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A Role of IgM Antibodies in Monosodium Urate Crystal Formation and Associated Adjuvanticity

by

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Abstract

Uric acid is a danger signal released from cells during cell death, or due to injury. Uric acid crystals are able to invoke strong inflammatory responses via activation of neutrophils to produce proinflammatory cytokines and chemokines. For these pathological events to occur, uric acid must crystallize. In this report, we verified that serum from MSU crystal immunized animals is able to precipitate MSU crystals in a supersaturated solution and that this activity is mediated by MSU specific IgM antibodies. These purified antibodies mediated the uric acid adjuvant effect in B cell deficient mice and general inflammatory responses to MSU crystals.

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Dedication

I would like to dedicate this thesis to my family for their ongoing support. Their help kept me going throughout my Bachelor of Science undergraduate degree and now my Master of Science graduate degree. They are both my cheerleaders and critics, but I would not be where I am without them.

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List of Symbols, Abbreviations and Nomenclature

51Cr = Chromium – 51

APCs = antigen presenting cells

Apo = apolipoprotein

APPs = acute phase protein

ASC = apoptosis-associated speck like protein

ATP = adenosine triphosphate

BCA = bicinchoninic acid

BCP = basic calcium phosphate

BIR = baculovirus inhibitor of apoptosis protein repeat

BMDCs = bone marrow derived Dendritic Cells

BMDMs = bone marrow derived macrophages

CAMs = cellular adhesion molecules

CARD = caspase recruitment domain

CCl4 = carbon tetrachloride

CD4, 8, 11b, 11c, 14, 16, 21, 35, 40, 62L, 80, 86 = cluster of differentiation 4, 8, 11b, 11c, 14, 16, 21, 35, 40, 62L 80, 86

COX-1, -2 = cyclooxygenase-1,-2

cPLA2 = cytosolic phospholipase A2

CPPD = calcium pyrophosphate dihydrate

CTLs = cytotoxic T cells

DAMPs = damage-associated molecular patterns

DG = diacylglycerol

DNA = deoxyribonucleic acid

E:T = effector: target ratio

ELISA = enzyme-linked immunosorbent assay

ESI-MS = electrospray ionization –mass spectrometry

F(ab)2 = variable regions of antibody

FACS = Fluorescent activated cell sorting

FBS = fetal bovine serum

Fc = constant region of antibody

FcR = constant region of antibody receptor

FDCs = follicular dendritic cells

FITC = fluorescein isothyacyanate

FPLC = fast protein liquid chromatography

GM-CSF = granulocyte macrophage – colony stimulating factor

HCl = hydrogen chloride

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMGB1 = high mobility group box protein 1

HPLC = High Performance Liquid Chromatography

HSPs = heat shock proteins

HTAB = hexadecytrimethyl ammonium bromide

i.p. = intraperitoneal

i.v. = intravenous

IC = immune complex

IFN-γ = interferon-gamma

IgA, E, G, M = immunoglobulin A, E, G, M

IKK = I kB kinase

IL-1b,-2,-4,-5,-6,-8,-10,-13,-18 = interleukin-1b, -2, -4,-5,-6, -8,-10,-13,-18

iNOS = inducible nitric oxide synthase

IP3 = inositol triphosphate

IRAK = IL-1 receptor kinase

ITAM = immunoreceptor tyrosine based activation motif

KC = keratinocyte chemoattractant

LRR = leucine rich repeat

LTB4 = leukotriene B4

mAb = monoclonal antibody

MAC = membrane attack complex

MBL = mannose binding lectin

MCP-1 = monocyte chemotactic protein-1

MgU = magnesium urate

MHC = major histocompatability complex

MIP-1a, -1b,-2 = macrophage inflammatory protein -1a,-1b, -2

MPO = myeloperoxidase

mRNA = messenger ribonucleic acid

MS-GC = mass spectroscopy-gas chromatography

MSU = monosodium urate

MyD88 = myeloid differentiation factor 88

Na+ = sodium

NaCl = sodium chloride

NaOH = sodium hydroxide

NF-kB = nuclear factor-kB

NLRPs = Nod like receptor family

NO = nitric oxide

NOD = nucleotide-binding oligomerization domain

OVA = ovalbumin

PAF = platelet activation factor

PAMPs = pathogen associated molecular patterns

PBS = phosphate buffer solution

PE = phycoerythrin

PGE-2 = prostaglandin E2

PI3K = phosphotidylinositol tyrosine kinase

PIP3 = phosphotidylinositol triphosphate

PKC = protein kinase C

PLCg = phospholipase C gamma

PLD = phospholipase D

PMN = polymorphonuclear leukocytes

PRRs = pattern recognition receptors

PYD = pyrin domain

RNA = ribonucleic acid

ROS = reactive oxygen species

RPAs = RNase protection assays

RPMI = Royal Park Memorial Institute

s.c. = subcutaneous

Syk = spleen tyrosine kinase

TD = thymus dependent

TGF-b = transforming growth factor - b

TI = thymus independent

TIR = Toll/ IL-1 receptor

TLR-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11 = Toll like receptor-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11

TNF-a = tumour necrosis factor - a

TRAF6 = TNF receptor associated factor 6

UBA = uric acid binding antibody

UV = ultraviolet

CHAPTER 1: LITERATURE REVIEW

1.1 Uric acid, a danger signal released from dying cells.

Cellular death has been described under many names and has been divided into different processes (1). During apoptosis, the cell undergoes rounding-up, loss of cellular volume, chromatin condensation and nuclear fragmentation; with some of these processes regulated by proenzymes called caspases. During autophagic cell death, there is a massive vacuolization of the cytoplasm. Necrosis is divided into two categories: necrosis and secondary necrosis. During necrosis, the cell gains cellular volume, followed by the rupture of the plasma membrane and the loss of intracellular contents. Usually necrosis is seen in pathological situations in vivo. Secondary necrosis is the process which follows apoptosis, and it has the same defining characteristics as necrosis. Pyroptosis is another form of cell death, which involves caspases, specifically caspase-1 and its activating complexes called inflammasomes, and is seen in pathological situations, involving intracellular bacteria. Whether it be apoptosis, autophagy, necrosis or pyroptosis, the cell ceases to perform its everyday functions, and cell death occurs. Still, the end result of cell death is not always the same. Besides the different processes which occur within the cell during cell death, the outcome upon the immune system can be defined as immunogenic, tolerogenic or silent.

While immunogenic or tolerogenic cell death must occur within the presence of a pathogen, non-immunogenic or silent cell death occurs within its absence. However, there are instances when an immunogenic cell death is more welcome, especially when an immune response to a tumour is desired. In other situations to reduce the possibility of autoimmunity, a silent cell death would be more appropriate. To know which factors of cellular death are involved in activating the immune system would increase our

understanding in the design of vaccines, treatment of chronic conditions and tumours. Green *et al* describe the complexity of determination of the immunogenicity of cellular death when they say that the nature of the immune response depends on several factors: which type of cell dies, how it dies, where it dies, whether any recognized antigens are associated with the cell and what type of cell cleans up the remains.(1) Still, they do acknowledge that the signals from dying cells may play a role on dendritic cells and the immune system, affecting the nature of the response.

Rock and Kono have further narrowed this topic to explain how cell death may initiate an immunogenic or inflammatory response (2). In their review, they explain the difference between necrosis and apoptosis and their potential for inciting an inflammatory response. Necrosis usually occurs in response to trauma, toxins, and is usually a result of a pathological process(2). While undergoing necrosis, a cell will lose its membrane integrity and will leak its intracellular components into the periphery, which may serve as danger signals to the immune system. These molecules are also referred to as damage-associated molecular patterns (DAMPs) (1). Apoptotic cells initially maintain their plasma membrane, which prevents release of intracellular components. Once these cells are phagocytosed by macrophages, the inflammatory response may be modulated by macrophages via the production of interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (3, 4). However, if the apoptotic cells are not cleared quickly, they may begin secondary necrosis and thereby initiating the necrotic inflammatory response.

During an infection, the presence of pathogen can elicit pathogen associated molecular patterns (PAMPs), such as bacterial cell wall components (peptidoglycan) or

viral ribonucleic acid (RNA) (1, 2). PAMPs can stimulate the immune system. This is the basis of Janeway's infectious-nonself model (5), a modification of Burnet's self-nonself model (6). The idea behind the self-nonself model is that lymphocytes on their surface contain many copies of a single receptor, specific for a single foreign antigen, which when activated through antigen-receptor binding will initiate an immune response. This model also suggests that self-reactive lymphocytes would be removed from the repertoire early in life of an organism. However, this model underwent many changes in order to explain the B cell development and hypermutation, T helper cells (7) and co-stimulation with antigen presenting cells (APCs) (8). The major change in the self-nonself model came with Janeway's suggestion of the existence of pattern recognition receptors (PRRs) on APCs, which are specific for conserved PAMPs (9). When bound these receptors would activate the APC, be it a dendritic cell or macrophage. These cells can then upregulate co-stimulatory molecules and travel to nearby lymph nodes to present the captured antigens to local cluster of differentiation 4 (CD4) + T cells.

However, more recently, another model has emerged called the danger hypothesis. Matzinger described this hypothesis as a paradigm shift in the field of immunology. Specifically, the focus changes from APCs directing the immune response through co-stimulation, to the tissues themselves responding to the pathogen and activating the APCs through danger signals (5, 10, 11). This model predicts the existence of molecules, which are released during injury, disease, toxin presence or mechanical damage, that are capable of stimulating APCs, and therefore initiating the immune response. Normal, healthy cells do not release these signals. This model also states that cells undergoing normal physiological death, as seen in apoptosis, would not release the

danger signals since they would be efficiently scavenged or phagocytosed, before undergoing secondary necrosis. In a study done by Gallucci *et al*, stressed cells that were virally infected or killed necrotically release endogenous signals that activated dendritic cells, as measured by the expression of several maturation markers (B7.1, B7.2, Major Histocompatability Complex (MHC) Class II and CD 40) (12). Cells undergoing apoptosis did not stimulate dendritic cells to the same degree as necrotic cells. When APCs cultured with apoptotic cells were used to stimulate a CD4+ T cell clone, the T cells did not achieve the same proliferation stage as those treated with APCs co-cultured with necrotic cells. This study confirmed the existence of endogenous adjuvants released during abnormal cell death. The same endogenous adjuvants may also play a role in the induction of an immune response in transplant rejection, spontaneous tumour rejection and even autoimmunity.

Over the past decade, other reports have emerged in support of the danger hypothesis. Shi *et al* (13) found that injection of cells with the particulate antigen provided a strong adjuvant effect on cytotoxic T cell (CTL) response to the antigen. The adjuvant activity was investigated and seen with different cell types, mouse strains and antigens. They were able to show that when the cells were treated with an irreversible protein inhibitor, emetine, the adjuvant effect on the CTLs was not decreased, meaning that the adjuvant activity in this situation is not mediated by a secreted protein factor. Still, they did suggest that the adjuvant activity is constitutively present within the cytoplasm of cells. When they tested the cytoplasm of apoptotic cells, it had the highest adjuvant activity on cytotoxic T cell response to ovalbumin (OVA) as compared to normal, tumour, or necrotic cells. They also showed that the adjuvant effect of apoptotic

cells injected with a particulate antigen (OVA or HIV gp120 conjugated beads) is systemic as well as local. This in vivo study provides a strong platform for the existence of danger signals released upon cell death.

A further examination of this phenomenon was done by Shi and Rock in 2002 (14). They tested the adjuvant activity of UV-treated cells by co-injecting them with HIV gp120-bound latex beads. CD4+ T cell responses were assessed by the measurement of their production of interleukin-2 (IL-2) using CTLL cells. The results showed that CD4+ T cell response is enhanced using UV-treated cytoplasm as adjuvant, with a particulate or cell-associated antigen. If particulate antigen is used, then this indicates that the adjuvant may act on APCs, instead of directly on T cells or other steps involved in the generation of an immune response to soluble antigens. After testing this hypothesis, they discovered that injections of fluorescent beads with cytoplasm from injured cells into the hind flanks of the mice, induced a migration of large CD11c+ and CD11c- cells containing fluorescent beads into the draining lymph nodes. These cells also had higher levels of B7.1 and B7.2 maturation markers as compared to other cells lacking fluorescent particles. This study indicates that APCs are affected by local adjuvant activity, which with phagocytosis of an antigen can cause them to mature and migrate to the lymph node, where they come in contact with T cells and B cells, to initiate the adaptive immune response. This data provides evidence for the Danger Model (10).

Still there are several conflicts between studies done by Gallucci and Shi, that should be considered (12-15). Firstly, even though the adjuvant activity in the studies was conferred by killed or injured syngeneic cells, the type of antigen used to stimulate the immune response was different. Specifically, soluble antigen was used in the study by

Gallucci *et al* while Shi *et al* used antigen conjugated to latex beads. The use of soluble antigen raises a question regarding the source of antigen - whether the cells admixed with the antigen injections were a source of antigen, due to possible adsorption of antigen on their cell surface or if they were the source of the adjuvant. Secondly, the Danger model predicts that only necrotic cells would release the danger signals because necrosis is usually seen in pathological situations, and the cell membrane during necrosis is ruptured releasing cell contents with possible adjuvant activities. During apoptosis, cell membrane integrity is maintained and the apoptotic bodies are cleared rapidly by phagocytosis. These predictions are maintained by the Gallucci study, where cells undergoing necrosis were found to have the highest adjuvant activity (12). However, they are incongruent with the results found by Shi, where apoptotic cells had a higher adjuvant effect on CTL responses in vivo than necrotic cells (12, 14).

Rock *et al* described several other studies that support either the use of necrotic cells or apoptotic cells as adjuvants to stimulate the immune response (15). Specifically, there were several studies showing that apoptotic cells did not stimulate dendritic cells to mature in vitro (12, 16-18), did not cause the production of anti-inflammatory cytokines (19-21) and did not induce tolerance to injected antigen admixed with the apoptotic cells (22, 23). These studies suggested that apoptotic cells did not contain endogenous adjuvant activity. On the other side, there are several studies which found that apoptotic cells can stimulate dendritic cell maturation in vitro (24-30). They were also able to stimulate T cell responses in vitro (31-35) when dendritic cells were pulsed with apoptotic cells prior to experiments and in vivo when apoptotic cells were injected by themselves (36-41). Other studies showed that apoptotic cells may be even more

immunogenic than those undergoing necrosis (23, 33, 35, 40). Due to the contrast between these findings, Rock *et al* suggested that in those studies where no immunogenicity was seen with apoptotic cells, they have not released their danger signals or intracellular components; they did not undergo secondary necrosis, preventing immune system stimulation (15). Overall, this issue has not been completely discerned, due to differences in model systems and experimental conditions among many of the studies.

Several substances found in the cytoplasm of cells have been identified as danger signals or DAMPs. They can activate neutrophils, dendritic cells and monocytes, the primary cellular level of the innate and adaptive immune responses, to produce proinflammatory cytokines and co-stimulatory molecules. High mobility group box 1 (HMGB1) protein is normally found in the nucleus, bound to chromatin. This protein can regulate gene transcription (42). In 2002, Scaffidi *et al* found that when HMGB1 is released from necrotic cells it can trigger the production of tumour necrosis factor - α (TNF-α) (a proinflammatory cytokine) in monocytes in vitro (43). This same protein was found to be able to cause dendritic cell maturation and activation, acting as an endogenous adjuvant (44, 45). Another type of DAMP was identified as heat shock proteins (HSPs). In 1991, Srivastava *et al* were trying to identify tumour-released antigens, and instead found HSPs, which were capable of inducing tumour-specific immunity if injected into the mice with murine sarcoma (46, 47). The specific HSPs identified from these two studies were HSP70, gp96 and HSP90. They were found to activate the nuclear factor-κB (NF-κB), a transcription factor with a large role in the regulation of the immune response to inflammatory stimuli, in dendritic cells (48). Wallin

et al later showed that these particular molecules also have the ability to cause activation and maturation of dendritic cells and macrophages (49).

In 2003, Shi *et al* identified a molecule from the lower molecular fraction of cytosol from ultraviolet(UV)-treated BALB/c 3T3 cells, purified using High Performance Liquid Chromatography (HPLC). This molecule conferred adjuvant activity on CTL responses in vivo and stimulated bone marrow derived dendritic cells (BMDC) to rapidly upregulate their expression of CD86 (B7.2) and CD80 (B7.1) maturation markers (50). After analyzing the fraction using mass spectrometry-gas chromatography (MS-GC) and electrospray ionization mass spectrometry (ESI-MS), the molecule isolated from dying cells was identified as uric acid. To further confirm the identity of this molecule, uricase, an enzyme which specifically breaks down uric acid to allantoin, was added to the fraction eliminating the adjuvant effect on CTLs (50).

Uric acid is a normal product of purine cellular catabolism, it comes mainly from digested RNA and deoxyribonucleic acid (DNA). It is a highly ubiquitous molecule and is found in the cell cytoplasm (15). It would be considered almost counterintuitive for a molecule that would be released constantly from dying cells to have strong proinflammatory properties. Shi *et al* also revealed that the concentrations of uric acid used to stimulate higher expression of CD86 on dendritic cells matched those at which uric acid could crystallize into monosodium urate (MSU) crystals (50). When the MSU crystals were mixed with BMDC cultures, the cell surface expression of CD80 and CD86 was higher than that seen for uric acid. Shi *et al* then suggested that the crystals were the more biologically active form of uric acid in vitro. The same effect maybe be seen in vivo as well, considering that a 4 mg/mL uric acid solution when injected would produce a

highly supersaturated situation at the injection site (50). To test their prediction, they used pre-formed MSU crystals as an adjuvant with gp120 conjugated latex beads in the CTL assay, and the results showed that MSU crystals also had adjuvant activity in vivo. This activity was specific to crystalline MSU, since other crystals of allopurinol, alum and basic calcium phosphate (BCP) did not increase the expression of CD86 marker and did not have adjuvant effect on the CTL response when used with injections of gp-120 conjugated latex beads(50). This particular study built a bridge between cellular death and inflammation by linking the release of uric acid, a danger signal, during cellular death to the subsequent activation of antigen presenting cells.

1.2 Monosodium urate crystals and their effect on the innate immune system.

Monosodium urate (MSU) crystals were detected in the synovial fluid of those suffering with gout by Anton van Leeuwenhoek in the 17th century. In the 18th century, Scheele and Wollaston identified uric acid as one of the components of gout associated stones. However, it was Garrod in the 19th century, who drew an association between hyperuricaemia and gout (51). Even though gout's clinical manifestations have been described by both ancient Egyptians and Hippocrates, only in the past few decades have the cellular mechanisms behind this disease been deciphered (52). Currently, this disease is coming back into the spotlight due to increased incidence of gout in the older population (53). Gout is an autoinflammatory disorder, which is characterized by the deposition of MSU crystals within the joints and periarticular tissues (54). This condition is very painful, due to both the physical presence of MSU crystals and the following inflammation. After the initial acute attack, which is considered self-limiting, gout may become chronic. It could also develop as a complication for medical conditions such as diabetes, renal disorders, hypertension or truncal obesity. Dalbeth and Haskard described the acute attack of gout as an example of the acute inflammatory response (55). Several events, such as trauma, surgery, excess alcohol intake or drugs that alter the serum urate levels, have been identified as triggers for the acute gout attacks.

In many cases, although not in all, hyperuricaemia is observed prior to the attack (56). To increase uric acid in the serum, cell death could occur on a larger scale, as it happens during trauma, surgery or recurrent illness, releasing uric acid into the extracellular space, to allow interaction with sodium (Na) ions to create monosodium urate. These events would aid in the formation of supersaturated solution conditions

required for crystallization of monosodium urate and initiating the inflammatory response to the crystals. Still, there is evidence of other factors besides the concentration of uric acid, that can contribute to uric acid crystallization. Besides providing the uric acid as the starting material for the MSU crystals, cells associated with the innate immunity, have also been implicated in both the induction and the resolution of gout (51).

During the induction of an acute gout attack, there is histological evidence showing infiltration of the synovial membrane by neutrophils, macrophages and lymphocytes (57). However, these cells must extravasate from the circulation, to appear in the tissues. Extravasation is a process of movement of circulating innate immune cells such as neutrophils and monocytes into the tissues. The steps include: the tethering and adhesion of the circulating cell to the vascular endothelial cells, activation of the tethered cell via chemoattractant stimulus, and the migration of the cell through the vascular endothelium and the vascular basement membrane into the tissues (58). The vascular endothelial cells play a large role in the processes of adhesion and tethering, through the expression of adhesion molecules such as cell adhesion molecules (CAMs), selectins, and integrins. These molecules are responsible for binding their respective partners on the circulating cell surface, allowing the cell to slow down in the blood vessel and tether to the endothelium. For the expression of these molecules to be upregulated, the endothelial cells must be activated, which can occur in several stages (51). First, the endothelial cells can be stimulated with agonists, such as histamine, thrombin, and complement component C5a, to move the Weibel-Palade bodies (organelles found in endothelial cells, containing pre-formed P-selectin, interleukin-8 (IL-8), and von Willebrand factor) to the luminal surface, and release their components (59-61). This event results in the

incorporation of P-selectin into the plasma membrane and the release of IL-8 (a chemoattractant), allowing for the initial binding and rolling of the leukocyte to occur. The second stage of activation is the *de novo* production of additional adhesion molecules such as E-selectin, and vascular cell adhesion molecule-1 (vCAM-1) along with chemokines, IL-8 and monocyte chemotactic protein-1 (MCP-1). Interleukin-1α/β (IL-1α/β) and tumour necrosis factor-α (TNF-α) were previously found to activate the endothelial cells at this stage (62).

The quick release of the Weibel-Palade bodies to the surface on the cells in the first stage of endothelial cell activation is similar to the degranulation of mast cells upon stimulation. Getting *et al* pointed out that if endogenous mast cells were removed from a model of MSU crystal-induced peritonitis, the neutrophil influx was significantly inhibited, indicating a role for the crystal induced degranulation of mast cells in MSU crystal mediated inflammation (63). Mast cell degranulation can occur via allergen cross-linkage of immunoglobulin E (IgE) bound by FcεRI, or anaphylotoxins such as C3a, C5a, and other mast cell receptors (58). The mast cells granule contents include: histamine, TNF-α, and enzymes such as tryptase, mast cell chymase and serine esterase (important inflammatory components). These molecules once released can act on the endothelial cells and neutrophils, playing a role in the downstream cascades in the inflammatory response (64). There is a notion of a positive feedback loop of inflammation involving the MSU crystals, their stimulation of mast cells to degranulate, the mast cell-produced histamine and IL-8 stimulation of the endothelial cells and the subsequent stimulation of neutrophils to extravasate into the tissues. Once near the MSU crystals neutrophils can produce pro-inflammatory mediators to induce more cellular infiltration.

IL-1β is involved in the activation of endothelial cells and was found to be a product of MSU crystal activated neutrophils (65, 66). The proinflammatory effects of IL-1β include stimulating the production of prostoglandin E-2 (PGE-2), interleukins-6, -8, (IL-6, IL-8), tumour growth factor β (TGF β) and TNF α (67-69). Other cell types activated by MSU crystals, such as synoviocytes, macrophages, monocytes, and platelets, were found to produce TNF-α, IL-6 and IL-8, promoting the acute inflammation during gouty arthritis (70, 71). PGE-2, specifically, was produced in both the rat air pouch model and in MSU stimulated human neutrophils, but its proinflammatory role was not investigated (72, 73). Normally, prostaglandins are involved in induction of neutrophil chemotaxis, increased vascular permeability, and increased vascular dilation - hallmarks of inflammation (58). Leukotriene (LT) B4, also produced by neutrophils in response to MSU crystals, has been defined as a neutrophil chemotaxis agent (74). Besides prostaglandins and leukotrienes and other arachidonic acid derivatives, MSU crystal activated neutrophils can produce oxygen free radicals to amplify the inflammatory response (75, 76).

In depth investigation of cytokine serum levels in gout patients revealed increases in plasma interleukin-18 (IL-18), which stimulates the production of interferon-γ (IFN-γ), a cytokine that can attract and activate macrophages during the acute inflammatory response, leading to chronic inflammation (58, 77). IL-18 was also found to have a role in induction of interleukin-4,-5,-10 and -13 (IL-4, IL-5, IL-10, and IL-13) from T cells and natural killer (NK) cells (78, 79). IL-18 seems to be involved not just in the development and continuation of the acute inflammatory response but also in the compensatory anti-inflammatory response (80). The other cytokines whose levels were found to be

increased in the serum of gout patients were IL-6 and IL-8. These results are indicative of a local inflammatory response. The repeated mentions of IL-1β, TNF-α and IL-6 are not surprising because these cytokines have many redundant functions such as fever induction, synthesis of acute phase proteins by the liver, increased vascular permeability as well as T and B cell activation (58). The same cells are being continually stimulated by each other via inflammatory cytokines and the MSU crystals; these feedback mechanisms add up to the painful inflammatory response seen in gout.

Other mediators of MSU crystal induced inflammation include macrophage produced chemokines and reactive oxygen species (ROS). In one particular study, B10R murine macrophages were stimulated with MSU crystals. The messenger ribonucleic acid (mRNA) expression of chemokines macrophage inflammatory protein -1β, - 1α, -2 (MIP-1β, MIP-1α, MIP-2) and MCP-1, was measured afterwards using RNase protection assays (RPAs). Their results showed that the levels of MIP-1α, MIP-1β and MCP-1 in the macrophages were increased even after two hours of incubation with the MSU crystals (81). This evidence shows that MSU stimulated macrophages are a source of chemoattractants and act to increase the amount of cell infiltrate in the affected tissues. Another study by Chen *et al*, showed that a murine monocyte/macrophage RAW 264.7 cell line responded by upregulating it's expression of the inducible nitric oxide synthase (iNOS) within the first hour of MSU crystal stimulation (82). The results clearly indicated the increase of expression, but did not delve into the significance of this finding in MSU crystal induced inflammation. There was mention that nitric oxide (NO) produced by the iNOS is an important mediator of inflammation with functions in vasodilation, neurotransmission, tissue homeostasis, wound repair and cytotoxicity (83,

84). The literature shows that NO can have either pro-inflammatory and anti-inflammatory properties, depending on the experimental conditions.

Other studies found that the differentiation of monocyte to macrophage would have an effect on the type of cytokines produced. Landis *et al*, found that even though undifferentiated peripheral blood monocytes produce proinflammatory cytokines (TNF-α, IL-1β and IL-6), differentiated macrophages lost the ability to produce these cytokines (85). Murakami *et al* used a system of retrovirally transfected macrophages, which overexpressed IL-10 (an anti-inflammatory cytokine) to show that IL-10 produced by macrophages could inhibit the production of proinflammatory molecules (TNF-α, MIP-1α, and MIP-1β) (86). But another study led by Yagnik showed that in vitro differentiated human macrophages did not produce a significant amount of IL-10 upon stimulation with MSU crystals (87). Even though MSU crystals can induce the initial production of proinflammatory cytokines from both neutrophils and monocytes/ macrophages, it is still uncertain whether differentiated macrophages could control the ensuing response with anti-inflammatory molecules.

Besides neutrophils, mast cells, and monocytes, the innate immune system consists of an interconnecting collection of acute phase proteins (APPs) and complement. These proteins circulate in the serum as zymogens (inactive enzymes) until proteolytic cleavage exposes their active site (58). The complement system has several basic functions including bacterial lysis, opsonization of particulate antigens, clearance of immune complexes, binding to complement receptors on various cells to induce inflammation, secretion of immunoregulatory molecules. There are several ways to activate the complement system: the classical pathway (C1 complement component binds

to an antigen antibody complex), the alternative pathway (C3 complement component is cleaved via an antibody independent step and the complement component C3b binds to a foreign cell surface constituent) and the lectin pathway (mannose binding lectin (MBL) binds to an antigen). All three of these pathways use the C3 and C5 convertases, which are important checkpoints of the complement cascade. In the synovial fluid from patients with acute gout symptoms, the activity of the complement system is greatly increased as compared to the uninflamed synovial fluid (88). Other studies found that MSU crystals are able to activate the classical and the alternative pathways in vitro, leading to the generation of complement components C3a and C5a, known chemoattractants for macrophages and neutrophils (55, 89, 90).

Due to the ability of the complement system to be recruited to immune complexed antigens, there was a study to address whether the classical complement pathway was involved with binding to an immunoglobulin (Ig) or to the crystals themselves to become activated. Giclas *et al* observed that the classical pathway complement components C1, C3 and C4 were depleted in the normal human serum. They also showed that C1 was activated without the presence of IgG (91). These findings supported earlier discoveries made by Byers and Naff (92, 93). One of the functions of the complement system is the formation of the membrane attack complex (MAC) on the surface of microbes to form pores in the membrane and thereby killing the pathogen. Tarmontini *et al* studied the effect of the C6 deficiency in the rabbit model of crystal induced arthritis and showed diminished swelling, lower numbers of neutrophils, and a decrease in the intraarticular IL-8 in C6 deficient rabbits (94). This study identifies a role for the MAC complex in the induction of inflammation during an acute gout attack while supporting previous reports,

which showed that the MAC can trigger the activation of endothelial cells and the expression of IL-8, MCP-1 and platelet activation factor (PAF) (95, 96). The recruitment of the complement system adds to the arsenal of the MSU crystal induced inflammation to aggravate the synovial joint tissues.

1.3 Cellular signalling pathways activated by MSU crystals.

In an acute gout attack, many inflammatory signals are produced in the forms of cytokines, chemokines, and complement proteins, that can activate neutrophils, monocytes and macrophages. Within these cells, there are intricate signalling pathways, leading to the transcription and translation of more pro-inflammatory proteins, which are then secreted outside the cell. Over the years, the body of research on the intracellular signals and molecules activated by the MSU crystals has grown, but no single signalling pathway has emerged dominant over the rest. In 1990, Bomalaski *et al* showed that MSU crystal interaction with peripheral blood monocytes stimulates the cytosolic phospholipase A2 (cPLA2) activity (97). Phospholipases are normally involved in the cleavage of membrane phospholipids into fatty acid derivatives, which are further modified by either cyclooxygenases (COX-1, COX-2) or lipooxygenases into the lipid mediators of inflammation, eicosanoids. PLA2 activity is specific for hydrolysing the sn-2 position of membrane glycerophospholipids, freeing arachidonic acid to be further processed into prostaglandins (PGs) and leukotrienes (LTs) (98). In gout, the increased production of PGE-2 and LT B4 by neutrophils is a direct consequence of the action of increased expression and production of PLA2. To continue the theme of phospholipase involvement in the MSU crystal interaction with neutrophils, Naccache *et al* tested whether MSU crystals could stimulate the activation of phospholipase D (PLD). By looking at this pathway, they tested if hydrolysis of phosphatidylcholine and the generation of the secondary messenger phosphatidic acid would lead to the generation of diacylglycerol (DG), an activator of protein kinase C (PKC) (99). After using three different measures to test the enzyme's activity, they concluded that PLD was activated

by MSU crystals; however, this signalling pathway was not the main pathway responsible for the functional consequences of the MSU crystal neutrophil interaction (100).

Another study by the same group focused on the tyrosine phosphorylation patterns, which occur during the co-incubation of MSU crystals with human peripheral blood neutrophils (101). The results revealed a distinct pattern of tyrosine phosphorylation, specific to MSU crystals, different from that of calcium pyrophosphate dihydrate (CPPD) crystals or other particulate and soluble antigens. One protein substrate, identified as pp70, showed most phosphorylation when MSU crystals were used. When colchicine, a toxic natural product, that binds to tubulin proteins and can prevent the formation of microtubules and cytoskeleton, was used, the phosphorylation pattern was inhibited. Uric acid was shown to be an ineffective stimulator of tyrosine phosphorylation (101). The results of this study suggested that another biochemical pathway was involved in the MSU crystal neutrophil interactions.

While looking for possible MSU crystal receptors on the surface of neutrophils, Barabe *et al*, suggested the investigation of a protein named spleen tyrosine kinase (Syk). Syk has been identified in the activation of phagocytes when their Fcγ receptors were engaged, would the same protein be involved upon the binding of FcγRIIIB (CD16) during stimulation by MSU crystals (102, 103)? They showed that upon stimulation, Syk phosphorylation increased significantly, but was inhibited in cells pre-incubated with antibodies against CD16, prior to MSU crystal stimulation (104). Upon further investigation of the Syk involvement in MSU crystal neutrophil interactions, Desaulniers *et al* showed that with the use of a Syk specific inhibitor (piceatannol), the phosphorylation pattern seen before, was inhibited in a concentration dependent manner

(101, 105). They were also able to decrease the downstream events of MSU crystal neutrophil interactions, such as intracellular calcium mobilization, the activation of PLD, and production of superoxides (100, 106, 107). The main idea derived from this study, is the Syk tyrosine kinase plays a central role in MSU crystals induced activation of neutrophils, and the described downstream events, including the activation of phosphotidylinositol tyrosine kinase, (PI3K) (108, 109). Earlier, PI3K was found to be activated upon MSU crystal stimulation, but now it is apparent that Syk can be found a step above PI3K in the signal transduction pathway (105, 110).

Besides the involvement of Syk tyrosine kinase in many of the downstream signalling pathways involved in the MSU crystal induced inflammation, other kinases were investigated as well. Popa-Nita *et al* found that PKC, a serine/ threeonine kinase, that was attributed with the initiation or regulation of many neutrophil responses, was activated by MSU crystals (111). Previously, the activation of PKC was implicated in the following neutrophil responses: degranulation, activation of the oxidative burst, activation of PLD, and PLA, redistribution of actin and the downregulation of L-selectin upon chemoattractant stimulation (112-116). This study found that MSU crystals activated PKC in a colchicine-sensitive manner, that PKC modulated the tyrosine phosphorylation through regulation of Syk, and that the phosphorylation of PI3K subunit p85 was also achieved through Syk. Specifically, PKC had translocated to the plasma membrane during neutrophil stimulation with MSU crystals, indicating its activation. When a PKC inhibitor was used in conjunction with MSU crystals, the degranulation and lysozyme release seen in the MSU control was diminished significantly. Additionally, the PKC inhibitor prevented the production of an unidentified chemotactic factor secreted by

neutrophils upon MSU crystal stimulation. But the most important finding of this paper was the hierarchy in signalling events, which occur during interactions of neutrophils with MSU crystals (111). Specifically, PKC gains the central role of regulation of MSU crystal associated neutrophil responses, since it can phosphorylate Syk, which in turn phosphorylates PI3K, producing secondary messengers (phosphotidylinositol triphosphate) PIP3 and phospholipase C γ (PLCγ) responsible for generating inositol triphosphate (IP3) and DG that are required for intracellular calcium mobilization.

Other avenues of intracellular signal transduction and activation were also explored in regards to MSU crystal induced inflammatory responses. In 2005, Liu-Bryan *et al* showed that MSU crystals could illicit NO generation in chondryocytes using Toll-like receptor-2 (TLR-2) (117). They also revealed that in bone marrow derived macrophages (BMDMs) deficiency in TLR2 and myeloid differentiation factor 88 (MyD88) resulted in lowered production of IL-1β, TNF-α, keratinocyte chemoattractant (KC) and TGFβ1. They also saw a decrease in the uptake of ^{14}Carbon-labelled MSU crystals in the TLR2, TLR4 and MyD88 deficient cells. They concluded that these molecules are required for the transduction of MSU crystal-induced inflammation(118). However, the data showed that the production of cytokines and the phagocytosis function of the cells was only partially diminished, implying that there may be other signalling pathways involved in the generation of the responses to MSU crystals. This is an interesting finding because TLRs are normally associated with binding to PAMPs, such as peptidoglycan, flagellin and double stranded RNA, and their ability to bind other substances was largely unexplored. TLRs are cell surface receptors, which contain three separate domains: the leucine-rich repeats (LRR) domain, required for the binding and

recognition of PAMPs, the transmembrane domain, and the cytoplasmic Toll/ IL-1 receptor (TIR) domain, required for the downstream signalling, that eventually activates the NF-κB pathway and proinflammatory molecule expression (119). The canonical pathway of signalling involves MyD88, IL-1 receptor activated kinases (IRAKs), and TNF receptor-associated factor 6 (TRAF6). This pathway leads to the activation of IκB kinase complex (IKKs) which upon phosphorylation releases NF-κB. NF-κB translocates into the nucleus and acts as a transcriptional activator for proinflammatory genes (58).

The possible overlap seen with MSU crystal induced inflammation, a danger signal, and TLR signalling, a PAMP associated receptor, warranted further investigation. A study by Scott *et al* hypothesized that if TLR2 and TLR4 were involved in the MSU crystal signalling, an adaptor molecule for these two TLRs could act as a binding agent for the crystals (120, 121). They investigated CD14, a pattern recognition receptor, that is able to bind peptidoglycan and LPS and to efficiently present it to the TLR2 and TLR4, respectively (122, 123). It appears in two forms, either soluble or membrane bound (124). Their results from cultures with BMDMs show that even though soluble CD14 could bind to the preformed MSU crystals, CD14 deficiency did not affect the phagocytosis of the crystals. However, the ability of CD14-/- BMDMs to produce proinflammatory molecules such as IL-1β, or KC was significantly diminished. This ability was restored if the MSU crystals were coated with sCD14 prior to the exposure to macrophages (120). They also used the murine subcutaneous air pouch model to show that CD14 deficiency decreased the numbers of leukocyte and neutrophil infiltration in the exudates. IL-1β and KC concentrations were also diminished in the CD14-/- deficient mice. Their main result

showed that CD14 may be required for the propagation of the MSU crystal signal within the cell and for the production of proinflammatory cytokines.

However, another study challenged these results by doing a thorough examination of the response of all TLR, MyD88, TRAF, and TRAM deficient mice to the injection of MSU crystals in the peritoneal cavity (125). The results were surprising because they showed that MSU crystal induced neutrophil infiltration of the peritoneal cavity was the same in TLR1, 2, 3, 4, 5, 6, 7, 9, and 11 deficient mice as it was in the similarly treated C57BL/6 mice. Another negative result was that in transfected HEK cells TLR2, 3, 4, 7, 8, 9, 10 and 11 did not respond to MSU crystal stimulation when the downstream NF-κB activation was measured. One of the adapter molecules in the signal transduction pathway from the TLRs was required for the MSU crystal induced inflammation: MyD88. MyD88 was also found to transduce signals downstream of the IL-1R, indicating that IL-1 is one of the major proinflammatory cytokines acting on neutrophils in an acute gout response in vivo. However, IL-1R is not used to bind to the crystal and signal to MyD88 directly. IL-1β was found to be produced in response to MSU crystals, but now it may also play the key role in the inflammation caused by the crystals. The cells upon which the IL-1β acts on are non bone marrow derived cells, including the vascular endothelium. In this case, the IL-1R and MyD88 mechanism would activate the cells to express the tethering mechanisms for circulating leukocytes, including vCAMs, selectins and integrins. This study summarized their findings in a figure, where uric acid interacts with the Na ions in the periphery to form MSU crystals. In the next step, the MSU crystals would then activate the tissue resident cells to produce proinflammatory cytokines, including TNF-α, IL-1β, IL-6, KC, MIP-2 and others. IL-1 specifically would

act on non-bone marrow derived cells (vascular endothelium) to signal through the IL-1R-MyD88 pathway, to activate the cells and increase the neutrophil infiltration form the circulation. The neutrophils once activated by the MSU crystals, would produce a new wave of pro-inflammatory cytokines. Thus, the feedback loop of MSU crystal induced inflammation would propagate itself in the acute attack.

With IL-1 and IL-1R being identified as major regulators and mediators of the inflammatory response in gouty arthritis, many turned to the production and expression of IL-1β in the cell to understand the underlying signalling events. According to Burns *et al,* the production of IL-1β involves three steps. Firstly, pro-IL-1β protein is produced. Secondly the proIL-1β is cleaved into the active form IL-1β. Lastly IL-1β must be secreted into the extracellular environment (126). The processing of the pro-IL-1β is performed by caspase-1, which must be activated in multiprotein caspase-1 activating complex, such as an inflammasome (127, 128). Proteins of the Nod-like receptor family (NLRPs), and the apoptosis-associated speck-like protein (ASC), are usually found within the inflammasome (129, 130). Most NLRPs consist of three domains: the N terminal region, which contains a protein interaction and binding domain such as caspase recruitment domain (CARD), pyrin domain (PYD) or baculovirus inhibitor of apoptosis protein repeat (BIR) domain, a nucleotide-binding oligomerization (NOD) domain in the center, and the C terminal usually houses a leucine-rich repeat (LRR) domain (131). The NLRPs and TLR share the LRR domain in their structure, uniting them as pattern recognition receptors (PRRs), that can bind and recognize DAMPs and PAMPs. The difference between them is the location of the proteins: the NLRPs are intracellular while the TLRs are associated with a membrane (129). In order for the caspase-1 to become

activated, the inflammasome must be assembled via interaction of the CARD and PYD of the NLRP and ASC proteins, which form a scaffold where the pro-caspase-1 can bind (127).

Various signals such as bacterial cell wall component muramyl dipeptide, extracellular adenosine triphosphate (ATP), and hypotonic stress were identified as culprits of inflammasome activation (128, 132, 133). Most recently, Martinon *et al* published a report showing evidence that the NLRP3 (NALP3) and ASC protein deficiency lowered the production of IL-1β in the human acute monocytic leukemia cell line (THP-1) cells, mouse macrophages, human monocytes during stimulation with MSU and CPPD crystals as compared to the controls. Also, their findings showed a reduction in neutrophil infiltration in the murine model of crystal-induced peritonitis (134). Even though the Western blots look accurate and convincing, the enzyme-linked immunosorbent assay (ELISA) data shows that some IL-1β was secreted even with a caspase-1 inhibitor z-YVAD-fmk. This occurrence could probably be attributed to the incomplete inhibitor action. This study was thorough in their use of different cell lines to show the effects of MSU crystal in culture and in vivo, Their findings show that the inflammasome has a clear role to play in the production of the IL-1β proinflammatory cytokine during the MSU crystal induced inflammatory response. The debate regarding the signal transduction mechanisms in neutrophils, macrophages, synoviocytes, and endothelial cells when they come in contact with MSU crystals continues. It may be that all of the aforementioned signalling molecules (PLA2, PLD, Syk, PKC, TLRs, MyD88, NLRP3 inflammasome) are involved in the initiation of the inflammatory state in the organism due to the presence of the MSU crystals. However, what has not been discussed

yet is how does the ubiquitous molecule uric acid crystallize into the highly inflammatory monosodium urate crystal.

CHAPTER 2: INTRODUCTION

2.1 Uric acid crystallization reaction.

Monosodium urate (MSU) crystals have been identified as the causative agent of gout for many years. They are highly inflammatory, evidenced by the proinflammatory substances secreted by neutrophils, monocytes, macrophages, synoviocytes and vascular endothelium when they come in contact with the MSU crystals. IL-1β has been identified as the main mediator of the inflammatory response in gout, but IL-6, IL-8, IL-18, TNF-α, and MIP-1 also have an additive effect. However, for the initiation of inflammation, the crystals must first precipitate in the synovial fluid. For this condition to occur, the serum uric acid concentration must be higher as seen in many gout patients who have hyperuricaemia (56). If uric acid is released during cell death, due to an injury, surgery or chronic illness and is allowed to reach its maximal solubility at 70 μg/mL, it could be at the risk of crystallization (135, 136). However, hyperuricaemia is only 36 % associated with the incidence of gout in patients. The most likely explanation for this weak association is that these individuals have differences in the ability to nucleate and grow the MSU crystals within the joints (51). Another line of evidence shows that the rate of precipitation of uric acid solution from a neutral buffer is very slow (more than 14 days) even from supersaturated starting solutions (135, 137). Thus, it was determined that other factors, besides the concentration of uric acid may be at play in the precipitation of MSU crystals (138).

Seegmiller and Howell suggested that pH may have a role in the precipitation reaction and Roberts described the potential of the sodium ion concentration to affect the precipitation rate (139, 140). Loeb described the effect of local temperature of the synovial fluid in the joint on the development of the reaction (138). As the temperature of

the solution decreased from 37°C, the solubility of urate ion decreased, increasing the propensity for uric acid to precipitate. Depending on the local temperature, there are places in the body where the plasma can become oversaturated even at normal uric acid plasma concentrations (30-60 μg/mL) (139). Kippen *et al* confirmed the influence of temperature on urate solubility, but their results showed that the sodium ion concentrations within the physiological range would not affect the uric acid solubility. They also found that physiological pH does not have an effect on the precipitation of uric acid (141). This study was one of the first to suggest that if MSU crystals are not present in the solution, then a stable supersaturated solution can be formed as uric acid does not precipitate. Until some mechanism causes the nucleation of the first crystals, the solution will remain supersaturated (141).

Tak *et al* revealed that synovial fluids from gout patients increased the nucleation of MSU crystals from supersaturated solutions (137). They concluded that a synovial fluid component, which is not uric acid and is not dialyzable, is responsible for the nucleation of uric acid from solution. Fiddis *et al* also discussed the various aspects of MSU crystals formation including supersaturation of the solution, nucleation, which sometimes may require small foreign particles and crystal growth rate, that is dependent on uric acid concentration (136). They confirmed that the presence of nucleating agents in the synovial fluid is possible; however, nucleation in hyperuricaemic synovial fluids has not been observed (136). Many of these studies show that the initial concentration as well as other conditions are important in the nucleation of the MSU crystal. Still, the possibility of nucleating agents acting to induce crystal formation was a new trail to

discover. If these nucleating agents could be found, could the crystal precipitation and the ensuing inflammatory response and disease, be prevented?

The identification of possible nucleating agents started with the study of proteins associated with MSU crystals in the synovial joint fluid. Terkeltaub *et al* used a 2-dimensional O'Farrell gel electrophoresis to map out proteins from plasma which bind to the MSU crystals (142). They found over 30 anionic and cationic polypeptides which were crystal bound. Since the charges of the polypeptides were different, this characteristic of the polypeptide is not the sole determinant for crystal binding. Albumin, transferrin and IgG were also found to bind the crystal but not as strongly as fibronectin, fibrinogen, and C1q, C1r and C1s. The results revealed that if MSU crystals could bind to C1, the initiator of the classical complement cascade without binding to IgG on the crystal, then the complement cascade could be activated in the absence of antibody (142). Another report from Terkeltaub *et al* found that apolipoproteins A-1, B and E were bound to the MSU crystals (143). Specifically, they also described an inhibitory effect on neutrophil activation of the MSU crystal bound by apolipoprotein (Apo) B.

The idea of crystal-bound proteins modulating the immune response to MSU crystals was investigated when the changes in protein coating of MSU crystals were measured during periods of active and resolving inflammation in the joints of gout patients and the subcutaneous rat air pouch model (144). During the inflammatory stage of joint-inflammation, the MSU crystals were coated mostly with IgG, but during the noninflammatory stage, Apo B and albumin were the main coating components of the MSU crystals. Other studies also described the binding of immunoglobulins (Igs) to MSU crystals, which could regulate the crystal and cell interactions as well as the

pathophysiology of the acute inflammatory response (145-147). Bardin *et al* were able to demonstrate using immunoelectron microscopy that IgA, IgM and IgG were found in the coating of the synthetic MSU crystals (148). The IgG molecule was bound to the crystals in an antigen-antibody (immune complex) formation, with its Fc fragments functionally available, confirming the results of another study by Kozin and McCarty (149). This finding may predict a functional role for the IgG coating in MSU crystal induced inflammation. Immunoglobulin structures were also found to bind MSU crystals by Gordon *et al* and Hashimoto *et al* discerned the amino acid sequence of the α, γ, and μ immunoglobulin heavy chains and κ light chain on MSU crystals and defined them as major co-precipitating matrix proteins (150, 151).

With antibodies binding to the crystal surfaces and potentially acting as modulators of the inflammatory response, the questions of antibody specificity to the crystals and their function arose. These questions led to an investigation by Kam *et al*. They hypothesized that certain antibody populations are amplified due to exposure to MSU crystals (152). The idea behind their experiments was that the presence of macromolecular molds specific for MSU crystals, would stabilize the initial crystal nuclei, lower the activating energy required for crystal formation, and catalyze further crystallization of the MSU. This same idea has been demonstrated before with other pathological crystallizations (153, 154). The results demonstrated that IgG samples isolated from synovial fluid of gout patients were able to increase the formation of MSU crystals from a supersaturated solution of monosodium urate. Also, after immunizing rabbits with MSU crystals, they developed specific IgG antibodies, that could catalyze crystal nucleation in vitro to a much larger extent than their pre-immune IgGs. This study

revealed that since urate is a small molecule, when bound by an antibody, it would not be available for the formation of sodium urate, preventing crystal lattice formation. However, in a supersaturated solution, the monosodium urate molecules can form an aggregate, resembling the mature crystal. If this aggregate is bound by the specific antibody, the antibody may act as a nucleating factor for the crystal (152). They concluded that MSU crystals can trigger the development of MSU crystal specific catalytic antibodies, capable of stabilizing MSU crystal nuclei. In a subsequent report, Kam *et al* were able to collect serum from rabbits injected with MSU, magnesium urate (MgU), or allopurinol crystals. The serum collected from rabbits injected with MSU crystals, when added to a supersaturated solution of uric acid, showed a higher precipitation rate of MSU crystals from solution. However, it did not have the same effect on the supersaturated MgU or allopurinol solutions. All of the serum collected had a higher precipitation rate in the solution of the immunizing crystal (155). These results indicate the presence of very specific antibodies, capable of binding to a crystal lattice and act as nucleating agents in a supersaturated solution.

2.2 The role of antibodies in the inflammatory reaction to MSU crystals.

Due to the presence of MSU crystal specific antibodies in the serum of the rabbits immunized with the MSU crystals, there is a possibility of formation of crystal antibody complexes, which would resemble antigen antibody complexes, also known as immune complexes (IC) (155). Previous research had shown that serum IgG could bind synthetic MSU crystals and had its Fc fragments available for further binding (148, 149). If immune complexes do form, they may trigger phagocytosis of the IC, the release of lysosomal enzymes, and the production of oxygen reactive compounds (156). They are deemed inflammatory and may be involved in causing tissue injury as seen in immune complex diseases: glomerulonephritis, arteritis, rheumatoid arthritis and others. Immune complexes can bind to the macrophages and polymorphonuclear (PMN) leukocytes via Fc receptors or through complement interaction with the immune complex and complement receptors on the cell. However, only the interaction through the Fc receptor can begin the endocytosis of the immune complex, seen with IgG immune complexes (157, 158). Furriel *et al* specifically showed that both IC with the IgM type and IgG type antibody were able to bind to rabbit neutrophils, but only ICs with IgG type antibody were phagocytosed (156). In gout, the immune complexes could form in the synovial fluid. The immune complex formation in the perivascular space could increase vascular permeability, cause the influx of PMN leukocytes, and tissue necrosis (159). Once PMN leukocytes are activated by the ICs, they would begin to release proinflammatory cytokines and arachidonic acid derivatives (prostaglandins, leukotrienes), aggravating the inflammatory response by activating the endothelium. But ICs could also cross-link Fc receptors on macrophages and mast cells, causing phagocytosis and the release of mast

cell granules containing histamine (160, 161). The possible existence of ICs in the joints could potentially increase the inflammatory potential of MSU crystals.

The ability of antibodies to act as nucleating agents for inflammatory MSU crystals is interesting, since it would seem that anyone who had MSU crystals in their joints would then develop MSU specific antibodies, in turn amplifying the inflammatory response of the immune system. However, clinical studies show that not every patient with hyperuricaemia develops gout and MSU crystals can be found in asymptomatic joints (56). The difference between patients who have gout and those who do not may be the ability to generate the specific nucleating antibodies. Crystals are not the common type of B cell activating antigens. Normally, antigens bound by B cells can be divided into thymus dependent (TD) antigens, and thymus independent (TI) antigens depending on whether the B cell can become activated with or without CD4+ T helper cells respectively (58). TD antigens are soluble proteins, able to induce isotype switching, affinity maturation and immunological memory, but they are very specific. TI antigens are further divided into two types. Type 1 TI antigens are polyclonal B cell activators or mitogens, such as bacterial cell wall components (lipopolysaccharide, LPS); they can activate B cells without B cell antigen specificity. They do not induce isotype switching, affinity maturation or immunological memory. Type 2 TI antigens can be polymeric proteins, or capsular polysaccharides, with highly repetitious components, which can be recognized by B cells with specific antigenicity. They do not cause affinity maturation or immunological memory. Still, type 2 TI antigens may induce limited isotype switching from initial IgM configuration to other antibody isotype (58). MSU crystals would belong to the TI type antigens, but more research would be needed to determine whether they

can act as mitogens and activate many B cells, regardless of their antigen specificity or cell maturity, or if they are more specific to particular B cells. However, it would take some time for lymphocytes to connect with the crystals and become activated, but the crystals have to precipitate using the antibody before they can be used as antigen. Does this incongruence predict the presence of antibodies in the serum prior to B cell activation, which can be used as precipitating matrices by the MSU crystals?

Both mice and humans contain ‘natural’ antibodies in their serum (162). These antibodies, mostly of the IgM isotype, are polyreactive with a variety of self and foreign antigens, but they compensate for their low affinity by the pentameric structure of the IgM antibody which increases their binding avidity (163). IgM is also a complement activator: a single molecule of IgM can bind to C1q and activate complement while 1000 molecules of IgG are needed to do the same (164). These natural antibodies are secreted without any antigenic stimulation, by long-lived, self-renewing B1 cells (165). B1 cells differentiate during fetal and neonatal development and localize in the pleural and peritoneal cavities, which is different from the conventional B2 cells (166, 167). Another difference is B1 cells have a restricted repertoire of antigens, which include phylogenetically conserved structures like nucleic acids, heat shock proteins, carbohydrates and phospholipids (162, 166, 167). Natural antibodies have several roles in the immune system (163). These roles include confinement of a bacterial or viral infection locally, directing the immune complexes to the secondary lymphoid tissues, and enhancement of the humoral, antibody response through binding to the follicular dendritic cells (FDCs) using complement receptors CD21/CD35, and complement activation (168-175). Even though a high proportion of natural IgM antibodies are self-

reactive and may have a role in antigen binding and immune complex formation with complement activation to stimulate a further autoantibody response, the exact role of the autoreactive IgM in the subsequent antibody response has not been investigated (162, 166, 167, 176).

The presence of these natural IgM antibodies, with low antigenic specificity and affinity but high avidity, could potentially have a role in the precipitation of MSU crystals from the supersaturated solution. The immune complexes also have some overlapping inflammatory functions with the MSU crystals, such as activation of the endothelial cells to produce adhesion molecules, recruitment of neutrophils in the tissues where they are deposited, activation of neutrophils to secrete proinflammatory cytokines and lipid derived mediators and activation of the complement cascade. It could be possible that these antibodies not only act as precipitating agents for the MSU crystals but also have a larger role in mediating the inflammatory responses to the crystals in an acute gout attack. The following ideas should be investigated to explain specific questions:

1. Do antibodies regulate the adjuvanticity of uric acid or MSU crystals in vivo?

2. If antibodies to MSU crystals can be created and act as precipitating agents in a uric acid supersaturated solution, what isotype are these antibodies?

3. What is the mechanism of antibody associated MSU crystal formation?

4. Do antibodies play a role in the inflammatory responses to MSU crystals?

The main hypothesis for this project is that if antibodies generated against MSU crystals, can bind and precipitate MSU crystals, the formation of the immune complexes would stabilize the crystal structure, activate the innate immune system and modulate the inflammatory response to the MSU crystals. By understanding the mechanisms of inflammation due to MSU crystals and the role of antibodies in these conditions, quick diagnostic tests, preventative measures and more effective and less toxic treatments can be devised (53, 177).

CHAPTER 3: RESULTS

3.1 Materials and Methods (reproduced with minor changes from the Journal of Immunology manuscript)

3.1.1 Mice, cells and reagents

All mouse strains were housed at the University of Calgary Animal Resource Centre under protocols approved by the University of Calgary Animal Research Review Committee. MuMT male and female mice were purchased from Jackson laboratories. Both male and female mice were used in the experiments when available. Control IgM antibodies were either purchased from Sigma or produced from similar MSU negative staining mAb hybridomas. CD62L (PE) and CD11b (FITC) antibodies were from Ebioscience. Secondary goat anti mouse IgG + IgM (H+L, 115-095-068), IgM (mu chain, 115-095-020), and F(ab')2 (115-096-146) antibodies were purchased from Jackson ImmunoResearch. IgM fragmentation kit was from Pierce. Secondary antibody for tissue section staining was from Vector Labs. SIINFEKL peptide was a gift from Dr. Kenneth Rock of University of Massachusetts Medical School. ^{51}Cr label was freshly ordered for each experiment from MP Biomedical. C3 complete ELISA kit was purchased from Kamiya Biomedica. All myeloperoxidase (MPO) measurement reagents were made in our laboratory from chemicals (all from Sigma). Dendritic cell cultures, cell lines, all other cytokine kits, antibodies, and reagents used in this work have been described previously (178). MSU crystals were produced as previously described (50). Briefly, 4 mg/ml uric acid (Sigma) was dissolved in 0.1 M borate buffer by continuously adjusting the pH to 8.0. The solution was filtered, the crystals precipitated after 7 days were washed twice with absolute alcohol, once with acetone, and air dried in a tissue culture hood prior to use. The fine structure of these crystals was depicted with electron

microscope in a separate report (179). Their immune activation effects in various cell types were not affected in TLR4 or other TLR deficient mice (50, 125, 179).

3.1.2 Immunization, monoclonal antibody production and HPLC immunoglobulin purification

C57BL/6 (B6) and Balb/c mice were immunized with 1 mg of suspended MSU crystals in PBS without any adjuvant, and were boosted with the same dose biweekly for 2 months. Anti-sera were collected at this time point for the FACS analysis. Tail blood (>100 ul) was mixed with 1 ul heparin and spun in a micro centrifuge to remove blood cells. Splenocytes were then harvested and fused with A3A tumour cells using a standard hybridoma protocol (180), with 10^7 tumour partners fused with 4 x 10^7 splenocytes. Between weeks 2 and 4, 25 ul of cell supernatants from growing hybridomas were used to stain 500 ug of MSU crystals to determine the binding. All clones with moderate to high binding were kept, along with several negative binding IgM controls.

(These experiments were performed by Dr. Yan Shi, Karen Dresser and Shelly Galusha)

3.1.3 Cytotoxic T cell lysis assay

C57BL/6 or muMT mice were s.c. immunized with 5 ug of OVA coated 1-um diameter latex beads in 100 ul PBS as previously described (50). For those that received the antibody infusion, 500 ug purified antibodies were injected twice via tail vein on the same day prior to the immunization. The immunized mice were rested for 7 days and were then sacrificed, and 5 x 10^7 splenocytes were stimulated in 10 ml of culture media in the presence of 10^{-7} M of SIINFEKL peptide. 5 days later, 5000 ^{51}Cr labelled EL4 cells pulsed with 10^{-6} M of SIINFEKL were used as target at the effector :

target (E:T) ratios indicated. Unpulsed EL4 cells were used as a background control, which produced negligible reading.

(These experiments were mostly performed by Dr. Yan Shi with antibody injections done by Uliana Kanevets)

3.1.4 Hybridoma culture, typing, fragmentation and purification

B cell hybridomas were cultured in RPMI with 10% FBS plus 1 mM of HEPES, 25 μM of 2 mercaptoethanol, penicillin/streptomycin antibiotics and HAT (hypoxanthine/ aminopterin/ thymidine) media. As a standard operation, the monoclonal antibody from the supernatant was isolated from 800 ml of the supernatants collected. An equal volume of the supersaturated solution of ammonium sulphate was slowly added to the supernatant to precipitate the antibody. The mixture was centrifuged and only the precipitate was collected and dissolved using 30-50 ml of PBS. This solution was then dialyzed 3 times against PBS overnight. The solution was then analyzed using HPLC. The HPLC analysis was performed with a Shimadzu Prominence system with a CBM-20-A controller, a LC-20AB fluid pump and an SPD-M20A diode array. 500 μL of the antibody solution was injected and analyzed on an Alltech Macrosphere GPC 300A 7u column (Alltech 88181) with an isocratic buffer containing 10 mM phosphate and 120 mM NaCl at pH 7.2. The samples were run at a rate of 1 ml/min over 40 min. The fractions collected were tested for the ability to bind MSU crystals and then the active fractions were collected. This mixture was then further subjected to concentration via centrifugation with a Macrosep 300 K filter (Pall Filtron). The leftover fraction was tested for protein concentration using the BCA protein assay kit (Pierce) and then reconstituted to the final concentration of 2 mg/ml. In some assays, the antibodies were

further purified with an UNO 12 column (BioRad) with a BioRad FPLC, with pH 9.6 10 mM ethanolamine as Buffer A and Buffer A plus 1.5 M NaCl as Buffer B, resolved at a rate of 1 ml/min in 25 minutes from 0% Buffer B to 80% Buffer B. IgM fragmentation and the associated analysis were performed with a kit and instructions from Pierce (ImmunoPure IgM Fragmentation Kit)

(Hybridoma cultures, HPLC analysis and antibody isolation were performed by Uliana Kanevets. Dr. Yan Shi performed the antibody fragmentation experiment)

3.1.5 Flow cytometric analysis of MSU crystals

The flow analysis for MSU crystals was developed in our laboratory by modifying standard cell-based FACS analysis. In the MSU crystal binding FACS assay, 100 ul of 1 mg/ml MSU crystal suspension was mixed with 100 ul monoclonal antibody (mAb) supernatant or 1 ug of purified mAb (or as indicated otherwise) and incubated at room temperature for 20 min. The crystals were then washed twice with PBS and incubated with 0.5 ul of 1 mg/ml 2nd antibody (FITC) in 100 ul for 10 min. The crystals were then washed again before FACS analysis. On a BD FACScan (BD Biosciences), MSU crystals showed a smaller size (about 5 - 10 % on a linear scale) on the forward scatter, and higher granularity (one to several folds higher on a linear scale) on the side scatter. Typically, 40,000 to 50,000 total events were collected with Cell Quest, and data were analyzed with Flowjo (Tree Star). Xanthine crystals were produced by first incrementally adding 1 N NaOH into 5 mg/ml xanthine suspension to dissolve the powder at high pH. Once completely dissolved, 1 N HCl was added to bring pH back to 7.0. The crystals thus precipitated were washed in 95% ethanol twice, and once in acetone. The crystals were dried prior to use.

(Initial experiments with MSU crystal binding were performed by Dr. Yan Shi and Karan Sharma, the latter experiments of testing hybridoma culture HPLC fragments for MSU binding were done by Uliana Kanevets. MSU and xanthine crystals were precipitated by Dr. Yan Shi)

3.1.6 Uric acid precipitation assay

A supersaturated uric acid solution (1 mg/ml) was incubated with various concentrations of a control protein (ovalbumin) and a purified (both by sizing and anion exchange column, about 99% pure by HPLC analysis) antibody, either UBA 11, UBA E6, UBA fragment or a control in 1 ml at the indicated concentrations for 6 hours. The supernatants were removed and wells flushed. 1 ml of 0.01 N NaOH solution was added to the wells, and 20 ul of the solution was added to 1 ml of water and read at 292 nm with a spectrophotometer. The conversion to the quantity of uric acid was achieved using a standard curve for UV absorbance from a set of uric acid solutions with known concentrations:

Y (uric acid in ug) = 0.0766 X (UV 292 absorption) + 0.0298; R^2 for the curve is 0.9937.

(These experiments were performed by Dr. Yan Shi)

3.1.7 Serum uric acid measurement

C57BL/6 (B6) and muMT mice were injected i.v. with 500 ug of indicated antibodies or PBS for 3 days, with or without the co-injection of 1 mg of soluble uric acid each day. 24 hours after the last injection, blood from mice was collected into vials with 1 ul of heparin and serum was isolated via centrifugation. 2 μL of serum was mixed with 198 μL of Buffer A which contained 10 mM Ethanolamine and 120 mM NaCl adjusted to

pH 9.6. This mixture was passed though a nanosep 30 K filter (Pall Filtron), which would allow the uric acid in the sample to pass through but would prevent possible protein contaminants from the sample. 100 μL of the sample was then injected into the HPLC. Uric acid was resolved on a PolyWAX LP column (The Nest Group) by a gradient of 0- 60% B (10 mM Ethanolamine and 1.12 M NaCl adjusted to pH 9.6) at 0.5 mL/min over 15 min. The HPLC analyses with EZ-start software were done at UV 292 nm. Peak area at 292 nm as a percentage of total area of the sample was used for the calculation of uric acid levels in the serum to minimize run to run or injection volume variability as reported previously (178).

(The i.v. injections, blood collection, HPLC sample preparation and analysis were performed by Uliana Kanevets)

3.1.8 Myeloperoxidase measurement

Myeloperoxidase measurements were performed by a method adapted from Bradley et al. on lung tissue isolated on day 4 from mice injected with antibody and uric acid solution (181). The tissue was homogenized with a 50mM potassium phosphate buffer, which contained 0.5 % hexadecytrimethyl ammonium bromide (HTAB; pH 6.0). The samples were then vortexed and the supernatant was centrifuged for 4 minutes at 5000 rpm. 7 μL supernatant samples were placed in a 96 well plate and 200 μL of 0.167 mg/ml o-dianisidine hydrochloride solution containing 0.0005 % (w/v) of hydrogen peroxide was added and the changes in absorbance at 450 nm were measured using a microplate reader. The absorbance measurements were then converted to MPO units. The myeloperoxidase values were generated by multiplying the UV reading by a factor of

0.2528 per a standard protocol. One MPO unit of activity is defined as amount of myeloperoxidase required to degrade 1 micromole of peroxide per minute at 25 °C.
(The MPO measurements were performed by Uliana Kanevets)

3.1.9 Complement C3 reading

The blood from mice intravenously injected with antibody and uric acid was isolated using the cardiac puncture technique, heparinised and spun down for 10 minutes at 13,000 rpm to collect serum. The serum samples were then prepared according to the manufacturer's protocol (Mouse Complement C3 ELISA kit, Kamiya Biomedical). The absorbance at 450 nm was measured using a microplate reader. The absorbance was then converted to C3 concentration in mg/ml using a standard curve fitting.
(The C3 measurement procedures were performed by Uliana Kanevets)

3.1.10 Neutrophil activation

Blood samples were collected from mice injected with UBA E6 or control antibody as in the serum uric acid reading, and were subjected to hemolysis treatment and then stained with CD11b (FITC) and CD62L (PE) antibodies. The samples were then analyzed with flow cytometry.
(Blood sample collection and FACS analysis were performed by Uliana Kanevets)

3.1.11 Statistical analysis

All results were reproduced in at least three independent assays. All error bars are one standard deviation of the test sample groups. Student T test (2 tail) was used to produce the p values shown in the graphs.
(The statistical analyses were performed by Dr. Yan Shi and Uliana Kanevets)

3.2 Results

3.2.1 The effect of B cell absence on uric acid adjuvant effect.

With previous research suggesting that serum from animals immunized with MSU crystals was able to precipitate a supersaturated uric acid solution specifically, the conjecture that antibodies could be involved in the binding of monosodium urate crystals seemed plausible (152, 155). To test the above-mentioned hypothesis, B cell deficient mice were used (182). These mice lack a protein domain in the Ig μ chain; therefore, they do not produce functional antibody. They are also impaired in the development of B cells, but are responsive to antigen challenge aimed at innate immunity and T cells. Using these mice, we tested whether B cells and antibodies were important in the effect of uric acid adjuvanticity. B cell deficient (muMT/ IgH) and wildtype (C57Bl6) mice were compared in cytolytic T cell activity after injection of antigen mixed with a specific adjuvant-like solution. The CTL assay procedure has been previously published (13, 50). The mice were immunized subcutaneously with OVA coated latex beads, which were mixed with three adjuvants: phosphate buffer solution (PBS), a supersaturated uric acid solution or MSU crystal solution. After 7 days, the splenocytes from the immunized mice were harvested and stimulated with the peptide epitope SIINFEKL (a CD8 T cell OVA epitope). Five days later, these same cells were used to target epitope pulsed, Cr-51 containing EL4 cells. Figure 3.1 shows that cytotoxic T cells from wildtype mice or antibody producing mice elicited an immune response to the target cells when uric acid or MSU crystals were used as adjuvants. B cell deficient mice, whose serum lacks antibody, failed to respond in a similar manner. When uric acid solution was used as adjuvant there was no response from the muMT CTLs. One possible reason for the absence of the

adjuvant effect may be that the uric acid is not recognized by the immune system. MSU crystals, when used as adjuvant, were able to elicit a slightly higher response than when uric acid solution was used in immunization. However, when compared to the response of wildtype CTLs, the muMT CTL response was significantly lower.

The following experiment was able to test the MSU crystal binding ability of serum from wildtype and muMT mice. Figure 3.2 shows that the serum from immunized muMT mice could not bind to MSU crystals in the MSU crystal binding FACS assay. This assay involves the use of MSU crystals as targets to which the serum or purified hybridoma antibodies are added. After a 20-minute period of binding, the secondary fluorescently tagged antibody is added, cross-recognizing the heavy chain of the IgG and IgM molecules. This antibody allows us to measure the binding between the serum and the crystals. The serum from MSU crystal immunized wildtype mice, containing antibodies, could bind to the crystals. Serum isolated from MSU crystal immunized B cell deficient mice showed no positive binding to the MSU crystals. This data indicates that antibodies can be produced by the immune system which can bind MSU crystals in the body.

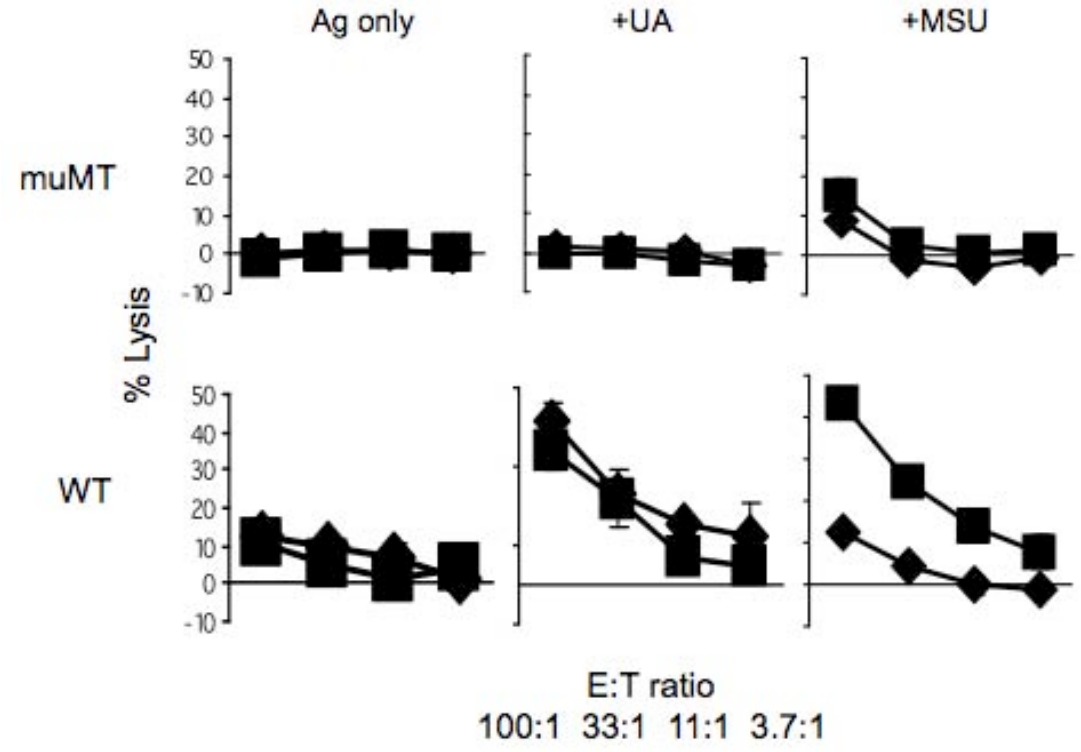

Figure 3.1. The muMT or B cell deficient mice do not sense the adjuvant effect of uric acid or MSU crystals.

This Cr51 cytotoxicity assay contains splenocytes isolated from wildtype and muMT (B cell deficient) mice, which were immunized subcutaneously with 5 µg of OVA coated latex beads. 100 µL PBS, 100 µg MSU crystals or uric acid solution were used as adjuvants. The immunized mice were sacrificed 7 days later and their spleens were collected. 50 million splenocytes were cultured and stimulated with the 10^{-10} M SIINFEKL peptide for 5 days. The remaining live cells were counted and mixed with the Cr-51 labelled, target EL-4 (H-2B) cells pulsed with SIINFEKL peptide, at the appropriate effector: target (E:T) ratio. The CTL assay was performed for 5 hours. The

two lines in the panels are the mean results ± SD of individual mice. The data are representative of three independent experiments.

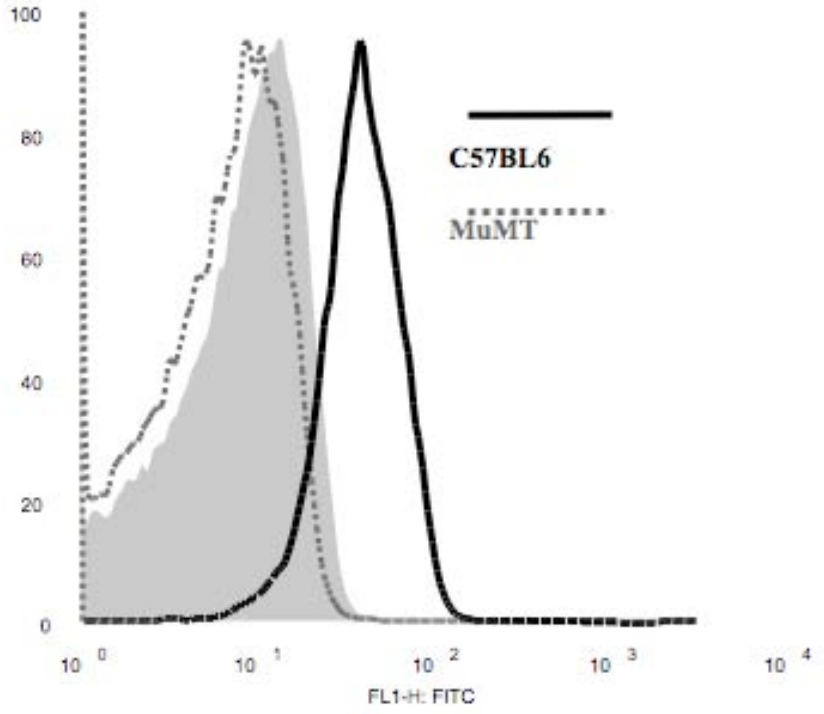

Figure 3.2. Serum from immunized C57Bl6 mice binds to MSU crystals, while serum from immunized muMT mice does not.

This is a representative FACS plot of the MSU crystal binding FACS assay. 100 µL of serum from an immunized C57Bl6 and muMT(B cell deficient) mice, was used to stain 100 µL of 1 mg/ mL solution of MSU crystals during a 20 minute incubation period at room temperature. After 2 washing steps, 0.5 µL of 1 mg/mL secondary FITC conjugated antibody was added for 10 minutes. The secondary antibody can cross-react with both heavy chain of the IgG antibody and the heavy chain of the IgM antibody. The crystals are labelled as events, but are found on a smaller scale than cells and at higher granularity. Approximately 25 000- 50 000 events were collected using the BD FACS Scan Cell Quest program. The data was then analyzed using the FlowJo

software (Tree Star). The serum from the immunized C57Bl6 mouse had positive binding for MSU crystals and the serum from the immunized muMT mouse did not show positive binding to the MSU crystals. The secondary antibody control is the lightly shaded area. The data shown are representative of ten independent experiments. This MSU crystal binding FACS assay was also performed at least twenty times as a quality control experiment for MSU binding antibodies used in the latter experiments. The antibodies were: E6C7 (positive for MSU crystal binding, IgM antibody) and cAB (negative for MSU crystal binding, IgM antibody).

3.2.2 MSU crystal binding antibodies in serum.

Using the MSU crystal binding FACS assay, we determined the nature of the antibodies in the serum of the immunized animals. In Figure 3.3 A, serum from individual C57Bl6 and Balb/c mice, immunized bi-weekly for 2 months with 1 mg of MSU crystals, was able to stain the MSU crystals; serum from unimmunized C57Bl6 mouse was also seen to bind to the crystals. This particular observation may indicate that there are natural antibodies present in the serum of the mice that have the ability to bind to the MSU crystals, although they may be present at a lower titre than in the immunized animals. Figure 3.3 B shows that the serum from the immunized animals is specific for MSU crystals. Xanthine crystals were used as a control for comparison with MSU crystals and did not show any antibody binding. These results reveal that the serum produced by the immunized animals is specific for the immunizing substance, which further supports the hypothesis that antibodies are involved in the binding of MSU crystals.

The next step was to generate hybridomas that could produce MSU crystal specific antibodies. Hybridomas were generated using an established protocol (180) with A3A cells used as fusion partners for the splenocytes from immunized animals. The hybridoma-generated antibodies were collected and tested for their ability to bind MSU crystals using the MSU crystal binding FACS assay. As Figure 3.4 A shows, the hybridoma antibodies or uric acid binding antibodies (UBAs) can bind to MSU crystals, and their binding ability to the crystals is similar to that of immunized serum from Balb/c and C57BL6 mice. In another comparison with muMT mice (Figure 3.4 B), the UBAs showed a greater binding ability for MSU crystals while the sera from B cell deficient

mice had a very low binding ability. Since the staining ability is above that of the secondary antibody alone control, it would indicate that other components of serum may also bind to MSU crystals. This data reaffirms the staining seen in Figure 3.2. Figure 3.4 C shows other antibodies such as W6/32 (anti human MHC Class II) and Y3 (anti-mouse MHC Class I) used to test the specificity of the immunized serum antibodies. The control antibodies did not stain the MSU crystals; therefore specificity is required for the antibodies to bind to MSU crystals, reiterating that antibodies can bind to crystals in a specific manner.

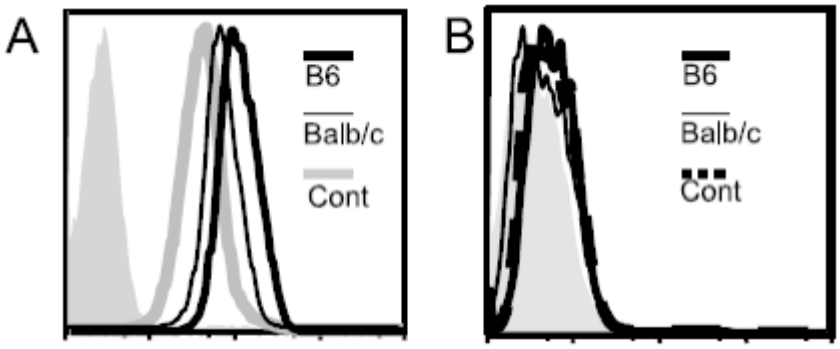

Figure 3.3. Serum from MSU crystal immunized C57Bl6 and Balb/c mice had a positive binding ability for MSU crystals (A) but not for xanthine crystals (B).

The MSU crystal binding FACS assay here, used 25 µl of serum from MSU crystal immunized individual mice (B6, Balb/c) to stain 500 µg MSU crystals (A) and 500 µg of xanthine crystals (B). Serum from the non-immunized mouse (Cont) can also bind MSU crystals. This observation could indicate the presence of natural MSU binding antibodies in the serum. The assay in Figure 3.3 B is using xanthine crystals in place of MSU crystals, but the serum is the same as in the previous figure. The lightly shaded area in this and following assays is 2nd antibody only control. The data are representative of two independent experiments.

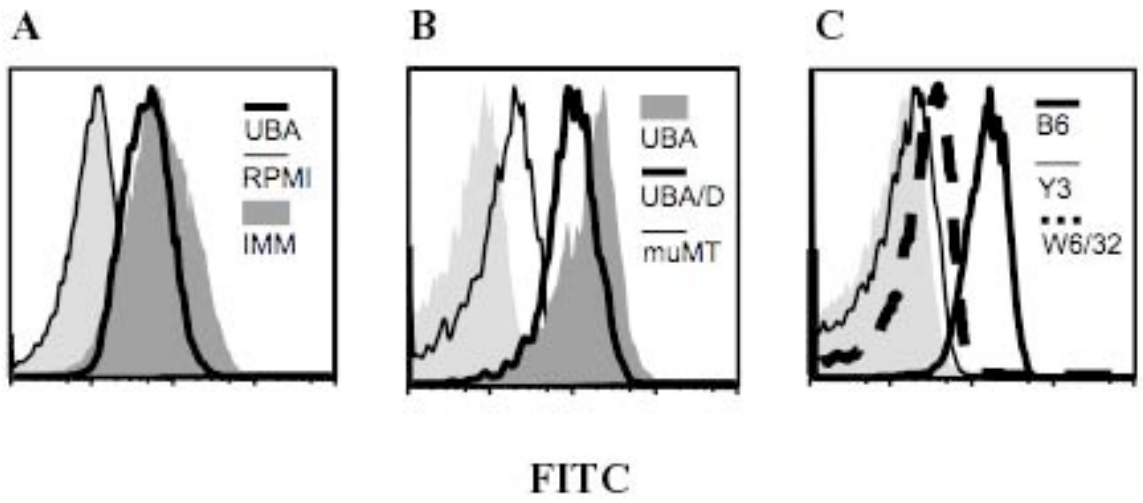

Figure 3.4. B cell hybridoma produced antibodies can bind MSU crystals with the same intensity as immunized serum.

The MSU crystal binding FACS assay was used to test the MSU crystal binding ability of B cell hybridoma supernatant. C57Bl6 mice were immunized intraperitoneal with 1 mg of suspended MSU crystals without any adjuvant. The same mice were boosted with the same dose bi-weekly for 2 months. Serum from the immunized mice was collected at the 2 month time point and tested for the ability to bind MSU crystals. Splenocytes were also harvested to be used in a standard hybridoma assay with A3A tumour cells, where 10 000 000 tumour cells were fused with 40 000 000 splenocytes. (A) The supernatant from a hybridoma culture (UBA) was tested along with the immunized serum (Imm) and cell culture media (RPMI) using the MSU crystal binding FACS assay. The hybridoma supernatant and the immunized serum showed a similar ability to bind MSU crystals. (B) Serum from MSU crystal immunized muMT mice was also compared with the hybridoma supernatant (UBA) and a 20 fold dilution of the supernatant (UBA/D). The hybridoma supernatant showed a higher MSU crystal

binding ability than the immunized muMT mice serum. (C) Human (W6/32) or mouse (Y3) MHC class I specific control antibodies do not stain MSU crystals to the same extent as the serum from immunized C57Bl6 mouse. These antibodies were considered to have a very low binding ability to MSU crystals when compared to the positive C57Bl6 serum. The data shown are representative of two independent experiments.

3.2.3 Hybridoma generated MSU binding antibodies (UBAs) can also cause the precipitation of uric acid from a uric acid solution.

The antibodies generated from B cell hybridomas have the ability to bind to MSU crystals in vitro. However could these same antibodies act to precipitate uric acid from a solution? An important feature of crystal induced arthropathies is the presence of crystals, which elicit an inflammatory response. How do the crystals precipitate and could their formation be aided by crystal specific antibodies? To answer these questions, we incubated a 1 mg/mL solution of uric acid in PBS (pH 8.0) with increasing concentrations of control ovalbumin protein and an uric acid binding antibody (UBA 11). Any formed crystals in the plates after 6 hours were washed with PBS and re-suspended with 0.01 N NaOH. The amount of precipitated uric acid was measured and quantified using control solutions of uric acid at UV absorbance at 292 nm. As seen in Figure 3.5, the uric acid solution had precipitated with increasing concentrations of the UBA and not the control protein (OVA). This experiment showed that antibody specific for MSU crystals can precipitate uric acid from solution. The data gives evidence that antibodies play a larger role in the inflammatory response to uric acid by aiding in the precipitation of the MSU crystals.

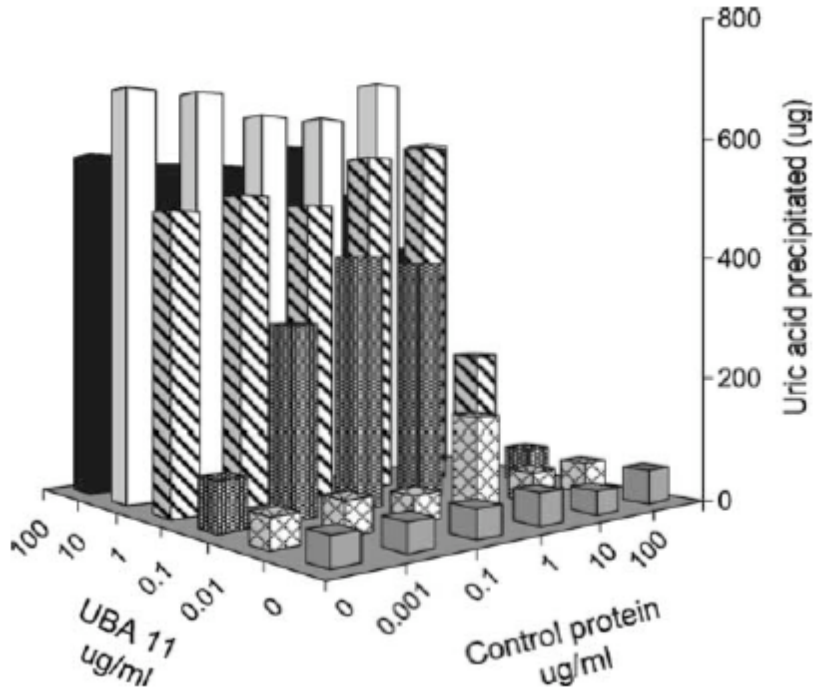

Figure 3.5. UBAs can precipitate soluble uric acid from solution.

The amount of precipitated uric acid from a uric acid solution (1 mg/mL, pH 8.0 in PBS, in 24-well tissue culture plates) was measured in the presence of ranging concentrations (0- 100 μg/ mL) of uric acid binding antibody (UBA 11) and control protein (ovalbumin). After 6 hours of incubation, the precipitated uric acid crystals were washed with PBS and resuspended in 0.01 N NaOH, and the UV absorption value at 292 nm of the solution was measured by a spectrophotometer to determine the uric acid concentration. The amount of crystal deposition in micrograms was converted from a standard UV absorption curve with known uric acid quantities. The data shown are representative of two independent experiments.

3.2.4 Characterization of the UBAs.

The antibodies produced by the hybridomas bind to MSU crystals. The next step was to discover the type of antibodies produced, how they bind to the MSU crystals and how they cause the precipitation of uric acid from solution. The antibodies that showed the highest ability to bind MSU crystals, as measured by the MSU crystal binding FACS assay, were subtyped using an antibody subtyping kit. According to Figure 3.6, the most common heavy chain and light chain combination seen in the antibodies specific for MSU crystals was the μ heavy chain and κ light chain. Approximately 85% of the antibodies were identified to have this particular IgM subtype. The other 15% were identified to be IgG type antibodies. The IgM antibodies are known to be pentameric, and this structure may help bring molecules of monosodium urate together to crystallize. The structure of the antibody may allow for the initial crystal lattice formation. The μ heavy chain is considered to be more efficient at starting complement reactions (164). The predominance of the mu subtype can also indicate the presence of a T cell independent antigen. Normally, B cells express the mu heavy chain; however, when they are stimulated via CD4 T cells to react to an antigen, the antibodies undergo a class switch from the mu heavy chain to another type. If the CD4 T cell interaction is not provided, the antibodies are produced with the mu heavy chain.

To discover how the MSU crystal specific antibody bind to the crystals, one of the hybridoma produced antibodies with a greater ability to bind MSU crystals, and of the IgM isotype, was digested using the Pierce ImmunoPure IgM Fragmentation Kit. The fragments were separated according to the provided protocol and used in the MSU crystal

binding FACS assay. From the assay in Figure 3.7, the variable regions of the antibody, labelled $F(ab)^2$, had a greater ability for binding to MSU crystals, similar to the whole UBA IgM molecule. The data showed that the antibody can bind to the MSU crystals with its variable regions; this is reminiscent of the binding seen between an antibody and antigen in the immune complex. These results are congruent with previous research with IgG molecules (148, 149). However, when the same fragments were tested in the uric acid precipitation assay in Figure 3.8, only the whole IgM molecule (UBA) was able to precipitate uric acid from the solution. Neither the variable regions $F(ab)^2$ nor the constant regions Fc5 of the fragmented UBA IgM could precipitate uric acid from solution. The findings indicate that the whole IgM molecule, is required for the precipitation of the uric acid from solution.

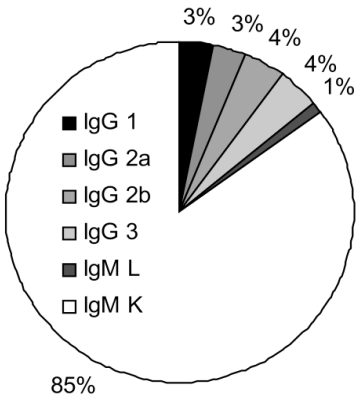

Figure 3.6. Distribution of classes of antibody produced by B cell hybridomas with a positive binding ability for MSU crystals.

Approximately 300 B cell hybridoma produced monoclonal antibodies were tested using the MSU crystal binding FACS assay. Approximately 30 % of these had a similar binding ability to the MSU crystals as compared to the positive C57Bl6 MSU crystal immunized serum. This subset of antibodies was subtyped using an antibody heavy/ light chain subtyping kit and 85 % of the subset were revealed to have a μ heavy chain and a κ light chain. The other 15 % had a γ heavy chain or a μ heavy chain with a λ light chain.

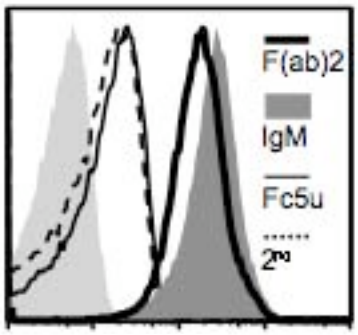

Figure 3.7. Fractions of UBA E6 IgM binding to MSU crystals.

The MSU crystal binding FACS assay was performed using fragments of the B cell hybridoma produced UBA E6. This antibody had previously shown a higher binding ability to MSU crystals as compared to the controls. An IgM fragmentation kit was used to generate the fragments (Pierce ImmunoPure IgM Fragmentation Kit). The starting IgM antibody was at a concentration of 1 mg/mL. Fc5u is the constant portion of the antibody. F(ab)2 is the portion containing the variable regions. IgM is the whole antibody molecule. It is evident that the variable regions of the antibody are responsible for binding of the antibody to the MSU crystals. This is the same configuration as of antibody and antigen immune complex. The data shown are representative of two independent experiments.

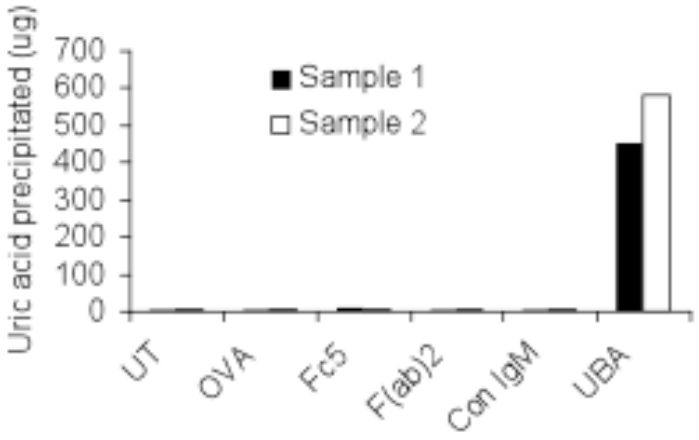

Figure 3.8. Uric acid precipitation data using the UBA IgM and its fragments.

The figure is showing results from a precipitation assay where fragments of the UBA E6 (generated for the previous figure) were tested for their ability to precipitate uric acid from solution (as in Figure 3.5). Other conditions include: untreated solution (UT), ovalbumin (OVA), a non specific protein, a control IgM (Con IgM (MOPC)), the constant portion of the antibody (Fc5) and the variable regions of the antibody (F(ab)2). The uric acid precipitation was measured via absorbance value at 292 nm after the precipitated crystals were solubilized using 0.01 N NaOH. The data shown is a composite of two independent experiments.

3.2.5 MSU crystals, UBAs and their interactions with the innate immune system's dendritic cells.

So far, the data showed that uric acid can be precipitated from solution by the MSU crystal specific antibodies. The antibody can bind to the MSU crystals with its variable $F(ab)^2$ regions, similar to the configuration seen in immune complexes. However, the whole IgM molecule is required for the uric acid to crystallize from the solution. Crystals can bind to antibodies as seen by the analysis of the MSU crystals from serum solutions (142, 144, 148, 151). Other properties have been attributed to the MSU crystals including complement activation (91, 94), and bone marrow dendritic cell (BMDCs) stimulation (50), seen in the first panel of Figure 3.9. The MSU crystals stimulated the DCs to produce higher levels of CD86, a maturation marker. The cells in this assay would be prepared for antigen presentation to T cells due to the expression of this co-stimulatory molecule on their surface. Without MSU crystals, the DCs did not show the same elevation in surface CD86, as measured by the elevation of fluorescence by FACS.

In the second panel of Figure 3.9, the MSU crystals were treated with the B cell hybridoma antibodies specific for MSU crystals while incubated with bone marrow derived GM-CSF/ IL-4 treated dendritic cells. This experiment was done to test whether antibody binding to crystals would prevent MSU crystal activation of dendritic cells. The cells still showed the same level of CD86 expression as did the stimulated cells in the first window. The conclusion was that the antibodies' presence in these cultures did not prevent the recognition of the MSU crystals and the stimulation of dendritic cells. The

antibodies do not present a physical obstacle to the binding of MSU crystals to antigen presenting cells and the subsequent APC activation.

The next panel in the figure shows that antibodies lacking specificity to MSU crystals (Y3 and W6/32) did not prevent the stimulation of dendritic cells and the expression of the CD86 surface maturation marker. These experiments reveal that there may be another mechanism involved in MSU crystal recognition by dendritic cells specifically, besides recognition via bound antibody. The IgM antibodies specific for MSU crystals, generated in these experiments, may be necessary for the precipitation of uric acid into MSU crystals and the initiation of the innate immune response. However, they may not be required for the activation of dendritic cells, which are potent initiators of the adaptive immune response.

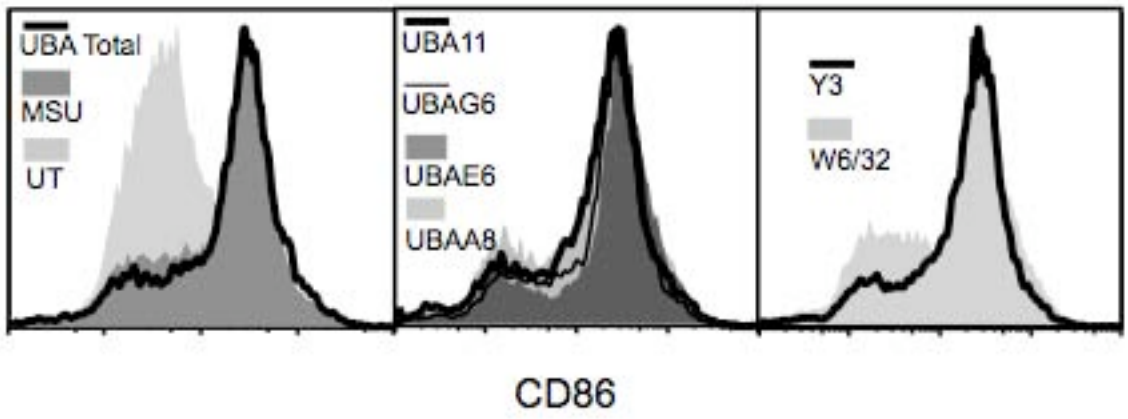

Figure 3.9. Dendritic cells (DCs), UBAs and MSU crystal interactions.

This FACS assay measured the intensity of the CD86 marker on cultured bone marrow GMCSF/IL-4 DCs that were stimulated with MSU crystals alone and with the addition of indicated UBA mAb supernatants for 6 hours. CD11c positive cells identified as dendritic cells were analyzed for their CD86 expression. The cells showed a higher expression of CD86 maturation marker when they were incubated with MSU crystals, and the expression was not altered by co-incubation of the cells with MSU crystal binding antibodies (UBA11, UBAG6, UBAE6, UBAA8) (second panel), or with antibodies which do not bind MSU crystals (Y3, W6/32) (third panel). The data shown are representative of two independent experiments.

3.2.6 The injection of the purified MSU crystals binding antibodies in vivo and the subsequent restoration of the uric acid adjuvant effect in muMT mice.

If the MSU crystal binding antibodies do not prevent the recognition of the MSU crystals and the activation of dendritic cells, then what is the possible connection between the lost uric acid adjuvant effect in B cell deficient mice and the inflammatory mechanism of the MSU crystals in vivo. To investigate this question, purified MSU crystal binding antibodies were injected into wildtype (C57Bl6) and B cell deficient (muMT) mice with 1 mg of uric acid in solution. The purpose of the injections was to increase the possibility of antibody mediated uric acid precipitation in vivo. The difficulty with using mice for these experiments is that mice produce an enzyme called uricase, which degrades uric acid into 5-hydroxyisourate and hydrogen peroxide (120). This enzyme prevents the accumulation and crystallization of uric acid in vivo. To circumvent this enzyme's action, a uric acid solution was injected into the mice with the MSU crystal binding antibodies.

If the antibodies form immune complexes with uric acid, then the serum uric acid levels would drop in the mice that were injected with antibody specific for MSU crystals. In Figure 3.10, the serum uric acid levels of C57Bl6 mice were measured using the HPLC. The serum was collected from tail blood after three days of injections of uric acid and antibodies. The serum was diluted and centrifuged through a 30K filter, preventing any protein particulates from contaminating the samples. The samples were then manually injected onto a weak anion exchange column. The uric acid peak at 292 nm was divided and recorded as a percentage of the total UV absorption of the specific run. This

figure shows that uric acid in the serum decreases slightly when E6C7, an MSU crystal binding antibody, is injected into the mice along with the uric acid solution. A control antibody, which poorly binds to MSU crystals did not show the same decrease of uric acid. On the contrary, the uric acid levels were higher in these mice. The data indicate that the uric acid was potentially sequestered from the serum, decreasing the serum uric acid levels, when purified UBA was injected with uric acid solution. The sequestration or possible precipitation of the uric acid would be observed as was seen in the in vitro experiments (Figure 3.5).

With the possibility of MSU crystal formation in vivo, other effects of the crystal's presence were assessed such as the activation of the complement system. Besides the MSU crystals' ability to activate the complement system (91, 94), the injected UBA antibodies when bound to their antigen, should be able to activate complement. The UBA used for the injections is an IgM molecule and its pentameric structure allows for several constant regions to be in close proximity with one another, which is required for complement activation. After three days of injections of uric acid solution and the purified hybridoma antibodies, the blood was collected using cardiac puncture and serum was isolated. The samples were then treated according to the protocol for Mouse complement C3 ELISA kit. This procedure allows for the measurement of serum complement component C3, one of the major proteins involved in the complement system (58, 183). The assay, however, does not measure the breakdown products of the C3 protein. After three days of injections with antibody and uric acid solution, complement activation was indirectly measured by quantifying the concentration of C3 complement component.

Figure 3.11 shows the results of the complement measurement assay. The levels of C3 in mice injected with uric acid solution and E6C7 (UBA) were higher than the serum C3 concentrations of the mice injected with PBS, uric acid solution (UA), control antibody (cAB) and uric acid solution in combination with control antibody (UA cAB). The complement C3 levels were variable when the IgM E6C7 alone was injected into the muMT mice. There is a statistically significant difference in the levels of C3 complement component between mice injected with the uric acid solution and the E6C7 UBA and the mice injected with the control antibody and the uric acid solution.

These results are counterintuitive in the sense that if complement is being activated, then there should be more breakdown of C3 complement component. Using this assay, only the whole C3 molecule was measured, thus decreases in the conditions, which induce the highest level of inflammation are expected. However, Manthei *et al* have found that C3 plasma concentrations may increase during an inflammatory reaction. Their reasoning was that C3 is one of the acute phase proteins, a group of plasma proteins whose concentrations can change in an acute inflammatory situation (184). Also, previous research showed that during an acute inflammatory reaction such as a *Schistosoma mansoni* infection in mice and an intramuscular injection of turpentine into mice, two fold increases in serum C3 concentrations were observed (184). In their acute inflammation experiments, Manthei *et al* observed an alteration in C3 distribution, and a five fold increase in C3 synthesis. However, they only skimmed the surface of the regulation of C3 synthesis, by stating that following the induction of local inflammation, a locally produced signal must reach the site of C3 production to induce the systemic acute phase response of increased synthesis of C3.

If micro MSU crystals have formed due to the presence of UBAs in vivo, these ensuing immune complexes may activate the complement system. Also if micro MSU crystals are inducing a local inflammatory reaction, another signal may reach the site of C3 production and induce an increase in C3 synthesis. Thus a local inflammatory response can then become a systemic acute phase response due to increases in C3 synthesis. In one of the conditions when E6C7 (UBA) was injected alone there was a variability in the results of C3 concentrations. This incidence could be due to the presence of endogenous uric acid in vivo, which potentially could form MSU microcrystals with the UBAs. However, when the control IgM antibody alone was injected, the levels of C3 were similar to the PBS control, indicating that antibody alone is not activating the complement system. The antibodies must have bound to their target, the uric acid lattice structure, for the MSU crystals to form. Both the crystals and the immune complexes may cause a local inflammatory response and complement activation thus causing another signal to induce increases in C3 synthesis. Still, all of these changes signify an inflammatory response occurring in vivo after injection of the UBA and uric acid solution.

Previous research, using BMDCs, showed that MSU crystals do interact with DCs and can stimulate them to express higher levels of their maturation marker CD86 (50). However, the presence of the antibodies did not affect the stimulation of the DCs, indicating a different pathway of activation for dendritic cells. The next step was to investigate whether the presence of the UBAs in vivo would affect other cell types involved in the inflammatory response. Past research suggests that neutrophils are involved in the induction of the inflammation in gout and could potentially be affected

by the presence of micro MSU crystals in vivo (65, 66, 72, 100, 101, 104, 105, 111, 185-188). After three days of injections of uric acid and UBA solutions, blood from the injected mice was isolated, hemolysed and fluorescently labelled with CD11b and CD62L antibodies. The CD11b positive cells were selected and analysed for their CD62L surface expression using FACS. CD62L is L-selectin molecule that tethers neutrophils on activated endothelial cells of the venule blood vessels. Figure 3.12 shows that CD11b positive cells, identified as neutrophils and isolated from blood of the mouse injected with uric acid and UBA solutions, had a decreased expression of CD62L. This data indicates that these cells were activated and have already started to lose the L-selectin molecule as compared to the control PBS injected mouse (111, 116). The mouse injected with the control antibody showed a slight decrease in the CD62L expression on the neutrophils. This occurrence could be a response to the presence of an antibody in the B cell deficient mice. The figure included serum from three mice, and was repeated in quadruplicate. The data shown is representative of those repeats.

Another marker of neutrophil activation was measured to verify the FACS data. The level of the myeloperoxidase enzyme, which converts peroxide to hypochlorite in reactive oxygen species production, is indicative of tissue inflammation (181, 189). Figure 3.13 shows the results of the myeloperoxidase assay done on the lung tissue of C57Bl6 mice injected with uric acid and antibody solutions for three days. The results demonstrate that the lung myeloperoxidase content increased significantly as compared to the other conditions when E6C7 MSU crystal specific antibody was injected alone or with the uric acid solution. In the case of the UBA injected with the uric acid solution, microscopic MSU crystals could have formed, activated the neutrophils, which

upregulated myeloperoxidase enzyme production. However, when the MSU crystal specific antibody was injected on its own, it may have caused the precipitation of the endogenous uric acid. The antibody injections also could have caused stress to the recipient increasing the output of uric acid in the animals. These two figures emphasize that the neutrophils become activated and increase the production of the myeloperoxidase enzyme. This data indicates that the mice, which received the uric acid and UBA solution injections, had undergone an increase in their general inflammatory state.

The injections of UBAs with the uric acid solutions were able to increase the general inflammatory state of the animals. The decrease of uric acid in the serum indicated a sequestration of the uric acid from the serum and the possibility of crystallization of the uric acid into MSU crystals. If the crystals were formed, they could also propagate an inflammatory response, which was indirectly evidenced by the increase in the C3 complement component and neutrophil activation. The main question then becomes whether the uric acid adjuvant effect can be restored in B cell deficient mice if MSU crystal specific antibodies were injected into the mice? The main hypothesis was tested by repeating the cytotoxic T cell assay, seen in Figure 3.1, with B cell deficient mice (muMT) being injected with OVA labelled latex beads mixed with the MSU crystal solution and the UBA E6C7 or control antibody. As seen in Figure 3.14, the MSU crystal specific antibody (E6) injected mice had an elevated cytotoxic T cell response to the Cr-51-containing SIINFEKL pulsed EL4 cells. This response was comparative to that seen in wildtype mice in Figure 3.1. The control antibody or PBS injections did not have the same effect in the B cell deficient mice. The CTL response in these conditions was minimal, which is similar to the response seen in muMT mice when uric acid solution or

MSU crystal solution were used as adjuvants in Figure 3.1. The addition of antibodies, that can bind to MSU crystals along with MSU crystal solution as adjuvant in B cell deficient mice, had complemented the CTL response to the level of wildtype mice which were injected with uric acid or MSU crystal solution as adjuvants. The MSU crystal specific antibodies' presence was able to replicate the uric acid adjuvant effect seen in wildtype mice, in B cell deficient mice.

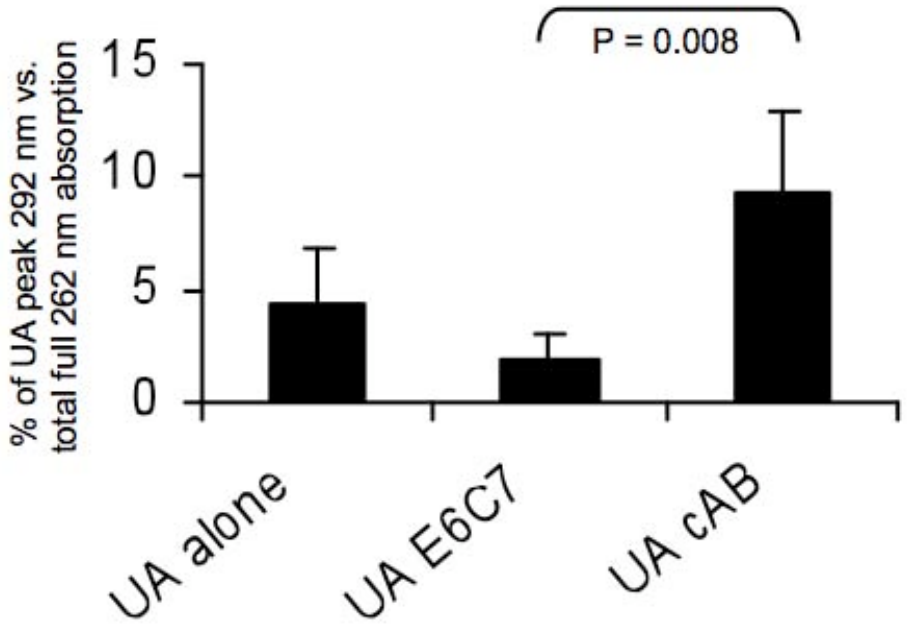

Figure 3.10. Serum uric acid measurement in C57Bl6 mice injected with uric acid solution and purified B cell hybridoma produced antibodies.

Serum uric acid measurements were performed on serum samples using the HPLC method. Two C57Bl6 mice were i.v. injected with 0.500 mL of 2.0 mg/mL uric acid solution and 0.5 mg of UBA (E6C7) or control antibody once per day for three days (6 mice used in an experiment, 2 per condition). UA: uric acid; E6C7: UBA; cAB: control IgM. The serum was collected from the tail vein, diluted, and centrifuged through a 30K filter to prevent protein contaminants. The samples were then injected onto a weak anion exchange column and the uric acid peak at 292 nm was compared with the total UV absorption of the run. The data shown are representative of two triplicate experiments and presented as a mean value ± SD. The P value between the UA E6C7 and UA cAB conditions is 0.008. This pair of conditions showed a significant difference according to the statistical calculations.

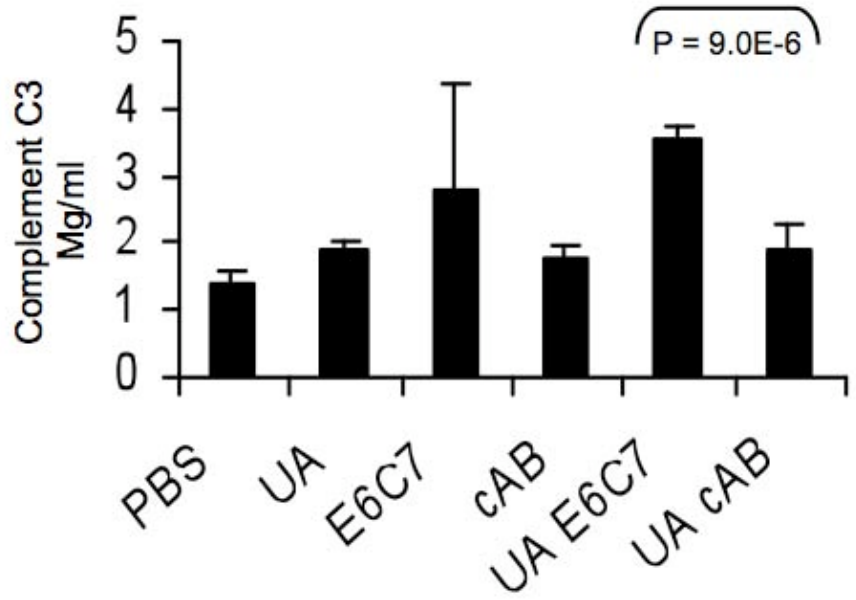

Figure 3.11. Serum complement C3 component concentration in muMT mice injected with uric acid solution and B cell hybridoma produced antibodies.

Two muMT mice were i.v. injected with 0.500 mL of 2.0 mg/mL uric acid solution and 0.5 mg of UBA (E6C7) or control antibody once per day for three days (12 mice used in an experiment, 2 per condition). The serum was collected as described in Materials and Methods. The complement measurement was done using the manufacturer's protocol (Mouse complement C3 ELISA kit, Kamiya Biomedical). The absorbance at 450 nm was measured using a microplate reader. The absorbance was then converted to the C3 concentration in mg/ml using a standard curve fitting procedure. The data shown are representative of two triplicate experiments and presented as a mean value ± SD. The P value between the UA E6C7 and UA cAB conditions is 9.9*10-6. This pair of conditions showed a significant difference according to the statistical calculations.

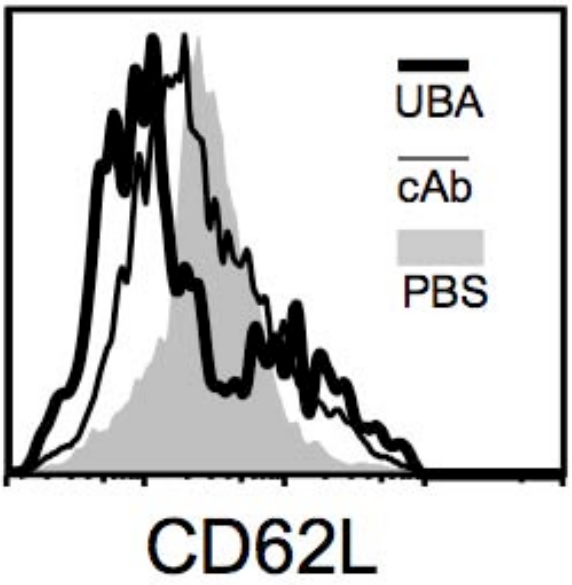

Figure 3.12. CD62 L expression on blood CD11b positive cells of muMT mice injected with uric acid and B cell hybridoma produced antibodies.

Two muMT mice were i.v. injected with 0.500 mL of 2.0 mg/mL uric acid solution and 0.5 mg of UBA (E6C7) or control antibody once per day for three days (6 mice were used in an experiment, 2 per condition). On day 4, the blood was collected, and hemolysed to remove blood cells. CD11b (FITC) and CD62L (PE) antibodies were used to stain activated neutrophils from the blood (eBioscience). Blood CD11b+ cells (neutrophils) were gated and analyzed for their CD62L expression. The data are from three individual mice but are representative of two duplicate experiments.

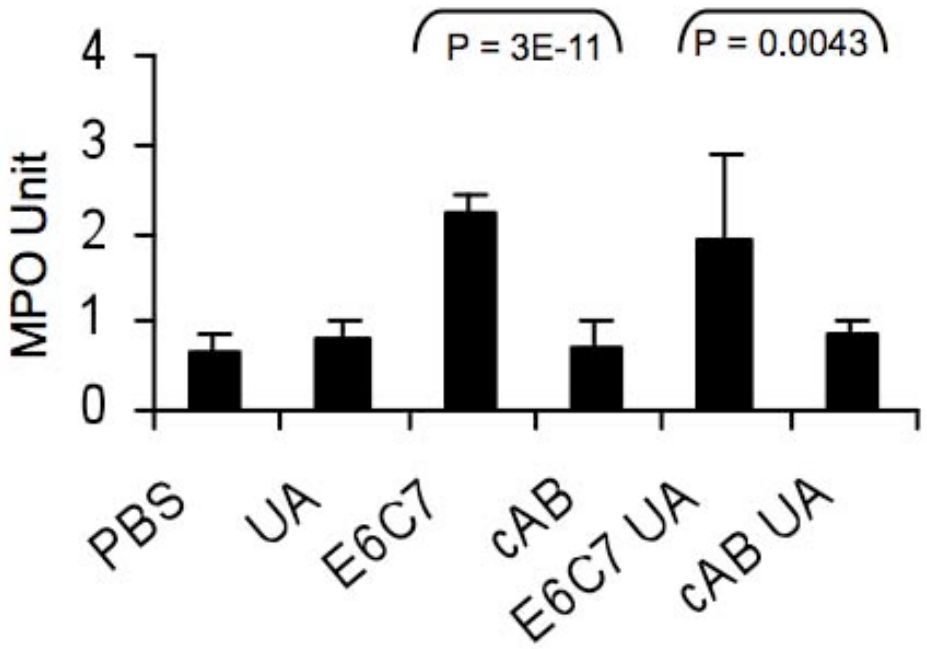

Figure 3.13. Myeloperoxidase measurements from the lung tissues of C57Bl6 mice injected with uric acid and UBA solutions.

Myeloperoxidase (MPO) measurement in the lung tissue of C57Bl6 mice i.v. injected with 0.500 mL of 2.0 mg/mL uric acid solution and 0.5 mg of UBA (E6C7) or control antibody once per day for three days (12 mice were used in an experiment, 2 per condition). On Day 4, lung tissue was collected and frozen to be used in the myeloperoxidase procedure as described in the Materials and Methods. The myeloperoxidase values were generated by multiplying the UV reading by a factor of 0.2528 per a standard protocol. The data shown are representative of two triplicate experiments. The P value between the E6C7 and cAB conditions is 3.0*10-11. The P value between E6C7 UA and cAB UA is 0.0043. These pairs of conditions showed a significant difference according to the statistical calculations.

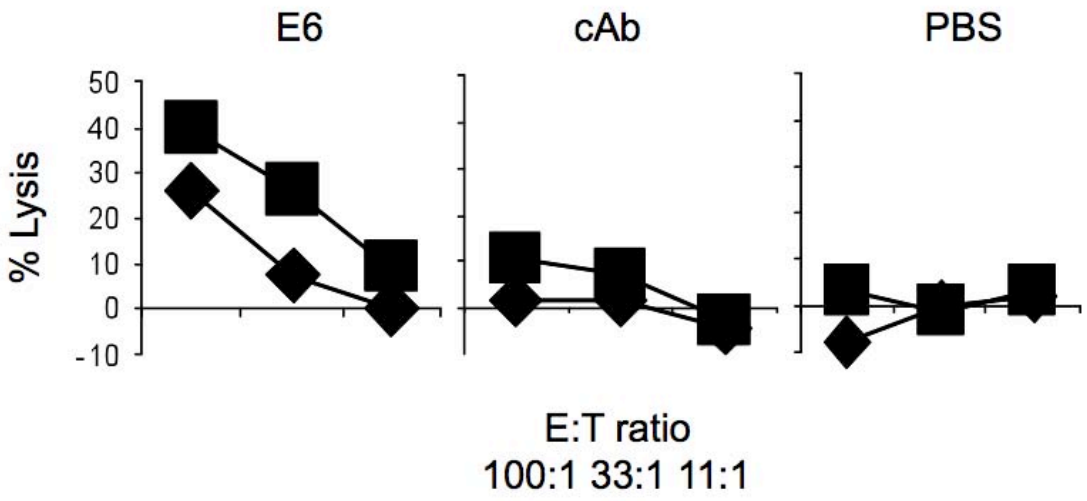

Figure 3.14. The cytotoxic T cell response of muMT mice after injections of UBA antibody were made prior to injections of OVA coated latex beads (antigen) mixed with MSU crystals (adjuvant) in suspension.

This Cr51 cytotoxicity assay contains splenocytes isolated from muMT (B cell deficient) mice, which were immunized subcutaneously with 5 µg of OVA coated latex beads. 100 µg MSU crystals was used as an adjuvant. Prior to immunization, these mice received 2 injections of 500 ug purified UBA E6C7 (E6), a control IgM (cAb) or PBS. The immunized mice were sacrificed 7 days later and their spleens were collected. 50 million splenocytes were cultured and stimulated with the 10^{-10} SIINFEKL peptide for 5 days. The remaining live cells were counted and mixed with the Cr-51 labelled, target EL-4 (H-2B) cells pulsed with SIINFEKL peptide, at the appropriate effector: target (E:T) ratio. The CTL assay was performed for 5 hours. The two lines in the panels are the mean results of individual mice. The data shown is a representative of three duplicate experiments and presented as a mean value ± SD.

CHAPTER 4: DISCUSSION

During the investigation of uric acid's adjuvanticity, several observations regarding the uric acid precipitation and the inflammatory response stimulated by the formed crystals were noted. B cell deficient (muMT/IgH) mice did not experience the uric acid adjuvant effect as the wildtype (C57BL6) mice. When uric acid or MSU crystals were used as adjuvants for the OVA coated latex beads (a particulate antigen), only the wildtype mice were able to respond to the antigen in a CTL assay. This lack of response could be attributed to the lack of antibodies in the serum. Specifically, the immunized muMT mice's serum did not have the same binding ability to MSU crystals as the serum from immunized C57BL6 mice. The serum from immunized mice was deemed specific for the MSU crystals because of lack of binding to the control xanthine crystals. The positive binding ability of serum for MSU crystals indicated that antibodies could be involved in mediating the binding to MSU crystals. Since antibody presence should be the only factor different between the two types of mice, then the antibodies may also mediate the uric acid adjuvant effect seen in wildtype mice.

Antibodies isolated from immunized animals have previously been shown to bind the immunizing crystals, furthering the idea that crystal binding antibodies could be generated in vivo (152, 155, 190). Another important observation was that serum from nonimmunized C57BL6 mice could also bind to the MSU crystals. The binding ability of serum in this situation must be mediated by antibodies present in the animal prior to the immunization with MSU crystals. These antibodies, defined as natural antibodies, are usually of the IgM isotype and do not have a high affinity, even though they strongly bind to their antigens due to their pentameric structure (162). These antibodies are

normally produced by the B1 B cell subset. Thus, crystal binding antibodies could be generated in immunized animals and can be found in non immunized animals.

The MSU crystal binding ability of the immunized serum was replicated in some B cell hybridoma generated antibodies. The uric acid binding antibodies (UBAs) from hybridomas were specific for MSU crystals and most importantly, demonstrated the ability to precipitate uric acid from solution. Almost 85 % of the UBAs that could bind MSU crystals were identified as IgM isotype. These results are consistent with the possibility that natural IgM antibodies are acting as nucleating agents for MSU crystals. Prior research has evidence that IgM is attracted to sites of injury and is a potent mediator of tissue damage (193, 194). At these sites the IgM molecule can interact with uric acid released from cells upon cell death. Uric acid precipitation may occur and inflammatory MSU crystals may form with the aid of natural IgM antibodies.

The UBAs generated by the B cell hybridomas were able to bind to the MSU crystals via their variable regions. These same regions are used to bind antigens during a conventional humoral immune response. These results were consistent with prior reports indicating that crystal bound IgG molecules used their variable regions to bind to the crystals, exposing the constant regions (148, 149). The configuration of the bound antibody was reminiscent of the antigen antibody immune complexes. However, for the crystals to begin precipitating from solution, the whole antibody molecule was required. Neither the variable regions or the constant regions or the non specific antibody were enough to precipitate uric acid from the solution. These results are important in highlighting the role of the antibody structure for the successful precipitation of MSU crystals.

Besides being able to activate neutrophils, endothelial cells and macrophages to produce proinflammatory cytokines, chemokines and lipid derived inflammatory mediators; MSU crystals incubated with dendritic cells were able to stimulate them to increase the surface expression of the maturation markers CD80 and CD86 (50). MSU crystal binding antibodies, when co-incubated with the crystals and DCs, did not prevent the activation of dendritic cells and the expression of the CD86 maturation marker on the cell surface. These observations are important because uric acid is normally found within the cytosol of cells and is released upon tissue cell death as a danger signal (DAMP) (50). Upon the interaction of a local supersaturated solution and a natural IgM antibody with the ability to bind to the uric acid lattice in solution, the MSU crystals can be formed. The antibodies bound to the crystal or the MSU immune complex, ensures the stability of the activating crystal. These crystals can act as endogenous adjuvants on DCs and other phagocytes, inducing an immune response during sterile or pathogenic cell death.

If the UBAs bound to MSU crystals do not act to increase the activation of dendritic cells, then how can they impact the CTL response of B cell deficient mice in vivo as shown in Figure 3.14? Immune complexes, composed of antigen, natural antibodies and complement have been found to activate a CD11b+ $CD11c^{lo}$, clodronate-sensitive phagocytes, to secrete IL-4 (191). These cells are regarded as pre-dendritic and may act to initiate the immune response (192). MSU crystals and the subsequent immune complexes formed due to UBAs may have a role in starting the immune response especially when injury, surgery or massive cell death release uric acid into the extracellular environment. The MSU crystals or the crystal-containing immune complexes would interact with immune cells including pre-dendritic, dendritic cells,

neutrophils and macrophages, causing the subsequent activation of these cells, thus propagating the immune response.

The mice injected with UBAs and a uric acid solution showed a decrease in the serum uric acid content as compared to those mice injected with uric acid solution alone. The sequestration or the possible precipitation of uric acid from the serum, and the ensuing inflammatory responses to the MSU crystals were expected. The presence of UBA E6C7 and uric acid in vivo caused the increase in C3 complement component, indicative of the increased generation of complement components and a general inflammatory state of the animal (184). UBA E6C7 alone also generated an increase in the C3 complement component, but the precipitation of the endogenous uric acid without the additional uric acid injection may have contributed (135). Due to this observation, the ANOVA statistical analysis showed that there was no statistically significant difference between the groups, however it is clear that the condition when both uric acid and E6C7 (UBA) are injected into the mice, the values for serum C3 complement concentration are higher than those seen in mice injected with PBS, uric acid solution alone (UA), control antibody alone (cAB), or uric acid and control antibody together (UA cAB).

Neutrophil activation was measured using the expression of CD62L on the cell surface and the myeloperoxidase content of tissue. The expression of CD62L on blood CD11b+ cells was decreased when the UBA was injected into the mice. The shedding of CD62L or L-selectin occurs due to activation of the neutrophils via the PKC intracellular signalling pathway, which can be activated in neutrophils by MSU crystal interactions (111, 116). Additionally, the myeloperoxidase (MPO) content of the lung tissue of the mice injected with the UBA E6C7 and uric acid was higher than in mice injected with

control antibody or uric acid solution alone. The same pattern was seen again with the MPO measurement as with the C3 complement component assay: the injection of E6C7 (UBA) alone showed a higher measurement of MPO in the lung as compared to the PBS, uric acid solution (UA), control antibody (cAB) and control antibody and uric acid solution (UA cAB). Endogenous uric acid may be present in the mice, which could be precipitated when a UBA is injected into the mouse, thus forming an inflammatory signal, the MSU crystal. Myeloperoxidase is an enzyme associated with neutrophil function and the release of ROS; thus, an increase of its content in tissue indicates the increase of neutrophil numbers in the tissue as well as a general inflammatory state of the organism (181, 189). The diminished uric acid content in UBA E6C7 and uric acid injected mice, increases in C3 complement component, shedding of the CD62L from CD11b+ blood cells, and increases in myeloperoxidase content, indirectly indicate the possible precipitation and formation of the MSU crystals in vivo, and induction of systemic inflammatory responses.

An important observation of an antibody requirement for MSU crystal recognition was made when B cell deficient mice were injected with a MSU crystal binding UBA and immunized with OVA coated latex beads mixed with an MSU crystal solution. The uric acid adjuvant effect seen in wildtype mice had then appeared in B cell deficient mice, where it had not been observed before. The presence of the UBA must have aided the recognition of the inflammatory MSU crystal signal by subsets of cells, including endothelial cells, neutrophils, monocytes and macrophages, via the binding of the MSU crystals. These cells would then be activated to secrete proinflammatory molecules (cytokines, chemokines, lipid mediators) that would act to activate T cells and increase

the cytotoxic T cells response after the MSU crystals were recognized. A general increase in the inflammatory state of the mice was observed after injection of the uric acid solution and UBA. Thus the formation of the MSU crystal immune complex in vivo may be a required signal for certain immune cells to begin secretion of proinflammatory molecules.

The results observed from this line of experiments were congruent with the hypothesis that MSU crystal binding antibodies have a role in uric acid precipitation, uric acid mediated adjuvant effect and the MSU crystal induced inflammatory responses. We were able to answer the questions posed in the introductory chapter by experimental observations:

1. Antibodies to MSU crystals can mediate the adjuvant effect of uric acid in vivo as seen in B cell deficient mice
2. The antibodies that could act as nucleating agents for MSU crystals were of the IgM isotype. They could bind to the crystals via their variable regions, leaving the constant regions functionally accessible
3. The antibodies and MSU crystals could potentially form immune complexes
4. An increase in the general inflammatory state of the animal was noted when the UBAs were injected along with uric acid solution, implying the formation of MSU crystals. The crystal formation may have subsequently caused the increase in inflammatory responses via the activation of complement and neutrophils

However, the results obtained cannot be viewed without some caution. Mice produce uricase, which degrades uric acid in their tissues. Therefore mice could not have

conditions associated with gout including hyperuricaemia, that may potentially result in MSU microcrystal formation. A uricase-knockout mice would be most useful in the model of MSU crystal induced inflammation; however, these mice are not viable due to acute renal failure (195). Thus, uric acid solution was injected to increase the uric acid serum content. Almost 15 % of the B cell hybridoma produced antibodies with a positive MSU crystal binding ability were IgG class. However, IgG antibodies were not used in any of the experiments due to a short in vitro half life. E6C7 (UBA) alone was able to induce an increase in the complement and myeloperoxidase measurements, rivalling that seen with the UBA E6C7 and uric acid injections. One possible explanation for this observation is that the injection of the antibody may have caused stress, increasing the endogenous serum uric acid content. The increases in the endogenous uric acid would act like an additional infusion of uric acid in the other animals; possible precipitation of uric acid could occur, resulting in the same inflammatory responses seen.

In addition, the control antibody could mediate the shedding of the CD62L from CD11b+ cells in vivo. The response was not as dramatic as that seen with the UBA E6C7, but this result could indicate that the presence of an antibody in B cell deficient (muMT) mice could regulate immune system activation. Also, many of the results obtained in vivo indirectly indicated MSU crystal formation. The most direct approach to verify our hypothesis would be to visualize the microscopic crystal deposition in the tissues. Histology could prove to be useful; however, preparations such as Gomori's methenamine-silver method are incompatible with the microcrystals due to dissolution or dismounting of the crystals during the preparatory steps (148). At this time, we do not possess the imaging technologies that are useful in detecting the presence of MSU

microcrystal deposits within tissue, but we remain hopeful that the formation of MSU crystals in vivo could be observed in the future.

Produced due to MSU crystal immunizations, IgM antibodies that are capable of precipitating supersaturated uric acid solution indicate the presence of a new class of antibodies that can act as nucleating agents and stabilize the crystal lattice formation. These antibodies were observed not only with MSU crystals but also with crystals of magnesium urate and allopurinol (155). The observed specificity shows that crystal binding serum proteins may be involved in crystal binding, precipitation and crystal associated pathology. This is especially important in humans and primates who have lost their ability to degrade uric acid (196, 197). 85 % of the monoclonal antibodies with a positive binding ability to MSU crystals, produced by the B cell hybridomas, were identified as IgM. Prior observations made with human MSU crystals showed that IgG was the most abundant immunoglobulin protein bound to the crystals (142, 144, 198). In humans, the class switch from IgM to IgG would be propagated by the natural IgM and by the proinflammatory cytokines produced (173, 174, 199). Mice do not normally see the local supersaturated solutions of uric acid, thereby producing no MSU crystals, which prevents the propagation of the antibody class switch. However, they still produce natural antibodies which could bind to the MSU crystals. Other groups observed that T15-IgM, a natural antibody that binds to apoptotic cells, would enhance dendritic cell phagocytosis of apoptotic cells, suppress TLR responses and inhibit inflammatory arthritis (200, 201). These results partially conflict with the main hypothesis that natural antibodies could be used as nucleating agents and inflammatory mediators to MSU crystals; however their natural antibody was specific for phosphorylcholine determinants and not for crystal

lattices (202). This does not affect our hypothesis since this information is indicative of different functions of the natural antibodies, that depend on their antigen specificity.

MSU crystals are the pathological agent identified in gout. Many studies have been devoted to deciphering the intracellular activation pathways and possible receptors for MSU crystal induced inflammation. Most recently, the NLRP3 inflammasome, a multi-protein complex capable of activating caspase-1, was found to be involved in producing IL-1β in response to MSU crystal stimulation (134). PLA2, PLD, PKC, Syk and PI3K have also been implicated in signal transduction when neutrophils interact with MSU crystals (100, 101, 105, 111, 185, 187). Ng *et al* had obtained similar results using dendritic cells where Syk dependent activation signals were measured during DC MSU crystal activation (179). Their results, however, contradicted the existence of a protein cell surface receptor for the MSU crystals. Using atomic force microscopy, they were able to show that MSU crystals could aggregate cholesterol components of the plasma membrane on individual dendritic cells and that Syk was involved in mediating the cell, MSU crystal interactions. The implication of this study is that MSU crystals can propagate a non-specific activation signal to the dendritic cells through surface contact on the plasma membrane and the sorting of lipid domains. The lipid domains, or rafts, contain receptors with immunoreceptor tyrosine based activation motifs (ITAMs) used for further signal transduction. However, they found no specific receptors on dendritic cells responsible for binding to the MSU crystals (179). These experiments would have to be repeated with macrophages and neutrophils to ascertain the non-specific cellular activation using the plasma membrane.

Other avenues of inflammatory cellular activation, such as through immune complexes and complement activation, have not been ruled out from causing the MSU crystal induced inflammatory responses. There have been investigations into the existence of a mu heavy chain Fc receptor on murine cells, which could allow the IgM immune complex to act the same as in other defined FcR-mediated inflammatory responses (203-206). Additionally, complement components C3a and C5a released during complement activation, possibly occurring during the formation of IgM immune complexes, are anaphylotoxins that trigger degranulation of mast cells (207).

Uric acid, a ubiquitous molecule in the cytosol, is released from cells undergoing cellular death as a danger signal; it is also a component of the inflammatory MSU crystals found in gout (50). The data implies the presence of antibodies with crystal nucleating capabilities would increase MSU crystal formation in the body. These findings are significant to the study of diagnosis of gout in clinical settings. If a patient has MSU crystal nucleating agents in their serum, as well as hyperuricaemia they should be considered in a higher risk category or those who can potentially develop gout. Besides diagnosis, these findings may allow for other treatment options to be developed against the MSU crystal nucleating antibodies. By preventing the accumulation of MSU crystal nucleating antibodies, the formation of MSU crystals can be prevented. Thus the subsequent inflammation caused due to MSU crystal presence in the body may also be prevented. Besides gout, uric acid has been found to promote tumour immune rejection, play a role in cytotoxic T cell activation in transplantation and autoimmune diabetes and act in the proliferation of carbon-tetrachloride (CCl_4) induced liver fibrosis (208-210). This new line of investigation may enhance understanding about other crystal-induced

arthropathies and uric acid related pathological conditions besides addressing the question of how endogenous MSU crystals are formed.

The current findings showed that uric acid must be in a local supersaturated solution and in the presence of MSU crystal specific IgM antibody to precipitate. The antibodies generated can bind to the MSU crystals in an immune complex formation and mediate systemic inflammatory responses in vivo, as seen by elevations in C3 complement component synthesis and neutrophil activation. In B cell deficient mice, antibodies were required for the uric acid/MSU crystal adjuvant effect to occur. Overall, this work implies that IgM antibodies can act as potential nucleating agents for the MSU crystals. This is a novel role for antibodies as their main activities have been described as opsonization, complement activation and antibody dependent cell-mediated cytotoxicity (58). Besides acting as nucleating agents, the antibodies may also stabilize the crystal and may have a role in mediating the subsequent adjuvanticity and inflammatory responses in vivo.

REFERENCES

1. Green, D. R., T. Ferguson, L. Zitvogel, and G. Kroemer. 2009. Immunogenic and tolerogenic cell death. *Nat Rev Immunol* 9:353-363.
2. Rock, K. L., and H. Kono. 2008. The inflammatory response to cell death. *Annu Rev Pathol* 3:99-126.
3. Chung, E. Y., S. J. Kim, and X. J. Ma. 2006. Regulation of cytokine production during phagocytosis of apoptotic cells. *Cell Res* 16:154-161.
4. Huynh, M. L., V. A. Fadok, and P. M. Henson. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest* 109:41-50.
5. Matzinger, P. 2002. The danger model: a renewed sense of self. *Science* 296:301-305.
6. Burnet, F. M. 1959. *The clonal selection theory of acquired immunity*. Vanderbilt University Press, Nashville.
7. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science* 169:1042-1049.
8. Lafferty, K. J., and A. J. Cunningham. 1975. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci* 53:27-42.
9. Janeway, C. A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1:1-13.
10. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol* 12:991-1045.

11. Matzinger, P. 1998. An innate sense of danger. *Semin Immunol* 10:399-415.

12. Gallucci, S., M. Lolkema, and P. Matzinger. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 5:1249-1255.

13. Shi, Y., W. Zheng, and K. L. Rock. 2000. Cell injury releases endogenous adjuvants that stimulate cytotoxic T cell responses. *Proc Natl Acad Sci U S A* 97:14590-14595.

14. Shi, Y., and K. L. Rock. 2002. Cell death releases endogenous adjuvants that selectively enhance immune surveillance of particulate antigens. *Eur J Immunol* 32:155-162.

15. Rock, K. L., A. Hearn, C. J. Chen, and Y. Shi. 2005. Natural endogenous adjuvants. *Springer Semin Immunopathol* 26:231-246.

16. Jenne, L., J. F. Arrighi, H. Jonuleit, J. H. Saurat, and C. Hauser. 2000. Dendritic cells containing apoptotic melanoma cells prime human CD8+ T cells for efficient tumor cell lysis. *Cancer Res* 60:4446-4452.

17. Sauter, B., M. L. Albert, L. Francisco, M. Larsson, S. Somersan, and N. Bhardwaj. 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 191:423-434.

18. Somersan, S., M. Larsson, J. F. Fonteneau, S. Basu, P. Srivastava, and N. Bhardwaj. 2001. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. *J Immunol* 167:4844-4852.

19. Morelli, A. E., A. T. Larregina, W. J. Shufesky, A. F. Zahorchak, A. J. Logar, G. D. Papworth, Z. Wang, S. C. Watkins, L. D. Falo, Jr., and A. W. Thomson. 2003.

Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood* 101:611-620.

20. Savill, J., I. Dransfield, C. Gregory, and C. Haslett. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat Rev Immunol* 2:965-975.

21. Stuart, L. M., M. Lucas, C. Simpson, J. Lamb, J. Savill, and A. Lacy-Hulbert. 2002. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. *J Immunol* 168:1627-1635.

22. Ferguson, T. A., J. Herndon, B. Elzey, T. S. Griffith, S. Schoenberger, and D. R. Green. 2002. Uptake of apoptotic antigen-coupled cells by lymphoid dendritic cells and cross-priming of CD8(+) T cells produce active immune unresponsiveness. *J Immunol* 168:5589-5595.

23. Steinman, R. M., S. Turley, I. Mellman, and K. Inaba. 2000. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J Exp Med* 191:411-416.

24. Buttiglieri, S., A. Galetto, S. Forno, M. De Andrea, and L. Matera. 2003. Influence of drug-induced apoptotic death on processing and presentation of tumor antigens by dendritic cells. *Int J Cancer* 106:516-520.

25. Feng, H., Y. Zeng, M. W. Graner, and E. Katsanis. 2002. Stressed apoptotic tumor cells stimulate dendritic cells and induce specific cytotoxic T cells. *Blood* 100:4108-4115.

26. Goldszmid, R. S., J. Idoyaga, A. I. Bravo, R. Steinman, J. Mordoh, and R. Wainstok. 2003. Dendritic cells charged with apoptotic tumor cells induce long-lived protective CD4+ and CD8+ T cell immunity against B16 melanoma. *J Immunol* 171:5940-5947.

27. Ignatius, R., M. Marovich, E. Mehlhop, L. Villamide, K. Mahnke, W. I. Cox, F. Isdell, S. S. Frankel, J. R. Mascola, R. M. Steinman, and M. Pope. 2000. Canarypox virus-induced maturation of dendritic cells is mediated by apoptotic cell death and tumor necrosis factor alpha secretion. *J Virol* 74:11329-11338.

28. Ishii, S., K. Hiroishi, J. Eguchi, and K. Mitamura. 2003. Dendritic cell maturation induced by delivery of ultraviolet-mediated apoptotic colorectal cancer cell lines. *Anticancer Res* 23:2457-2463.

29. Rovere, P., C. Vallinoto, A. Bondanza, M. C. Crosti, M. Rescigno, P. Ricciardi-Castagnoli, C. Rugarli, and A. A. Manfredi. 1998. Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. *J Immunol* 161:4467-4471.

30. Rovere, P., M. G. Sabbadini, C. Vallinoto, U. Fascio, V. S. Zimmermann, A. Bondanza, P. Ricciardi-Castagnoli, and A. A. Manfredi. 1999. Delayed clearance of apoptotic lymphoma cells allows cross-presentation of intracellular antigens by mature dendritic cells. *J Leukoc Biol* 66:345-349.

31. Chang, J. W., M. Peng, J. E. Vaquerano, Y. M. Zhou, R. A. Clinton, W. C. Hyun, M. A. Giedlin, and S. P. Leong. 2000. Induction of Th1 response by dendritic cells pulsed with autologous melanoma apoptotic bodies. *Anticancer Res* 20:1329-1336.

32. Ferlazzo, G., C. Semino, G. M. Spaggiari, M. Meta, M. C. Mingari, and G. Melioli. 2000. Dendritic cells efficiently cross-prime HLA class I-restricted cytolytic T lymphocytes when pulsed with both apoptotic and necrotic cells but not with soluble cell-derived lysates. *Int Immunol* 12:1741-1747.

33. Hoffmann, T. K., N. Meidenbauer, G. Dworacki, H. Kanaya, and T. L. Whiteside. 2000. Generation of tumor-specific T-lymphocytes by cross-priming with human dendritic cells ingesting apoptotic tumor cells. *Cancer Res* 60:3542-3549.

34. Kotera, Y., K. Shimizu, and J. J. Mule. 2001. Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. *Cancer Res* 61:8105-8109.

35. Schnurr, M., C. Scholz, S. Rothenfusser, P. Galambos, M. Dauer, J. Robe, S. Endres, and A. Eigler. 2002. Apoptotic pancreatic tumor cells are superior to cell lysates in promoting cross-priming of cytotoxic T cells and activate NK and gammadelta T cells. *Cancer Res* 62:2347-2352.

36. Chen, Z., T. Moyana, A. Saxena, R. Warrington, Z. Jia, and J. Xiang. 2001. Efficient antitumor immunity derived from maturation of dendritic cells that had phagocytosed apoptotic/necrotic tumor cells. *Int J Cancer* 93:539-548.

37. Kim, K. W., S. H. Kim, J. G. Shin, G. S. Kim, Y. O. Son, S. W. Park, B. H. Kwon, D. W. Kim, C. H. Lee, M. Y. Sol, M. H. Jeong, B. S. Chung, and C. D. Kang. 2004. Direct injection of immature dendritic cells into irradiated tumor induces efficient antitumor immunity. *Int J Cancer* 109:685-690.

38. Nowak, A. K., R. A. Lake, A. L. Marzo, B. Scott, W. R. Heath, E. J. Collins, J. A. Frelinger, and B. W. Robinson. 2003. Induction of tumor cell apoptosis in vivo

increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J Immunol* 170:4905-4913.

39. Ronchetti, A., P. Rovere, G. Iezzi, G. Galati, S. Heltai, M. P. Protti, M. P. Garancini, A. A. Manfredi, C. Rugarli, and M. Bellone. 1999. Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. *J Immunol* 163:130-136.

40. Scheffer, S. R., H. Nave, F. Korangy, K. Schlote, R. Pabst, E. M. Jaffee, M. P. Manns, and T. F. Greten. 2003. Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response in vivo. *Int J Cancer* 103:205-211.

41. Strome, S. E., S. Voss, R. Wilcox, T. L. Wakefield, K. Tamada, D. Flies, A. Chapoval, J. Lu, J. L. Kasperbauer, D. Padley, R. Vile, D. Gastineau, P. Wettstein, and L. Chen. 2002. Strategies for antigen loading of dendritic cells to enhance the antitumor immune response. *Cancer Res* 62:1884-1889.

42. Landsman, D., and M. Bustin. 1993. A signature for the HMG-1 box DNA-binding proteins. *Bioessays* 15:539-546.

43. Scaffidi, P., T. Misteli, and M. E. Bianchi. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418:191-195.

44. Rovere-Querini, P., A. Capobianco, P. Scaffidi, B. Valentinis, F. Catalanotti, M. Giazzon, I. E. Dumitriu, S. Muller, M. Iannacone, C. Traversari, M. E. Bianchi, and A. A. Manfredi. 2004. HMGB1 is an endogenous immune adjuvant released by necrotic cells. *EMBO Rep* 5:825-830.

45. Messmer, D., H. Yang, G. Telusma, F. Knoll, J. Li, B. Messmer, K. J. Tracey, and N. Chiorazzi. 2004. High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization. *J Immunol* 173:307-313.

46. Srivastava, P. K., and M. Heike. 1991. Tumor-specific immunogenicity of stress-induced proteins: convergence of two evolutionary pathways of antigen presentation? *Semin Immunol* 3:57-64.

47. Srivastava, P. K., and R. G. Maki. 1991. Stress-induced proteins in immune response to cancer. *Curr Top Microbiol Immunol* 167:109-123.

48. Basu, S., R. J. Binder, R. Suto, K. M. Anderson, and P. K. Srivastava. 2000. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol* 12:1539-1546.

49. Wallin, R. P., A. Lundqvist, S. H. More, A. von Bonin, R. Kiessling, and H. G. Ljunggren. 2002. Heat-shock proteins as activators of the innate immune system. *Trends Immunol* 23:130-135.

50. Shi, Y., J. E. Evans, and K. L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425:516-521.

51. Haskard, D. O., and R. C. Landis. 2002. Interactions between leukocytes and endothelial cells in gout: lessons from a self-limiting inflammatory response. *Arthritis Res* 4 Suppl 3:S91-97.

52. Akahoshi, T., Y. Murakami, and H. Kitasato. 2007. Recent advances in crystal-induced acute inflammation. *Curr Opin Rheumatol* 19:146-150.

53. Wallace, K. L., A. A. Riedel, N. Joseph-Ridge, and R. Wortmann. 2004. Increasing prevalence of gout and hyperuricemia over 10 years among older adults in a managed care population. *J Rheumatol* 31:1582-1587.

54. Martinon, F., and L. H. Glimcher. 2006. Gout: new insights into an old disease. *J Clin Invest* 116:2073-2075.

55. Dalbeth, N., and D. O. Haskard. 2005. Mechanisms of inflammation in gout. *Rheumatology (Oxford)* 44:1090-1096.

56. Hall, A. P., P. E. Barry, T. R. Dawber, and P. M. McNamara. 1967. Epidemiology of gout and hyperuricemia. A long-term population study. *Am J Med* 42:27-37.

57. Schumacher, H. R. 1975. Pathology of the synovial membrane in gout. Light and electron microscopic studies. Interpretation of crystals in electron micrographs. *Arthritis Rheum* 18:771-782.

58. Kindt, T. J., R. A. Goldsby, B. A. Osborne, and J. Kuby. 2007. *Kuby immunology*. W.H. Freeman, New York.

59. Wagner, D. D. 1993. The Weibel-Palade body: the storage granule for von Willebrand factor and P-selectin. *Thromb Haemost* 70:105-110.

60. Utgaard, J. O., F. L. Jahnsen, A. Bakka, P. Brandtzaeg, and G. Haraldsen. 1998. Rapid secretion of prestored interleukin 8 from Weibel-Palade bodies of microvascular endothelial cells. *J Exp Med* 188:1751-1756.

61. Wolff, B., A. R. Burns, J. Middleton, and A. Rot. 1998. Endothelial cell "memory" of inflammatory stimulation: human venular endothelial cells store interleukin 8 in Weibel-Palade bodies. *J Exp Med* 188:1757-1762.

62. Pober, J. S., and R. S. Cotran. 1990. Cytokines and endothelial cell biology. *Physiol Rev* 70:427-451.

63. Getting, S. J., R. J. Flower, L. Parente, R. de Medicis, A. Lussier, B. A. Woliztky, M. A. Martins, and M. Perretti. 1997. Molecular determinants of monosodium urate crystal-induced murine peritonitis: a role for endogenous mast cells and a distinct requirement for endothelial-derived selectins. *J Pharmacol Exp Ther* 283:123-130.

64. Meng, H., M. G. Tonnesen, M. J. Marchese, R. A. Clark, W. F. Bahou, and B. L. Gruber. 1995. Mast cells are potent regulators of endothelial cell adhesion molecule ICAM-1 and VCAM-1 expression. *J Cell Physiol* 165:40-53.

65. Roberge, C. J., J. Grassi, R. De Medicis, Y. Frobert, A. Lussier, P. H. Naccache, and P. E. Poubelle. 1991. Crystal-neutrophil interactions lead to interleukin-1 synthesis. *Agents Actions* 34:38-41.

66. Roberge, C. J., R. de Medicis, J. M. Dayer, M. Rola-Pleszczynski, P. H. Naccache, and P. E. Poubelle. 1994. Crystal-induced neutrophil activation. V. Differential production of biologically active IL-1 and IL-1 receptor antagonist. *J Immunol* 152:5485-5494.

67. Dinarello, C. A. 1991. Interleukin-1 and interleukin-1 antagonism. *Blood* 77:1627-1652.

68. Guerne, P. A., R. Terkeltaub, B. Zuraw, and M. Lotz. 1989. Inflammatory microcrystals stimulate interleukin-6 production and secretion by human monocytes and synoviocytes. *Arthritis Rheum* 32:1443-1452.

69. Terkeltaub, R., C. Zachariae, D. Santoro, J. Martin, P. Peveri, and K. Matsushima. 1991. Monocyte-derived neutrophil chemotactic factor/interleukin-8 is a potential mediator of crystal-induced inflammation. *Arthritis Rheum* 34:894-903.

70. Di Giovine, F. S., S. E. Malawista, G. Nuki, and G. W. Duff. 1987. Interleukin 1 (IL 1) as a mediator of crystal arthritis. Stimulation of T cell and synovial fibroblast mitogenesis by urate crystal-induced IL 1. *J Immunol* 138:3213-3218.

71. di Giovine, F. S., S. E. Malawista, E. Thornton, and G. W. Duff. 1991. Urate crystals stimulate production of tumor necrosis factor alpha from human blood monocytes and synovial cells. Cytokine mRNA and protein kinetics, and cellular distribution. *J Clin Invest* 87:1375-1381.

72. Gilbert, C., P. E. Poubelle, P. Borgeat, M. Pouliot, and P. H. Naccache. 2003. Crystal-induced neutrophil activation: VIII. Immediate production of prostaglandin E2 mediated by constitutive cyclooxygenase 2 in human neutrophils stimulated by urate crystals. *Arthritis Rheum* 48:1137-1148.

73. Gordon, T. P., I. C. Kowanko, M. James, and P. J. Roberts-Thomson. 1985. Monosodium urate crystal-induced prostaglandin synthesis in the rat subcutaneous air pouch. *Clin Exp Rheumatol* 3:291-296.

74. Serhan, C. N., U. Lundberg, G. Weissmann, and B. Samuelsson. 1984. Formation of leukotrienes and hydroxy acids by human neutrophils and platelets exposed to monosodium urate. *Prostaglandins* 27:563-581.

75. Simchowitz, L., J. P. Atkinson, and I. Spilberg. 1982. Stimulation of the respiratory burst in human neutrophils by crystal phagocytosis. *Arthritis Rheum* 25:181-188.

76. Abramson, S., S. T. Hoffstein, and G. Weissmann. 1982. Superoxide anion generation by human neutrophils exposed to monosodium urate. *Arthritis Rheum* 25:174-180.

77. Okamura, H., H. Tsutsi, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, and et al. 1995. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 378:88-91.

78. Vervoordeldonk, M. J., and P. P. Tak. 2002. Cytokines in rheumatoid arthritis. *Curr Rheumatol Rep* 4:208-217.

79. Kashiwamura, S., H. Ueda, and H. Okamura. 2002. Roles of interleukin-18 in tissue destruction and compensatory reactions. *J Immunother* 25 Suppl 1:S4-11.

80. Inokuchi, T., Y. Moriwaki, H. Tsutsui, A. Yamamoto, S. Takahashi, Z. Tsutsumi, T. Ka, K. Nakanishi, and T. Yamamoto. 2006. Plasma interleukin (IL)-18 (interferon-gamma-inducing factor) and other inflammatory cytokines in patients with gouty arthritis and monosodium urate monohydrate crystal-induced secretion of IL-18. *Cytokine* 33:21-27.

81. Jaramillo, M., M. Godbout, P. H. Naccache, and M. Olivier. 2004. Signaling events involved in macrophage chemokine expression in response to monosodium urate crystals. *J Biol Chem* 279:52797-52805.

82. Chen, L., M. S. Hsieh, H. C. Ho, Y. H. Liu, D. T. Chou, and S. H. Tsai. 2004. Stimulation of inducible nitric oxide synthase by monosodium urate crystals in macrophages and expression of iNOS in gouty arthritis. *Nitric Oxide* 11:228-236.

83. Miyasaka, N., and Y. Hirata. 1997. Nitric oxide and inflammatory arthritides. *Life Sci* 61:2073-2081.

84. Stichtenoth, D. O., and J. C. Frolich. 1998. Nitric oxide and inflammatory joint diseases. *Br J Rheumatol* 37:246-257.

85. Landis, R. C., D. R. Yagnik, O. Florey, P. Philippidis, V. Emons, J. C. Mason, and D. O. Haskard. 2002. Safe disposal of inflammatory monosodium urate monohydrate crystals by differentiated macrophages. *Arthritis Rheum* 46:3026-3033.

86. Murakami, Y., T. Akahoshi, S. Kawai, M. Inoue, and H. Kitasato. 2002. Antiinflammatory effect of retrovirally transfected interleukin-10 on monosodium urate monohydrate crystal-induced acute inflammation in murine air pouches. *Arthritis Rheum* 46:2504-2513.

87. Yagnik, D. R., B. J. Evans, O. Florey, J. C. Mason, R. C. Landis, and D. O. Haskard. 2004. Macrophage release of transforming growth factor beta1 during resolution of monosodium urate monohydrate crystal-induced inflammation. *Arthritis Rheum* 50:2273-2280.

88. Pekin, T. J., Jr., and N. J. Zvaifler. 1964. Hemolytic Complement in Synovial Fluid. *J Clin Invest* 43:1372-1382.

89. Doherty, M., J. T. Whicher, and P. A. Dieppe. 1983. Activation of the alternative pathway of complement by monosodium urate monohydrate crystals and other inflammatory particles. *Ann Rheum Dis* 42:285-291.

90. Hasselbacher, P. 1979. C3 activation by monosodium urate monohydrate and other crystalline material. *Arthritis Rheum* 22:571-578.

91. Giclas, P. C., M. H. Ginsberg, and N. R. Cooper. 1979. Immunoglobulin G independent activation of the classical complement pathway by monosodium urate crystals. *J Clin Invest* 63:759-764.

92. Byers, P. H., P. A. Ward, R. W. Kellermeyer, and G. B. Naff. 1973. Complement as a mediator of inflammation in acute gouty arthritis. II. Biological activities generated from complement by the interaction of serum complement and sodium urate crystals. *J Lab Clin Med* 81:761-769.

93. Naff, G. B., and P. H. Byers. 1973. Complement as a mediator of inflammation in acute gouty arthritis. I. Studies on the reaction between human serum complement and sodium urate crystals. *J Lab Clin Med* 81:747-760.

94. Tramontini, N., C. Huber, R. Liu-Bryan, R. A. Terkeltaub, and K. S. Kilgore. 2004. Central role of complement membrane attack complex in monosodium urate crystal-induced neutrophilic rabbit knee synovitis. *Arthritis Rheum* 50:2633-2639.

95. Kilgore, K. S., E. Schmid, T. P. Shanley, C. M. Flory, V. Maheswari, N. L. Tramontini, H. Cohen, P. A. Ward, H. P. Friedl, and J. S. Warren. 1997. Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-kappa B activation. *Am J Pathol* 150:2019-2031.

96. Kilgore, K. S., P. A. Ward, and J. S. Warren. 1998. Neutrophil adhesion to human endothelial cells is induced by the membrane attack complex: the roles of P-selectin and platelet activating factor. *Inflammation* 22:583-598.

97. Bomalaski, J. S., D. G. Baker, L. M. Brophy, and M. A. Clark. 1990. Monosodium urate crystals stimulate phospholipase A2 enzyme activities and the synthesis of a phospholipase A2-activating protein. *J Immunol* 145:3391-3397.

98. Murakami, M., and I. Kudo. 2002. Phospholipase A2. *J Biochem* 131:285-292.

99. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* 257:7847-7851.

100. Naccache, P. H., S. Bourgoin, E. Plante, C. J. Roberge, R. de Medicis, A. Lussier, and P. E. Poubelle. 1993. Crystal-induced neutrophil activation. II. Evidence for the activation of a phosphatidylcholine-specific phospholipase D. *Arthritis Rheum* 36:117-125.

101. Gaudry, M., C. J. Roberge, R. de Medicis, A. Lussier, P. E. Poubelle, and P. H. Naccache. 1993. Crystal-induced neutrophil activation. III. Inflammatory microcrystals induce a distinct pattern of tyrosine phosphorylation in human neutrophils. *J Clin Invest* 91:1649-1655.

102. Indik, Z. K., J. G. Park, S. Hunter, and A. D. Schreiber. 1995. The molecular dissection of Fc gamma receptor mediated phagocytosis. *Blood* 86:4389-4399.

103. Indik, Z. K., J. G. Park, X. Q. Pan, and A. D. Schreiber. 1995. Induction of phagocytosis by a protein tyrosine kinase. *Blood* 85:1175-1180.

104. Barabe, F., C. Gilbert, N. Liao, S. G. Bourgoin, and P. H. Naccache. 1998. Crystal-induced neutrophil activation VI. Involvment of FcgammaRIIIB (CD16) and CD11b in response to inflammatory microcrystals. *FASEB J* 12:209-220.

105. Desaulniers, P., M. Fernandes, C. Gilbert, S. G. Bourgoin, and P. H. Naccache. 2001. Crystal-induced neutrophil activation. VII. Involvement of Syk in the responses to monosodium urate crystals. *J Leukoc Biol* 70:659-668.

106. Burt, H. M., J. K. Jackson, and H. Salari. 1994. Inhibition of crystal-induced neutrophil activation by a protein tyrosine kinase inhibitor. *J Leukoc Biol* 55:112-119.

107. Marcil, J., D. Harbour, M. G. Houle, P. H. Naccache, and S. Bourgoin. 1999. Monosodium urate-crystal-stimulated phospholipase D in human neutrophils. *Biochem J* 337 (Pt 2):185-192.

108. Raeder, E. M., P. J. Mansfield, V. Hinkovska-Galcheva, J. A. Shayman, and L. A. Boxer. 1999. Syk activation initiates downstream signaling events during human polymorphonuclear leukocyte phagocytosis. *J Immunol* 163:6785-6793.

109. von Willebrand, M., S. Williams, P. Tailor, and T. Mustelin. 1998. Phosphorylation of the Grb2- and phosphatidylinositol 3-kinase p85-binding p36/38 by Syk in Lck-negative T cells. *Cell Signal* 10:407-413.

110. Jackson, J. K., R. Lauener, V. Duronio, and H. M. Burt. 1997. The involvement of phosphatidylinositol 3-kinase in crystal induced human neutrophil activation. *J Rheumatol* 24:341-348.

111. Popa-Nita, O., S. Proulx, G. Pare, E. Rollet-Labelle, and P. H. Naccache. 2009. Crystal-induced neutrophil activation: XI. Implication and novel roles of classical protein kinase C. *J Immunol* 183:2104-2114.

112. Naccache, P. H., T. F. Molski, P. Borgeat, J. R. White, and R. I. Sha'afi. 1985. Phorbol esters inhibit the fMet-Leu-Phe- and leukotriene B4-stimulated calcium

mobilization and enzyme secretion in rabbit neutrophils. *J Biol Chem* 260:2125-2131.

113. Gilbert, C., M. Gaudry, and P. H. Naccache. 1992. Rapid priming of calcium mobilization and superoxide anion production in human neutrophils by substimulatory concentrations of phorbol esters: a novel role for protein kinase C and tyrosine phosphorylation in the up-modulation of signal transduction. *Cell Signal* 4:511-523.

114. Kanaho, Y., K. Takahashi, U. Tomita, T. Iiri, T. Katada, M. Ui, and Y. Nozawa. 1992. A protein kinase C inhibitor, staurosporine, activates phospholipase D via a pertussis toxin-sensitive GTP-binding protein in rabbit peritoneal neutrophils. *J Biol Chem* 267:23554-23559.

115. Lopez-Lluch, G., M. M. Bird, B. Canas, J. Godovac-Zimmerman, A. Ridley, A. W. Segal, and L. V. Dekker. 2001. Protein kinase C-delta C2-like domain is a binding site for actin and enables actin redistribution in neutrophils. *Biochem J* 357:39-47.

116. Alexander, S. R., T. K. Kishimoto, and B. Walcheck. 2000. Effects of selective protein kinase C inhibitors on the proteolytic down-regulation of L-selectin from chemoattractant-activated neutrophils. *J Leukoc Biol* 67:415-422.

117. Liu-Bryan, R., K. Pritzker, G. S. Firestein, and R. Terkeltaub. 2005. TLR2 signaling in chondrocytes drives calcium pyrophosphate dihydrate and monosodium urate crystal-induced nitric oxide generation. *J Immunol* 174:5016-5023.

118. Liu-Bryan, R., P. Scott, A. Sydlaske, D. M. Rose, and R. Terkeltaub. 2005. Innate immunity conferred by Toll-like receptors 2 and 4 and myeloid differentiation factor 88 expression is pivotal to monosodium urate monohydrate crystal-induced inflammation. *Arthritis Rheum* 52:2936-2946.

119. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu Rev Immunol* 21:335-376.

120. Scott, P., H. Ma, S. Viriyakosol, R. Terkeltaub, and R. Liu-Bryan. 2006. Engagement of CD14 mediates the inflammatory potential of monosodium urate crystals. *J Immunol* 177:6370-6378.

121. Pugin, J., I. D. Heumann, A. Tomasz, V. V. Kravchenko, Y. Akamatsu, M. Nishijima, M. P. Glauser, P. S. Tobias, and R. J. Ulevitch. 1994. CD14 is a pattern recognition receptor. *Immunity* 1:509-516.

122. Fitzgerald, K. A., D. C. Rowe, and D. T. Golenbock. 2004. Endotoxin recognition and signal transduction by the TLR4/MD2-complex. *Microbes Infect* 6:1361-1367.

123. Palsson-McDermott, E. M., and L. A. O'Neill. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113:153-162.

124. Landmann, R., B. Muller, and W. Zimmerli. 2000. CD14, new aspects of ligand and signal diversity. *Microbes Infect* 2:295-304.

125. Chen, C. J., Y. Shi, A. Hearn, K. Fitzgerald, D. Golenbock, G. Reed, S. Akira, and K. L. Rock. 2006. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. *J Clin Invest* 116:2262-2271.

126. Burns, K., F. Martinon, and J. Tschopp. 2003. New insights into the mechanism of IL-1beta maturation. *Curr Opin Immunol* 15:26-30.

127. Franchi, L., T. Eigenbrod, R. Munoz-Planillo, and G. Nunez. 2009. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* 10:241-247.

128. Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10:417-426.

129. Yu, H. B., and B. B. Finlay. 2008. The caspase-1 inflammasome: a pilot of innate immune responses. *Cell Host Microbe* 4:198-208.

130. Tschopp, J., F. Martinon, and K. Burns. 2003. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 4:95-104.

131. Martinon, F., and J. Tschopp. 2005. NLRs join TLRs as innate sensors of pathogens. *Trends Immunol* 26:447-454.

132. Mariathasan, S., K. Newton, D. M. Monack, D. Vucic, D. M. French, W. P. Lee, M. Roose-Girma, S. Erickson, and V. M. Dixit. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430:213-218.

133. Martinon, F., L. Agostini, E. Meylan, and J. Tschopp. 2004. Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. *Curr Biol* 14:1929-1934.

134. Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237-241.

135. Kanevets, U., K. Sharma, K. Dresser, and Y. Shi. 2009. A role of IgM antibodies in monosodium urate crystal formation and associated adjuvanticity. *J Immunol* 182:1912-1918.

136. Fiddis, R. W., N. Vlachos, and P. D. Calvert. 1983. Studies of urate crystallisation in relation to gout. *Ann Rheum Dis* 42 Suppl 1:12-15.

137. Tak, H. K., S. M. Cooper, and W. R. Wilcox. 1980. Studies on the nucleation of monosodium urate at 37 degrees c. *Arthritis Rheum* 23:574-580.

138. Loeb, J. N. 1972. The influence of temperature on the solubility of monosodium urate. *Arthritis Rheum* 15:189-192.

139. Seegmiller, J. E., and R. R. Howell. 1962. The old and new concepts of acute gouty arthritis. *Arthritis Rheum* 5:616-623.

140. Roberts, W. 1892. *On the chemistry and therapeutics of uric acid gravel and gout*. Smith, Elder, & co., London,.

141. Kippen, I., J. R. Klinenberg, A. Weinberger, and W. R. Wilcox. 1974. Factors affecting urate solubility in vitro. *Ann Rheum Dis* 33:313-317.

142. Terkeltaub, R., A. J. Tenner, F. Kozin, and M. H. Ginsberg. 1983. Plasma protein binding by monosodium urate crystals. Analysis by two-dimensional gel electrophoresis. *Arthritis Rheum* 26:775-783.

143. Terkeltaub, R., L. K. Curtiss, A. J. Tenner, and M. H. Ginsberg. 1984. Lipoproteins containing apoprotein B are a major regulator of neutrophil responses to monosodium urate crystals. *J Clin Invest* 73:1719-1730.

144. Ortiz-Bravo, E., M. S. Sieck, and H. R. Schumacher, Jr. 1993. Changes in the proteins coating monosodium urate crystals during active and subsiding

inflammation. Immunogold studies of synovial fluid from patients with gout and of fluid obtained using the rat subcutaneous air pouch model. *Arthritis Rheum* 36:1274-1285.

145. Wallingford, W. R., and D. J. McCarty. 1971. Differential membranolytic effects of microcrystalline sodium urate and calcium pyrophosphate dihydrate. *J Exp Med* 133:100-112.

146. Kozin, F., and D. J. McCarty. 1976. Protein adsorption to monosodium urate, calcium pyrophosphate dihydrate, and silica crystals: relationship to the pathogenesis of crystal-induced inflammation. *Arthritis Rheum* 19 Suppl 3:433-438.

147. Hasselbacher, P., and H. R. Schumacher. 1978. Immunoglobulin in tophi and on the surface of monosodium urate crystals. *Arthritis Rheum* 21:353-361.

148. Bardin, T., P. Varghese Cherian, and H. R. Schumacher. 1984. Immunoglobulins on the surface of monosodium urate crystals: an immunoelectron microscopic study. *J Rheumatol* 11:339-341.

149. Kozin, F., and D. J. McCarty. 1980. Molecular orientation of immunoglobulin G adsorbed to microcrystalline monosodium urate monohydrate. *J Lab Clin Med* 95:49-58.

150. Gordon, T. P., M. J. Ahern, C. Reid, and P. J. Roberts-Thomson. 1985. Studies on the interaction of rheumatoid factor with monosodium urate crystals and case report of coexistent tophaceous gout and rheumatoid arthritis. *Ann Rheum Dis* 44:384-389.

151. Hashimoto, Y., K. Kohri, Y. Hayashi, A. Moriyama, M. Iguchi, K. Asai, and T. Kato. 1997. Specific detection of kappa light chain in uric acid stones. *Life Sci* 61:249-253.

152. Kam, M., D. Perl-Treves, D. Caspi, and L. Addadi. 1992. Antibodies against crystals. *FASEB J* 6:2608-2613.

153. Perl-Treves, D., and L. Addadi. 1988. A structural approach to pathological crystallizations. Gout: the possible role of albumin in sodium urate crystallization. *Proc R Soc Lond B Biol Sci* 235:145-159.

154. Harvey, P. R., G. A. Upadhya, and S. M. Strasberg. 1991. Immunoglobulins as nucleating proteins in the gallbladder bile of patients with cholesterol gallstones. *J Biol Chem* 266:13996-14003.

155. Kam, M., D. Perl-Treves, R. Sfez, and L. Addadi. 1994. Specificity in the recognition of crystals by antibodies. *J Mol Recognit* 7:257-264.

156. Furriel, R. P., Y. M. Lucisano, and B. Mantovani. 1992. Precipitated immune complexes of IgM as well as of IgG can bind to rabbit polymorphonuclear leucocytes but only the immune complexes of IgG are readily phagocytosed. *Immunology* 75:528-534.

157. Mantovani, B., M. Rabinovitch, and V. Nussenzweig. 1972. Phagocytosis of immune complexes by macrophages. Different roles of the macrophage receptor sites for complement (C3) and for immunoglobulin (IgG). *J Exp Med* 135:780-792.

158. Mantovani, B. 1975. Different roles of IgG and complement receptors in phagocytosis by polymorphonuclear leukocytes. *J Immunol* 115:15-17.

159. Fernandez, N., S. Jancar, and M. Sanchez Crespo. 2004. Blood and endothelium in immune complex-mediated tissue injury. *Trends Pharmacol Sci* 25:512-517.

160. Sylvestre, D. L., and J. V. Ravetch. 1996. A dominant role for mast cell Fc receptors in the Arthus reaction. *Immunity* 5:387-390.

161. Lee, D. M., D. S. Friend, M. F. Gurish, C. Benoist, D. Mathis, and M. B. Brenner. 2002. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* 297:1689-1692.

162. Boes, M. 2000. Role of natural and immune IgM antibodies in immune responses. *Mol Immunol* 37:1141-1149.

163. Manson, J. J., C. Mauri, and M. R. Ehrenstein. 2005. Natural serum IgM maintains immunological homeostasis and prevents autoimmunity. *Springer Semin Immunopathol* 26:425-432.

164. Cooper, N. R. 1985. The classical complement pathway: activation and regulation of the first complement component. *Adv Immunol* 37:151-216.

165. Hamilton, A. M., A. Lehuen, and J. F. Kearney. 1994. Immunofluorescence analysis of B-1 cell ontogeny in the mouse. *Int Immunol* 6:355-361.

166. Kantor, A. B., and L. A. Herzenberg. 1993. Origin of murine B cell lineages. *Annu Rev Immunol* 11:501-538.

167. Hardy, R. R., and K. Hayakawa. 1994. CD5 B cells, a fetal B cell lineage. *Adv Immunol* 55:297-339.

168. Ochsenbein, A. F., and R. M. Zinkernagel. 2000. Natural antibodies and complement link innate and acquired immunity. *Immunol Today* 21:624-630.

169. Wichterman, K. A., A. E. Baue, and I. H. Chaudry. 1980. Sepsis and septic shock--a review of laboratory models and a proposal. *J Surg Res* 29:189-201.

170. Boes, M., A. P. Prodeus, T. Schmidt, M. C. Carroll, and J. Chen. 1998. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp Med* 188:2381-2386.

171. Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, and R. M. Zinkernagel. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* 286:2156-2159.

172. Baumgarth, N., O. C. Herman, G. C. Jager, L. E. Brown, L. A. Herzenberg, and J. Chen. 2000. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med* 192:271-280.

173. Boes, M., C. Esau, M. B. Fischer, T. Schmidt, M. Carroll, and J. Chen. 1998. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol* 160:4776-4787.

174. Ehrenstein, M. R., T. L. O'Keefe, S. L. Davies, and M. S. Neuberger. 1998. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc Natl Acad Sci U S A* 95:10089-10093.

175. Applequist, S. E., J. Dahlstrom, N. Jiang, H. Molina, and B. Heyman. 2000. Antibody production in mice deficient for complement receptors 1 and 2 can be induced by IgG/Ag and IgE/Ag, but not IgM/Ag complexes. *J Immunol* 165:2398-2403.

176. Casali, P., and A. L. Notkins. 1989. CD5+ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. *Immunol Today* 10:364-368.

177. Sutaria, S., R. Katbamna, and M. Underwood. 2006. Effectiveness of interventions for the treatment of acute and prevention of recurrent gout--a systematic review. *Rheumatology (Oxford)* 45:1422-1431.

178. Desrosiers, M. D., K. M. Cembrola, M. J. Fakir, L. A. Stephens, F. M. Jama, A. Shameli, W. Z. Mehal, P. Santamaria, and Y. Shi. 2007. Adenosine deamination sustains dendritic cell activation in inflammation. *J Immunol* 179:1884-1892.

179. Ng, G., K. Sharma, S. M. Ward, M. D. Desrosiers, L. A. Stephens, W. M. Schoel, T. Li, C. A. Lowell, C. C. Ling, M. W. Amrein, and Y. Shi. 2008. Receptor-independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells. *Immunity* 29:807-818.

180. Springer, T. A. 1985. *Hybridoma technology in the biosciences and medicine.* Plenum Press, New York.

181. Bradley, P. P., D. A. Priebat, R. D. Christensen, and G. Rothstein. 1982. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 78:206-209.

182. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 350:423-426.

183. Dodds, A. W. 2002. Which came first, the lectin/classical pathway or the alternative pathway of complement? *Immunobiology* 205:340-354.

184. Manthei, U., R. C. Strunk, and P. C. Giclas. 1984. Acute local inflammation alters synthesis, distribution, and catabolism of third component of complement (C3) in rabbits. *J Clin Invest* 74:424-433.

185. Naccache, P. H., M. Grimard, C. J. Roberge, C. Gilbert, A. Lussier, R. de Medicis, and P. E. Poubelle. 1991. Crystal-induced neutrophil activation. I. Initiation and modulation of calcium mobilization and superoxide production by microcrystals. *Arthritis Rheum* 34:333-342.

186. Popa-Nita, O., L. Marois, G. Pare, and P. H. Naccache. 2008. Crystal-induced neutrophil activation: X. Proinflammatory role of the tyrosine kinase Tec. *Arthritis Rheum* 58:1866-1876.

187. Popa-Nita, O., E. Rollet-Labelle, N. Thibault, C. Gilbert, S. G. Bourgoin, and P. H. Naccache. 2007. Crystal-induced neutrophil activation. IX. Syk-dependent activation of class Ia phosphatidylinositol 3-kinase. *J Leukoc Biol* 82:763-773.

188. Roberge, C. J., M. Gaudry, R. de Medicis, A. Lussier, P. E. Poubelle, and P. H. Naccache. 1993. Crystal-induced neutrophil activation. IV. Specific inhibition of tyrosine phosphorylation by colchicine. *J Clin Invest* 92:1722-1729.

189. Chen, C. J., H. Kono, D. Golenbock, G. Reed, S. Akira, and K. L. Rock. 2007. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med* 13:851-856.

190. Behrens, M. D., W. M. Wagner, C. J. Krco, C. L. Erskine, K. R. Kalli, J. Krempski, E. A. Gad, M. L. Disis, and K. L. Knutson. 2008. The endogenous danger signal, crystalline uric acid, signals for enhanced antibody immunity. *Blood* 111:1472-1479.

191. Stager, S., J. Alexander, A. C. Kirby, M. Botto, N. V. Rooijen, D. F. Smith, F. Brombacher, and P. M. Kaye. 2003. Natural antibodies and complement are endogenous adjuvants for vaccine-induced CD8+ T-cell responses. *Nat Med* 9:1287-1292.

192. O'Keeffe, M., H. Hochrein, D. Vremec, B. Scott, P. Hertzog, L. Tatarczuch, and K. Shortman. 2003. Dendritic cell precursor populations of mouse blood: identification of the murine homologues of human blood plasmacytoid pre-DC2 and CD11c+ DC1 precursors. *Blood* 101:1453-1459.

193. Austen, W. G., Jr., L. Kobzik, M. C. Carroll, H. B. Hechtman, and F. D. Moore, Jr. 2003. The role of complement and natural antibody in intestinal ischemia-reperfusion injury. *Int J Immunopathol Pharmacol* 16:1-8.

194. Zhang, M., W. G. Austen, Jr., I. Chiu, E. M. Alicot, R. Hung, M. Ma, N. Verna, M. Xu, H. B. Hechtman, F. D. Moore, Jr., and M. C. Carroll. 2004. Identification of a specific self-reactive IgM antibody that initiates intestinal ischemia/reperfusion injury. *Proc Natl Acad Sci U S A* 101:3886-3891.

195. Wu, X., M. Wakamiya, S. Vaishnav, R. Geske, C. Montgomery, Jr., P. Jones, A. Bradley, and C. T. Caskey. 1994. Hyperuricemia and urate nephropathy in urate oxidase-deficient mice. *Proc Natl Acad Sci U S A* 91:742-746.

196. Wu, X. W., D. M. Muzny, C. C. Lee, and C. T. Caskey. 1992. Two independent mutational events in the loss of urate oxidase during hominoid evolution. *J Mol Evol* 34:78-84.

197. Kissel, P., M. Lamarche, and R. Royer. 1968. Modification of uricaemia and the excretion of uric acid nitrogen by an enzyme of fungal origin. *Nature* 217:72-74.

198. Landis, R. C., and D. O. Haskard. 2001. Pathogenesis of crystal-induced inflammation. *Curr Rheumatol Rep* 3:36-41.

199. Zhang, K. 2003. Accessibility control and machinery of immunoglobulin class switch recombination. *J Leukoc Biol* 73:323-332.

200. Chen, Y., Y. B. Park, E. Patel, and G. J. Silverman. 2009. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol* 182:6031-6043.

201. Chen, Y., S. Khanna, C. S. Goodyear, Y. B. Park, E. Raz, S. Thiel, C. Gronwall, J. Vas, D. L. Boyle, M. Corr, D. H. Kono, and G. J. Silverman. 2009. Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis. *J Immunol* 183:1346-1359.

202. McDaniel, L. S., W. H. Benjamin, Jr., C. Forman, and D. E. Briles. 1984. Blood clearance by anti-phosphocholine antibodies as a mechanism of protection in experimental pneumococcal bacteremia. *J Immunol* 133:3308-3312.

203. Sakamoto, N., K. Shibuya, Y. Shimizu, K. Yotsumoto, T. Miyabayashi, S. Sakano, T. Tsuji, E. Nakayama, H. Nakauchi, and A. Shibuya. 2001. A novel Fc receptor for IgA and IgM is expressed on both hematopoietic and non-hematopoietic tissues. *Eur J Immunol* 31:1310-1316.

204. Shimizu, Y., S. Honda, K. Yotsumoto, S. Tahara-Hanaoka, H. J. Eyre, G. R. Sutherland, Y. Endo, K. Shibuya, A. Koyama, H. Nakauchi, and A. Shibuya. 2001. Fc(alpha)/mu receptor is a single gene-family member closely related to

polymeric immunoglobulin receptor encoded on Chromosome 1. *Immunogenetics* 53:709-711.

205. Aderem, A., and D. M. Underhill. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 17:593-623.

206. Underhill, D. M., and A. Ozinsky. 2002. Phagocytosis of microbes: complexity in action. *Annu Rev Immunol* 20:825-852.

207. Hawlisch, H., M. Wills-Karp, C. L. Karp, and J. Kohl. 2004. The anaphylatoxins bridge innate and adaptive immune responses in allergic asthma. *Mol Immunol* 41:123-131.

208. Hu, D. E., A. M. Moore, L. L. Thomsen, and K. M. Brindle. 2004. Uric acid promotes tumor immune rejection. *Cancer Res* 64:5059-5062.

209. Shi, Y., S. A. Galusha, and K. L. Rock. 2006. Cutting edge: elimination of an endogenous adjuvant reduces the activation of CD8 T lymphocytes to transplanted cells and in an autoimmune diabetes model. *J Immunol* 176:3905-3908.

210. Novobrantseva, T. I., G. R. Majeau, A. Amatucci, S. Kogan, I. Brenner, S. Casola, M. J. Shlomchik, V. Koteliansky, P. S. Hochman, and A. Ibraghimov. 2005. Attenuated liver fibrosis in the absence of B cells. *J Clin Invest* 115:3072-3082.