2.3 CD11b^{+}CD27^{-} NK cells

Prior to induction of inflammation there were few most mature CD11b^{-}CD27^{+} NK cells in both the control and the depleted groups, although their number were 2-3 fold higher than the number of immature NK cells (Figure 6C). Their numbers remained low at all time-points following induction of inflammation but increased at 48 h in the control group. At 6 h after induction of inflammation there were 50% fewer NK cells in the depleted group than the control group and at 48 h there was a tendency for these NK cell to be lower in the depleted group than in the control group. Although there was no difference between the depleting and control groups at other time-points there was an overall effect of the treatment with the depleting antibody (P=0.0008) between the depleted and the control groups.

4.3 Effects of NK cell depletion on the number of peritoneal cells

There was no difference in the number of total peritoneal cells between the control and the depleted groups at all time-points (Figure 7). The number of peritoneal cells in both groups had increased 48 h after induction of inflammation.

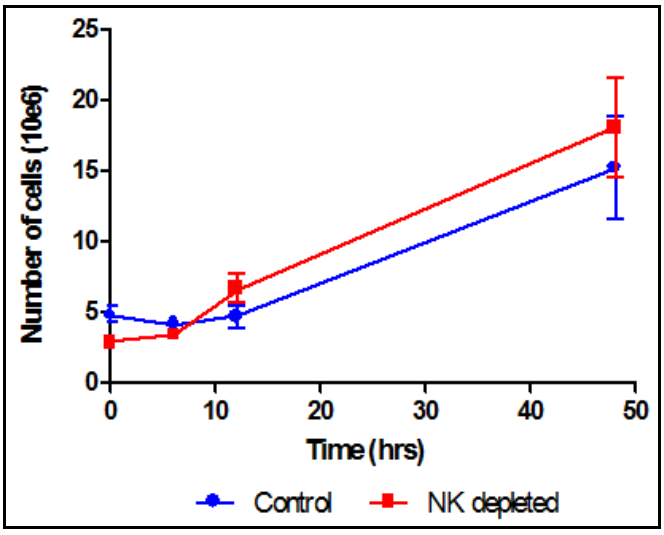

Figure 7. Effects of NK cell depletion on the number of total peritoneal cells. Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and peritoneal cells counted using Countess automated cell counter and stained with monoclonal antibodies and analysed by flow cytometer. Values are means ± SEM, n = 6-8.

4.4 Effects of NK cell depletion on the number of innate cells

4.4.1 Neutrophils

There were no neutrophils present in the peritoneum prior to peritonitis induction either in the control group or the depleted group (Figure 8). The number of peritoneal neutrophils increased after induction of inflammation and peaked in the control group at 6 h, whereas the number of peritoneal neutrophils in the depleted group peaked at 12 h and was twofold that in the control group. By 48 h the number of neutrophils in the control group had almost returned back to baseline but in the depleted group the number of neutrophils had not decreased and was almost 14 fold higher than that in the control group. There was an overall effect of the treatment with the depleting antibody (P=0.005) between the depleted and the control groups.

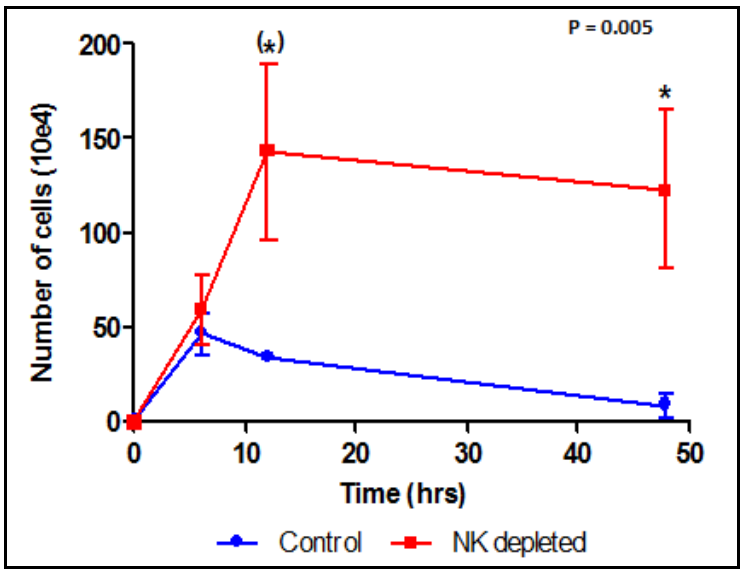

Figure 8. Effects of NK cell depletion on the number of peritoneal neutrophils. Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and peritoneal cells counted using Countess automated cell counter and stained with monoclonal antibodies and analysed by flow cytometer. P value on right hand corner represents the overall P value for the effect of treatment with the depleting antibody in both groups. Values are means ± SEM, n = 6-8. * Different from control at the same time-point, P<0.05. (*) Tendency towards being different from control at the same time-point, P<0.1.

4.4.2 Eosinophils

There were few eosinophils in the peritoneum of mice in the both groups prior to induction of inflammation, but their numbers had increased in both groups at 12 h and were further increased by 48 h (Figure 9A). At 12 h after induction of inflammation the number of eosinophils in the depleted group had a tendency towards being higher than that in the control group but their numbers were similar in both groups at all other time-points. There was no overall effect of the treatment with the depleting antibody between the two groups.

There was no difference in the mean expression levels for CD11b for the eosinophils between the two groups prior to and after induction of inflammation (data not shown).

4.4.3 Macrophages

There was no difference in the number of resident peritoneal macrophages between the control and the depleted groups (Figure 9B). The number of macrophages decreased after induction of inflammation and was lowest at 12 h but had increased again by 48 h. There was no difference in the number of macrophages between the control and the depleted groups at any time-point after induction of inflammation. There was no overall effect of the treatment with the depleting antibody between the two groups.

There was no difference in the mean expression levels for CD11b for the macrophages between the two groups prior to and after induction of inflammation (data not shown).

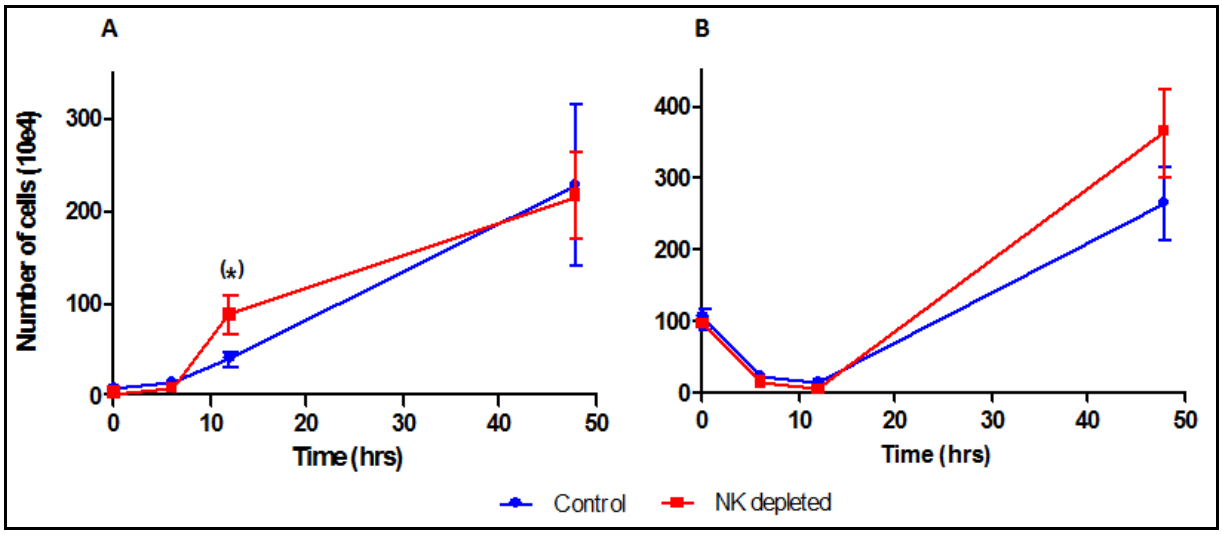

Figure 9. Effects of NK cell depletion on the number of peritoneal eosinophils (A) and macrophages (B). Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and peritoneal cells counted using Countess automated cell counter and stained with monoclonal antibodies and analysed by flow cytometer. Values are means ± SEM, n = 6-8. (*) Tendency towards being different from control at the same time-point. P<0.1.

4.5 Effects of NK cell depletion on the number of adaptive cells

4.5.1 B Lymphocytes

There was a tendency towards fewer peritoneal B cells being present and there were fewer peritoneal B cells present in the depleted group than in the control group prior to and 6 h after peritonitis induction, respectively (Figure 10A). At 48 h after induction of inflammation the number of peritoneal B cells had increased in both groups. There was no overall effect of the treatment with the depleting antibody between the two groups.

4.5.2 T Lymphocytes

There was a tendency towards fewer peritoneal T cells being present in the depleted group than in the control group prior to peritonitis induction, whereas there were more peritoneal T cells in the depleted group than in the control group 12 h after induction of peritonitis (Figure 10B). At 48 h after induction of peritonitis the number of peritoneal T cells had increased in both groups. There was no overall effect of the treatment with the depleting antibody between the two groups.

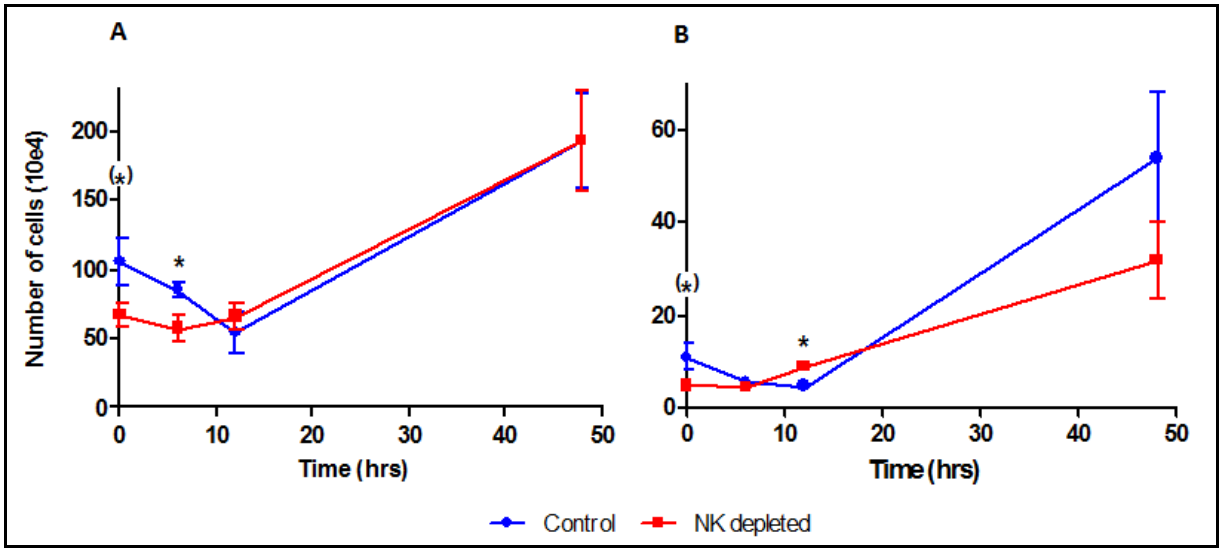

Figure 10. Effects of NK cell depletion on the number of B cells (A) and T cells (B). Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and peritoneal cells counted using Countess automated cell counter and stained with monoclonal antibodies and analysed by flow cytometer. Values are means ± SEM, n = 6-8. * Different from control at the same time-point, $P<0.05$. (*) Tendency towards being different from control at the same time-point, $P<0.1$.

4.6 Effects of NK cell depletion on NK cell receptors

4.6.1 NKG2D

Between 80-90% of the mature NK cells expressed the activating NKG2D receptor prior to and 6 h after induction of inflammation (Figure 11 B). However, at 12 and 48 h after induction of inflammation only 25-40% of the mature NK cells expressed this receptor. At 48 h a lower proportion of the mature NK cells in the depleted group expressed this receptor than in the control group. Around 70-90% of the immature NK cells (Figure 11A) and 60-70% of the most mature NK cells (Figure 11C) expressed NKG2D prior to induction of inflammation and remained relatively stable at all time-points following induction of inflammation. Less proportion of the immature cells in the depleted group expressed NKG2D than in the control group prior to induction of inflammation and the same was also true for the most mature cells 48 h after induction of inflammation. There was an overall effect of the treatment

with the depleting antibody between the two groups for the immature NK cells but not for the mature and most mature NK cells.

The mean expression levels of the NKG2D receptor were measured for the NK cell subtypes and was low (MFI=2.0) prior to the induction of inflammation for both the depleted and the control groups and did not change during the inflammation (data not shown).

4.6.2 NKp46

Between 25-35% of the mature NK cells expressed the activating NKp46 receptor prior to and 6 h after induction of inflammation (Figure 11E). At 12 and 48 h after induction of inflammation less than 15% of the mature NK cells expressed this receptor. The proportion of the mature NK cells expressing the NKp46 receptor was higher in the depleted group 12 h after induction of peritonitis and a tendency for the reverse 48 h after induction of peritonitis. Around 100% of immature NK cells expressed the NKp46 receptor prior to induction of inflammation with no change occurring during inflammation. However, the proportion of the immature NK cells expressing the NKp46 receptor was lower for the depleted group than the control group for all time-points (Figure 11D). Around 20% of most mature NK cells expressed the NKp46 receptor prior to induction of inflammation, with an increase in the control group after induction of peritonitis but no change in the depleted group (Figure 11F). There was an overall effect of the treatment with the depleting antibody between the two groups for the immature NK cells but not for the mature and most mature NK cells.

The mean expression levels for the NKp46 receptor was measured for the NK cell subtypes and was low (MFI=1.0) prior to the induction of inflammation for both the depleted and the control groups and did not change much during the inflammation (data not shown).

4.6.3 CD43

More or less all the mature, immature and most mature NK cells expressed the CD43 prior to and following the induction of inflammation (Figure 11G-11I) with no difference between the two groups at any time-point. There was no overall effect of the treatment with the depleting antibody between the two groups in all 3 NK cell populations.

The mean expression levels for CD43 was measured for the NK cell subtypes and was low (MFI=2.2) prior to the induction of inflammation for both the depleted and the control groups and did not change significantly during the inflammation (data not shown).

4.6.4 CD107a

Between 80-95% of the mature NK cells and around 60-70% of most mature NK cells in both groups expressed CD107a prior to induction of inflammation with no changes occurring during the inflammation and there was no difference between the two groups at any time-point (Figure 12B-12C). Around 10-40% of immature NK cells expressed CD107a prior to and after induction of inflammation in both groups with higher proportion in the depleted group expressing CD107a than in the control group at 12 and 48 h (Figure 12A). There was an overall effect of the treatment with the depleting antibody between the two groups for the immature NK cells but not for the mature and most mature NK cells.

The mean expression levels for CD107a was measured for the NK cell subtypes and was low (MFI=2.4) prior to the induction of inflammation for both the depleted and the control groups and rose slightly during the inflammation (MFI=6.8 at 48 h) but there was no difference the two groups (data not shown).

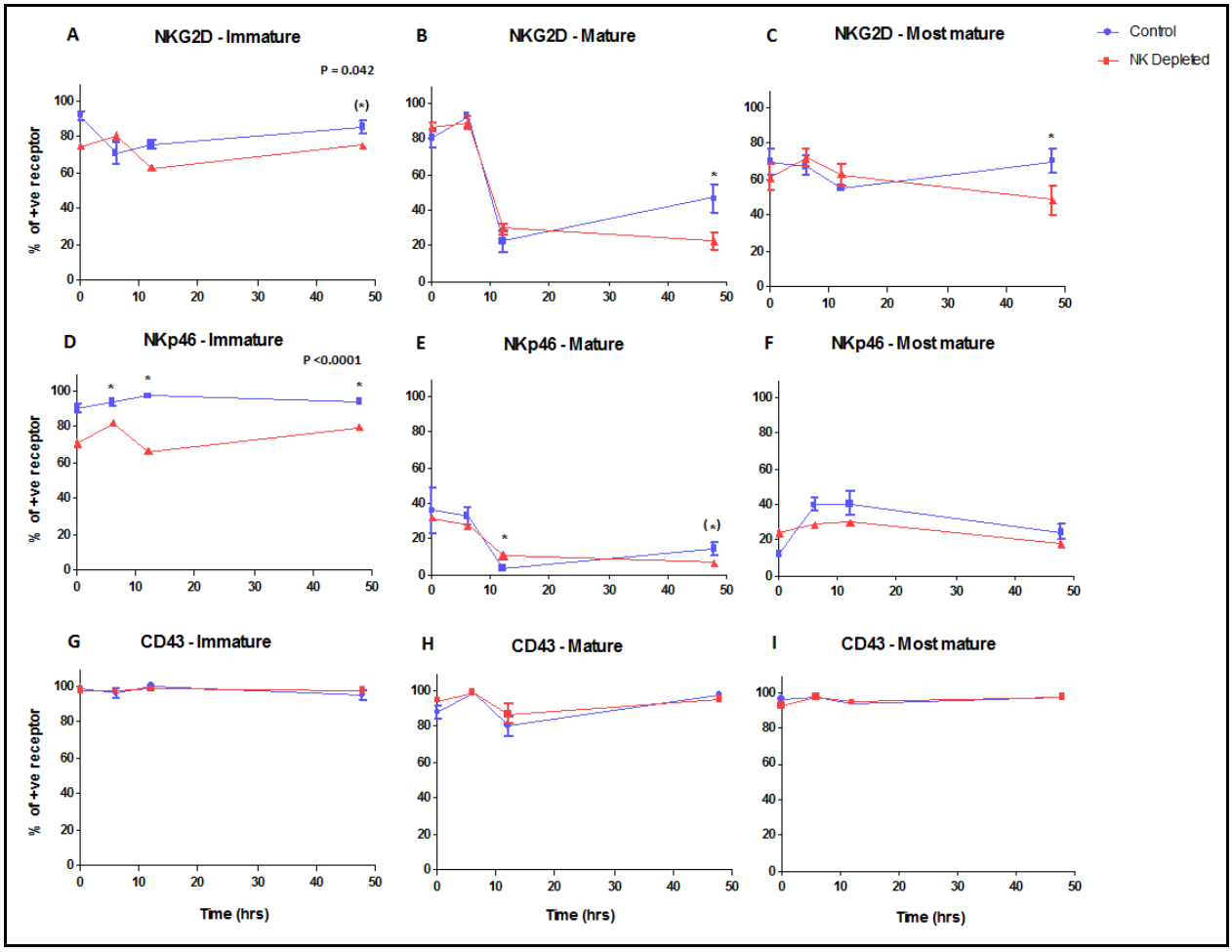

Figure 11. Percentage of positive receptors expressed on the immature, mature and most mature NK1.1 NK cells. Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and peritoneal cells counted using Countess automated cell counter and stained with monoclonal antibodies and analysed by flow cytometer. P value on right hand corner represents the overall P value for the effect of treatment with the depleting antibody in both groups. Values are means ± SEM, n = 6-8. * Different from control at the same time-point, P<0.05. (*) Tendency towards being different from control at the same time-point, P<0.1.

4.6.5 CMKLR1

Between 95-100% of the mature NK cells expressed the CMKLR1 receptor prior to and 6 h after induction of inflammation in both groups (Figure 12E). At 12 h there was a decline in the percentage of the mature NK cells expressing this receptor, but had increased again a 48 h, at which time there was a tendency towards a lower percentage of the mature NK cells expressing this receptor in the depleted

group than in the control group. Around 10-55% of immature NK cells and 45-65% of the most mature NK cells expressed the CMKLR1 receptor prior to and after induction of inflammation with no difference between the two groups at any time-point (Figure 12D and 12F). There was an overall effect of the treatment with the depleting antibody between the two groups for the mature NK cells but not for the immature and most mature NK cells.

The mean expression levels fir the CMKLR1 receptor were measures for the NK cell subtypes and was high (MFI=40.2) prior to the induction of inflammation for both the depleted and the control groups but declined dramatically during the inflammation with no difference between the two groups (data not shown).

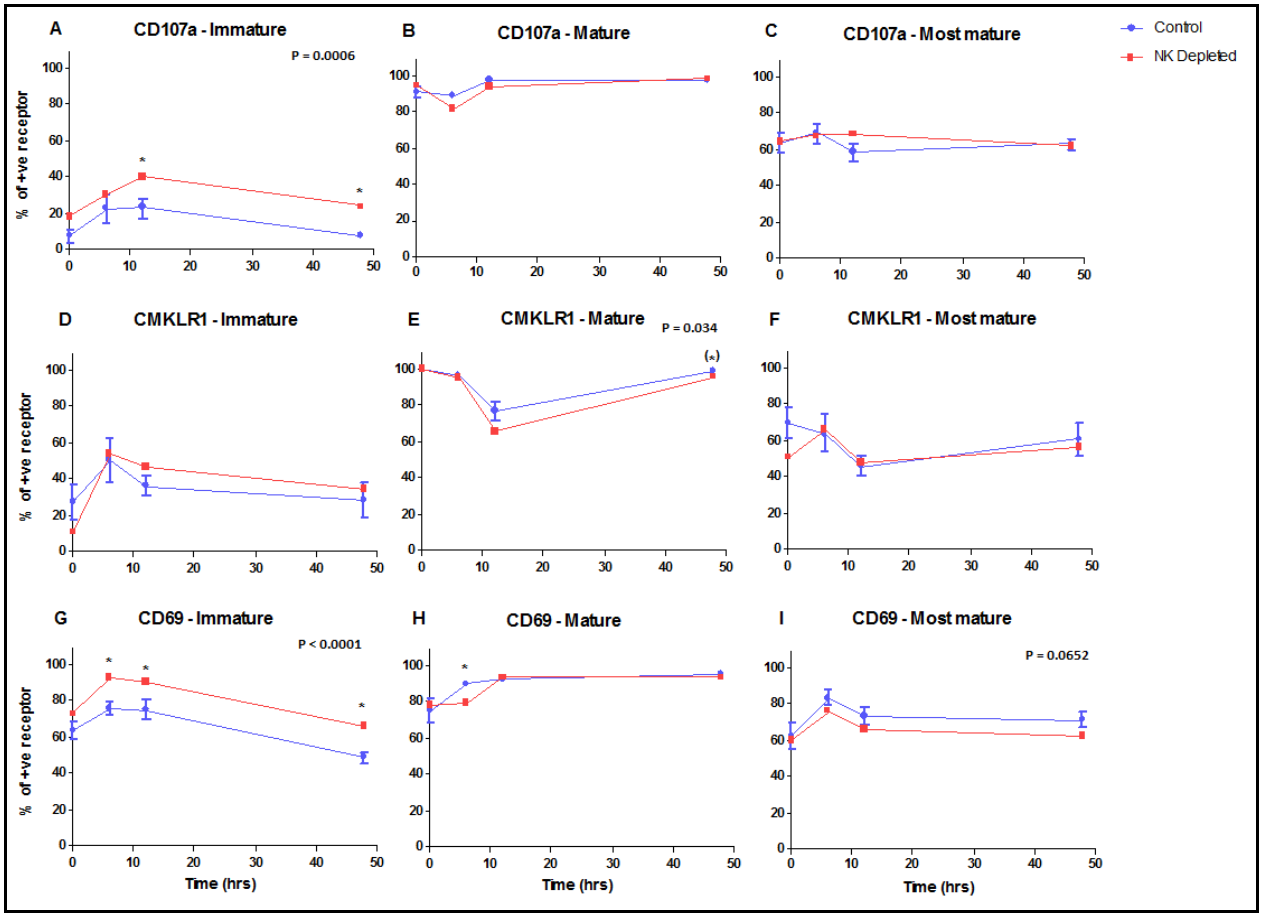

Figure 12. Percentage of positive receptors expressed on the immature, mature and most mature NK1.1 NK cells. Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and peritoneal cells counted using Countess automated cell counter and stained with monoclonal antibodies and analysed by flow cytometer. P value on right hand corner represents the overall P value for the effect of treatment with the depleting antibody in both groups. Values are means ± SEM, n = 6-8. * Different from control at the same time-point, P<0.05. (*) Tendency towards being different from control at the same time-point, P<0.1.

4.6.6 CD69

Between 75-95% of the mature NK cells expressed CD69 prior to and after induction of inflammation with a lower proportion of mature NK cells expressing CD69 at 6 h in the depleted group than the control group (Figure 12H). Around 65-75% of the immature NK cells expressed CD69 prior to induction of inflammation, with the levels increasing at 6 and 12 h and declining again at 48 h after induction of inflammation (Figure 12G). A higher proportion of the immature NK cells expressed CD69 at 6, 12 and 48 h in the depleted group than in the control group. Around 60-85% of the most mature NK cells expressed CD69 prior to and after induction of inflammation with no difference between the two groups (Figure 12I). There was an overall effect and a tendency of an effect of the treatment with the depleting antibody between the two groups for the immature NK cell and the most mature NK cells, respectively, but not for the mature NK cells.

The mean expression levels for CD69 were measured for the NK cell subtypes and was low (MFI=1.4) prior to the induction of inflammation for both the depleted and the control groups and did not change much during the inflammation (data not shown).

4.7 Effects of NK cell depletion on pro- and anti-inflammatory mediators in the peritoneal fluid

Peritoneal concentration of the pro-inflammatory mediator IL-6 was not measurable prior to induction of inflammation but rose sharply and peaked at 6 h after induction of inflammation in both the control and the depleted groups with no difference in peak IL-6 levels between the groups (Figure 13A). In the control group the IL-6 concentration had returned back to basal levels by 12 h but was still around half peak concentration in the depleted group. However, by 48 h the concentration of IL-6 was also back to basal levels in the depleted group. There was no overall effect of the treatment with the depleting antibody between the two groups (Figure 13A).

The peritoneal concentration of IL-12p40 in the control group peaked at 6 h and had returned to basal levels by 12 h (Figure 13B). In the depleted group the IL-12p40 concentration peaked at 12 h and at 48 h the IL-12p40 concentration had decreased again (Figure 13B). At 12 h the concentration of IL-12p40 in the depleted group was more than 50% higher than the peak concentration in the control group (6 h) and more than twofold its concentration at 12 h. There was a tendency towards an overall effect of the treatment with the depleting antibody (P=0.0576) between the depleted and the control groups (Figure 13B).

The peritoneal concentration of CXCL1 peaked at 6 h after induction of inflammation in the control group and was more that twofold higher than in the depleted group (Figure 13C). In the control group the CXCL1 concentration had return back to basal levels by 12 h, whereas the concentration was back to basal levels at 48 h in the depleted group (Figure 13C). There was no overall effect of the treatment with the depleting antibody between the two groups.

The peritoneal concentration of G-CSF in the control group peaked at 6 h and was close to basal levels by 12 h (Figure 13D). In the depleted group the concentration of G-CSF peaked at 12 h and was back to basal levels by 48 h (Figure 13D). At the 12 h time-point the G-CSF concentration in the depleted group had a tendency towards being higher than the G-CSF concentration in the control

group. There was an overall effect of the treatment with the depleting antibody (P=0.0426) between the depleted and the control groups.

Peritoneal concentration of the anti-inflammatory mediator IL-1ra peaked later in mice in the depleted group (12 h) than in the control group (6 h) (Figure 14A). There was a difference in the concentration of IL-1ra between the two groups at 6 and 12 h after induction of peritonitis, with the depleted group being lower at 6 h but higher at 12 h. There was also a tendency towards an overall effect of the treatment with the depleting antibody (P=0.0509) between the two groups.

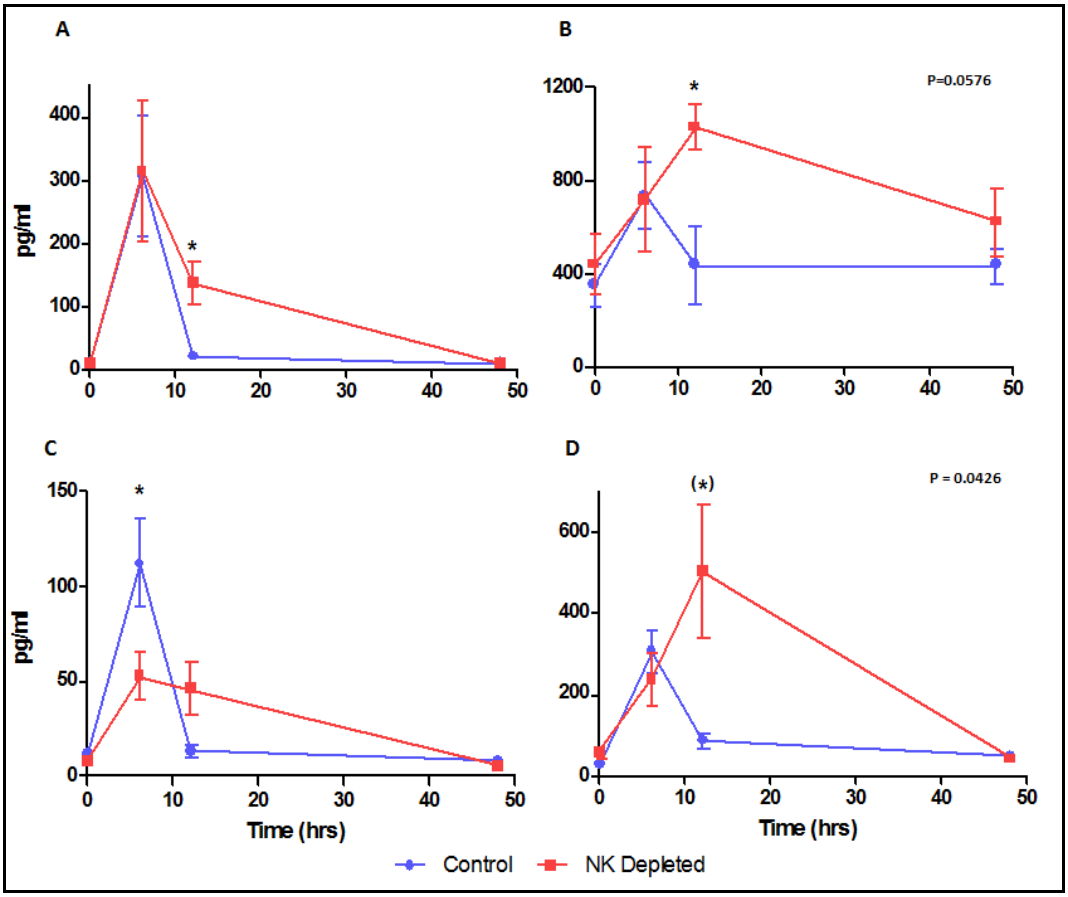

Figure 13. Effects of NK cell depletion on peritoneal concentrations of IL-6 (A), IL-12p40 (B), CXCL1 (C) and G-CSF (D). Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and concentration of the cytokine, chemokines and growth factors measured with ELISA. P value on right hand corner represents the overall P value for the effect of treatment with the depleting antibody in both groups. Values are means ± SEM, n = 6-8. * Different from control at the same time-point, P<0.05. (*) Tendency towards being different from control at the same time-point, P<0.1.

Peritoneal concentration of the anti-inflammatory mediator sIL-6R was similar in both groups prior to and 6 h after induction of peritonitis. At 12 and 48 h after induction of peritonitis the sIL-6R concentration had increased in both groups with no significant difference between the groups (Figure 14B). There was no overall effect of the treatment with the depleting antibody between the two groups.

Peritoneal concentration of TGF-β was low in both the depleted and the control groups prior to and at 6 and 12 h after induction of inflammation with no difference in the concentration between the two groups at these time-points (Figure 14C). The TGF-β concentration had increased at 48 h in both groups with a tendency towards being higher in the depleted group than in the control group. There was a tendency towards an overall effect of the treatment with the depleting antibody (P=0.0804) between the two groups.

Peritoneal concentration of IL-10 and IFN-γ was measured but the levels were below detection limits.

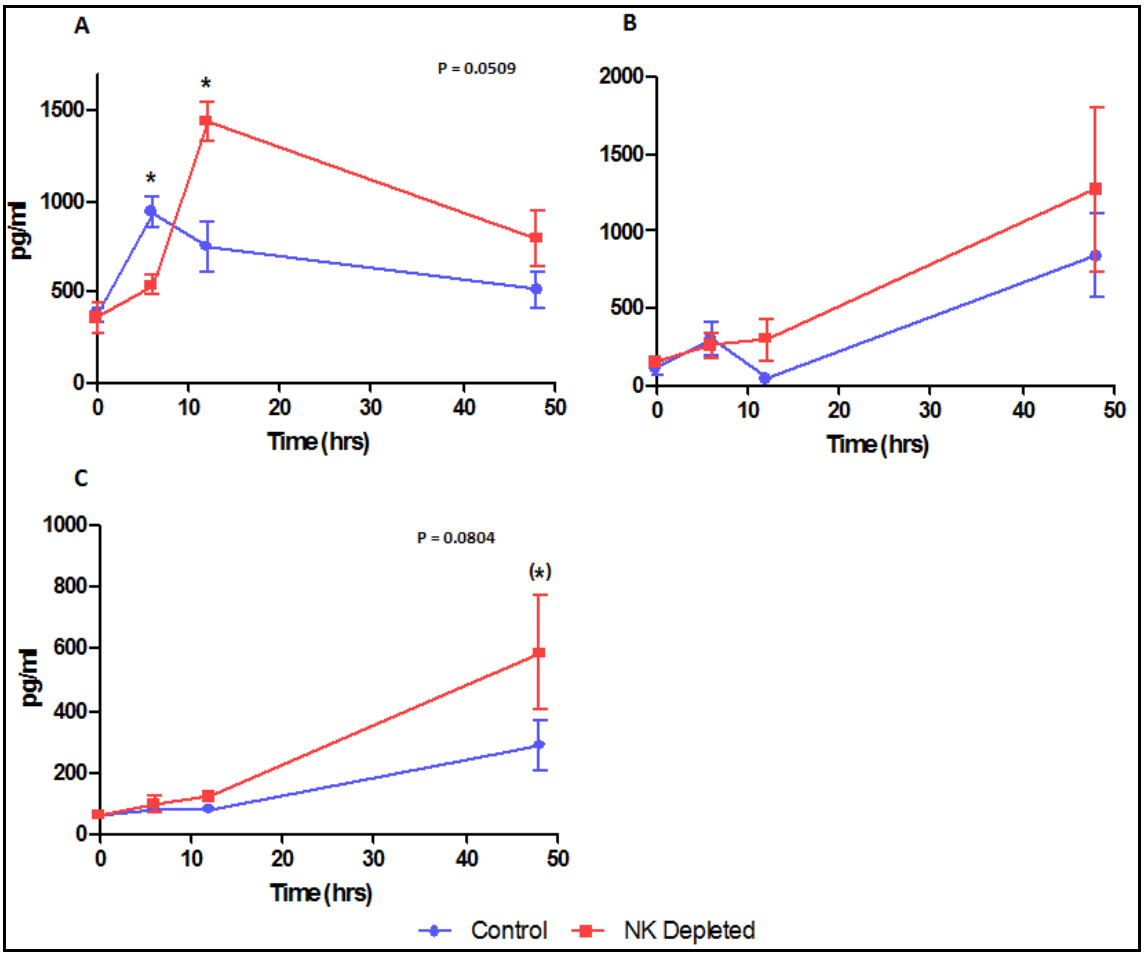

Figure 14. Effects of NK cell depletion on IL-1ra (A), sIL-6R (B) and TGF-β (C). Immunized mice were injected i.v. with either anti-asialo GM1 antibody (NK depleted) or normal rabbit serum (control) 24 h prior to induction of peritonitis with mBSA (0 h time-point). Mice were sacrificed at the indicated time-points, peritoneal fluid collected and concentration of IL-1ra, sIL-6R and TGF-β measured with ELISA. P value on right hand corner represents the overall P value for the effect of treatment with the depleting antibody in both groups. Values are means ± SEM, n = 6-8. * Different from control at the same time-point, P<0.05. (*) Tendency towards being different from control at the same time-point, P<0.1.

5 Discussion

The results from this study demonstrate that NK cells affect the inflammation process of antigen-induced inflammation and are indispensable for the resolution of the inflammation. The results showed that administration of the NK cell depletion antibody led to a 30-50% reduction in the number of NK cells in the induction phase of the inflammation. Depletion of the NK cells increased the number of neutrophils at their peak and prevented reduction in their numbers in the resolution phase of the inflammation. The increase in the concentration of pro- and anti-inflammatory cytokines in the depleted group may help explain the increased number of neutrophils.

The NK cell depletion antibody (anti-asialo GM1) did not completely deplete the peritoneal NK cells but decreased their numbers by up to 50% in the first 36 h after its injection i.v. (12 h after induction of inflammation). Others have used the anti-asialo GM1 antibody to deplete NK cells from spleen (46), lymph nodes (33), lungs (30, 33) and blood (31). In these studies the NK cells were reduced in numbers by up to 50% in the lymph nodes (33) and 30% in the lungs (33) and circulating blood (31). In order to maintain the depletion, the depletion antibody is usually injected every 4-5 days. In the current study, the effect of the NK cells early in the inflammation process was being investigated and, therefore, the antibody was not administered again and the NK cell numbers were similar in the depleted and the control groups 48 h after induction of inflammation.

Three sub-populations of murine NK cells have been described previously and in the current study all three types were observed: the immature, the mature and the most mature NK cells. The majority of the NK cells belonged to the mature NK cells ($CD11b^{+}CD27^{+}$) in the present study and they decreased in numbers similarly to that observed for the total peritoneal NK cell numbers, suggesting that they are the main subtype involved in the inflammatory process. This is in concordance with the results from the study by Haworth *et al.* showing that this subtype was the major NK cell subtype functioning in the resolution phase (33). However, in that study they did not determine the effect of the NK cell depletion on this population of NK cells. The NK cell depletion in the present study also caused a reduction in the numbers of the most mature NK cells ($CD11b^{+}CD27^{-}$) and completely prevented an increase in the number of immature NK cells ($CD11b^{-}CD27^{+}$), which was seen during the later time-points in the control group. However, as the numbers of both the immature and the mature NK cells were very low compared with the number of the mature NK cells it is doubtful that reduction in their numbers affected the inflammatory process.

NK cell depletion had a major effect on the number of neutrophils in the peritoneum. In the depleted group the number of peritoneal neutrophils continued to increase at a time when they had passed their peak and started to decrease in the control group. At that time-point (12 h after induction of inflammation) the neutrophil number in the depleted group was three times higher than in the control group. In addition, when the number of neutrophils reached pre-inflammation levels in the control group (48 h after induction of inflammation) the number of neutrophils had not deceased in the depleted group and was eight times higher than that in the control group. These results indicate that depletion of the NK cells leads to increased recruitment of neutrophils into the peritoneum during inflammation and, in addition, that the NK cells are indispensable for the clearance of neutrophils from

the inflamed site. These results are in concordance with results that have shown NK cell depletion leading to an increase in the recruitment of neutrophils to the peritoneum after septic peritonitis (31), and to the lungs during *Pseudomonas aeruginosa* infection (47). In addition, NK cell depletion was shown to up-regulate neutrophil function and cause an increase in neutrophil number during a dextran sodium sulphate-induced colitis in mice (48). Others have shown that gene knock-out mice with impaired NK cell development or function also had increased neutrophil number in their lungs during lung inflammation/infection (49, 50). However, others have shown a decrease in neutrophil number following NK cell depletion. These reports have shown NK cell depletion causing a decrease in neutrophil number in the liver during acetaminophen-induced liver injury (51), in liver and lungs during sepsis (52), in nasal-associated lymphoid tissue and cervical lymph nodes in mice receiving intra-nasal treatment with an antigen and mucosal adjuvant from *Mycobacterium tuberculosis* (53), and also impaired neutrophil recruitment to the lungs in knock-out mice during a *Mycobacterium tuberculosis* infection (54). Thus, NK cell depletion either increases or decreases neutrophil recruitment following infection/inflammation and in a recent review by Costantini *et al.* it is stated that the effect of NK cell depletion on neutrophil recruitment following infection/inflammation depends on the infection and the site of infection (55).

The increase in peritoneal neutrophil number observed in the depleted group in the present study can be attributed to signalling from the cytokines produced. The NK cell depletion led to an increase in the concentration of IL-12p40, IL-6 and G-CSF at the 12 h time-point. IL-12p40 is a well-known pro-inflammatory cytokine and has been shown to be critical for neutrophil activation during sepsis (56) and neutrophils have also been shown to be producers of IL-12 (57). Thus, the higher number of neutrophils in the depleted group could account for the higher concentration of IL-12 which may have promoted the pro-inflammatory conditions observed. IL-6 has been linked to neutrophil recruitment (58) and G-CSF stimulates survival, proliferation, differentiation and function of neutrophils (8-10). Taken together, IL-6 and G-CSF could be major players in the increased number of neutrophils 12 h after induction of inflammation in the depleted group. Together the increased concentrations of IL-12p40, IL-6 and G-CSF in the depleted group could have led to increased and prolonged pro-inflammatory conditions and mediated the increased recruitment and activation of peritoneal neutrophils.

In the depleted group in the present study, the high number of neutrophils could, in addition to being caused by an increase in neutrophil recruitment, be because of a decrease in neutrophil apoptosis and removal from the peritoneum. Increased concentration of IL-6 as well as a delay in the increase in IL-1ra concentration could both play a part in mediating this effect, as IL-6 has been shown to delay neutrophil apoptosis *in vitro* (59) and IL-1ra is an antagonist for IL-1, which can delay neutrophil apoptosis (34).

Apoptosis and removal of neutrophils from the inflamed site may also be dependent on cell to cell contact as NK cell mediated neutrophil apoptosis has been shown to be partly mediated by the NKp46 and the NKG2D receptors (32, 33). In the present study, NK cell depletion did not have much effect on the proportion of NK cells expressing different NK cell receptors, including NKp46 and NKG2D. However, the decrease in the number of NK cells also included NK cells expressing these receptors

and, therefore, cell mediated pathways for induction of neutrophil apoptosis involving these receptors would be expected to be less effective in the depleted group. The decrease in the number of mature NK cells expressing the NKG2D and the NKp46 receptors could, therefore, have contributed to the lack of removal of peritoneal neutrophils in the depleted group.

The decreased number of mature NK cells expressing CD43, CD107a and CD69 in the depleted group in the present study may have contributed to the delayed apoptosis and removal of neutrophils as these surface molecules play a role in NK cell proliferation, activation, degranulation, and cytotoxicity (60-63) and may also be involved in their function in resolution of inflammation (33). Although there was no difference in the proportion of NK cells expressing these surface molecules in the control and depleted groups the lower number of mature NK cells in the depleted group compared with that in the control group led to a lower number of cells expressing CD43, CD107a and CD69. This lower number of NK cells expressing these surface molecules could possibly have led to reduced capacity for cytotoxicity and resolution function of the NK cells, contributing to the delayed apoptosis and removal of neutrophils in the depleted group.

Resolution of inflammation is an active process and requires participation of a number of receptors and mediators. Among these are the resolution inducing lipid mediator RvE1 and its receptor CMKLR1, previously shown to be expressed by mature NK cells and NK cell depletion was previously shown to decrease RvE1s mediated resolution of allergic inflammation (33). The decreased number of mature NK cells expressing CMKLR1 in the present study could have led to less response to RvE1 in the peritoneum and, therefore, be one of the factors delaying the resolution process in the depleted group. TGF-β is one of the cytokines that is thought to play a role in resolution of inflammation. As mentioned previously, NK cells can produce TGF-β but macrophages can also be a source of TGF-β (34). In the current study, a higher concentration of TGF-β was observed in the depleted group 48 h after induction of inflammation. The higher TGF-β level in the depleted group would be expected to aid in resolving the inflammation, but that was not the case as there was no decrease in neutrophil numbers at that time-point. The higher TGF-β level in the depleted group may, however, be a result of the peritoneal cells trying to counteract the high concentration of pro-inflammatory cytokines. Such a scenario can e.g. be seen in inflamed joints of rheumatoid arthritis patients where the anti-inflammatory cytokine IL-10 is found in high levels at the same time as pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6 (64).

Depletion of the NK cells had little effect on the recruitment or clearance of the other cell types in the peritoneum. NK cells have been shown in co-culture to regulate eosinophil function by inducing their activation and apoptosis (65) and eosinophils have been shown to promote resolution of inflammation by production of pro-resolving mediators (14). In the present study, no major effect of NK cell depletion was seen on the number or activation of eosinophils, macrophages and lymphocytes and, therefore, these cells are not thought to play a key role in the NK cell depletion-induced effects on the inflammatory and resolution processes in the present study.

6 Conclusion

In conclusion, the results from this project demonstrate that NK cells recruited to the inflamed site early in the induction phase of inflammation play a major role in shaping both the induction and the resolution phases of the immune response. They seem to affect both neutrophil infiltration into the peritoneum as well as their removal during resolution of the inflammation. These effects are likely to be mediated by production of cytokines and other soluble factors as well as expression of specific NK cell receptors.

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