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VOIES DE SIGNALISATION ACTIVÉES PAR LES CRISTAUX D'URATE MONOSODIQUE DANS LES NEUTROPHILES HUMAINS

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Résumé court

Les polymorphonucléaires neutrophiles sont les principales cellules effectrices du système immunitaire inné. Les cristaux d'urate monosodique sont l'agent étiologique de l'arthrite goutteuse. L'interaction directe entre les neutrophiles humains et les cristaux d'UMS est essentielle pour le déclenchement de la crise de goutte aigue.

Le principal objectif de ce projet de recherche est d'identifier les voies de signalisation activées par les cristaux d'UMS et de caractériser leurs fonctions dans les réponses des neutrophiles humains.

Résumé long

Représentant 50% à 70% des globules blancs totaux dans le sang, le neutrophile est le leucocyte le plus abondant dans la circulation sanguine. Les neutrophiles sont les principales cellules effectrices du système immunitaire inné mais les neutrophiles humains peuvent aussi servir de lien entre l'immunité innée et l'immunité acquise grâce à leur capacité de présenter des antigènes et d'induire la maturation des cellules dendritiques.

L'acide urique est le produit final de dégradation des purines. Divers facteurs génétiques et environnementaux peuvent engendrer une hyperuricémie qui est une des conditions prérequises pour la précipitation des cristaux d'UMS, l'agent étiologique de l'arthrite goutteuse.

L'interaction directe entre les neutrophiles humains et les cristaux d'UMS est essentielle pour le déclenchement de la crise de goutte aigue. Un élément important de la pathogénie de l'arthrite goutteuse est l'afflux, dans le synovium, de neutrophiles aux capacités destructrices. Les neutrophiles représentent plus de 95% des cellules infiltrées dans le liquide synovial et sont présents dès les étapes précoces de l'inflammation. Le rôle primordial des neutrophiles dans l'arthrite goutteuse est mis en évidence par l'absence de réponse inflammatoire aux injections de cristaux d'UMS chez les souris déplétées en neutrophiles ainsi que par le fait que les molécules inhibant les fonctions du neutrophile sont efficaces pour le traitement de la goutte.

Les résultats présentés dans cette thèse montrent que la stimulation des neutrophiles humains par les cristaux d'urate monosodique comprend l'activation séquentielle des Src kinases, Syk et PI3Ks ainsi que la baisse de l'activité phosphatase de SHP-1. Le produit des PI3Ks, PIP3 sert d'ancre à la membrane plasmique pour Tec qui, phosphorylée par les Src (plus précisément Lyn), contrôle ensuite des réponses précoces et tardives qui sont en partie responsables de l'inflammation de l'arthrite goutteuse.

Le rôle majeur que la tyrosine kinase Tec joue dans les réponses des neutrophiles humains aux cristaux d'UMS permet de proposer que cette enzyme soit une cible pharmacologique pour le traitement de l'arthrite goutteuse.

Avant-propos

Le mérite pour cette thèse appartient à ma mère. C'est elle qui a toujours veillé à ce que l'impossible devienne possible pour ma sœur, mon père et moi. Je lui dois, en grande partie, tout ce que je suis et tout ce que je réussis.

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Je n'ai pas plus de mérite qu'une éponge d'avoir écrit cette thèse. Je n'ai fait qu'« absorber » à partir du moment où j'ai pris conscience de la vie qui m'entoure.

Je vous remercie d'exister.

Contribution dans les articles

Chapitre II : Crystal-induced neutrophil activation.IX.Syk-dependent activation of class Ia phosphatidylinositol 3-kinases

- technique : 85%
- conception : 75%
- écriture : 90%

Chapitre III : Crystal-induced neutrophil activation.X.Pro-inflammatory role of the tyrosine kinase Tec

- technique : 90%
- conception : 90%
- écriture : 90%

Annexe II : Characterization of an activation factor released from human neutrophils after stimulation by triclinic monosodium urate crystals

- technique : 15%
- conception : 15%

Annexe III : Activation of human neutrophils by MSU crystals

- conception : 90%
- écriture : 90%.

Liste des abréviations

$^{1}O_2$	Oxygène singulet
ACTH	Adrenocorticotropic hormone
ADN	Acide désoxyribonucléique
AINS	Anti-inflammatoires non stéroïdiens
ARN	Acide ribonucléique
ATP	Adenosine triphosphate
BCR	B-cell receptor
Btk	Burton's tyrosine kinase
C5a	Fragment C5a du complément
C3a	Fragment C3a du complément
CCF	Crystal Chemotactic Factor
CMH	Complexe majeur d'histocompatibilité
COX-1	Cyclooxygénase-1
COX-2	Cyclooxygénase-2
DRM	Detergent-resistent membranes
fMLP	formylMethionine-Leucine-Phenylalanine
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
H_2O_2	Peroxyde d'hydrogène
HBSS	Hank's balanced salt solution
HLA-DR	Human leukocyte antigen-DR
HOCl	Acide hypochloreux
IFN	Interféron
Ig	Immunoglobuline
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
IL	Interleukine
ICAM	Intercellular Adhesion Molecule
LAD	Leukocyte Adhesion Deficiency
JAM-C	Junctional adhesion molecule-C
LFM-A13	Analogue A13 du leflunomide
LFM-A11	Analogue A11 du leflunomide
LPS	Lipopolysaccharide
LTB_4	Leucotriène B_4
MAP	Mitogen Activating Protein
MAPK	Mitogen Activating Protein Kinase
MC 3-R	Melanocortin 3-receptor
MIP	Macrophage Inflammatory Protein
MRP	Myeloid-related proteins
MSU	Monosodium urate
MTP	Métatarsophalangien
Na_3VO_4	Orthovanadate de Sodium
OH°	Radical hydroxyle
PAF	Platelet Activating Factor
PAMP	Pathogen-associated microbia pattern
PECAM-1	Platelet endothelial cell adhesion molecule-1
PGE_2	Prostaglandine E_2
PH	Pleckstrin homology

PI3K	Phosphatidyl-inositol-3 kinase
PIP3	Phosphatidyl-inositol-3-4-5 triphosphate
PPARγ	Peroxisome proliferator-activated receptor γ
PTEN	Phosphatase and TENsin homolog mutated in multiple advanced cancers 1
PKC	Protéine Kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol-Myristate Acétate
PMN	PolyMorphoNucléaire
PMSF	Phenylmethylsulfonyl fluoride
PP2	4-Amino-5-(4-cholrophenyl)-7-(t-butyl)pyrazolo-(3-4-d) pyrimidine
PP3	4-Amino-7-phenylpyrazolo(3-4-d)pyrimidine
PSGL-1	P-selectin glycoprotein ligand-1
PVDF	Polyvinylidene fluoride
SH1	Src homology 1
SHIP	SH2-containing 5' inositol phosphatase
SHP-1	SH2-containing tyrosine phosphatase 1
SOD	Superoxide dismutase
Syk	Spleen tyrosine kinase
TCR	T-Cell Receptor
TLR	Toll-Like Receptor
TNF	Tumor necrosis factor
UMS	Urate monosodique
VCAM-1	Vascular cell adhesion molecule-1
XLA	X-linked agammaglobulinemia

Note : il est a noter que, dans le présent ouvrage, les mots anglais ont été utilisés lorsqu'une traduction courante n'était pas disponible, ceci afin de rendre cette thèse plus lisible.

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Chapitre I : Introduction

1. Neutrophile humain

a) Formation, maturation, élimination et changements morphologiques du neutrophile humain

Représentant 50% à 70% des globules blancs totaux dans le sang, le polymorphonucléaire (PMN) neutrophile humain est le leucocyte le plus abondant dans la circulation sanguine. Les neutrophiles sont les principales cellules effectrices du système immunitaire inné mais les neutrophiles humains peuvent aussi servir de lien entre l'immunité innée et l'immunité acquise grâce à leur capacité de présenter des antigènes et d'induire la maturation des cellules dendritiques (Megiovanni, Sanchez et al. 2006).

Comme les autres cellules du système immunitaire, le neutrophile est formé dans la moelle osseuse à partir d'une cellule souche hématopoïétique. Selon les concentrations des facteurs de croissance qui l'entourent, cette cellule peut se différencier et devenir une cellule progénitrice myéloïde menant à la formation des monocytes, des macrophages et des neutrophiles ou se transformer en cellule lymphoïde menant à la formation des lymphocytes. La présence combinée de GM-CSF (*Granulocyte Macrophage-Colony Stimulating Factor*) et d'interleukine-3 (IL-3) favorise la différenciation de la lignée myéloïde des granulocytes/macrophages. Ce précurseur doit ensuite être en présence de GM-CSF et de G-CSF et subir quelques étapes de maturation supplémentaires pour finalement donner un neutrophile prêt à passer dans la circulation sanguine et à défendre l'organisme. Les neutrophiles matures sont continuellement libérés de la moelle osseuse d'une manière très contrôlée afin de maintenir leur niveau homéostatique dans le sang et de répondre à d'éventuels stress physiologiques. Le nombre de neutrophiles libérés de la moelle osseuse augmente rapidement dans le cas d'une réponse à des *stimuli* inflammatoires (Migliaccio, Migliaccio et al. 1988).

Les neutrophiles en circulation et en périphérie ont une durée de vie relativement courte. Une fois passés de la moelle osseuse dans la circulation sanguine, ils patrouillent environ 7 à 12 heures. La majorité des neutrophiles vont être à des niveaux plus ou moins avancés d'apoptose après 24-48 heures d'incubation *in vitro*. Les neutrophiles apoptotiques expriment à leur surface des marqueurs, comme la phosphatidylsérine ainsi que des sucres

et acides aminés cationiques, qui faciliteront leur ingestion et élimination par les macrophages (Savill, Wyllie et al. 1989). Par contre, dans un contexte inflammatoire, l'apoptose des neutrophiles est retardée et ils peuvent survivre plusieurs jours (Baran, Guzik et al. 1996; Jimenez, Watson et al. 1997; Sweeney, Nguyen et al. 1998).

Une des premières étapes de l'inflammation est l'infiltration des tissus par les neutrophiles. Cette infiltration est déclenchée à la suite de l'activation de l'endothélium par des cytokines secrétées au niveau du site inflammatoire. Cette stimulation provoque l'apparition de molécules d'adhésion (P- et E-sélectines, ICAM-1 (*intercellular adhesion molecule-1*) et VCAM-1 (*vascular cell adhesion molecule-1*)) sur les cellules endothéliales des vaisseaux. Les P- et E-sélectines endothéliales interagissent d'abord avec les molécules d'adhésion des leucocytes, principalement avec PSGL-1 (*P-selectin glycoprotein ligand-1*) et la L-sélectine présentes de façon constitutive à la surface des neutrophiles. Ces liaisons sont de faible affinité et insuffisantes pour arrêter le flux des neutrophiles. Elles sont cependant suffisantes pour les ralentir en les faisant rouler le long de l'endothélium. Les leucocytes sont ensuite immobilisés grâce à une interaction de plus forte affinité entre les intégrines leucocytaires (CD11a/CD18 ou LFA-1 et CD11b/CD18 ou Mac-1) et les molécules ICAM-1 sur l'endothélium. Ensuite, la diapédèse à travers l'endothélium se fait par l'intermédiaire des molécules PECAM-1 (*Platelet entothelial cell adhesion molecule-1* ou CD31) et CD99, qui sont exprimées à la fois à la jonction endothéliale et sur les neutrophiles. Le contact endothélium – neutrophile pendant la transmigration fait aussi intervenir l'interaction entre JAM-C (*junctional adhesion molecule-C*), molécule exprimée à la jonction des cellules endothéliales et l'intégrine Mac-1 exprimée à la surface des neutrophiles (Chavakis, Keiper et al. 2004). Ce passage prépare les neutrophiles à leur migration ultérieure à travers la matrice extracellulaire, en déclenchant l'apparition des intégrines nécessaires $\alpha2\beta1$ et $\alpha6\beta1$ et la sécrétion de métalloprotéinases capables de dégrader la matrice extracellulaire et/ou de faire apparaître sur celle-ci des sites cryptiques reconnaissables par les intégrines (Ley, Laudanna et al. 2007).

Au cours de la diapédèse à travers l'endothélium et de leur migration subséquente vers le site inflammatoire, les neutrophiles répondent à des signaux émis par les cellules endothéliales mais aussi par les cellules des tissus activés *in situ* par des cytokines pro-inflammatoires ou par des produits bactériens comme le LPS. Des modèles *in vitro* ont

montré que les leucocytes naviguent en suivant des gradients complexes d'agents chimiotactiques et migrent en répondant successivement à une source puis à une autre de ces agents (Witko-Sarsat, Rieu et al. 2000). Les chimiokines sont des petites protéines basiques de 8 à 17 kDa qui, après sécrétion, sont immobilisées en raison de leur charge sur les protéoglycanes des membranes cellulaires ou de la matrice extracellulaire. Les principales chimiokines agissant sur les neutrophiles sont des membres de la famille des CXC-chimiokines dont CXCL-8 (IL-8) est la plus importante. Leur structure se caractérise par la présence d'un motif constitué de deux cystéines séparées par un acide aminé quelconque. Les récepteurs cellulaires responsables des effets des CXC-chimiokines sur les neutrophiles font partie de la famille des récepteurs à sept domaines transmembranaires couplés aux protéines G (Haribabu, Richardson et al. 2000).

À la suite de la migration transendothéliale vers le site de l'inflammation, les principales fonctions de défense de l'organisme que le neutrophile exerce sont : la phagocytose, la dégranulation et la sécrétion des formes réactives de l'oxygène (Witko-Sarsat, Rieu et al. 2000).

Récemment, des indications de changements morphologiques, physiologiques et phénotypiques du neutrophile ont été rapportées lors de l'incubation de neutrophiles dans des conditions inflammatoires et en présence de certaines cytokines (GM-CSF, IL-4, TNFα (*tumor necrosis factor-α*)). Des neutrophiles incubés en présence de GM-CSF, IL-4 et TNFα survivent jusqu'à 10 jours, présentent un noyau rond plutôt que multilobé et expriment HLA-DR (*human leukocyte antigen-DR*), HLA-DQ, CD40, CD54 et éventuellement CD80, CD86 et le complexe majeur d'histocompatibilité (CMH) de classe II, des récepteurs classiquement associés aux cellules présentatrices d'antigène. Les neutrophiles ayant ce phénotype présentent une forte ressemblance avec les cellules dendritiques et peuvent activer la prolifération des lymphocytes T (Oehler, Majdic et al. 1998). De plus, l'expression de certains marqueurs classiques des neutrophiles comme le CD16b et la lactoferrine est diminuée. Il a été aussi observé que les neutrophiles isolés de patients souffrant de la maladie de Wegener ou de vasculite expriment CD80, CD86 et CMH de classe II, ce qui voudrait dire que les neutrophiles acquérant des caractéristiques des cellules dendritiques pourraient être responsables des inflammations chroniques (Iking-Konert, Vogt et al. 2001; Iking-Konert, Wagner et al. 2002).

Les neutrophiles peuvent donc, à la fois, vivre plus longtemps, modifier leur phénotype en diminuant l'expression des molécules de phagocytose et en exprimant les molécules nécessaires pour la présentation d'antigènes et migrer vers les organes lymphoïdes (Maletto, Ropolo et al. 2006).

b) Fonctions du neutrophile humain

Les neutrophiles humains constituent un des plus puissants systèmes de défense innée de l'homme contre les agents pathogènes qui franchissent la barrière cutanéo-muqueuse et contre toutes les structures reconnues comme étrangères telles que les cellules et molécules endogènes altérées. Les principales fonctions que le neutrophile exerce au site inflammatoire sont : la phagocytose, la dégranulation, la production et la sécrétion de cytokines et de formes réactives de l'oxygène (Witko-Sarsat, Rieu et al. 2000).

Phagocytose

La phagocytose est le principal mécanisme qui permet au neutrophile d'ingérer et de détruire les agents pathogènes. Le neutrophile reconnaît préférentiellement les pathogènes opsonisés (recouverts par des anticorps ou des fragments du complément, essentiellement C3). Ces opsonines sont reconnues par les récepteurs FcγRIIa (CD32a) et FcγRIIIb (CD16b) ou par les récepteurs du complément (CR3 ou Mac-1 ou CD11b/CD18). Le neutrophile exprime aussi des récepteurs pour certains composants bactériens comme, par exemple, les récepteurs pour les carbohydrates bactériens (mannose ou glucan) ou les récepteurs CD14, Toll-like (TLR) ou *scavenger* pour des lipides ou d'autres composants dérivés des pathogènes. Dans le neutrophile, la stimulation de ces récepteurs aboutit à un réarrangement du cytosquelette d'actine permettant l'extension de pseudopodes qui englobent la particule dans un phagosome. Le phagosome fusionne ensuite avec le lysosome pour former un phagolysosome dans lequel le pathogène sera digéré grâce aux enzymes contenues dans les granules spécifiques qui fusionnent avec le phagolysosome et à la production de radicaux libres oxygénés par la NADPH oxydase membranaire (Allen and Aderem 1996; Vieira, Botelho et al. 2002; Lee, Harrison et al. 2003).

Dégranulation

Au cours de la maturation des neutrophiles, apparaissent successivement les granules azurophiles (primaires) dans les promyélocytes, les granules spécifiques (secondaires) dans les myélocytes et les granules contenant de la gélatinase (tertiaires) au stade du neutrophile immature. Enfin, les vésicules sécrétoires apparaissent plus tardivement vers la fin de la maturation. Les granules sont classées selon leur taille, leur morphologie, leur densité en microscopie électronique ainsi que par leur contenu en protéines qui jouent un rôle important dans les fonctions physiologiques et pathologiques du neutrophile (Lew, Monod et al. 1986; Barrowman, Cockcroft et al. 1987). Une des particularités du neutrophile est donc d'être une cellule compartimentée en granules contenant des molécules « prêtes à l'emploi ». La dégranulation permet de déclencher rapidement les fonctions effectrices du neutrophile. Par ailleurs, la mobilisation des granules contenant des récepteurs stockés dans leur membrane induit une fusion entre les membranes plasmiques et granulaires pour augmenter ainsi l'expression membranaire de ces récepteurs. De plus, la dégranulation permet une libération dans le phagosome et dans le milieu extracellulaire de molécules présentes dans la matrice des granules : molécules bactéricides, myéloperoxydase, enzymes protéolytiques telle que la gélatinase intervenant dans le passage transendothélial, médiateurs de l'inflammation, cytokines pro- et anti-inflammatoires et certains de leurs récepteurs solubles. D'une manière générale, ces molécules participent à la défense de l'hôte contre des agents pathogènes et interviennent dans les réparations tissulaires : ainsi l'expression régulée des molécules d'adhérence permet une migration adaptée vers le foyer de l'inflammation ; la libération d'enzymes protéolytiques permet le passage transendothélial ; les formes réactives de l'oxygène sont des molécules avec un potentiel oxydant capables d'induire des altérations des lipides, des protéines et des acides nucléiques des agents pathogènes (Falloon and Gallin 1986; Middelhoven, Ager et al. 1997; Chertov, Yang et al. 2000; Middelhoven, Van Buul et al. 2001; Faurschou and Borregaard 2003).

Production des formes réactives de l'oxygène

Un des mécanismes utilisés par les neutrophiles pour éliminer les pathogènes consiste à produire des formes réactives de l'oxygène à partir des anions superoxydes (O_2^-). Ces anions sont formés par le complexe multimérique de la NADPH oxydase, situé à la

membrane cytoplasmique, et sont libérés à la face externe de cette membrane. Ainsi, les ions peuvent agir à l'extérieur du neutrophile et à l'intérieur du phagosome, la face externe de la membrane plasmique devenant la face interne du phagosome lors de l'invagination phagocytaire. L'anion superoxyde est un précurseur de plusieurs autres formes de l'oxygène fortement réactives et plus toxiques pour le pathogène: le peroxyde d'hydrogène (H_2O_2), le radical hydroxyle (OH^o) et l'oxygène singulet ($^1O_2$). Le peroxyde d'hydrogène peut être transformé en acide hypochloreux (HOCl), un composé bactéricide, par la myéloperoxydase libérée dans le phagosome. Si ces produits sont libérés de manière exagérée dans le milieu extracellulaire, ils peuvent engendrer des dommages tissulaires dus à la peroxydation lipidique, causant une désorganisation membranaire, ou encore à l'altération de protéines et d'acides nucléiques (Gougerot-Pocidalo, el Benna et al. 2002; Roos, van Bruggen et al. 2003).

c) Disfonctionnements du neutrophile humain

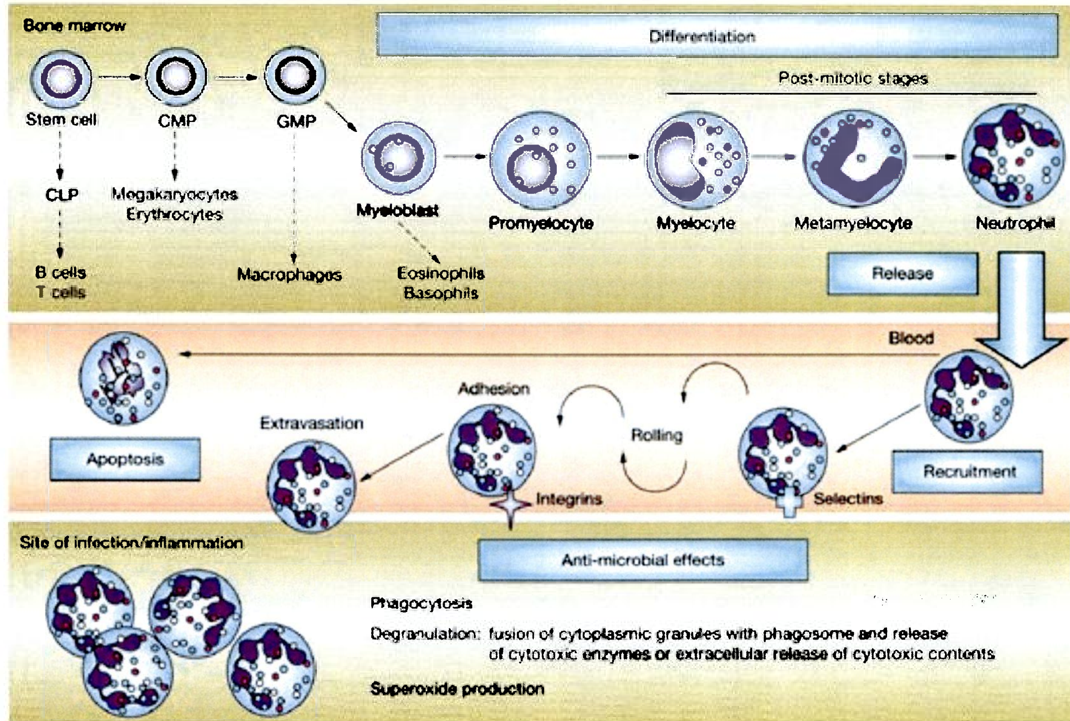
Le neutrophile est une cellule destructrice de pathogènes qui possède plusieurs moyens de protéger l'organisme. Un faible niveau de neutrophiles ou, encore, une déficience dans leur fonctions, que ce soit au niveau des granules (Chediak-Higashi, leucémie aiguë myéloïde) (Gallin 1985; Introne, Boissy et al. 1999), de la NADPH oxydase (granulomatose septique familiale) (Curnutte 1993) ou des récepteurs d'adhésion (*Leukocyte Adhesion Deficiency*) (Lau, Low et al. 1991; Harlan 1993), accroît la susceptibilité aux infections.

D'autre part, une activation prolongée ou une accumulation exagérée des neutrophiles peut engendrer une réponse excessive qui causera des dommages tissulaires. Les enzymes nécessaires pour la dégradation des bactéries sont aussi très efficaces pour détruire des tissus et cellules de l'hôte (Weiss 1989). Les élastases, les collagénases ou les formes réactives de l'oxygène sont tous des produits nocifs pour les bactéries aussi bien que pour l'hôte (White and Gallin 1986). Plusieurs pathologies comme l'arthrite rhumatoïde, la goutte, l'infarctus du myocarde, la dermatose neutrophilique, les vasculites, les maladies inflammatoires de l'intestin et l'asthme sont associées à une suractivation des neutrophiles. Les mécanismes de contrôle qui limitent la suractivation des neutrophiles représentent donc

de possibles cibles thérapeutiques pour le traitement de ces maladies (Malech and Gallin 1987).

d) Remarque concernant le *priming* du neutrophile humain

L'état de pré-activation (*priming*) du neutrophile ayant traversé la couche de cellules endothéliales activées et migré vers le site de l'inflammation est souvent négligé expérimentalement. Les résultats rapportés dans la littérature concernant l'activation des neutrophiles en réponse à divers *stimuli* sont majoritairement obtenus avec des neutrophiles fraîchement isolés à partir du sang. Ces neutrophiles ont certainement un phénotype différent de ceux ayant migré aux sites inflammatoires, ce qui pourrait changer leurs réponses fonctionnelles. Les modèles murins *in vivo* utilisés pour l'étude des inflammations neutrophiliques présentent l'avantage de prendre en compte cet état de *priming* du neutrophile quoique les neutrophiles murins présentent des divergences dans l'expression de certaines protéines avec les neutrophiles humains, par exemple en ce qui concerne les récepteurs Fcγ (souris : FcγRIIb et FcγRIIIa ; humain : FcγRIIa et FcγRIIIb). La mise au point des protocoles de *priming* des neutrophiles fraîchement isolés avant leur stimulation est donc nécessaire pour valider les observations décrites jusqu'à maintenant.



Tirée de Eyles et al., 2006, Nature Clinical Practice Rheumatology

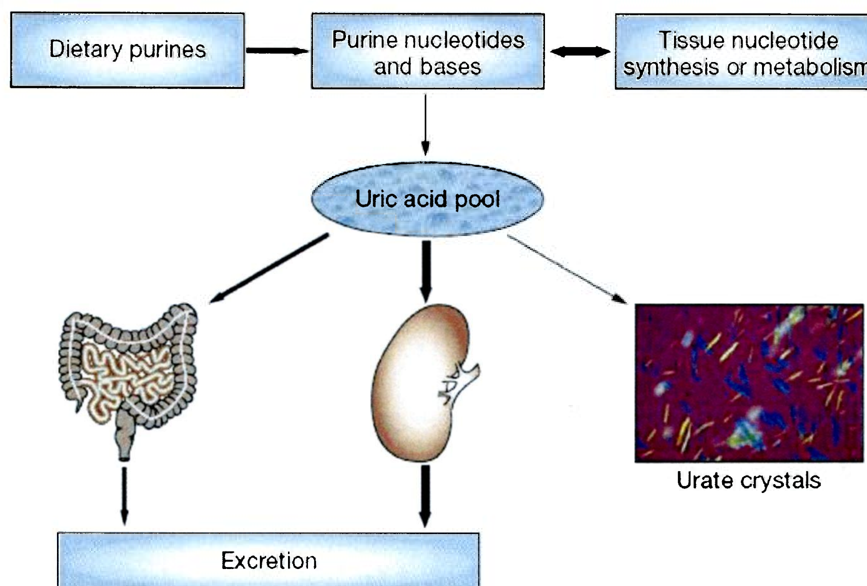
Figure 1 : Différenciation, libération, recrutement et activation des neutrophiles humains. Le neutrophile se différencie dans la moelle osseuse et les neutrophiles matures sont libérés dans le sang. Dans le cas d'une inflammation, le nombre de neutrophiles matures (et immatures) augmente significativement. En condition normale, les neutrophiles ont une durée de vie d'environ 7 à 12 heures après quoi ils entrent en apoptose. Dans un contexte inflammatoire, l'apoptose des neutrophiles est retardée et ils sont rapidement recrutés dans les tissus en réponse à des *stimuli* chimiotactiques tels le C5a ou l'IL-8. Les sélectines (faible affinité) amorcent le roulement du neutrophile le long de l'endothélium. L'adhésion ferme se fait par l'intermédiaire des intégrines (forte affinité) qui interagissent avec les molécules d'adhésion exprimées à la surface des cellules endothéliales. Ensuite, les neutrophiles migrent vers le site inflammatoire où ils exercent leurs fonctions principales (phagocytose, dégranulation et production de formes réactives de l'oxygène).

2. Cristaux d'urate monosodique (UMS) et arthrite goutteuse

a) Formation et précipitation des cristaux d'UMS

L'acide urique est le produit final de dégradation des purines. Il a été décrit comme possédant un rôle antioxydant dû à sa capacité de lier des radicaux libres (Waring, Webb et al. 2001) et d'empêcher la dégradation de la superoxide dismutase (SOD), enzyme nécessaire au maintien des fonctions endothéliales et vasculaires (Hink, Santanam et al. 2002; Watanabe, Kang et al. 2002). L'espèce humaine ne possédant pas d'enzyme de dégradation de l'acide urique (uricase), le taux sanguin d'acide urique fait l'objet d'un contrôle rigoureux pour maintenir sa concentration dans les limites de la solubilité (70mg/L, à 37°C *in vitro*) (Loeb 1972). Il existe trois sources métaboliques de l'acide urique : le catabolisme des acides nucléiques cellulaires, la dégradation des acides nucléiques alimentaires et la synthèse des purines dite *de novo* au niveau du foie (à partir de ribose-5-phosphate). Divers facteurs génétiques et environnementaux entraînent une hyperuricémie (taux sérique d'acide urique > 70 mg/L) en diminuant l'excrétion de l'acide urique et/ou en augmentant sa production. Lorsque les concentrations d'acide urique augmentent dans les liquides biologiques, l'urate monosodique peut cristalliser dans les tissus sursaturés, principalement dans et autour des articulations, mais aussi dans la peau ou d'autres structures comme les ligaments ou l'os. En plus de la concentration sérique d'acide urique, d'autres composants spécifiques du liquide synovial peuvent favoriser la nucléation de cristaux d'UMS. Ainsi, la chondroïtine sulfate, les protéoglycanes ou le collagène de type I induisent la cristallisation de l'urate monosodique *in vitro*. Les sites les plus souvent touchés, comme la membrane synoviale, le cartilage, les tendons, la peau et le tissu interstitiel rénal, sont particulièrement riches en tissu conjonctif, suggérant l'intervention de certaines protéines de la matrice extracellulaire comme le collagène de type I dans les mécanismes de cristallisation *in vivo*. D'autres facteurs peuvent influencer le dépôt de cristaux d'UMS. Ils comprennent une température locale basse (expliquant la formation des cristaux dans la partie distale du membre inférieur), l'hydratation des tissus (expliquant la précipitation nocturne des cristaux), la présence de protéoglycanes, une diminution du pH,

un traumatisme et l'âge (Fam 2000; Kim, Ralph Schumacher et al. 2003; Pascual and Pedraz 2004; Masseoud, Rott et al. 2005; Abeles, Park et al. 2007).



Tirée de Roddy et al., 2007, Nature Clinical Practice Rheumatology

Figure 2 : La production et la sécrétion de l'acide urique. Deux tiers de la quantité d'acide urique proviennent de la production endogène et un tiers de l'alimentation. Deux tiers vont être secrétés par le rein et un tiers par l'intestin. Un déséquilibre entre la production et la sécrétion d'acide urique peut entraîner une condition d'hyperuricémie qui est un prérequis pour la précipitation des cristaux d'UMS.

b) Arthrite goutteuse

Les cristaux d'UMS sont l'agent étiologique de l'arthrite goutteuse. On distingue quatre stades d'évolution de la goutte : l'hyperuricémie asymptomatique, la crise de goutte aiguë, les intervalles libres et la goutte chronique (Revaz and Dudler 2007). Un accès de goutte se caractérise par les signes classiques de l'inflammation aiguë (douleur, rougeur, chaleur, gonflement), typiquement dans une articulation comme la première métatarsophalangienne (MTP), mais cette inflammation peut également surgir dans tout tissu où les cristaux d'UMS sont déposés comme on l'observe dans la goutte tophacée. Le volume du liquide synovial est augmenté et il contient diverses cytokines pro-inflammatoires et de nombreux

neutrophiles. Un élément important de la pathogénie de l'arthrite goutteuse est l'afflux, dans le synovium, de neutrophiles aux capacités destructrices. Les neutrophiles représentent plus de 95% des cellules infiltrées dans le liquide synovial et sont présents dès les étapes précoces de l'inflammation (Agudelo and Schumacher 1973). Le rôle primordial des neutrophiles dans l'arthrite goutteuse est mis en évidence par l'absence de réponse inflammatoire aux injections de cristaux d'UMS chez les souris déplétées en neutrophiles (Seegmiller and Howell 1962) ainsi que par le fait que les molécules inhibant les fonctions du neutrophile sont efficaces pour le traitement de la goutte (Phelps 1969). Malgré son intensité et sa brutalité, la crise de goutte est caractérisée par une résolution spontanée survenant environ 7-10 jours après son déclenchement.

La goutte chronique tophacée se caractérise par le développement synovial, sous-cutané et sous-chondral de nodules de cristaux d'UMS enchâssés dans une matrice constituée de lipides, de protéines et de débris calciques. Ces nodules prennent le nom de « tophi ». Dans les stades avancés, les dépôts de cristaux d'UMS finissent par produire des déformations cartilagineuses et osseuses permanentes. De plus, les modifications radiologiques de la goutte tophacée peuvent progresser malgré une baisse de l'uricémie. Ceci souligne l'implication directe des dépôts de cristaux d'UMS dans la dégradation tissulaire (Gutman 1973; Dalbeth, Collis et al. 2007).

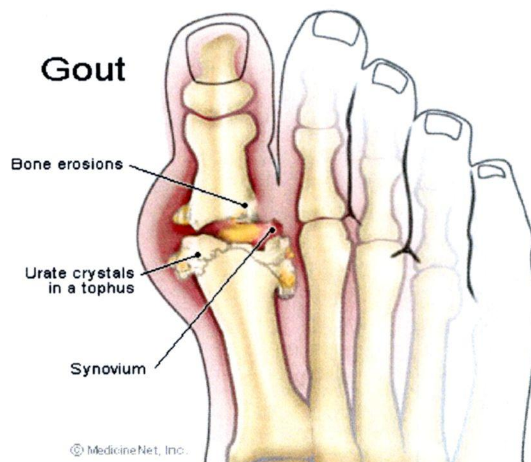


Figure 3 : Arthrite goutteuse. La précipitation des cristaux d'UMS dans les articulations provoque une crise de goutte aiguë. Dans les stades avancés de la goutte chronique les

dépôts de cristaux d'UMS (« tophi ») produisent des déformations cartilagineuses et osseuses permanentes.

c) Traitements de l'arthrite goutteuse

Peu d'études d'envergure et contrôlées ont évalué les différents traitements de l'arthrite goutteuse. Il s'agit le plus souvent de petites études observationnelles sur une population hétérogène. En absence de contre-indication rénale ou digestive, les anti-inflammatoires non stéroïdiens (AINS) sont le traitement de premier choix. L'efficacité de différents AINS, qu'ils soient ou non sélectifs de la cyclooxygenase-2 (COX-2), est comparable pour autant que la posologie prescrite soit suffisante (Smyth and Percy 1973; Weiner, White et al. 1979; Altman, Honig et al. 1988; Maccagno, Di Giorgio et al. 1991; Shrestha, Morgan et al. 1995). Le traitement anti-inflammatoire doit être poursuivi jusqu'à la résolution complète de l'arthrite goutteuse aiguë puis diminué progressivement (Emmerson 1996).

Les corticostéroïdes sont également rapidement efficaces quel que soit leur mode d'administration et ils représentent une alternative thérapeutique pour les malades de goutte ayant des contre-indications pour les AINS (Groff, Franck et al. 1990; Werlen, Gabay et al. 1996; Fernandez, Noguera et al. 1999).

L'hormone synthétique adrénocorticotrope (ACTH) représente une autre alternative aux AINS en cas de contre-indication rénale ou digestive. L'ACTH est produite par l'hypophyse et agit, par l'intermédiaire de l'adényl-cyclase, sur la libération des corticostéroïdes par les glandes surrénales. L'ACTH agit sur le récepteur des mélanocortines 3 (MC 3-R) qui a des propriétés anti-inflammatoires dans l'arthrite goutteuse (Getting, Lam et al. 2006). Son efficacité est comparable à celle des corticostéroïdes et des AINS, mais plusieurs cas d'allergie et d'effet « rebond » à l'arrêt du traitement ont été décrits.

Enfin, la colchicine, un alcaloïde extrait du crocus automnal est utilisée depuis plus de 2000 ans dans le traitement des maladies inflammatoires et reste très utile notamment dans le cas d'intolérance aux AINS et aux corticostéroïdes (Molad 2002). De nombreuses propriétés pharmacologiques (inhibition de l'assemblage des microtubules (Margolis and Wilson 1977), de l'excrétion de facteurs chimiotactiques (Serhan, Lundberg et al. 1984;

Matsukawa, Yoshimura et al. 1998) et de l'expression de protéines de surface comme le récepteur du TNF-α (Spilberg, Mandell et al. 1979; Cronstein, Molad et al. 1995)) expliquent son effet anti-inflammatoire. Plus récemment, il a été montré que la colchicine, à concentration relativement élevée, inhibe également l'activation de l'inflammasome qui joue un rôle essentiel dans la réponse inflammatoire de la goutte (Martinon, Petrilli et al. 2006). Il faut toutefois rappeler la marge thérapeutique étroite de la colchicine : les effets secondaires, surtout digestifs, surviennent souvent avant ou simultanément à l'effet thérapeutique souhaité.

Lorsqu'un traitement de fond est nécessaire, l'administration d'un inhibiteur de la xanthine oxydase (enzyme responsable de la conversion de la xanthine en acide urique) (Fam 1998) ou uricosurique (qui induit l'excrétion de l'acide urique) (Agudelo and Wise 2000) est classiquement retenue selon les comorbidités du patient. Un changement des habitudes alimentaires, une perte pondérale chez le sujet obèse et une diminution de la consommation d'alcool sont également recommandés.

d) Comorbidités de l'arthrite goutteuse

Des associations entre l'hyperuricémie et différentes maladies telles l'hypertension (Gavin and Struthers 2003), la mortalité cardiovasculaire (Anker, Doehner et al. 2003), l'athérosclérose (Gavin and Struthers 2003), le diabète (Gavin and Struthers 2003) et l'atteinte rénale chronique ont été mises en évidence (Vazquez-Mellado, Alvarez Hernandez et al. 2004).

Choi *et al.* ont récemment décrit les résultats d'une étude de la relation entre la goutte et le risque de mort et infarctus du myocarde chez 51297 hommes sur une période de 12 ans. Cette étude suggère que les hommes souffrant de goutte présentent un risque accru de maladies cardiovasculaires et d'infarctus du myocarde. Ces personnes devraient donc être traitées préventivement pour diminuer tous les autres facteurs de risque pour des maladies cardiovasculaires (Choi and Curhan 2007).

e) Remarque concernant l'opsonisation des cristaux d'UMS

Les expériences avec des cristaux d'UMS reposent sur leur ajout aux cellules dont les réponses moléculaires et fonctionnelles sont ensuite étudiées. *In vivo*, les cristaux d'UMS sont connus pour absorber à leur surface diverses protéines dont les propriétés physiques peuvent influencer leurs propriétés phlogistiques (Brandt 1974; Cherian and Schumacher 1986; Burt, Jackson et al. 1989; Ortiz-Bravo, Sieck et al. 1993). Ainsi, par exemple, les cristaux recouverts par des IgGs sont les plus susceptibles d'induire une réaction inflammatoire puissante. Inversement, les cristaux ayant adsorbé des apolipoprotéines sont peu inflammatoires. De plus, il est clair que les cristaux d'UMS favorisent une réaction inflammatoire, mais la raison pour laquelle de tels cristaux sont retrouvés dans le liquide synovial de patients asymptomatiques reste inconnue. La nature et la quantité des protéines recouvrant les cristaux pourraient affecter leur capacité à induire une crise aiguë. Les protéines recouvrant les cristaux d'UMS seront donc caractéristiques de chaque étape de la crise de goutte. L'opsonisation des cristaux d'UMS est le plus souvent négligée lors des études *in vitro* où les cristaux d'UMS sont utilisés sous leur forme « nue ». La connaissance détaillée de la composition du liquide synovial de goutte, de la dynamique d'adsorption des protéines sur les cristaux d'UMS ainsi que de la nature de l'étude effectuée (déclenchement *vs* résolution de l'inflammation) devraient déterminer les paramètres d'une opsonisation optimale.

Les modèles murins in vivo utilisés pour l'étude de l'inflammation engendrée par les cristaux d'UMS (ex. : injection des cristaux d'UMS dans une poche à l'air qui mime les principales caractéristiques de la cavité synoviale (Edwards, Sedgwick et al. 1981; Wilkinson, Moore et al. 1993; Generini, Matucci-Cerinic et al. 2001)) présentent l'avantage de prendre en compte l'opsonisation des cristaux quoique, contrairement aux humains, les souris expriment l'uricase (enzyme de dégradation de l'acide urique) ce qui prévient l'hyperuricémie et donc une éventuelle précipitation « naturelle » des cristaux d'UMS.

3. Déclenchement de la crise de goutte aiguë

a) Reconnaissance moléculaire des cristaux d'UMS

Les charges présentes à leur surface et la fixation de protéines sur cette surface sont des facteurs importants du pouvoir phlogogène des cristaux d'UMS. Par l'intermédiaire de liaisons hydrogènes ou électrostatiques, les protéines peuvent se fixer à la surface chargée négativement des cristaux d'UMS. La liaison de protéines est accompagnée par une modification des propriétés phlogogènes des cristaux. Les IgGs, en particulier, se lient aux cristaux d'UMS et contribuent à l'inflammation aiguë *in vivo* (Kozin and McCarty 1977; Skosey, Kozin et al. 1977). Les cristaux d'UMS peuvent aussi activer le facteur Hageman (facteur XII de coagulation) et le système des kinines provoquant une vasodilatation et une augmentation de la perméabilité vasculaire qui favorise l'afflux de leucocytes, la douleur, l'œdème et l'augmentation de la température locale (Kellermeyer 1965; Kellermeyer and Breckenridge 1966; Kellermeyer 1968; Ginsberg, Jaques et al. 1980). Il a aussi été montré que les cristaux d'UMS pouvaient activer la voie classique du complément *in vitro* (Byers, Ward et al. 1973; Giclas, Ginsberg et al. 1979; Hasselbacher 1979) et plusieurs composants du complément (C1q, C1r et C1s) ont été élués à partir de cristaux d'UMS incubés avec du plasma (Terkeltaub, Tenner et al. 1983). L'activation des voies du complément pourrait contribuer au recrutement des neutrophiles dans la cavité synoviale pendant la crise de goutte. En accord avec cette hypothèse, il a été montré que l'activité du complément était augmentée dans les liquides synoviaux des patients atteints de goutte (Pekin and Zvaifler 1964).

L'identité des récepteurs (spécifiques ou opportunistes) des cristaux d'UMS n'est pas encore entièrement identifiée. Plusieurs études utilisant des anticorps bloquants suggèrent une implication du FcγRIIIB et de l'intégrine β2 CD11b dans l'interaction des cristaux d'UMS avec les neutrophiles humains (Barabe, Gilbert et al. 1998; Ryckman, Gilbert et al. 2004). Ces études ne fournissent pourtant pas une liste exhaustive de déterminants moléculaires de la reconnaissance des cristaux d'UMS puisque les inhibitions obtenues ne sont pas complètes et aussi puisque des cellules n'exprimant pas ces récepteurs (fibroblastes synoviaux, monocytes) sont activées par ces particules.

Des études utilisant des modèles expérimentaux murins suggèrent que les récepteurs TLR-2 et TLR-4 ainsi que la molécule adaptatrice CD14 sont nécessaires pour que les réponses fonctionnelles aux cristaux d'UMS soient déclenchées (Liu-Bryan, Scott et al. 2005; Scott, Ma et al. 2006). Les récepteurs TLR sont des récepteurs présents à la surface des cellules du système immunitaire chez l'homme et les mammifères et sont homologues au produit du gène Toll de la drosophile. Ces récepteurs très conservés au cours de l'évolution jouent un rôle important dans l'immunité innée et notamment dans les défenses contre les microorganismes. Ils peuvent reconnaître des motifs moléculaires uniquement présents chez ces microorganismes pathogènes dénommés PAMP pour *pathogen-associated microbia pattern* (des composants des parois bactériennes comme les lipopolysaccharides (LPS) et les peptidoglycanes ou la flagelline composant du flagelle de certaines bactéries, de l'ADN bactérien, de l'ARN viral double brin). Il a aussi été proposé que les TLRs pouvaient reconnaître des signaux de danger tels que des molécules sécrétées dans le milieu extracellulaire par des cellules nécrotiques (Sabroe, Parker et al. 2008) ou des molécules hydrophobes (Seong and Matzinger 2004). Une des principales substances capables d'induire ce signal de danger qui détermine la maturation de cellules dendritiques a été identifiée comme étant de l'acide urique sous sa forme cristalline (Shi, Evans et al. 2003). Les concentrations tissulaires d'acide urique étant proches de la saturation, cela explique pourquoi les cellules du système immunitaire ne sont pas sensibles à la forme soluble de l'acide urique. Un excès de production d'acide urique (dû, par exemple, à sa sécrétion par des cellules endommagées) peut donc induire rapidement la précipitation de cristaux d'UMS hydrophobiques qui sont perçus comme un signal de danger activateur de la réponse immunitaire. De plus, il a été récemment montré que l'acide urique sous forme cristalline pouvait aussi augmenter les réponses immunes humorales (augmentation de la production d'IgG1 contre des tumeurs ou contre l'ovalbumine) (Behrens, Wagner et al. 2008).

b) Activation des cellules résidentes de la cavité synoviale par les cristaux d'UMS

Les interactions des cristaux d'UMS avec les cellules résidentes de la cavité synoviale (synoviocytes, monocytes/macrophages) précèdent l'afflux de neutrophiles. Ces

interactions résultent en l'activation de ces cellules qui vont ensuite synthétiser et sécréter des facteurs pro-inflammatoires dont des cytokines et chimiokines. La sécrétion de ces facteurs pro-inflammatoires aura comme conséquences : l'activation des cellules environnantes, l'amplification du recrutement des neutrophiles et l'amplification de la réponse inflammatoire dans l'articulation affectée.

L'inflammation articulaire causée par les cristaux d'UMS résulte des interactions entre ces cristaux et les synoviocytes de type B (fibroblastiques), les synoviocytes de type A (macrophagiques) et les leucocytes infiltrants (Agudelo and Schumacher 1973). Ces interactions créent un environnement pro-inflammatoire en induisant la sécrétion d'IL-6, d'IL-1β, de TNF-α ou d'IL-8 (Di Giovine, Malawista et al. 1987; Guerne, Terkeltaub et al. 1989; di Giovine, Malawista et al. 1991; Roberge, Grassi et al. 1991; Terkeltaub, Zachariae et al. 1991; Hachicha, Naccache et al. 1995). Ces cytokines ont une action pyrogène endocrine sur le centre de régulation thermique de l'hypothalamus et provoquent de la fièvre. La synthèse de la protéine réactive C, qui est une protéine de l'inflammation, est activée par l'IL-1β. L'IL-1β et le TNF-α peuvent agir sur les fibroblastes, les ostéoclastes et les chondrocytes pour produire de la prostaglandine E2 qui est un important médiateur de l'inflammation aiguë et de la collagénase qui favorise le remodelage tissulaire (Cheung, Halverson et al. 1981; Hasselbacher, McMillan et al. 1981; Hasselbacher 1982; Wigley, Fine et al. 1983).

L'IL-8 est une chimiokine puissante pour les neutrophiles et elle joue un rôle majeur dans la réaction inflammatoire mettant en jeu ces cellules (Mukaida 2003). L'IL-8 est généralement abondante dans le liquide synovial de patients atteints de goutte aiguë (Hachicha, Naccache et al. 1995; McNearney, Baethge et al. 2004) et elle joue un rôle primordial dans le recrutement des neutrophiles dans des modèles expérimentaux d'inflammation aiguë goutteuse *in vivo* (Nishimura, Akahoshi et al. 1997; Terkeltaub, Baird et al. 1998). L'IL-8 n'est pas seulement un facteur chimiotactique pour les neutrophiles. Cette chimiokine est aussi capable d'induire la synthèse par les neutrophiles d'autres facteurs chimiotactiques incluant les leukotriènes et le PAF (*platelet activating factor*) (Hoch, Schraufstatter et al. 1996). En activant les intégrines exprimées par les neutrophiles, l'IL-8 stimule aussi directement l'adhésion des neutrophiles aux cellules endothéliales (Detmers, Lo et al. 1990). De plus, l'IL-8 active le neutrophile (relargage de

protéases lysosomales, activation de la NADPH oxydase) (Hoch, Schraufstatter et al. 1996). L'IL-8 peut ainsi potentialiser la dégradation du cartilage articulaire dans les arthrites microcristallines (Matsukawa, Yoshimura et al. 1995). L'expression d'IL-8 module donc directement le potentiel arthritogénique des cristaux d'UMS. A ce titre, la neutralisation de l'IL-8 apparaît comme une stratégie thérapeutique. Un anticorps anti-IL-8 a été développé et s'est révélé capable d'inhiber l'arthrite microcristalline induite par les cristaux d'UMS chez le lapin (Nishimura, Akahoshi et al. 1997). D'autres agents thérapeutiques potentiels dans l'inflammation goutteuse pourraient être des inhibiteurs des étapes de la signalisation impliquées dans l'expression de l'IL-8 (inhibiteurs de la famille des tyrosine kinases Src ou des MAP (*mitogen-activated protein*) kinases) (Liu, O'Connell et al. 2000).

4. Interaction entre les cristaux d'UMS et les neutrophiles humains

a) Voies de signalisation activées par les cristaux d'UMS dans les neutrophiles humains

Les cristaux d'UMS activent de nombreuses voies de signalisation intracellulaires qui conduisent à l'activation et au recrutement cellulaire. La phosphorylation sur résidus tyrosine est caractéristique des réponses des neutrophiles humains stimulés par les cristaux d'UMS. Des études effectuées dans notre laboratoire ont établi une corrélation entre le profil de phosphorylation sur résidus tyrosine caractéristique des cristaux d'UMS et leurs propriétés phlogogènes (Gaudry, Roberge et al. 1993). La régulation du niveau de phosphorylation sur résidus tyrosine est vraisemblablement le résultat d'un équilibre entre des activités kinase et phosphatase.

Plusieurs familles de protéines, dont les tyrosine kinases Src (Gaudry, Gilbert et al. 1995), Syk (Desaulniers, Fernandes et al. 2001) et Tec (Popa-Nita, Marois et al. 2008) ainsi que les phosphatidylinositol 3-kinases (PI3Ks) (Popa-Nita, Rollet-Labelle et al. 2007) ont été identifiées comme intervenant dans la cascade de signalisation activée par les cristaux d'UMS et leurs activités sont régulées par des mécanismes complexes.

Les Src kinases participent à la régulation de plusieurs fonctions cellulaires telles que la prolifération, la migration, la différenciation et la survie (Bolen 1991). Elles agissent en catalysant le transfert d'un phosphate de l'ATP sur un résidu tyrosine. Les membres de la famille des Src kinases (Lyn, Hck, Fgr, Src, Fyn, Yes et Yrk) sont formés d'un domaine Src homology 1 (SH1) possédant l'activité kinase, un domaine SH2 avec une affinité pour les résidus tyrosine phosphorylés, un domaine SH3 permettant les interactions avec les régions riches en résidus proline, et finalement le domaine SH4 qui joue un rôle dans l'association de ces kinases aux membranes cytoplasmiques et granulaires (Feldman, Hanafusa et al. 1980; Welch and Maridonneau-Parini 1997).

Le neutrophile humain exprime Lyn, Hck, Fgr et Yes. Les modèles de souris déficientes en une ou plusieurs de ces Src kinases révèlent le rôle important que ces protéines jouent dans la migration, la phagocytose et la dégranulation des neutrophiles puisque des neutrophiles déficients en Hck et Fgr présentent une diminution de leur migration induite par le LPS

dans un modèle murin (Lowell and Berton 1998) ainsi qu'une diminution de leur adhésion sur ICAM-1 ou sur la matrice extracellulaire (Lowell, Fumagalli et al. 1996) et de la dégranulation en réponse à cette adhésion (Mocsai, Ligeti et al. 1999). De plus, il a été rapporté que les patients souffrant de granulomatose chronique présentent une diminution de l'activation de Lyn et Fgr (Yan and Berton 1996).

Il a été précédemment montré dans notre laboratoire qu'un des premiers signes d'activation du neutrophile humain par les cristaux d'UMS était la phosphorylation sur résidu tyrosine de la Src kinase Lyn (Gaudry, Gilbert et al. 1995). Ensuite, la cinétique d'activation de Lyn a été corrélée avec l'augmentation du niveau global de phosphorylation sur résidus tyrosine caractéristique des cristaux d'UMS dans les neutrophiles humains.

L'inhibition marquante des réponses des neutrophiles humains aux cristaux d'UMS par des inhibiteurs pharmacologiques des Src kinases (PP1, PP2) met en évidence leur rôle majeur dans les voies de signalisation activées par ces particules (Majeed, Caveggion et al. 2001; Piccardoni, Sideri et al. 2001; Gaudreault, Thompson et al. 2005). A la suite de la stimulation des neutrophiles humains par les cristaux d'UMS, les Src kinases sont nécessaires à l'activation de la tyrosine kinase Syk qui a été identifiée comme étant essentielle au déclenchement de la cascade de signalisation (Desaulniers, Fernandes et al. 2001).

Syk est une tyrosine kinase cytoplasmique ubiquitaire de 72 kDa, de la famille de ZAP-70 (principalement nécessaire pour la signalisation du récepteur des cellules T), avec deux domaines SH2 en tandem présentant une forte affinité pour l'ITAM (*immunoreceptor tyrosine-based activation motif*) des Fcγ récepteurs. La région C-terminale contient le domaine catalytique. Syk est phosphorylée sur plusieurs résidus tyrosine, soit Tyr130 entre les domaines SH2, Tyr317, Tyr342, et Tyr346 dans la région « linker ». Les Tyr519 et Tyr520 avec Tyr317 régulent négativement l'activité de Syk dans le domaine catalytique (Keshvara, Isaacson et al. 1998; Ottinger, Botfield et al. 1998). Les souris *syk -/-* meurent périnatalement à la suite d'hémorragies massives, démontrant le rôle vital de Syk (Turner, Mee et al. 1995). Un rôle prépondérant pour Syk dans la phagocytose et l'adhésion a aussi été observé dans plusieurs modèles expérimentaux (Greenberg, Chang et al. 1996). Il a été précédemment montré dans notre laboratoire que Syk est activée lors de la phagocytose de cristaux d'UMS par les neutrophiles et que le piceatannol, un inhibiteur pharmacologique

de Syk inhibe l'internalisation des cristaux ainsi que la production d'anions superoxydes, l'activation de la phospholipase D (PLD) et le profil général de phosphorylation sur résidus tyrosine (Desaulniers, Fernandes et al. 2001). Nous avons montré récemment que Syk est aussi nécessaire à l'activation de la classe Ia des PI3Ks en réponse à la stimulation des neutrophiles humains par les cristaux d'UMS (Popa-Nita, Rollet-Labelle et al. 2007).

Les PI3Ks forment une famille de lipides kinases qui phosphorylent la position 3-hydroxyl du groupement inositol des phosphatidylinositols (PtdIns). Le principal substrat des PI3Ks *in situ* est le PtdIns(4,5)P2. Le PtdIns(3,4)P2 et le PtdIns(3,4,5)P3 (PIP3) sont pratiquement absents des cellules au repos mais se retrouvent à la membrane plasmique en réponse à plusieurs agonistes (IL-2, IL-3, IL-6, IL-7, IL-15, GM-CSF, fMLP, IL-8). Il existe trois classes de PI3K, soit les classes I, II et III. La classe IA est composée d'hétérodimères d'une sous-unité catalytique de 110 kDa (α,β,δ) et d'une sous-unité régulatrice p85 (α,β), p55 (α,γ) ou p50α. La classe IB ne comporte qu'un seul membre formé d'une sous-unité catalytique p110γ et d'une sous-unité régulatrice p101, et est exprimée majoritairement dans les cellules du système immunitaire. Les PI3K de classe I transforment le PtdIns(4,5)P2 en PIP3. La classe II forme principalement du PtdIns(3)P et du PtdIns(3,4)P2 alors que la classe III produit du PtdIns(3)P et est surtout active dans le trafic vésiculaire. L'activation des PI3K de classe IA se fait principalement par des voies dépendantes de tyrosine kinases alors que la classe IB est activée d'une manière dépendante des protéines G (Cantrell 2001).

Nous avons montré récemment que l'activité PI3 kinase associée à des immunoprécipités p85 était augmentée dans les neutrophiles stimulés par les cristaux d'UMS et que cette activité ainsi que la phosphorylation d'AKT (reconnue comme indicatrice de l'activation des PI3 kinases) étaient inhibées par le piceatannol, un inhibiteur spécifique de Syk (Popa-Nita, Rollet-Labelle et al. 2007). Ces résultats sont en accord avec des études précédentes qui avaient montré que des cellules déficientes en Syk présentaient une diminution de l'accumulation de PIP3 (Beitz, Fruman et al. 1999). Dans les neutrophiles humains, les PI3Ks régulent l'activation de la PLD ainsi que la dégranulation déclenchées par les cristaux d'UMS (Popa-Nita, Rollet-Labelle et al. 2007).

Lors de la stimulation du neutrophile, le niveau de PIP3 fait l'objet d'une régulation complexe comprenant un équilibre entre les activités des PI3Ks qui catalysent sa

production et les activités des lipides phosphatases qui l'hydrolysent. Les lipides phosphatases SHIP1 (*Src homology 2-containing inositol 5-phosphatase -1*) et PTEN (*Phosphatase and TENsin homolog mutated in multiple advanced cancers 1*) ont été précédemment étudiées dans notre laboratoire dans le contexte de la stimulation du FcγRIIa (Vaillancourt, Levasseur et al. 2006). SHIP1 (une 5'-phosphatase) et PTEN (une 3'-phosphatase) hydrolysent l'Ins(1,3,4,5)P4 et le PIP3. SHIP1 a été initialement décrite pour les rôles joués lors de la stimulation des récepteurs des cellules B et T (BCR, TCR) (Damen, Liu et al. 1996) alors que PTEN est connue pour son rôle dans le contrôle tumoral qu'elle exerce en régulant négativement la voie des PI3Ks (Wu, Senechal et al. 1998).

Le PIP3, second messager produit par les PI3Ks, agit en tant qu'ancre dans la membrane plasmique pour des protéines régulatrices de la signalisation qui s'y lient *via* leur domaine *pleckstrin homology* (PH) (Lemmon 2008). En accord avec ce modèle, des études de notre laboratoire ont montré que la tyrosine kinase Tec était activée suite à la stimulation des neutrophiles humains par les cristaux d'UMS (Popa-Nita, Marois et al. 2008).

La famille des tyrosine kinases Tec est une famille de protéines kinases non-couplées aux récepteurs dont la plupart est exprimée dans le cytoplasme des cellules hématopoïétiques au repos (Okoh and Vihinen 1999; Schmidt, Boucheron et al. 2004). Plusieurs études documentent le rôle de ces molécules dans la croissance et la différenciation cellulaires, la mobilisation du calcium intracellulaire, l'apoptose, la réorganisation du cytosquelette d'actine et la régulation des MAPKs (August and Dupont 1995; Uckun 1998; Yao, Janmey et al. 1999). La famille de Tec kinases est composée de cinq membres : Bmx/Etk, Btk, Itk/Emt/Tsk, Tec et Txk/Rlk. Nous avons montré précédemment que le neutrophile humain exprime Tec, Btk et Bmx (Lachance, Levasseur et al. 2002). Btk est la plus étudiée pour son rôle dans la XLA (*X-linked agammaglobulinemia*), maladie génétique caractérisée par une déficience en lymphocytes B matures (Velickovic, Prasad et al. 2004). La structure de ces protéines se caractérise par cinq domaines distincts. Dans la région N-terminale, le domaine PH est responsable d'interactions lipide – protéine et permet l'association des membres de la famille Tec aux membranes cytoplasmiques par l'intermédiaire du PIP3. La famille des Tec kinases est la seule famille de tyrosine kinases à posséder ce type de domaine. Le domaine PH est aussi responsable d'interactions protéine – protéine, les

membres de la famille Tec pouvant s'associer via leur domaine PH à diverses protéines G ainsi qu'à la F-actine, à diverses isoformes de la PKC (*protein kinase C*), à FAK, à Fas et à la PLC (phospholipase C). Le domaine PH est suivi d'un domaine TH (*Tec-homology*) qui se divise en deux parties : un motif Btk et une région riche en résidus proline. Le motif Btk en association avec le domaine PH est responsable de la liaison de Btk aux différentes sous-unités des protéines G. La région riche en prolines permet des interactions avec le domaine SH3 des Src kinases (Gomez-Rodriguez, Readinger et al. 2007). Il a été en effet montré que Lyn et Hck ont la capacité de phosphoryler le domaine kinase de Tec et Btk en se liant préalablement au domaine TH (Mano, Sato et al. 1994; Vihinen, Nilsson et al. 1994; Mano, Yamashita et al. 1996). Le domaine TH est suivi d'un domaine SH3 qui permet des interactions avec la région riche en prolines du domaine TH régulant ainsi l'activité des kinases Tec. Il a été montré (pour Tec, Btk et Itk) que la liaison SH3 – région riche en prolines maintient les kinases inactives et les stabilise (Hansson, Smith et al. 2001). La région SH3 permet aussi des interactions de membres de la famille Tec avec Vav, Sam-68, EWS, CD28 et WASP. Le domaine SH2 des Tec, en se liant à des protéines contenant des résidus tyrosine phosphorylés, permet le recrutement de molécules adaptatrices et de substrats spécifiques (SLP-65, BRDG1, PLCγ et SLP-76). Finalement, un domaine kinase forme la région C-terminale. Les Src kinases phosphorylent les membres de la famille Tec dans ce domaine sur la tyrosine 519 (pour Tec) et la tyrosine 552 (pour Btk).

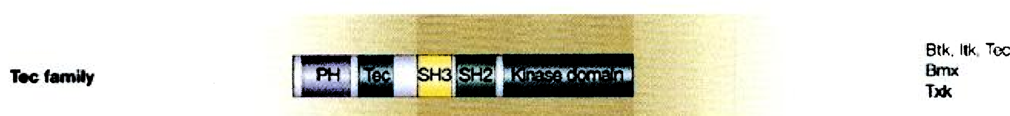


Figure 4 : Structure des membres de la famille des Tec kinases. PH – pleckstrin-homology; Tec (TH) : Tec – homology; SH2,3 – Src-homology 2,3.

Des études de notre laboratoire ont récemment mis en évidence une activation de la tyrosine kinase Tec à la suite de la stimulation des neutrophiles humains par les cristaux d'UMS (Popa-Nita, Marois et al. 2008). L'activité kinase de Tec est augmentée dans des cellules stimulées avec des cristaux d'UMS et cette augmentation est complètement inhibée par le PP2 suggérant que les kinases Src régulent l'activation de Tec. Nous avons

aussi montré que l'inhibition pharmacologique de Tec est corrélée avec une diminution des réponses des neutrophiles humains aux cristaux d'UMS, telles que la génération d'une activité chimiotactique ou la sécrétion d'IL-8 et d'IL-1β, cytokines nécessaires au développement de la réponse inflammatoire de l'arthrite goutteuse. Ces résultats suggèrent que Tec pourrait représenter une cible thérapeutique spécifique pour la goutte.

Le rôle que les tyrosine phosphatases jouent dans les voies de signalisation activées par les cristaux d'UMS dans les neutrophiles humains n'est que très peu connu. Des études en cours dans notre laboratoire semblent indiquer que l'activité phosphatase de la *SH2-containing tyrosine phosphatase 1* (SHP-1) pourrait être régulée par les cristaux d'UMS (Popa-Nita *et al.*, en préparation). SHP-1 est une tyrosine phosphatase exprimée principalement dans les cellules hématopoïétiques et décrite pour ses fonctions dans la différenciation et la croissance cellulaire, l'inflammation, la migration et la survie cellulaire (Chong and Maiese 2007). Son absence, dans des souris « moth-eaten » est associée avec une prolifération et une infiltration excessives des granulocytes et lymphocytes qui provoquent la mort à la suite d'une inflammation pulmonaire (Bignon and Siminovitch 1994; Shultz, Rajan et al. 1997). SHP-1 a été décrite comme modulant négativement la signalisation des cellules du système immunitaire (D'Ambrosio, Hippen et al. 1995; Dong, Siminovitch et al. 1999; Tamir, Dal Porto et al. 2000) en interagissant, entre autre, avec les kinases Src (Somani, Bignon et al. 1997) ou les PI3 kinases (Cuevas, Lu et al. 1999). Son activité est contrôlée négativement par une phosphorylation sur la serine 591 (Zhao, Shen et al. 1994) et positivement par une phosphorylation sur la tyrosine 536 (Zhang, Shen et al. 2003). Nos données préliminaires montrent que SHP-1 est phosphorylée sur la serine 591 d'une manière dépendante de la PKC à la suite de la stimulation des neutrophiles humains par les cristaux d'UMS. Ces résultats sont en accord avec des études précédentes qui avaient montré que la PKC régule la phosphorylation sur serine 591 de SHP-1 dans des cellules HL-60 ainsi que dans les neutrophiles (Zhao, Shen et al. 1994; Brumell, Chan et al. 1997). Une diminution de l'activité phosphatase de SHP-1 à la suite de la stimulation des neutrophiles humains par les cristaux d'UMS pourrait contribuer à l'augmentation du niveau global de phosphorylation sur résidus tyrosine caractéristique de l'activation initiée par ces particules.

b) Réponses fonctionnelles déclenchées par les cristaux d'UMS dans les neutrophiles humains

Après une vie relativement courte, les neutrophiles entrent spontanément en apoptose, ce qui conduit à leur phagocytose et élimination par les macrophages. Cependant, en cas d'arthrite goutteuse, l'inhibition de l'apoptose des neutrophiles en contact avec les cristaux d'UMS pourrait prolonger leur capacité de réponse pro-inflammatoire et, de ce fait, prolonger l'inflammation. Un retard de l'apoptose peut conduire à leur accumulation excessive et mener à des lésions tissulaires. En accord avec cette hypothèse, il a été montré que les cristaux d'UMS inhibent l'apoptose spontanée ou celle induite par le TNF-α dans les neutrophiles humains et que cette répression de l'apoptose passe par l'inhibition de la caspase-3 (Tudan, Jackson et al. 2000).

D'autre part, un mécanisme de lyse de l'intérieur (*rupture from within*) des cellules ayant phagocyté des cristaux d'UMS a été décrit il y a très longtemps (Shirahama and Cohen 1974). Pourtant il n'existe pas d'études récentes qui le valident. Il a été rapporté que des petits cristaux d'UMS (d'une longueur d'environ 2 μm) seraient ingérés par les neutrophiles humains plus rapidement et en plus grand nombre que des cristaux plus longs (environ 15 μm) (Schumacher, Fishbein et al. 1975). La phagocytose des cristaux d'UMS serait ensuite suivie par la fusion des phagosomes contenant des cristaux avec des lysosomes. Les cristaux d'UMS lyseraient ensuite la membrane phagolysosomale, ce qui causerait la libération des enzymes lysosomales dans le cytosol et la mort cellulaire. En plus des cristaux d'UMS, ces cellules nécrotiques participeraient à la prolongation et à l'amplification de l'inflammation (Shirahama and Cohen 1974).

L'activation des neutrophiles humains par les cristaux d'UMS mène à la sécrétion précoce de facteurs pro-inflammatoires tels que des enzymes lysosomales (Ginsberg, Kozin et al. 1977), des radicaux oxygénés (Abramson, Hoffstein et al. 1982), d'un (ou plusieurs) facteur(s) chimiotactique(s) resté(s) inconnu(s) (Spilberg, Mandell et al. 1974; Desaulniers, Marois et al. 2006), des eicosanoïdes (Serhan, Lundberg et al. 1984; Poubelle, De Medicis et al. 1987), des protéines S100A8/A9 (Ryckman, Gilbert et al. 2004) et à la production immédiate de la prostaglandine E2 (PGE_2) (Gilbert, Poubelle et al. 2003). Plus tardivement après leur stimulation avec des cristaux d'UMS, les neutrophiles humains secrètent aussi de

l'IL-1β (Roberge, Grassi et al. 1991) et de l'IL-8 (Hachicha, Naccache et al. 1995). La libération de ces facteurs permet le recrutement et l'activation d'autres neutrophiles au site inflammatoire ce qui pourrait expliquer en partie la prolongation et l'amplification de l'inflammation de la crise de goutte ainsi que les dommages tissulaires qui s'ensuivent.

Des études antérieures avaient suggéré que l'interaction des neutrophiles humains avec les cristaux d'UMS mène à la sécrétion d'un facteur chimiotactique de poids moléculaire d'environ 8,4 kDa et dont l'expression semble dépendre de la synthèse protéique *de novo* (Spilberg, Mandell et al. 1974). D'autre part, des études récentes de notre laboratoire ont mis en évidence l'apparition précoce (après moins de 15 minutes de stimulation) d'un facteur chimiotactique dans les surnageants des neutrophiles humains activés par des cristaux d'UMS. Ce facteur qui reste, lui aussi, inconnu à ce jour semble être de nature protéique (puisque inactivé par la trypsine) avec un poids moléculaire d'environ 30-50 kDa. Son expression ne semble pas dépendre de la synthèse protéique *de novo* (Desaulniers, Marois et al. 2006). De plus, nous avons récemment montré que sa sécrétion est dépendante de la tyrosine kinase Tec (Popa-Nita, Marois et al. 2008).

Les protéines S100A8 et S100A9, appelées aussi *myeloid-related proteins* (MRP8 et MRP14), sont de petites protéines de liaison du calcium fortement exprimées dans le cytoplasme des neutrophiles et des monocytes (Roth, Vogl et al. 2003). Il a été montré que les cristaux d'UMS entraînent le relargage de S100A8/A9 par les neutrophiles et que cette sécrétion est dépendante des tyrosine kinases de la famille Src ainsi que de Syk (Ryckman, Gilbert et al. 2004). S100A8, S100A9 et S100A8/A9 ont été détectées à de fortes concentrations dans des exsudats de souris ayant reçu des injections de cristaux d'UMS et dans les liquides synoviaux des patients souffrant de goutte. L'immunisation passive avec des anticorps anti-S100A8 et anti-S100A9 avant l'injection de cristaux d'UMS inhibe significativement le recrutement des neutrophiles dans un modèle de poche à l'air, suggérant un rôle important de ces molécules dans la migration des neutrophiles (Ryckman, McColl et al. 2003). La neutralisation de S100A8 et S100A9 ou le blocage des voies de signalisation menant à leur sécrétion (en utilisant des inhibiteurs des tyrosine kinases Src et Syk) pourraient ainsi constituer des cibles thérapeutiques potentielles dans l'inflammation causée par les cristaux d'UMS.

La vasodilatation, l'érythème et la douleur sont caractéristiques des articulations subissant une crise de goutte et ces symptômes sont causés en partie par des kinines, des peptides dérivés du complément, par l'histamine et par le PGE_2. Il a été déjà observé que la concentration de PGE_2 dans les liquides synoviaux des malades de goutte était augmentée (Wigley, Fine et al. 1983). Des résultats de notre laboratoire indiquent que la stimulation des neutrophiles humains par les cristaux d'UMS est suivie rapidement par la synthèse et la sécrétion de PGE_2 *via* une voie dépendante de la cyclooxygénase 2 (COX-2) constitutive de neutrophiles (Gilbert, Poubelle et al. 2003). Des études supplémentaires sont requises pour établir le rôle exact que le PGE_2 joue dans l'arthrite goutteuse puisque cette prostaglandine peut aussi avoir des effets anti-inflammatoires (Weissmann, Smolen et al. 1980).

Le neutrophile est tenu en partie responsable du dommage tissulaire survenu lors des maladies inflammatoires telles que l'arthrite rhumatoïde et la goutte à cause de la libération excessive du contenu cytolytique de ses granules dans le milieu extracellulaire (Ginsberg, Kozin et al. 1977). Nous avons donc étudié la dégranulation des neutrophiles humains en utilisant le lysozyme comme marqueur des granules primaires, secondaires et tertiaires et l'albumine pour les vésicules sécrétoires (Faurschou and Borregaard 2003). Nous avons ainsi mis en évidence que l'activité des PI3Ks est nécessaire à la dégranulation engendrée par les cristaux d'UMS dans les neutrophiles humains (Popa-Nita, Rollet-Labelle et al. 2007). Des études non publiées effectuées pendant ce projet de recherche ont aussi montré que la dégranulation des vésicules sécrétoires est dépendante de la tyrosine kinase Tec (Figure supplémentaire 1, Annexe 1), en accord probablement avec le rôle que cette enzyme joue dans la réorganisation du cytosquelette d'actine (Jog, Rane et al. 2007).

Il a été montré dans notre laboratoire que la stimulation des neutrophiles humains par les cristaux d'UMS entraîne la production et la sécrétion d'IL-1β (Roberge, Grassi et al. 1991). En même temps, la sécrétion de l'antagoniste du récepteur de l'IL-1 (IL-1Ra) est diminuée par les cristaux d'UMS, ce qui entraîne un ratio d'ensemble pro-inflammatoire (Roberge, de Medicis et al. 1994). La sécrétion d'IL-1β a été partiellement diminuée par un inhibiteur pharmacologique de la kinase Tec (LFM-A13) (Popa-Nita, Marois et al. 2008) ainsi que par la colchicine (Roberge, Grassi et al. 1991) et la molécule active du leflunomide (Popa-Nita, Marois et al. 2008), médicaments utilisés actuellement pour le traitement de la goutte aiguë (Molad 2002) et de l'arthrite rhumatoïde respectivement (Breedveld and Dayer 2000). Cette

observation est un autre argument en faveur du rôle crucial que l'interaction neutrophiles–cristaux d'UMS joue dans l'inflammation goutteuse puisqu'il a été récemment montré que l'IL-1β est essentielle au développement de la réponse immunitaire dans la crise de goutte (Chen, Shi et al. 2006). De plus, l'inhibition de cette cytokine s'est avérée efficace pour le traitement de l'arthrite goutteuse (So, De Smedt et al. 2007).

L'IL-8, un puissant agent chimiotactique pour les neutrophiles, a été décrit comme étant nécessaire au développement des plusieurs conditions inflammatoires dépendantes des neutrophiles (Mukaida 2003). L'IL-8 a été montré comme étant essentielle au déclenchement de l'inflammation et à la migration des neutrophiles dans des modèles expérimentaux de goutte (Terkeltaub, Baird et al. 1998). De plus, la concentration d'IL-8 dans des liquides synoviaux des patients souffrant d'arthrite goutteuse a été rapportée comme étant augmentée (Terkeltaub, Zachariae et al. 1991). Des études effectuées dans notre laboratoire ont montré que les neutrophiles humains stimulés par les cristaux d'UMS produisent de l'IL-8 mais pas de MIP-1α (*macrophage inflammatory protein 1 alpha*), ce qui expliquerait le recrutement presque exclusif des neutrophiles aux sites de l'inflammation engendrée par ces particules (Hachicha, Naccache et al. 1995). De plus, comme dans le cas de l'IL-1β, nous avons récemment montré que la sécrétion d'IL-8 dépend de la tyrosine kinase Tec (Popa-Nita, Marois et al. 2008). Cette kinase pourrait donc représenter une cible thérapeutique efficace pour le traitement de l'arthrite goutteuse.

5. Résolution spontanée de la crise de goutte

Même si les cristaux d'UMS sont reconnus comme étant l'agent étiologique de l'arthrite goutteuse depuis très longtemps, le fait que certaines personnes demeurent asymptomatiques en dépit de la présence de cristaux d'UMS dans leurs articulations et que les crises de goutte se résolvent spontanément indépendamment de la persistance de cristaux d'UMS demeure incompris (Liote and Ea 2007). Plusieurs mécanismes qui expliquent la résolution spontanée de la crise de goutte ont été proposés.

Les modifications subies par les cristaux pendant la crise de goutte fournissent une première explication : réduction de la taille et de la charge électrique de surface, phagocytose par les macrophages et changement de leur revêtement protéique. Les apolipoprotéines E et B peuvent pénétrer dans la cavité synoviale pendant une crise de goutte et il a été montré qu'en se liant aux cristaux d'UMS, ces protéines inhibent de façon significative les réponses (phagocytose et production d'anions superoxyde) des neutrophiles (Terkeltaub, Dyer et al. 1991; Ortiz-Bravo, Sieck et al. 1993).

Les phagocytes mononucléaires jouent aussi un rôle dans la résolution de l'inflammation synoviale faisant pencher la balance d'un état asymptomatique à une inflammation aiguë et vice-versa, selon l'état de différenciation du monocyte en macrophage (Yagnik, Hillyer et al. 2000; Haskard and Landis 2002; Landis, Yagnik et al. 2002). Le changement monocyte – macrophage s'accompagne d'une perte de capacité à produire des cytokines pro-inflammatoires (IL-1, IL-6, TNF-α). Au contraire, les macrophages produisent des cytokines anti-inflammatoires telles que l'IL-10 et le TGF-β après avoir phagocyté des cristaux d'UMS ou des cellules apoptotiques (Liote, Prudhommeaux et al. 1996; Yagnik, Evans et al. 2004). Le TGF-β peut aussi réduire l'activation des cellules endothéliales limitant ainsi le recrutement des neutrophiles et des monocytes dans la cavité synoviale (Bertolino, Deckers et al. 2005).

Le PPARγ (*peroxisome proliferator-activated receptor γ*), un membre de la famille des récepteurs nucléaires d'hormones (Kersten, Desvergne et al. 2000), a été proposé comme un régulateur négatif de l'inflammation puisque sa stimulation réduit l'expression des gènes codant pour différentes cytokines pro-inflammatoires (IL-1β, IL-6 et TNF-α) (Ricote, Huang et al. 1999). En accord avec cette hypothèse, les monocytes présentent une

expression accrue de PPARγ suite à leur stimulation par les cristaux d'UMS (Akahoshi, Namai et al. 2003). De plus, l'expression de cette molécule a été observée comme étant augmentée dans les tissues synoviaux des malades d'arthrite, suggérant qu'elle pourrait participer à la résolution de l'inflammation goutteuse (Kawahito, Kondo et al. 2000).

Les mélanocortines (hormones dérivées de la pro-opiomélanocortine) naturelles ou synthétiques ont été proposées parmi les molécules qui pourraient favoriser l'arrêt de l'inflammation goutteuse (Lipton and Catania 1997). Ainsi, une étude clinique a montré l'efficacité de l'administration intramusculaire de l'ACTH1-39 (le prototype des mélanocortines (O'Donohue and Dorsa 1982)) pour le traitement de l'arthrite goutteuse (Ritter, Kerr et al. 1994). Plus récemment, un modèle murin a permis la mise en évidence du rôle anti-inflammatoire du MC3-R ce qui lui conférerait un éventuel potentiel en tant que cible thérapeutique pour la goutte (Getting, Lam et al. 2006).

6. Objectif

Le principal objectif de ce projet de recherche est d'identifier les voies de signalisation activées par les cristaux d'UMS et de caractériser leurs fonctions dans les réponses des neutrophiles humains.

La précipitation des cristaux d'UMS dans les articulations des personnes souffrant de l'hyperuricémie engendre une puissante réaction inflammatoire connue sous le nom d'arthrite goutteuse qui est caractérisée par les signes classiques de l'inflammation aiguë (douleur, rougeur, chaleur, gonflement). Les neutrophiles représentent plus de 95% des cellules infiltrées dans le liquide synovial et sont présents dès les étapes précoces de l'inflammation (Agudelo and Schumacher 1973). Le rôle primordial des neutrophiles dans l'arthrite goutteuse est mis en évidence par l'absence de réponse inflammatoire aux injections de cristaux d'UMS chez les souris déplétées en neutrophiles (Seegmiller and Howell 1962) ainsi que par le fait que les molécules inhibant les fonctions du neutrophile sont efficaces pour le traitement de la goutte (Phelps 1969).

La phosphorylation sur résidus tyrosine est un des premiers signes caractéristiques de l'activation des neutrophiles humains par les cristaux d'UMS (Gaudry, Roberge et al. 1993). La Src kinase Lyn et la tyrosine kinase Syk sont parmi les premières protéines phosphorylées sur résidus tyrosine à la suite de la stimulation des neutrophiles par les cristaux d'UMS (Gaudry, Gilbert et al. 1995; Desaulniers, Fernandes et al. 2001). L'inhibition marquante des réponses des neutrophiles humains aux cristaux d'UMS par des inhibiteurs pharmacologiques des Src kinases (PP1, PP2) ou Syk (piceatannol) met en évidence leur rôle majeur dans les voies de signalisation activées par ces particules (Desaulniers, Fernandes et al. 2001; Majeed, Caveggion et al. 2001). Des études précédentes ont montré que Syk est nécessaire pour l'activation des PI3 kinases en réponse à différents *stimuli* tels que l'activation du récepteur des cellules B (Beitz, Fruman et al. 1999) ou des récepteurs Fcγ des macrophages ou neutrophiles (Okada, Maeda et al. 2000). Ainsi, des cellules déficientes en Syk présentent une diminution de l'accumulation de PIP3, le principal produit des PI3 kinases (Beitz, Fruman et al. 1999). De plus, il a été aussi montré que la sous-unité régulatrice des PI3 kinases de classe Ia, la p85, pouvait interagir directement avec Syk dans un essai de double hybride chez la levure (Moon, Post et al.

2005). La première hypothèse de ce projet est donc que les cristaux d'UMS activent les PI3 kinases d'une manière dépendante de la protéine kinase Syk (Chapitre II).

Le PIP3, second messager produit par les PI3 kinases, agit en tant qu'ancre dans la membrane plasmique pour des protéines régulatrices de la signalisation qui s'y lient *via* leur domaine PH telles que les membres de la famille des Tec kinases (Lemmon 2008). Des études précédentes de notre laboratoire avaient montré que la tyrosine kinase Tec est activée à la suite de la stimulation du CD16b (Fernandes, Lachance et al. 2005). Ce récepteur Fcγ est en partie responsable de la reconnaissance des cristaux d'UMS par les neutrophiles humains (Barabe, Gilbert et al. 1998). La deuxième hypothèse de ce projet est donc que les cristaux d'UMS activent la tyrosine kinase Tec dans les neutrophiles humains (Chapitre III).

Chapitre II : L'activation des phosphatidylinositol 3-kinases par les cristaux d'UMS dans les neutrophiles humains dépend de la tyrosine kinase Syk

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Résumé

Le dépôt des cristaux d'urate monosodique (UMS) dans les articulations provoque une puissante réaction inflammatoire (goutte) caractérisée par une infiltration massive des neutrophiles. L'interaction directe entre les neutrophiles et les cristaux d'UMS entraîne l'activation d'une voie de signalisation dépendante de la tyrosine kinase Syk et des phosphatidylinositol 3-kinases (PI3Ks). L'étude présente analyse le rôle que les PI3Ks jouent dans la régulation des réponses des neutrophiles humains aux cristaux d'UMS, l'agent étiologique de la goutte. Les résultats présentés ici montrent que la stimulation des neutrophiles humains par les cristaux d'UMS entraîne l'activation des PI3Ks de la classe Ia via un mécanisme dépendant de la tyrosine kinase Syk. La stimulation des neutrophiles est accompagnée par une association de la sous-unité régulatrice des PI3Ks de la classe Ia, la protéine p85, avec des fractions cellulaires enrichies en cytosquelette et domaines membranaires résistants au détergent (DRMs). De plus, l'activation des neutrophiles humains entraîne la formation d'un complexe protéique contenant Syk et p85. Finalement, ces résultats montrent aussi que les PI3Ks jouent un rôle majeur dans les réponses fonctionnelles (activation de la phospholipase D (PLD) et dégranulation) des neutrophiles humains. Ces réponses sont responsables en partie des dommages tissulaires associés à l'arthrite goutteuse.

Crystal-induced neutrophil activation. IX. Syk-dependent activation of class Ia phosphatidylinositol 3-kinase[1]

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Running title: Syk-dependent activation of PI3Ks by MSU crystals

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Abstract

The deposition of monosodium urate (MSU) crystals in the joints of humans leads to an extremely acute inflammatory reaction, commonly known as gout, characterized by a massive infiltration of neutrophils. Direct interactions of MSU crystals with human neutrophils and inflammatory mediators are crucial to the induction and perpetuation of gout attacks. The intracellular signalling events initiated by the physical interaction between MSU crystals and neutrophils depend on the activation of specific tyrosine kinases (Src and Syk in particular). Additionally, phosphatidylinositol 3-kinases (PI3Ks) may also be involved. The present study investigates the involvement of the PI3K family in the mediation of the responses of human neutrophils to MSU crystals. The results obtained indicate that the interaction of MSU crystals with human neutrophils leads to the stimulation of class Ia PI3Ks by a mechanism which is dependent on the tyrosine kinase Syk. We also found an increase in the amount of p85 associated with the NP40-insoluble fraction derived from MSU crystal-stimulated human neutrophils. Furthermore, MSU crystals induce the formation of a complex containing p85 and Syk which is mediated by the Src family kinases. Finally, evidence is also obtained indicating that the activation of PI3Ks by MSU crystals is a critical element regulating PLD activation and degranulation of human neutrophils. The latter response is likely to be involved in the joint and tissue damage that occurs in gouty patients.

Introduction

The deposition of needle-like monosodium urate (MSU) crystals in the joints of humans suffering from hyperuricaemia leads to an extremely acute inflammatory reaction, commonly known as gout or gouty arthritis, which is characterized by acute and intense symptoms of swelling, redness and pain, the hallmarks of inflammation [1].

Neutrophils play a crucial role in host defence against injury and infection as well as in inflammatory responses by virtue of their ability to mount a series of effector responses. MSU crystals cause a massive infiltration of neutrophils in both the joint fluid and synovial membrane, where they actively phagocytose crystals [2]. If animals are depleted of polymorphonuclear leukocytes, the inflammatory response to injections of MSU crystals is suppressed [3] and moreover, a number of agents that suppress neutrophil functions prevent and/or terminate acute gouty inflammation [4]. Direct MSU crystal interactions with neutrophils and inflammatory mediators that potentiate neutrophil activation are therefore crucial to the induction and perpetuation of gout attacks. MSU crystals induce secretion of a variety of cytokines (reviewed in [5]), prostanoids [6, 7], chemotactic factors (including IL-8, MCP-1 [8] and unidentified factors (crystal-induced chemotactic factor [9] and others [10])) that drive the inflammatory process. These cytokines can amplify the inflammatory process through increased inflammatory cell recruitment by upregulation of adhesion molecules and stimulation of the acute phase response.

One of the earliest events observed upon stimulation of neutrophils with MSU crystals is an increase in the level of tyrosine phosphorylation of various proteins [11]. The protein tyrosine kinase Syk, which has been largely associated with the phagocytic response by Fc receptors [12] and with spreading mediated by integrins [13], has been previously identified in our laboratory as one of the major proteins tyrosine-phosphorylated and activated in human neutrophils upon stimulation with MSU crystals [14]. Syk is a 72 kDa protein kinase that plays a central role in coupling immune recognition receptors to multiple downstream signalling pathways in haematopoietic cells. Our previous results showed that piceatannol, a specific Syk inhibitor, diminished the tyrosine phosphorylation patterns, the mobilization of intracellular calcium, the production of superoxide anions and the PLD activity stimulated by MSU crystals [14].

Together with Syk, Src family kinases play crucial roles in multiple neutrophil intracellular signalling pathways. A hierarchical activation of Src family kinases and Syk has been proposed following FcγRIIa engagement [15].

The phosphoinositide 3-kinases (PI3Ks) phosphorylate PI, PI(4)P and $PI(4,5)P_2$ to form PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ respectively. These lipids serve as phospholipase substrates for the generation of soluble (inositol phosphate) and membrane-associated (diacylglycerol) second messengers; they also interact directly with intracellular proteins, affecting their location and/or activity and, finally, they can alter the local membrane topology by electrostatic interactions [16]. Class Ia enzymes of the PI3K family are heterodimeric proteins, each of which consists of a catalytic subunit of 110-120 kDa and an associated regulatory subunit called p85 proteins α and β. The tyrosine phosphorylation of p85 is indicative of its activation. Under resting conditions, p85 serves to stabilize and inactivate the p110 catalytic subunit [17].

Syk is required for the activation of the PI3K family in response to a variety of signals including engagement of the B cell antigen receptor (BCR) [18] and macrophage or neutrophil Fcγ receptors [19]. Moreover, p85 PI3K subunit was the major Syk-binding protein identified in yeast two-hybrid screens using libraries from two different sources [20].

Although activation of PI3Ks has been well documented for other stimuli [21, 22], little is known about its involvement in the responses of human neutrophils to MSU crystals. Increased PI3K activity has been reported in tyrosine phosphorylated immunoprecipitates from plasma-opsonised MSU crystal-stimulated human neutrophils [23]. However, the signal transduction pathways leading to the activation of PI3Ks in response to MSU crystals and its functional significance remain to be investigated.

We show here that MSU crystals induce the activation of class Ia PI3Ks in human neutrophils and that this activation depends on the kinase activity of Syk. Our results also indicate that activated Syk and p85 are both found in specialized microdomains insoluble in the non-ionic detergent NP40 upon MSU crystal stimulation. Moreover, we show that MSU crystals induce the formation of a Syk/p85 complex that is dependent on Src family kinases. Our results also indicate that the stimulation of the activity of PLD and of the degranulation response induced by MSU crystals depends on the Syk-mediated activation

of PI3Ks. These events are likely to be relevant to the tissue damage that occurs in gouty arthritis.

Material and Methods

1. Antibodies and Chemicals

The monoclonal anti-phosphotyrosine antibody 4G10 (16-101) and the anti-p85 antibody (06-195) were purchased from Upstate Biotechnology (Lake Placid, NY). The anti-phospho-p85 (sc-12929R) was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). The anti-Syk antibody (MAB88906) was purchased from Chemicon (Temecula, CA). Antibodies against phospho-AKT (9271S) and AKT (9272) were purchased from Cell Signalling Technology (Beverly, MA). The anti-flotillin-1 antibody (610821) was from BD Biosciences (Franklin Lakes, NJ). The anti-LDH antibody (20-LG22) was from Fitzgerald Industries International Inc (Concord, MA). The peroxidase-conjugated sheep anti-mouse IgG (NXA931), donkey anti-goat IgG (sc-2056) and donkey anti-rabbit IgG (711-035-152) were obtained from GE Healthcare (Buckinghamshire,UK), Santa Cruz Biotechnology Inc (Santa Cruz, CA) and Jackson Immuno Research Laboratories (West Grove, PA), respectively.

Dextran T-500 and Ficoll-Paque were purchased from Wisent (Saint-Bruno, QC, Canada).

1-O-[^{3}H]alkyl-2-lyso-phosphatidylcholine was from Perkin Elmer (Woodbridge, ON, Canada). Wortmannin and piceatannol were purchased from Biomol (Plymouth Meeting, PA). LY294002 (440204), PP_2 (529573) and PP_3 (529574) were purchased from Calbiochem (La Jolla, CA). Protein A Sepharose beads (71-5280-04) were purchased from Amersham Biosciences (Baie d'Urfé, QC, Canada). NP40 was from Calbiochem (La Jolla, CA). CHAPS, aprotinin and leupeptin were obtained from Roche Diagnostics (Indianapolis, IN). Di-isopropylfuorophosphate (DFP) was from Serva Electrophoresis (Heidelberg, Germany). 4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP) and PMSF were purchased from Sigma Aldrich (Oakville, ON, Canada).

Triclinical monosodium urate monohydrate crystals were synthesized and characterized as described previously [24].

2. Neutrophil purification

Venous blood was collected from healthy adult volunteers in isocitrate anticoagulant solution. Neutrophils were separated as previously described [25]. Briefly, whole blood was centrifuged at 180g for 10 min and the resulting platelet rich plasma was discarded. Leukocytes were obtained following sedimentation in 2% Dextran T-500. Mononuclear

cells were removed by centrifugation on Ficoll-Paque cushions and contaminating erythrocytes in the neutrophil pellets were removed by a 20 seconds hypotonic lysis. Neutrophils were resuspended at 40x10₆ cells/ml in HBSS 1X, pH 7.4 containing 1.6 mM Ca^{2+} but no Mg^{2+}.

3. Cell stimulation

When indicated, neutrophils (40x10^6 cells/ml) were pre-incubated at 37°C for 10 min with the specified concentrations of inhibitors (40 μM piceatannol; 10 μM PP_2, PP_3 or LY294002; 30 nM wortmannin) or an equal volume of dimethyl sulfoxide (Me_2SO). The Me_2SO concentration never exceeded 0.1%. In cell-based assays these concentrations of piceatannol, wortmannin and LY294002 have been previously described as exhibiting a relatively high selectivity for Syk and PI3Ks respectively [16, 26]. Neutrophil suspensions were then stimulated at 37°C with 3 mg/ml of MSU crystals for the indicated periods of time.

4. Western Blotting

For whole cell analysis, stimulations were stopped by transferring 100 μl of the cell suspension to an equal volume of boiling 2X Laemmli's sample buffer (1X is 62.5 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM pNPP, 12.5 μg/ml leupeptin, 12.5 μg/ml aprotinin, 0.00125% bromophenol blue) and boiled for 7 min. Samples were then loaded onto 7.5-20% gradient SDS-polyacrylamide gels. Separated proteins were transferred from the gels to Immobilon polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). After incubations with the desired antibodies (mouse anti-phosphotyrosine : 1:4000 dilution; mouse anti-flotillin-1 and goat anti-LDH : 1:2000 dilution; rabbit anti- phospho-p85, anti-p85, anti-phospho-AKT (Thr308), anti-phospho-AKT (Ser473), anti-AKT and mouse anti-Syk : 1:1000 dilution) and the corresponding second antibody (1:20000 dilution) the membranes were revealed using the ECL detection system.

5. Preparation of NP40-insoluble fractions

Neutrophils were processed and stimulated with MSU crystals as described above. The reactions were stopped at the indicated times by a 10 s centrifugation at 6000g. The supernatants were discarded and the cells were lysed in 1 ml of ice-cold hypotonic lysis buffer (20 mM Tris-HCL, 0.1% NP40, 10 mM NaCl, 2 mM EDTA, 10 μg/ml aprotinin and

leupeptin, 2 mM Na_3VO_4, 10 mM pNPP, 2 mM PMSF, 3 mM DFP, pH 7.2) as described previously [27]. The samples were vortexed, kept on ice for 5 min and then centrifuged at 16000g at 4°C for 5 min. An aliquot of the supernatants was transferred to the same volume of 2X Laemmli's sample buffer and boiled for 7 min. The insoluble pellets were resuspended in 0.1% NP40 lysis buffer and transferred to the same volume of 2X Laemmli's sample buffer. When loading the SDS-polyacrylamide gels, adjustments were made for all samples to be equivalent with respect to cell number.

6. Immunoprecipitations under native conditions

Neutrophils were processed and stimulated with MSU crystals as described above. The stimulations were stopped by transfer to an ice bath followed by a 10 s spin in a microcentrifuge at 6000g. The pellets were resuspended in cold lysis buffer (10 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, 0.6% CHAPS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM sodium orthovanadate, 250 μg/ml soybean trypsin inhibitor, 3 mM DFP, 1 mM PMSF). After 5 min on ice, the cell lysates were centrifuged at 16000g for 5 min at 4 °C. The supernatants were incubated at 4°C with gentle rotation for 3 h in the presence of pre-washed protein-A Sepharose beads linked to an anti-p85 antibody (4 μg for 50 μl beads) or to an anti-Syk antibody (0.8 μg for 50 μl beads). For p85, the immunoprecipitations were preceded by one hour of preclearing with protein-A Sepharose beads. The beads were then collected and washed three times with cold lysis buffer. 50 μl of Laemmli's sample buffer were added to the beads, which were boiled for 7 min.

7. Immunoprecipitations under denaturing conditions

This protocol has already been described in our laboratory as being useful for preserving the stability of the tyrosine phosphorylated proteins of various molecular weights and their subsequent immunoprecipitation and identification [28, 29]. Briefly, neutrophils were processed and stimulated with MSU crystals as described above. The total lysates and the soluble NP40 fractions were transferred in the same volume of 2X denaturing Lysis Buffer (125 mM Tris-HCl, 6% SDS, 2% β-mercaptoethanol, 5 mM Na_3VO_4, 25 μg/ml aprotinin and leupeptin, 0.0025% bromophenol blue at pH 6.8) and boiled for 10 min. The insoluble NP40 fractions were resuspended in NP40 Lysis Buffer, transferred in the same volume of 2X denaturing Lysis Buffer and boiled for 10 min. The samples were filtered through sephadex G-10 columns to remove the denaturing agents. The denatured samples were

incubated with the anti-Syk antibody (0.8 µg bound to 50 µl of protein-A Sepharose beads) for 3 h at 4°C on a rotator platform. The beads were then collected and washed twice with ice-cold NP40 buffer (62.5 mM Tris-base, 1% NP40, 1% glycerol, 137 mM NaCl, 2 mM Na_3VO_4, 10 µg/ml aprotinin and leupeptin at pH 8.0). The beads were boiled 10 min in 100 µl of 2X Laemmli's sample buffer.

8. Assay for class Ia PI3K activity

PI3K activity was determined *in vitro* using a competitive PI3K ELISA kit (K-1000) from Echelon Biosciences Inc (Salt Lake City, UT). This kit measures PI3K activity as a conversion of $PI(4,5)P_2$ into $PI(3,4,5)P_3$. We used this kit in conjunction with p85 immunoprecipitates (see Immunoprecipitations under native conditions). Thus, we specifically determined the class Ia activity of PI3K. Briefly, PI3K bound to the protein-A Sepharose beads was incubated for 1h with 10 µM diC_8 PI(4,5)P2 substrate at room temperature in 50 µl of buffer containing 4 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4), 10 mM NaCl and 25 µM ATP. The beads were removed by centrifugation and the supernatant, or known concentrations of $PI(3,4,5)P_3$ were incubated for 1h with 50 µl of $PI(3,4,5)P_3$-binding reagent then transferred to a detection plate coated with $PI(3,4,5)P_3$. Plate-bound binding reagent was quantified by using a secondary detection reagent, peroxidase, and peroxidase substrate with reaction product measured by absorbance at 450 nm.

9. Phospholipase D measurements – PEt formation

Neutrophils were labelled with 1-O-[^{3}H]alkyl-2-lyso-phosphatidylcholine (2 µCi/10^7 cells) for 90 min as previously described [30]. The cells were then washed and resuspended at $10x10^6$ cells/ml in HBSS. Cell suspensions (0.5 ml) were stimulated with 3 mg/ml MSU crystals for 15 min in the presence of 1% ethanol. Incubations were stopped by adding 1.8 ml of chloroform/methanol/HCl (50:100:1) and unlabeled phosphatidylethanol (PEt) as a standard. Lipids were extracted and dried under nitrogen. The lipid extracts were dissolved in 40 µl of chloroform/methanol (2:1) and spotted on pre-washed silica gel TLC plates. [^{3}H]PEt was separated from the other lipids using the solvent mixture chloroform/methanol/acetic acid (65:15:2). Lipids were visualized by Coomassie Brilliant Blue staining (0.03% dye; 35% MeOH; 200mM NaCl) and the different lipid classes were scraped off the plates. Radioactivity in PEt was monitored by liquid scintillation counting and the results corrected for background radioactivity and quenching.

10. Degranulation

Neutrophils were processed and stimulated with MSU crystals as described above. The stimulations were stopped by a quick spin (15 s, 6000g), and the supernatants were harvested, filtered on 0.22 μm nylon syringe filters and kept on ice. The extent of lysozyme release was assessed by adding 100 μl supernatant to 900 μl of a 0.25 mg/ml *Micrococcus lysodeikticus* solution prepared in a 0.1 M PO_4 buffer. The loss of absorbance was then read at 450 nm for a period of 2 min. To obtain a percentage value for MSU crystal-induced degranulation, the absorbance values were compared, and the maximal loss of absorbance was obtained by lysing the cells with 0.1% Triton X-100.

Human albumin levels in MSU crystal-stimulated neutrophil supernatants were determined by capture ELISA (BETHYL Laboratories Inc, Montgomery, TX) according to the manufacturer's recommendations. The amount of total albumin released was obtained by lysing the cells with 0.1% Triton X-100. All samples were measured in triplicate, and the results are expressed as the mean ± SEM percentage of total albumin released in at least 5 experiments.

11. Densitometric analysis

Densitometric analysis was performed using Image J Software on the scanned Western Blot results. The measurements of pixel intensity of each blot lane were plotted using GraphPad Prism 4 Software.

12. Statistical analysis

Statistical analyses were performed using the Student's paired *t*-test (two-tailed) using GraphPad Prism 4 Software on the non-processed data. Every inhibitory condition was compared to the control condition and significance was considered to be attained when *P* was less than 0.05.

Results

MSU crystals activate the PI3K pathway in human neutrophils in a Syk-dependent manner

Recent studies have shown that PI3Ks play a major role in the control of multiple neutrophil responses including phagocytosis, superoxide anion generation and chemotaxis (reviewed in [31]). Although MSU crystals are known to stimulate several PI3K-dependent responses in neutrophils and increased PI3K activity has been detected in tyrosine phosphorylated immunoprecipitates from plasma opsonised MSU crystal-stimulated human neutrophils [23], the signal transduction pathways relating these observations have not been characterized yet.

The tyrosine phosphorylation of the p85 regulatory subunit of class Ia PI3Ks is indicative of its activation [17]. As shown in Fig.1A (left panel), MSU crystals induce a transient tyrosine phosphorylation of p85 with a peak of intensity observed between 2 and 5 min of stimulation. Since it was previously shown in our laboratory that Syk tyrosine kinase plays a key role in the responses of human neutrophils to stimulation with MSU crystals [14], we investigated the effect of piceatannol, a Syk specific inhibitor [26], on the tyrosine phosphorylation of p85 induced by MSU crystals. The cells were incubated for 10 min at 37°C with 40 μM piceatannol prior to MSU crystal stimulation. These conditions were previously observed to be sufficient to significantly diminish the stimulation of tyrosine phosphorylation of Syk and its tyrosine kinase activity in neutrophils stimulated with MSU crystals [14]. As shown in Fig.1A (right panel), piceatannol significantly inhibited the stimulation of tyrosine phosphorylation of p85 induced by MSU crystals in human neutrophils. A quantitative evaluation of these results (by densitometry) is provided in the lower panel of Figure 1A.

We next assessed the effect of piceatannol on the activity of class Ia PI3Ks. Using p85 immunoprecipitates derived from neutrophils stimulated by MSU crystals, we measured the class Ia catalyzed conversion of PI(4,5)P_2 into PI(3,4,5)P_3. Three independent measurements performed on neutrophils obtained from different donors revealed that MSU crystals increased PI3K activity approximately 13-fold over basal levels, an effect that was significantly inhibited by piceatannol (Fig.1B).

The serine/threonine kinase AKT is a major target of PI3Ks. Phosphoinositides activate phosphoinositide-dependent kinases 1 (PDK1) and 2 (PDK2) that phosphorylate AKT on Thr 308 and Ser 473 respectively [32]. We therefore examined the state of activation of AKT following the stimulation of neutrophils by MSU crystals. As shown in Fig.2A and Fig.2B, MSU crystals induced a transient phosphorylation of AKT on Thr308 and Ser473. As observed for p85 (Fig.1A), piceatannol drastically diminished the MSU crystal-induced phosphorylation of AKT (Fig.2A and Fig.2B). The inactive analogue of piceatannol, transtilbene, did not have any effect on the phosphorylation of p85 or AKT (data not shown) confirming that the effect of piceatannol was due to its inhibitory effect on Syk.

Taken together, these results strongly support the conclusion that MSU crystals induce the activation of class Ia PI3Ks in a Syk-dependent manner in human neutrophils.

Tyrosine-phosphorylated p85 and Syk are located in NP40-insoluble fractions in human neutrophils stimulated with MSU crystals

Specialized microdomains, insoluble in non-ionic detergents, rich in cholesterol and sphingolipids have been shown to concentrate signalling molecules such as tyrosine kinases and adapter proteins and are believed to be involved in the activation of signalling cascades and in membrane trafficking in a variety of cells including neutrophils [27, 33, 34]. Accordingly, tyrosine phosphorylated substrates and enzyme activities were found to be increased and concentrated in NP40-insoluble fractions upon stimulation of human neutrophils with MSU crystals [29].

We therefore monitored the distribution of p85 between the NP40-soluble and -insoluble fractions following stimulation of human neutrophils with MSU crystals. Neutrophils (stimulated or not with MSU crystals) were lysed in NP40 and the NP40-soluble and -insoluble fractions were prepared and immunoblotted for p85 as described in Material and Methods. The results summarized in Fig.3A show that the amount of p85 in the NP40-insoluble fractions more than doubled following stimulation of the cells with MSU crystals. Flotillin, a detergent-resistant membranes (DRMs) marker, was found to be contained nearly exclusively within the NP40-insoluble fraction.

Having established that the activation of PI3Ks by MSU crystals was Syk-dependent, we then evaluated the effect of piceatannol on the insolubility of p85 at various times. As shown in Fig.3B, the insolubilization of p85 induced by MSU crystals was not affected by

the Syk inhibitor at any of the times monitored (up to 10 minutes) suggesting that the tyrosine phosphorylation of p85 by Syk is not a requirement for its insolubility.

We next investigated the distribution of the phosphorylated p85 and Syk in the NP40-soluble and -insoluble fractions following stimulation with MSU crystals. These experiments were carried out as described above except that immunoblotting was achieved using an anti-phospho-p85 or an anti-phosphotyrosine antibody following the immunoprecipitation under denaturing conditions of Syk in the various fractions (the experimental details are described in Material and Methods). We observed that the phosphorylated form of p85 is found predominantly in the NP40-insoluble fraction following stimulation with MSU crystals (Fig.4A). The distribution of phospho-p85 among these fractions therefore closely parallels that of p85. As shown in Fig.4B, the NP40-insoluble fractions also contained nearly all the tyrosine phosphorylated Syk.

It can also be observed that whereas p85 translocates to the NP40-insoluble fractions upon stimulation with MSU crystals, a large amount (nearly 70%) of Syk is already insoluble in unstimulated cells (Fig.4B, bottom panel).

MSU crystal stimulation induces an interaction between the p85 regulatory subunit of PI3Ks and Syk in human neutrophils

CHAPS is a zwitterionic detergent used to solubilize proteins. Zwitterions are polar and usually have a high solubility in water and a poor solubility in most organic solvents. CHAPS is used as a non-denaturing solvent in the process of protein purification and is especially useful in purifying membrane proteins which are often sparingly soluble or insoluble in aqueous solutions due to their natively hydrophobic cellular environment [35]. As shown in Fig.5 and contrary to what was observed using NP40 (see Figs. 3 and 4), Syk and p85 are nearly completely soluble in CHAPS even after stimulation with MSU crystals, thus making it possible to perform immunoprecipitations under native conditions and to study protein-protein interactions.

In haematopoietic cells, Syk couples multiple membrane-associated receptors to intracellular signalling pathways. Our data indicate that the kinase activity of Syk is necessary for the activation of the class Ia PI3Ks family in response to MSU crystal stimulation (Fig.1). We have also shown that tyrosine phosphorylated Syk and p85 are both

found into cytoskeleton and/or DRMs-containing domains in response to MSU crystal stimulation (Fig.4).

We next assessed whether an interaction between p85 and Syk could be detected following the stimulation of human neutrophils by MSU crystals. By a two-way CHAPS co-immunoprecipitation technique (details in Material and Methods, Immunoprecipitations under native conditions), we show here that stimulation of human neutrophils with MSU crystals induces the time-dependent formation of a complex containing both p85 and Syk (Fig.6A).

To further investigate the transduction pathway leading to the MSU crystal-induced interaction between p85 and Syk, we tested the effect of piceatannol on the stimulated co-immunoprecipitation of these two proteins. Piceatannol did not diminish the interaction between p85 and Syk despite diminishing the tyrosine-phosphorylation of Syk (Fig.6B) suggesting that this interaction does not depend on Syk kinase activity. The same results were obtained when p85 was immunoprecipitated (data not shown).

Together with Syk, Src family kinases have crucial roles in multiple neutrophil intracellular signalling pathways. In many cases, they seem to operate together, with the general hypothesis being that activation of Src family kinases precedes and modulates the activation of Syk tyrosine kinase [36]. We therefore hypothesized that the MSU crystal-induced co-immunoprecipitation between p85 and Syk could depend on the Src-mediated activation of Syk. In order to confirm this hypothesis, we evaluated the potential effects of PP_2, a Src kinase specific inhibitor [37], on the interaction between p85 and Syk induced by MSU crystals. As shown in Fig.6C, PP_2 completely abolished the tyrosine phosphorylation of Syk and nearly completely inhibited the co-immunoprecipitation of p85 and Syk. We did not observe any effect of PP_2 on the MSU crystal-induced insolubilization of p85 (data not shown). Together with the inhibition of the MSU crystal-induced p85/Syk interaction by PP_2, this result suggests that the localisation of p85 to detergent-resistant domains is not sufficient for it to interact with Syk. The initial, Src-mediated tyrosine phosphorylation of Syk is on the other hand essential for the formation of the p85/Syk complex.

When compared to Me_2SO, the inactive analogue of PP_2, PP_3, affected neither the phosphorylation of Syk nor its association to p85 (data not shown). The same results were obtained when p85 was immunoprecipitated (data not shown).

Functional consequences of the MSU crystal-induced PI3Ks activation in human neutrophils

It was previously described in our laboratory that MSU crystal stimulation induces the activity of PLD in human neutrophils [38]. In view of the above results linking stimulation of human neutrophils to the activation of PI3Ks, we investigated the role of the latter in the stimulation of the activity of PLD. Neutrophils were pre-incubated with the two unrelated PI3Ks inhibitors, wortmannin and LY294002, which are cell-permeable, low-molecular-weight compounds with *in vitro* IC_{50} of around 5 nM and 1 μM, respectively. The results of these studies, summarized in Fig.7, show that both compounds inhibited the stimulation of the activity of PLD in a concentration-dependent manner (IC_{50} of 8 nM and 2 μM for wortmannin and LY294002, respectively). Maximal inhibition (80%) of the MSU crystal-induced PLD activity was obtained with about 20 nM of wortmannin and 10 μM of LY294002. No further inhibition of MSU crystal-induced PLD activity was observed by increasing the concentrations of wortmannin and LY294002 up to 1 μM and 100 μM, respectively.

PLD has been shown to play an important role in regulating the degranulation of human neutrophils in response to a variety of stimuli [39]. Since we showed that the MSU crystal-induced PLD activity is dependent on PI3Ks (Fig.7), we monitored next the potential effects of wortmannin and LY294002 on the degranulation of the four types of neutrophil granules induced by MSU crystals. Lysozyme was used as a marker of primary, secondary and tertiary granules [40] and albumin for secretory granules [41]. As shown in Fig.8, MSU crystals induce the extracellular secretion of up to 18% of the specific, secondary, and tertiary granular contents (Fig.8A) and close to 30% of the secretory granules (Fig.8B). Wortmannin and LY294002 significantly diminished the extent of degranulation induced by MSU crystals (Fig.8A and B). It should be noted that the effects of wortmannin on the stimulated release of albumin could not be determined as wortmannin interferes with the wavelength at which the albumin ELISA plates are analysed. Neutrophil degranulation induced by MSU crystals was also found to be inhibited by PP_2 and piceatannol (data not shown). It is relevant to note that the ability of PI3Ks inhibitors to decrease the degranulation of human neutrophils induced by MSU crystals provides strong evidence that this response is not due to the physical lysis of the cells by these particulate agonists. To

further confirm that the release of lysozyme or albumin was not a result of the MSU crystal-induced cellular lysis, neutrophils were stimulated with MSU crystals and the presence of lactate dehydrogenase (LDH) was evaluated in the supernatant by immunoblotting using an anti-LDH antibody. We observed less than 2.5 % of the total LDH in the supernatant of MSU crystal-stimulated neutrophils (data not shown). This indicates that MSU crystals induced a minimal loss of viability under our experimental conditions.

Discussion

The present study examined the involvement of the PI3Ks family in the mediation of the responses of human neutrophils to MSU crystals, the etiological agent of gout. The results indicate that the interaction of MSU crystals with human neutrophils, an event which is necessary for the perpetuation of the acute gout crisis, leads to the stimulation of class Ia PI3Ks by a mechanism which is dependent on the tyrosine kinase Syk. Moreover, evidence was also obtained indicating that the activation of PI3Ks by MSU crystals is involved in the sequence of events leading to the MSU crystal-induced stimulation of PLD activity and of the degranulation of human neutrophils. The latter response is likely to be involved in the joint and tissue damage that occurs in gouty patients.

The major characteristics of gout include rapid onset, self-limitation and phagocytosis of the MSU crystals by polymorphonuclear leukocytes within the synovial cavity, resulting in the release of inflammatory mediators such as leukotriene B_4 (LTB_4), platelet activating factor (PAF), prostaglandin E_2 (PGE_2), interleukin-8 (IL-8), lysosomal proteases, superoxide and a still unidentified chemotactic factor [6, 8, 10, 42].

Gout has become more common and more clinically complex in recent years, particularly in older subjects [43]. In addition, an association of hyperuricaemia with premature cardiovascular disease and mortality has become more evident over time [44]. The understanding of the molecular mechanisms involved in generating the pathologic effects of MSU crystals is essential to the ongoing search for more effective therapies for gouty arthritis since the lack of growth in available therapeutic options for chronic hyperuricaemia has led to a limited number of support options [45].

Recent studies have shown that PI3Ks play major roles in phagocytosis, superoxide anion production and chemotaxis (reviewed in [31]). We show here that MSU crystals induce the tyrosine phosphorylation of p85 regulatory subunit of class Ia PI3Ks in a Syk tyrosine kinase-dependent manner since this phosphorylation is diminished by piceatannol. We also show that, in human neutrophils, MSU crystals increase PI3K activity and this effect is significantly inhibited by piceatannol. As the PI3K activity was determined using p85 immunoprecipitates, these results identify class Ia PI3K as the target of stimulation by MSU crystals. The effects of MSU crystals on PI3K were Syk-dependent since piceatannol

inhibited both the activation of PI3K as well as that of the PI3K effector, AKT (as monitored by the Thr308 and the Ser473 phosphorylations of AKT).

The translocation of signalling proteins into non-ionic detergent-resistant domains has already been described in human neutrophils stimulated, among others, by cross-linking of FcγRIIa or FcγRIIIb. For example, we have recently observed that the cross-linking of FcγRIIIb, which mediates at least in part the responses of human neutrophils to MSU crystals [46], induces a significant increase in the amount of the receptor in high-density DRMs [47]. Moreover, tyrosine phosphorylation profiles and enzyme activities were found to be increased and concentrated in NP40-insoluble fractions upon stimulation of human neutrophils [29]. Accordingly, we found here an increase in the amount of p85 associated with the cytoskeleton and/or DRM-containing NP40-insoluble fraction derived from human neutrophils stimulated with MSU crystals. Furthermore, we observed that piceatannol did not diminish the MSU crystal-induced p85 translocation into these detergent-resistant structures suggesting that neither the kinase activity of Syk nor the tyrosine phosphorylation of p85 are required for the insolubility of p85. Finally, we found that upon stimulation by MSU crystals, tyrosine phosphorylated p85 and Syk are both localized into NP40-insoluble fraction whose functions appear to be related to signal transmission and membrane trafficking. These data provide additional evidence that detergent-resistant microdomains function as signalling platforms in human neutrophils. Furthermore, they underline the similarities between the signal transduction pathways activated following the engagement of FcγRIIIb and by MSU crystals.

The insoluble status of p85 and Syk in NP40 precluded any attempts at examining potential interactions between these two proteins by co-immunoprecipitations in this detergent. On the other hand, we found Syk and p85 to be predominantly soluble in CHAPS even after stimulation by MSU crystals, and were thus able to show a co-immunoprecipitation of these two proteins.

The formation of Syk and p85 containing complexes has previously been described as necessary for the FcγR-mediated phagocytosis of opsonised particles in COS-1 cells stably transfected with FcγRIIa [48]. The signalling pathway activated by MSU crystals appears to be similar as MSU crystals induced the formation of a complex containing p85 and Syk which was not affected by piceatannol but which was completely abrogated by PP_2, a Src

family kinase inhibitor, suggesting that Src-mediated tyrosine phosphorylation of Syk is essential for it to interact with p85. Our results are in accordance with recent studies which proposed that p85 interacted directly with Syk and these interactions were mediated by the SH2 domains of p85, binding to specific phosphotyrosines on Syk thus providing a mechanism by which signals could be transmitted directly from activated, phosphorylated Syk to p85 [20]. On the other hand, neither the tyrosine kinase activity of Syk nor its autophosphorylation appear to be necessary for p85 to interact with Syk since piceatannol affected neither the co-immunoprecipitation nor the insolubilization of p85 induced by MSU crystals. However, the tyrosine kinase activity of Syk and/or its autophosphorylation are essential for the subsequent MSU crystal-induced activation of PI3Ks as shown by the effects of piceatannol on the phosphorylation of p85 and the PI3Ks major target, AKT and on the PI3K activity.

Evidence for the functionality of the signalling events described above was also obtained. It was previously described in our laboratory that MSU crystals stimulate the activity of PLD in human neutrophils [38] and that this activation is Syk-dependent [14]. We show here that the activation of PLD by MSU crystals is dependent on PI3Ks since wortmannin and LY294002 significantly inhibited it. This result suggests that the previously observed effects of piceatannol on MSU crystal-induced PLD activation are exerted indirectly *via* the inhibition of one or more members of the class Ia PI3Ks family.

Previous studies have identified a role for PLD in the regulation of neutrophil degranulation in response to different stimuli [39, 49]. In inflammatory diseases such as rheumatoid arthritis and gout, the neutrophil is thought to be largely responsible for the tissue damage caused by the excessive release of its granules' cytolytic enzymes into the synovial fluid. Moreover, we have recently shown that MSU crystal-stimulated neutrophils release a potent activation factor within 15 min of stimulation [10]. This still unidentified activation factor is thought to amplify and prolong the gouty inflammation. We show here that MSU crystal induce a significant degranulation of human neutrophils as measured by the amounts of lysozyme and albumin (markers of the azurophil, specific, gelatinase, and secretory granules, respectively) released into the extracellular milieu. PI3Ks seem to play an important role in the MSU crystal-induced neutrophil degranulation since wortmannin and LY294002 significantly diminished it. Further studies of the MSU crystal-induced

degranulation of human neutrophils are required since the granules' contents are various and exert contradictory effects. While the cytolytic enzymes contained within the neutrophil granules could be largely responsible for the tissue damage occurring in patients suffering from gout, the opioid peptides contained within the same granules counteract the inflammatory pain in the early stages of inflammation thus having a beneficial role [50]. Moreover, the secretory vesicles represent a specialized membrane-like intracellular compartment of human neutrophils enriched in GPI-anchored proteins such as FcγRIIIb and CD14, both of which have been shown to play a role in the recognition of MSU crystals [41, 46, 51].

In conclusion, this is the first report of the activation of class Ia PI3Ks by MSU crystals in human neutrophils and of the dependence of this response on the tyrosine kinase Syk. Moreover, the results of this study also document the degranulation that accompanies the stimulation of human neutrophils with MSU crystals and show that the Syk-PI3Ks axis is critical for this response. Further studies of the intracellular signal transduction pathways activated by MSU crystals are required in order to identify the complex of receptors with which MSU crystals interact (FcγRIIIb, CD14, TLRs...) which still remains largely uncharacterized and the elucidation of which could provide critical clues as to the cellular mechanisms involved in the inflammatory reaction characteristic of gout and, in so doing, indicate novel and more efficient therapeutic targets for the treatment of gouty arthritis.

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Disclosures

The authors have no financial conflict of interest.

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Figures

Figure 1 : MSU crystal stimulation induces PI3K activation in human neutrophils in a Syk-dependent manner

Neutrophils (40×10^6 cells/ml) were pre-incubated with 1 mM DFP for 10 min at room temperature. When indicated, piceatannol (40 µM) or an equal volume of its solvent (Me_2SO) was added for 10 min at 37°C. The neutrophils were then stimulated with 3 mg/ml of MSU crystals and the stimulations were stopped at the indicated times. (A) Cells were lysed in non denaturing lysis buffer containing 0.6% CHAPS. The 16000g supernatants were immunoprecipitated for p85 protein and revealed with an anti-phosphotyrosine antibody (pY) or with an anti-p85 antibody (p85) as described in Material and Methods. (B) The p85 immunoprecipitates were also used for performing PI3K activity ELISA according to the manufacturer's recommendations. Class Ia PI3K activity was determined as a conversion of $PI(4,5)P_2$ into $PI(3,4,5)P_3$. The results shown are representative of at least three independent experiments.

Figure 2 : MSU crystal stimulation induces AKT phosphorylation in human neutrophils in a Syk-dependent manner

Neutrophils (40×10^6 cells/ml) were pre-incubated with 1 mM DFP for 10 min at room temperature. When indicated, piceatannol (40 µM) or an equal volume of its solvent (Me_2SO) was added for 10 min at 37°C. The neutrophils were then stimulated with 3 mg/ml of MSU crystals and the stimulations were stopped at the indicated times. The whole cell lysates were analysed by Western blotting for Thr308 (A) or Ser473 (B) phosphorylated AKT (pAKT) and for AKT as loading control. The results shown are representative of at least three independent experiments.

Figure 3 : p85 translocates into NP40-insoluble fraction upon MSU crystal stimulation

Neutrophils (40×10^6 cells/ml) were pre-incubated with 1 mM DFP for 10 min at room temperature. Where indicated, neutrophils were incubated with piceatannol (40 µM) or with an equal volume of Me_2SO for 10 min at 37°C. The cells were then stimulated with 3 mg/ml MSU crystals and the stimulations were stopped on ice at the indicated times. The NP40-insoluble fractions were prepared as described in Material and Methods. (A) The total cell lysates, the soluble and insoluble fractions were probed with an anti-p85 antibody (p85) or an anti-flotillin antibody (flotillin). (B) The insoluble fractions were probed with

an anti-p85 antibody (p85). When loading the SDS-polyacrylamide gels, adjustments were made in order for all samples to be equivalent with respect to cell number. The results shown are representative for at least three different experiments.

Figure 4 : Tyrosine phosphorylated p85 and Syk are located in NP40-insoluble fraction upon MSU crystal stimulation

Neutrophils ($40x10^6$ cells/ml) were pre-incubated with 1 mM DFP for 10 min at room temperature. The cells were then stimulated with 3 mg/ml MSU crystals and the stimulations were stopped after 5 min. The NP40-insoluble fractions were prepared as described in Material and Methods and analysed with an anti-phospho-p85 or an anti-p85 antibodies (A). For Syk, the samples were immunoprecipitated under denaturing conditions and probed with an anti-phosphotyrosine or an anti-Syk antibody (B). When loading the SDS-polyacrylamide gels, adjustments were made in order for the samples to be equivalent with respect to cell number. The results shown are representative of three independent experiments.

Figure 5 : p85 and Syk are entirely soluble in the zwitterionic detergent CHAPS

Neutrophils ($40x10^6$ cells/ml) were pre-incubated with 1 mM DFP for 10 min at room temperature. The cells were then stimulated with 3 mg/ml MSU crystals and the stimulations were stopped on ice after 5 min. The CHAPS-soluble and -insoluble fractions were prepared as described in Material and Methods (Immunoprecipitations under native conditions) and analysed with an anti-p85 or an anti-Syk antibody. When loading the SDS-polyacrylamide gels, adjustments were made in order for the samples to be equivalent with respect to cell number. The results shown are representative of at least five different experiments.

Figure 6 : MSU crystal stimulation induces an interaction between the class Ia PI3Ks regulatory subunit and Syk in human neutrophils

(A) Neutrophils ($40x10^6$ cells/ml) were pre-incubated with 1 mM DFP for 10 min at room temperature. The cells were then stimulated with 3 mg/ml MSU crystals and the stimulations were stopped on ice at the indicated times. The CHAPS p85 and Syk immunoprecipitations were performed as described in Material and Methods and probed with an anti-phosphotyrosine, an anti-Syk or an anti-p85 antibody. (B) Prior to stimulation, neutrophils were pre-incubated with piceatannol (40 μM) or with an equal volume of

Me_2SO and then treated as described above. (C) Prior to stimulation, neutrophils were preincubated with PP_2 (10 μM) or with an equal concentration of PP_3 and then treated as described above. The results shown are representative of five independent experiments.

Figure 7 : MSU crystal-induced PLD activity depends on PI3Ks

Neutrophils were pre-incubated with wortmannin or LY294002 at the indicated concentrations. The cells were stimulated with 3 mg/ml MSU crystals for 15 min and the samples were then processed for PLD activity as described in Material and Methods. The radioactivity was monitored by liquid scintillation counting and the results were corrected for background radioactivity and quenching. The results shown are the mean value of three independent experiments.

Figure 8 : MSU crystal-induced neutrophil degranulation depends on PI3Ks

Neutrophils ($40x10^6$ cells/ml) were pre-incubated with wortmannin (30 nM), LY294002 (10 μM) or an equal volume of Me_2SO as vehicle for 10 min and then stimulated with 3 mg/ml of MSU crystals for 15 min at 37°C. The stimulations were stopped by a quick spin (15 sec, 16000g) and the supernatants were harvested and analysed, as described in Material and Methods, for the amount of lysozyme released (A) or for the amount of albumin released (B). The results shown are the mean value of six independent experiments.

Fig. 1

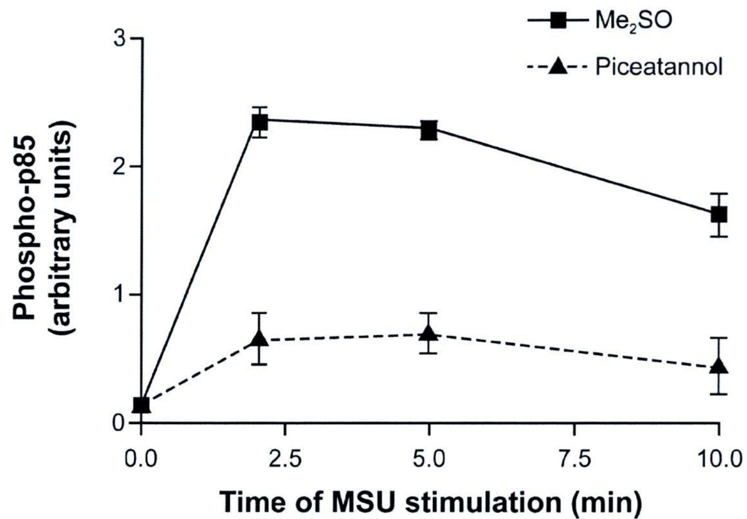
A.

IP : p85

WB : pY

WB : p85

MSU (min)	0	2	5	10	0	2	5	10
Piceatannol	-	-	-	-	+	+	+	+



B.

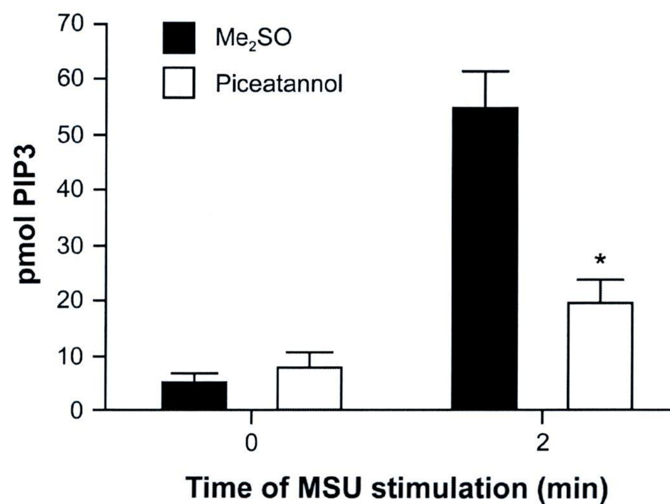
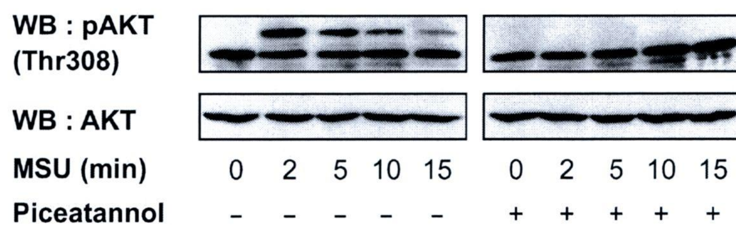
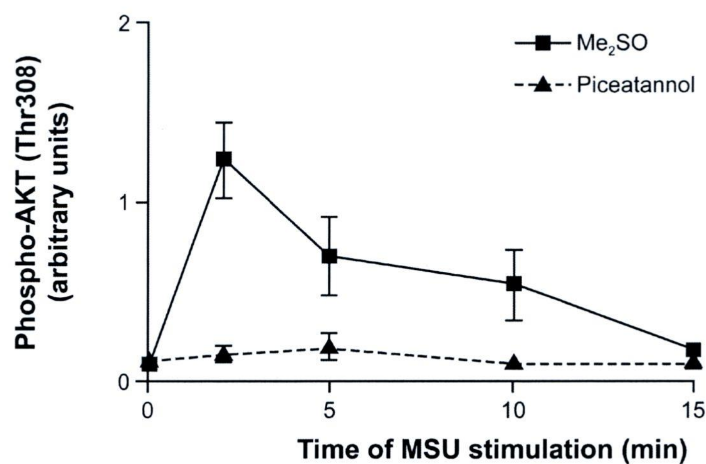


Fig. 2

A.





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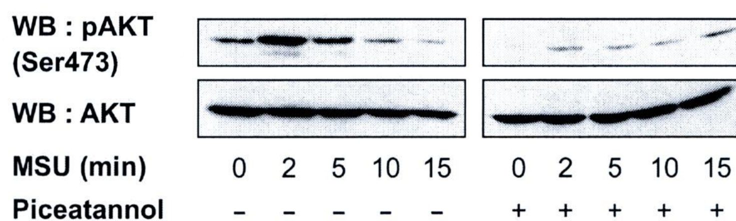


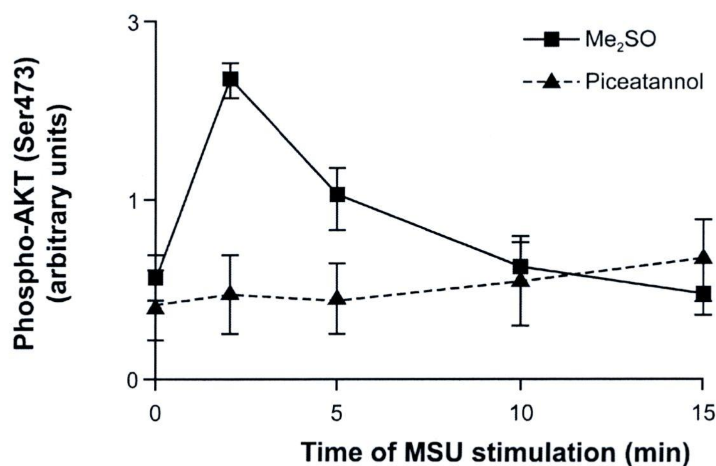
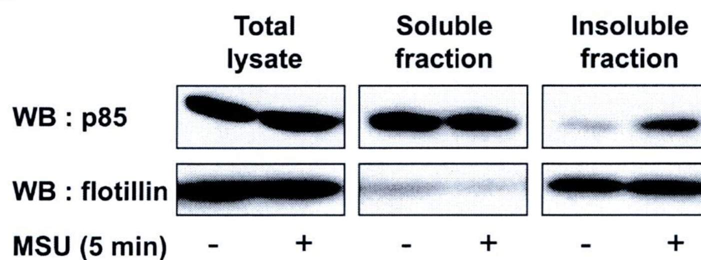
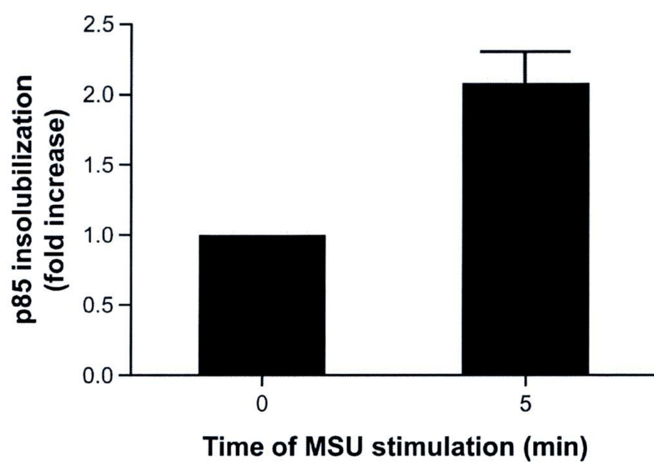


Fig. 3

A.





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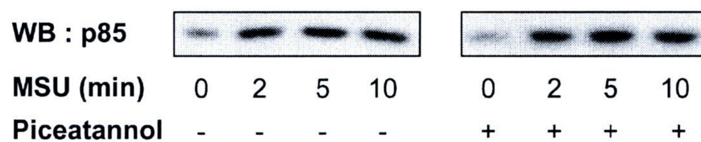


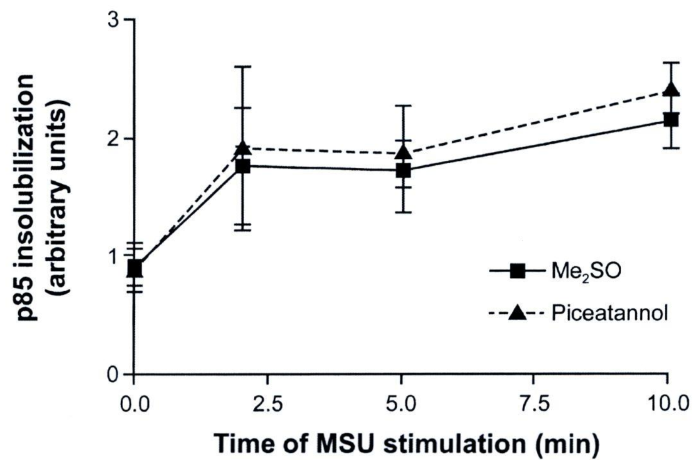
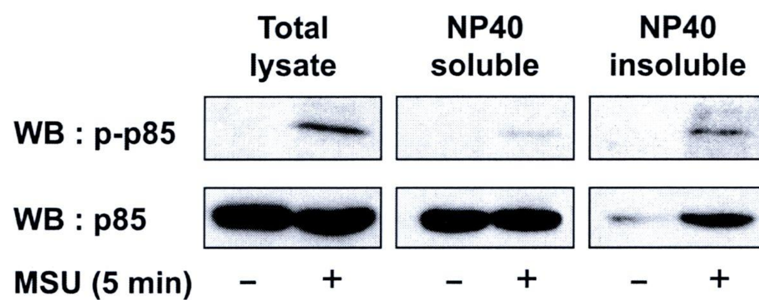


Fig. 4

A.



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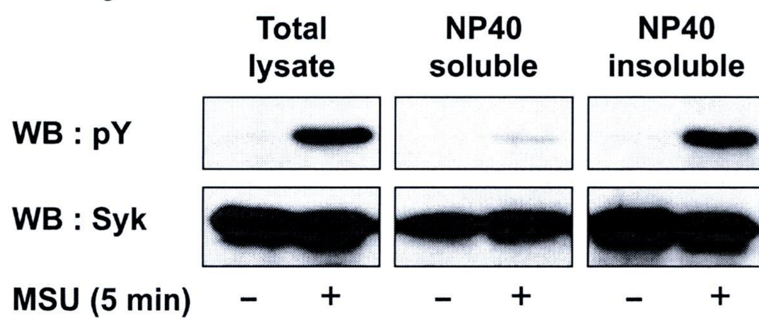


Fig. 5

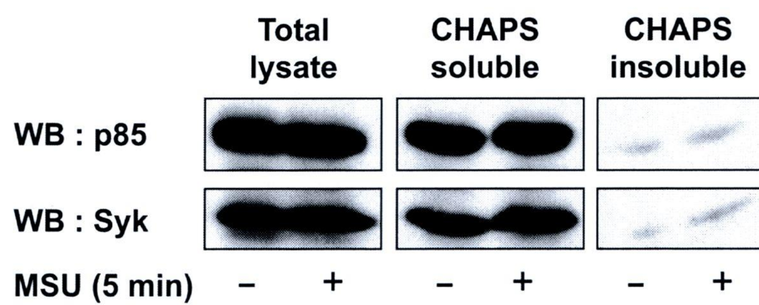


Fig. 6

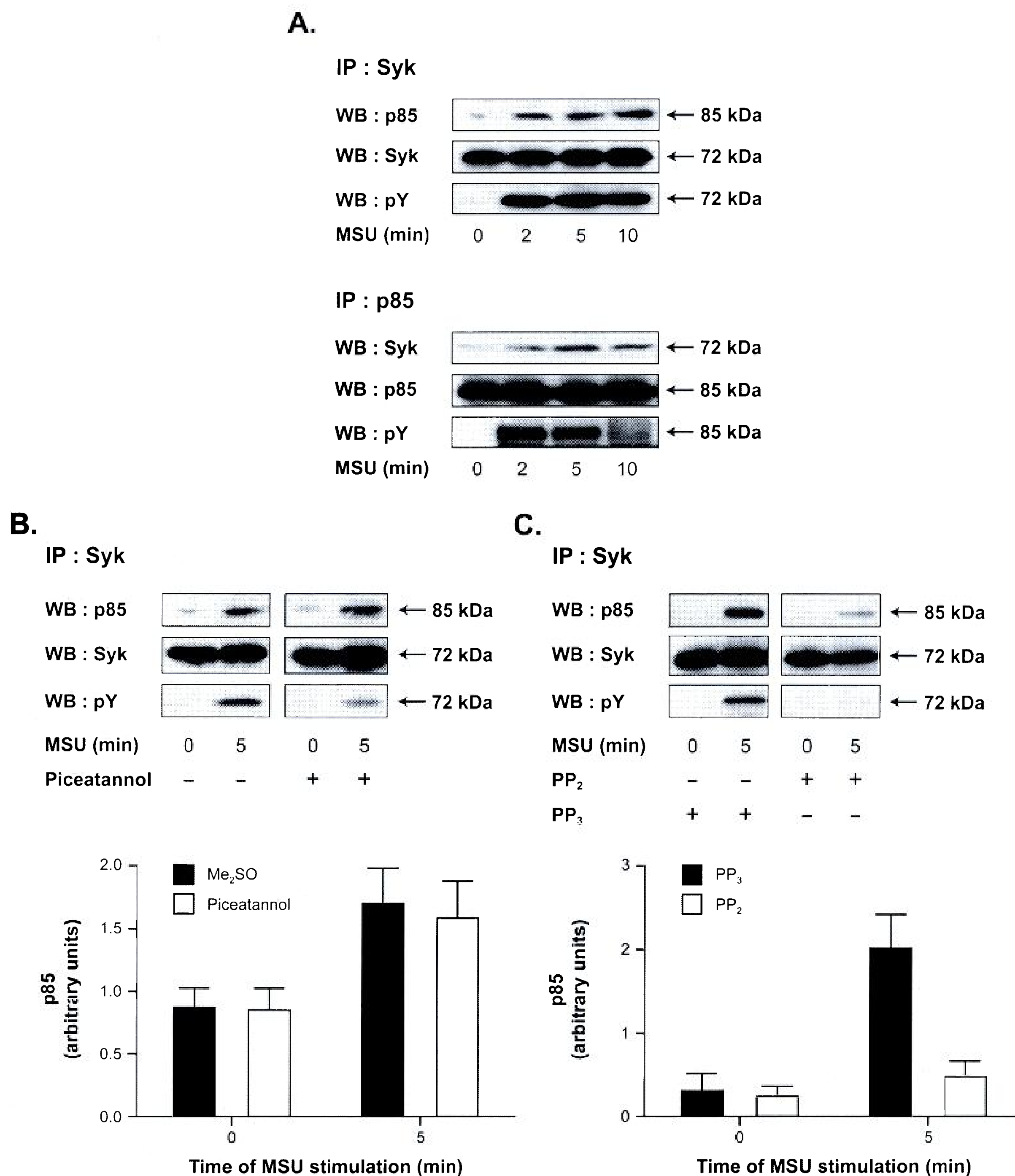


Fig.7

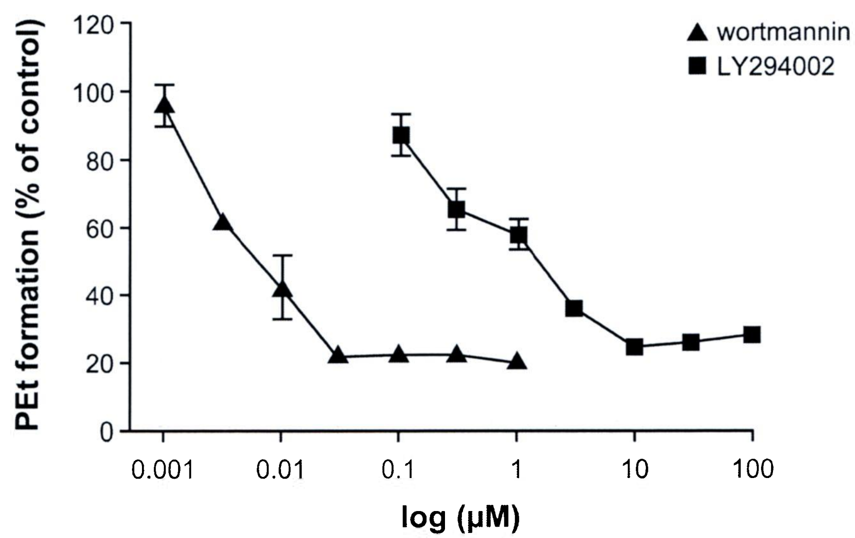
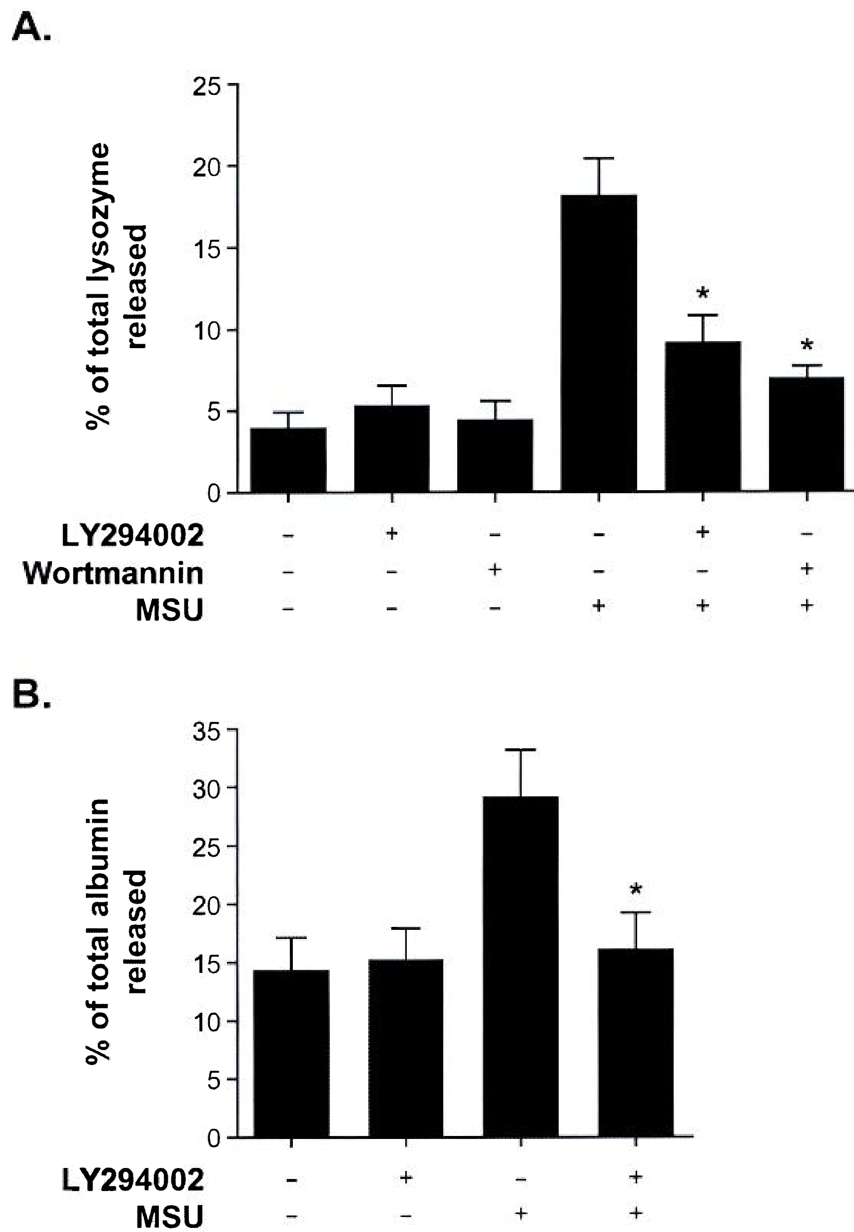


Fig. 8



Chapitre III : Caractérisation et étude fonctionnelle de l'activation de la protéine tyrosine kinase Tec par les cristaux d'UMS dans le neutrophile humain

L'article présenté dans ce chapitre a été publié dans Arthritis and Rheumatism, 2008, Juin 58(6) : 1866-76.

Résumé

La précipitation de l'acide urique et le dépôt des cristaux d'urate monosodique (UMS) dans les articulations provoquent une puissante réaction inflammatoire connue sous le nom de goutte. La crise de goutte est caractérisée par une infiltration massive des neutrophiles dont l'activation semble responsable des dommages tissulaires puisque ces leucocytes relâchent des espèces réactives oxygénées et des enzymes cytolytiques contenues dans leurs granules. L'interaction entre les neutrophiles et les cristaux d'UMS est absolument nécessaire au déclenchement de l'arthrite goutteuse comme le prouvent les études mettant en évidence l'absence de réponse inflammatoire aux injections de cristaux d'UMS lorsque les neutrophiles sont déplétés. De plus, des molécules supprimant les fonctions des neutrophiles se sont avérées efficaces pour le traitement de la goutte. Les membres de la famille de tyrosine kinases Tec participent à plusieurs voies de signalisation régissant l'activation des mitogen-activated protein (MAP) kinases, la réorganisation du cytosquelette d'actine, la régulation transcriptionnelle ou la survie cellulaire.

Les résultats présentés ici montrent que la stimulation des neutrophiles humains par les cristaux d'UMS entraîne une activation de la tyrosine kinase Tec qui est dépendante de l'activation des kinases Src. Cette activation s'avère nécessaire à la sécrétion d'IL-1β et d'IL-8 et à l'activité chimiotactique du surnageant des neutrophiles stimulés par les cristaux d'UMS. Ces réponses du neutrophile humain sont responsables en partie du déclenchement et de la prolongation de l'arthrite goutteuse et des dommages articulaires et osseux qui y sont associés.

Crystal-induced neutrophil activation. X. Pro-inflammatory role of the tyrosine kinase Tec[1].

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Abstract

Objective

MSU crystals are among the most potent pro-inflammatory stimuli and an innate immune inflammatory response to the crystal surface is involved in the pathology of gouty arthritis. Release of the crystals into the joint cavity promotes an acute inflammation characterized by a massive infiltration of neutrophils that leads to tissue damage. The aim of the present study was to assess the involvement of the tyrosine kinase Tec in MSU crystal-initiated transduction events in human neutrophils.

Methods

Immunoprecipitation and immunoblotting techniques were used for the cellular signaling studies. Chemotaxis and ELISA techniques were used for the functional studies. Silencing of Tec expression using specific siRNA was also performed.

Results

MSU crystals induce the phosphorylation and the activation of Tec in a Src-dependent manner. This activation is necessary for the MSU crystal-induced secretion of IL-1β and IL-8 and for the generation of a chemotactic activity in supernatants of MSU crystal-stimulated neutrophils. We also provide evidence that colchicine, an effective drug for the treatment of gout inhibits the MSU crystal-induced tyrosine phosphorylation of Tec thus modulating its kinase activity.

Conclusion

We show here that Tec is the principal kinase of the Tec family which plays a major role in responses of human neutrophils to MSU crystals which are likely to be involved in the initiation and perpetuation of gout. Our results suggest that the specific inhibition of Tec during the acute phase of MSU crystal-induced inflammation may be considered for the treatment of gouty arthritis.

Introduction

The deposition of monosodium urate (MSU) crystals into the joint cavity of hyperuricaemic individuals results in acute gouty arthritis. MSU crystal-promoted inflammation manifests as massive infiltration of neutrophils into the joints leading to tissue damage (1). The crucial role of neutrophils in MSU crystal-induced inflammation is underscored by studies that documented the suppression of the inflammatory response to injections of MSU crystals in animals depleted of neutrophils (2). The interaction of MSU crystals with human neutrophils leads to the production of inflammatory mediators such as interleukin-8 (IL-8) (3), leukotriene-B_4 (LTB_4) (4), prostaglandin E_2 (PGE_2) (5) and interleukin-1β (IL-1β) (6) that can play a role in the recruitment of neutrophils and their over-activation.

The Tec family kinases represent the second largest family of mammalian non-receptor protein tyrosine kinases. Tec kinases influence a wide range of signaling pathways controlling activation of mitogen-activated protein kinases (MAPKs) (7), actin reorganization (8), transcriptional regulation (9), cell survival (10) and cellular transformation (11). Tec family kinases are expressed in a variety of cells of the myeloid lineage (reviewed in (12)) and we have previously reported the expression of Tec, Btk and Bmx in human neutrophils (13). However, with the exception of B lymphocytes, mast cells and platelets, their biological role in the myeloid system is only poorly understood. Previous studies of our laboratory showed that Tec is activated in human neutrophils upon cross-linking of CD16b (14). This Fcγ receptor mediates, at least in part, the interaction of MSU crystals with human neutrophils (15). Tec family members are, among more than 80 tyrosine-specific kinases, the only ones that carry a pleckstrin homology (PH) domain in their protein structure, which mediates physical and functional interactions with phospholipid-dependent signaling pathways (16). We recently documented the stimulation of class Ia phosphoinositide-3 kinases (PI3Ks) upon interaction of MSU crystals with human neutrophils (17). Thus, MSU crystal-mediated PI3K stimulation might lead to activation and regulation of Tec tyrosine kinases.

In the present study, we investigated the role of Tec kinases in MSU crystal-induced signaling. We show here that MSU crystals induce the phosphorylation and the activation of Tec and that this activation is necessary for the MSU crystal-induced secretion of IL-1β and IL-8 and for the generation of the chemotactic activity in supernatants from MSU

crystal-stimulated neutrophils. We also provide evidence that colchicine, an effective drug for the treatment of gout, inhibits the MSU crystal-induced tyrosine phosphorylation of Tec thus modulating its kinase activity.

Materials and Methods

Antibodies and Chemicals

The monoclonal anti-phosphotyrosine 4G10 (16-101) and the polyclonal anti-Tec antibodies (06-561) were from Upstate Biotechnology (Lake Placid, NY). Glutathione affinity chromatography purified Sam68 (sc-4249) and the polyclonal anti-Sam68 (sc-333), anti-Tec (sc-1109), anti-Btk (sc-1107), anti-Bmx (sc-8873), anti-Lyn (sc-15), the monoclonal anti-Lyn (sc-7274) and the donkey anti-goat (sc-2056) antibodies were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). The peroxidase-conjugated sheep anti-mouse IgG (NXA931) and the donkey anti-rabbit IgG (711-035-152) were from GE Healthcare (Buckinghamshire, UK) and Jackson Immuno Research Laboratories (West Grove, PA), respectively.

Calcein-AM (206700), α-Cyano-β-hydroxy-β-methyl-N-(3-fluorophenyl)propenamide (LFM-A11) (435301), α-Cyano-β-hydroxy-β-methyl-N-(2,5-fdibromophenyl)propenamide (LFM-A13) (435300), N-(4-Trifluoromethylphenyl)-2cyano-3-hydroxycrotoamide (A771726) (100128), PP2 (529573) and PP3 (529574) were from Calbiochem (San Diego, CA).

Triclinical monosodium urate monohydrate crystals were synthesized and characterized as described previously (18) and endotoxin contamination was ruled out by Limulus amebocyte lysate assay.

PLB-985 cells

The myeloid cell line PLB-985 was grown in RPMI 1640 containing 10% FBS at 37°C in a 5% CO_2 humidified atmosphere. To induce differentiation to a neutrophil-like phenotype (19), PLB-985 cells were cultured in medium supplemented with 0.3 mM dibutyryl cAMP for 3 days before each experiment.

Transfection of dPLB-985 cells

48h following differentiation, PLB-985 cells were transfected using the Nucleofector®II system from Amaxa Biosystems (Cologne, Germany). After centrifugation, 2 x 10^6 cells were resuspended in 100 µl of nucleofection buffer (25 mM Hepes, 120 mM KCl, 2 mM $MgCl_2$, 10 mM K_2HPO_4, 5 mM L-cysteine) containing 1 µg of siRNA and transfections were performed with the program U-002. The Tec (SI02223165) and control (1027280) siRNAs were from Qiagen Sciences Inc. (Germantown, MD). After nucleofection, the cells

were transferred into RPMI 1640 containing 0.3 mM dibutyryl cAMP, 10 mM Hepes, 1 mM Na-pyruvate. 24h after nucleofection, the cells were harvested and resuspended at 40 x 10^6 cells/ml in Mg^{2+}-free HBSS containing 1.6 mM $CaCl_2$.

Neutrophil purification

Venous blood was collected from healthy adult volunteers in isocitrate anticoagulant solution. Neutrophils were separated as previously described (20). Briefly, whole blood was centrifuged at 180g for 10 min and the resulting platelet rich plasma was discarded. Leukocytes were obtained following sedimentation in 2% Dextran T-500. Mononuclear cells were removed by centrifugation on Ficoll-Paque cushions and contaminating erythrocytes were removed by a 20 seconds hypotonic lysis. Neutrophils were resuspended at 40x10^6 cells/ml in HBSS containing 1.6 mM Ca^{2+} but no Mg^{2+}.

Immunoprecipitations

MSU crystal-stimulations were stopped by transfer to an ice bath followed by a 10 s spin in a microcentrifuge. The pellets were resuspended in cold lysis buffer (10 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, 0.6% CHAPS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM sodium orthovanadate, 250 μg/ml soybean trypsin inhibitor, 3 mM DFP, 1 mM PMSF). After 5 min on ice, the cell lysates were centrifuged at 16000g for 5 min at 4 °C. The supernatants were incubated at 4°C for 3 h in the presence of protein A Sepharose beads linked to an anti-Tec (4 μg/50 μl beads) or an anti-Lyn (1.5 μg/50 μl beads) antibody. The beads were then washed three times with cold lysis buffer. 50 μl of 2X Laemmli's sample buffer (1X is 62.5 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM pNPP, 12.5 μg/ml leupeptin, 12.5 μg/ml aprotinin, 0.00125% bromophenol blue) were added to the beads, which were then boiled for 7 min.

***In vitro* kinase activity assay towards Sam68**

The Tec immunoprecipitates were resuspended in kinase buffer (50 mM Hepes pH 7.4, 10 mM $MnCl_2$, 2 mM $MgCl_2$, 25 μM pNPP, 10 μM sodium orthovanadate, 50 μM ATP) containing 0.5 μg of Sam68-GST and transferred to 30°C for 10 min. The supernatants of the kinase assays were collected and incubated for 1 h at 4°C in lysis buffer with 50 μl glutathione-Sepharose beads. The glutathione-Sepharose beads were then washed twice and boiled for 7 min in 2X Laemmli's sample buffer.

Neutrophil migration

The supernatants from MSU crystal-stimulated neutrophils or transfected dPLB-985 cells were obtained as previously described (21). Chemotaxis of neutrophils was then assessed as previously described (22). Briefly, neutrophils were resuspended at $10x10^6$ cells/ml in HBSS with 10% fetal FBS and were pre-incubated with 5 µg/ml calcein-AM at 37°C for 30 minutes. The cells were then washed and resuspended in HBSS/FBS at $5x10^6$ cells/ml. Neutrophil migration was monitored using a 96-well ChemoTX disposable chemotaxis system (101-8) from NeuroProbe Inc (Gaithersburg, MD).

Enzyme Linked ImmunoSorbent Assays (ELISAs)

The assessment of the extracellular IL-1β and IL-8 was performed using commercially available ELISA kits (88-7010-22 and CHC1303 respectively) from eBioscience (San Diego, CA) and Biosource International Inc (Camarillo, CA) respectively. All samples were measured in triplicate.

Densitometry analysis

Densitometry analysis was performed using ImageJ Software on the scanned immunoblotting results. The measurements of pixel intensity of each blot lane were plotted using GraphPad Prism4 Software.

Statistical analysis

Statistical analyses were performed using the Student's paired *t*-test (two-tailed) using GraphPad Prism4 Software on the non-processed data. Every inhibitory condition was compared to the control condition and significance was considered to be attained when P was less than 0.05.

Results

Src-dependent activation of Tec tyrosine kinase by MSU crystals in human neutrophils

The tyrosine phosphorylation level of the Tec family of tyrosine kinases is a widely-used index of their activation status (23). We therefore investigated the effect of MSU crystal stimulation on the tyrosine phosphorylation of Tec in human neutrophils. The cells were stimulated with MSU crystals for the indicated periods of time and Tec was immunoprecipitated as described in Materials and Methods. The data shown in Figure 1A indicate that stimulation of human neutrophils by MSU crystals induces a time-dependent tyrosine phosphorylation of Tec. This response is Src-dependent since PP2, a potent Src family kinase inhibitor, completely abolished it. PP3, an inactive analog of PP2, had no effect on the stimulation of the tyrosine phosphorylation of Tec by MSU crystals.

We next examined whether the MSU crystal-induced tyrosine phosphorylation of Tec modulated its kinase activity. Neutrophils were stimulated with MSU crystals for the indicated periods of time and Tec was immunoprecipitated as described in Materials and Methods. The immunoprecipitates were then resuspended in kinase buffer, and the kinase activity of Tec was monitored by its ability to phosphorylate an exogenous substrate, Sam68-GST. The latter was chosen as it has previously been shown to associate with and to be phosphorylated by members of the Tec family (24). The results of these experiments indicate that stimulation of human neutrophils by MSU crystals significantly increases the kinase activity of Tec towards an exogenous substrate as evidenced by the increase in the level of tyrosine phosphorylation of Sam68 (Figure 1B). Our observations also indicate that an initial, Src-dependent, tyrosine phosphorylation of Tec is required for the MSU crystal-induced increase of its kinase activity since PP2 significantly inhibited it.

On the other hand, under our experimental conditions, we were not able to detect any MSU crystal-induced tyrosine phosphorylation of the other members of the Tec family, Btk and Bmx (data not shown) which are also expressed in human neutrophils (13). Taken together, these results suggest that Tec is the principal kinase of the Tec family activated in MSU crystal-stimulated human neutrophils in a Src kinase-dependent manner.

MSU crystal stimulation induces an interaction between the Src kinase Lyn and Tec

Previous studies have shown that the N-terminal domain of Tec contained a binding site for the SH3 domain of the Src kinase Lyn (25) and that Lyn could phosphorylate tyrosine residues of the Tec protein, thereby activating Tec in 3T3 fibroblasts (26). Having established that the MSU crystal-induced activation of Tec was Src-dependent and since we have previously shown that the stimulation of human neutrophils by MSU crystals leads to the activation of Lyn (27), we therefore investigated whether the MSU crystal stimulation resulted in an interaction between Tec and Lyn. By a co-immunoprecipitation technique, we show here that stimulation of human neutrophils by MSU crystals induces the transient formation of a complex containing both Tec and Lyn (Figure 2). Furthermore, we did not detect any interaction between Tec and the Src-related proteins Fgr and Hck (data not shown) which are also expressed in human neutrophils (28).

Functional relevance of the MSU crystal-induced Tec activation in human neutrophils

LFM-A13 was previously described as a Btk inhibitor without any effect on Src family kinases (29). We recently showed that LFM-A13 also inhibited the kinase activity of Tec in an in vitro kinase assay performed on immunoprecipitated Tec (14) and we validated this result specifically for the MSU crystal stimulation of neutrophils pre-incubated with LFM-A13 (data not shown). LFM-A13 is a metabolic analog of the prodrug leflunomide which is used to improve outcomes in rheumatoid arthritis (30). We also observed that the active metabolite of leflunomide, A771726, inhibited the kinase activity of Tec in an in vitro kinase assay performed on immunoprecipitated Tec (data not shown).

We therefore used LFM-A13 and A771726 to investigate the functional significance of the activation of Tec with respect to early and late responses of neutrophils to MSU crystals.

Involvement of Tec in the MSU crystal-induced release of a chemotactic factor from human neutrophils

We have recently reported that MSU crystal-stimulated neutrophils rapidly liberate a potent activation signal for naïve cells (21). The supernatant from neutrophils stimulated by MSU crystals is chemotactic for, induces a mobilization of intracellular calcium and increases the level of the global tyrosine phosphorylation in freshly isolated neutrophils suggesting that this activation factor could further stimulate surrounding neutrophils and contribute to the

prolongation and the amplification of the inflammatory responses induced by MSU crystals.

We therefore examined the effect of LFM-A13 and A771726 on the chemotactic activity of supernatants from MSU crystal-stimulated neutrophils. The cells were pre-incubated for 15 min at 37°C with 50 μM LFM-A13 or its inactive analog, LFM-A11, or with 50 μM A771726 or the same volume of its solvent Me_2SO. Supernatants were collected after 15 min of incubation with MSU crystals and their chemotactic activity towards freshly isolated neutrophils was assessed as described in Materials and Methods. LFM-A13 and A771726 significantly inhibited (50% and 30%, respectively) the generation of the chemotactic activity in supernatants from MSU crystal-stimulated human neutrophils (Figure 3A).

To confirm the specificity of this result obtained using pharmacological inhibitors and to rule out the involvement of other members of the Tec family kinases expressed in human neutrophils, we next silenced the expression of Tec by siRNA transfections of dibutyryl cAMP-differentiated, neutrophil-like PLB-985 (dPLB-985) cells (19). This human myeloid cell line was chosen as it reproduces the tyrosine phosphorylation pattern and the transduction events that we have previously published (17, 31) and furthermore, we also observed the same kinetics of the Src-dependent tyrosine phosphorylation of Tec in dPLB-985 as in MSU crystal-stimulated human neutrophils (data not shown). Following nucleofection with specific siRNAs, the level of expression of Tec was significantly decreased as shown in Figure 3B. The expression of the other members of the Tec family, namely Btk and Bmx, was unaffected by the Tec-specific siRNA.

We next examined the effect of the Tec-specific siRNA on the production of the chemotactic activity in supernatants from MSU crystal-stimulated dPLB-985. Supernatants were prepared as described above and their chemotactic activity towards freshly isolated human neutrophils was assessed. Figure 3C indicates that the decreased level of Tec expression leads to a significant inhibition (60%) of the chemotactic activity which is in accordance with the pharmacological effects monitored in Figure 3A. Moreover, we also observed that a Btk-specific siRNA had no effect on the MSU crystal-induced release of chemotactic activity (data not shown).

Taken together, these results suggest that Tec is the principal kinase of the Tec tyrosine kinase family which plays a role in the MSU crystal-induced secretion of a chemotactic activity from human neutrophils.

Involvement of Tec in the MSU crystal-induced production of IL-8 in human neutrophils

IL-8 has been shown to be essential for the occurrence of neutrophilic inflammation in experimental in vivo models of MSU crystal-induced gouty arthritis (32, 33). In our laboratory, MSU crystals were previously shown to increase the secretion of IL-8 by human neutrophils (3). This observation suggests that neutrophils will not only be recruited to the synovial cavity during a gouty attack and be activated when exposed to inflammatory microcrystals, but also that they will significantly contribute to their own recruitment because of their ability to release high levels of IL-8. It should be noted that IL-8 is not the chemotactic factor released from human neutrophils within a few minutes of stimulation with MSU crystals (21) as its secretion, as opposed to that of the latter, requires longer incubation times and is dependent on de novo protein synthesis (3).

We therefore examined the effect of the inhibition of Tec on the MSU crystal-induced secretion of IL-8 from human neutrophils. The cells were incubated for 15 min at 37°C with 50 μM LFM-A13 or its inactive analog, LFM-A11, or with 50 μM A771726 or the same volume of its solvent Me_2SO prior to the addition of MSU crystals. The levels of secreted IL-8 were monitored after 3 hours of stimulation using a commercially available ELISA kit. As shown in Figure 4A, LFM-A13 and A771726 significantly diminished (50% and 30%, respectively) the secretion of IL-8 induced by MSU crystals.

We next investigated the effect of the Tec-specific siRNA on the MSU crystal-induced secretion of IL-8 from dPLB-985 cells. As shown in Figure 4B, the secretion of IL-8 is significantly inhibited in MSU crystal-stimulated dPLB-985 transfected with the Tec-specific siRNA when compared to cells transfected with a negative control siRNA.

Involvement of Tec in the MSU crystal-induced production of IL-1β in human neutrophils

We have previously shown that MSU crystals induced a time-dependent, colchicine-sensitive synthesis and release of IL-1β in human neutrophils (6) and evidence has recently been provided indicating that IL-1β was essential for the MSU crystal-induced

inflammatory response (34, 35). Furthermore, the naturally occurring IL-1 receptor antagonist (IL-1Ra) was shown to be an effective therapy for acute gouty arthritis (36).

We therefore examined the involvement of Tec in the MSU crystal-induced secretion of IL-1β. The cells were pre-incubated for 15 min at 37°C with 50 μM LFM-A13 or its inactive analog, LFM-A11, or with 50 μM A771726 or the same volume of its solvent Me_2SO. The secreted IL-1β concentrations were monitored after 8 hours of stimulation using a commercially available ELISA kit. As shown in Figure 5A, LFM-A13 and A771726 significantly diminished (40% and 25%, respectively) the MSU crystal-induced secretion of IL-1β. Furthermore, no inhibition of the secretion of IL-1Ra by LFM-A13 or A771726 was observed (data not shown).

We next investigated the effect of the Tec-specific siRNA on the MSU crystal-induced secretion of IL-1β from dPLB-985 cells. As shown in Figure 5B, the IL-1β secretion is diminished in MSU crystal-stimulated dPLB-985 transfected with the Tec-specific siRNA when compared to cells transfected with a negative control siRNA.

Taken together, these results suggest a major pro-inflammatory role of the Tec tyrosine kinase in early (generation of chemotactic activity) as well as in late (secretion of IL-8 and IL-1β) responses of neutrophils stimulated by MSU crystals.

MSU crystal-induced tyrosine phosphorylation of Tec is colchicine-sensitive

Colchicine has been used to treat gout for years (37) and is very effective in slowing down or eliminating the development of the inflammatory reaction associated with the deposition of MSU crystals in joints. We have previously shown that, in human neutrophils, colchicine inhibited the tyrosine phosphorylation pattern (38) and the secretion of IL-1β (6) induced by MSU crystals. Since we show here that the kinase activity of Tec is necessary for the MSU crystal-induced secretion of IL-1β (Figure 5), we next investigated the effect of colchicine on the MSU crystal-induced tyrosine phosphorylation of Tec which is required for its kinase activity (Figure 1B). The cells were incubated with 10 μM colchicine or the same volume of its solvent Me_2SO before stimulation with MSU crystals for the indicated periods of time and Tec was immunoprecipitated as described in Materials and Methods. As shown in Figure 6, colchicine significantly inhibited the MSU crystal-induced tyrosine phosphorylation of Tec suggesting that the alkaloid may down-regulate the kinase activity

of Tec which is required for the pro-inflammatory role of the latter in MSU crystal-stimulated human neutrophils.

Discussion

The present study examined the involvement of the tyrosine kinase Tec in the mediation of the responses of human neutrophils to MSU crystals. The results obtained indicate that the interaction of MSU crystals with human neutrophils, a necessary event for the initiation of the acute gouty crisis, leads to the stimulation of Tec in a Src kinase-dependent manner. Moreover, evidence was also obtained indicating that the activation of Tec is involved in the sequence of events leading to the rapid release of a chemotactic factor as well as to the secretion of IL-8 and IL-1β in human neutrophils stimulated by MSU crystals. These responses are likely to play significant roles in the initiation and perpetuation of acute gouty arthritis and its associated joint and bone destruction. Furthermore, we also provide evidence that colchicine inhibits the MSU crystal-induced tyrosine phosphorylation of Tec.

Gout has become more common and more clinically complex in recent years particularly in older subjects (39). Moreover, all of the drugs used to treat gout can have serious side effects (40). In addition, evidence was recently provided that men with gout had an increased mortality risk which was the result of an elevated risk of cardiovascular diseases, prticularly coronary heart diseases (41). The understanding of the molecular mechanisms involved in generating the pathological effect of MSU crystals is thus essential to the ongoing search for more effective therapies for gouty arthritis.

One of the characteristic features of the activation of the Tec family of tyrosine kinases is their level of in situ tyrosine phosphorylation. We show here that MSU crystals induce the rapid tyrosine phosphorylation of Tec in a Src-dependent manner since this phosphorylation is abolished by PP2. We also show that MSU crystals increase the activity of Tec towards an exogenous substrate Sam-68 and that this effect is also significantly inhibited by PP2, suggesting that the initial Src-dependent tyrosine phosphorylation of Tec is required for the increase in its kinase activity. We next show that stimulation of human neutrophils by MSU crystals induces the formation of a complex containing both Tec and the Src tyrosine kinase Lyn (but not Hck or Fgr). This result is in accordance with previous studies which indicated that Tec was an effector molecule of Lyn (26). It is also noteworthy that we have been unable to obtain any evidence for the activation of the other members of the Tec kinase family (or of their implication in the functional responses monitored) subsequently to stimulation of human neutrophils by MSU crystals. These results indicate

that Tec fulfills specific functions in human neutrophils that are not redundant with those of the other members of this family.

We next investigated the functional significance of the MSU crystal-induced Tec activation using two pharmacological inhibitors, LFM-A13 and the active metabolite of leflunomide A771726, as well as siRNA silencing of the expression of Tec.

The rapid liberation of a chemotactic factor by MSU crystal-stimulated neutrophils might serve to modulate neutrophil recruitment and responses during the initial stages of inflammation and it may also play a role in the amplification of the inflammatory reaction accompanying acute gouty arthritis (21). We show here that both the pharmacological inhibition as well as the decreased expression of Tec (but not the decreased expression of Btk) almost completely inhibited the chemotactic activity in the supernatants of MSU crystal-stimulated neutrophils suggesting a critical role for Tec in the release of an activation factor that may be involved in the over-activation of human neutrophils in gouty inflammation.

Functional IL-8, a potent neutrophil chemoattractant, was shown to be abundant in the synovial fluid of patients with acute gout (42). Furthermore, a critical role of IL-8 in neutrophil ingress in experimental models of acute gouty arthritis in vivo has been also documented (32). In our laboratory, MSU crystals were previously shown to increase the secretion of IL-8 by human neutrophils in a protein synthesis-dependent manner (3). We show here that Tec is necessary for the MSU crystal-induced secretion of IL-8 from human neutrophils as the latter event was decreased when the activity of Tec or its expression were reduced.

Molecular evidence has recently been provided indicating that IL-1β was essential for the MSU crystal-induced inflammatory response (35). Once released, IL-1β must contend with competition for receptor occupancy with the naturally occurring IL-1Ra which has recently been shown to be an effective therapy for acute gouty arthritis (36). We have previously shown that MSU crystals induced the synthesis and release of IL-1β in human neutrophils (6). We show here that both the pharmacological inhibition as well as the decreased expression of Tec inhibited the MSU crystal-induced secretion of IL-1β. No inhibition of the secretion of IL-1Ra was observed suggesting that the inhibition of Tec leads to an

overall anti-inflammatory condition (decreased IL-1β/IL-1Ra ratio) in MSU crystal-stimulated human neutrophils.

It is worthy to mention the anti-inflammatory effect of A771726, the active metabolite of leflunomide, a drug that is already used for the treatment of rheumatoid arthritis. Even though the slow mode of action of leflunomide might not be appropriate for ameliorating the clinical symptoms of gouty arthritis, these data indicate that further studies characterizing and exploiting the anti-inflammatory effect of interference with the Tec signaling pathway in gout are warranted.

Colchicine is an effective treatment used traditionally for ameliorating clinical symptoms in patients experiencing acute attacks of gout (37). We have previously reported that the alkaloid decreased the stimulation of the tyrosine phosphorylation pattern induced by MSU crystals in human neutrophils (31) but without identifying specific targets. The present data show that one of the proteins whose level of tyrosine phosphorylation is decreased in the presence of colchicine is the tyrosine kinase Tec. The ability of colchicine to significantly inhibit the stimulation of the tyrosine phosphorylation of Tec induced by MSU crystals reinforces the link between the kinase activity of Tec and the phlogistic activity of MSU crystals.

In conclusion, this is the first report of the activation of Tec by MSU crystals in human neutrophils and of the dependence of this response on the Src kinases, most probably Lyn since we show here an interaction between Tec and Lyn upon MSU crystal stimulation. Moreover, the results of this study also document the major role played by Tec in responses of human neutrophils which are likely to be involved in the amplification and the prolongation of the gouty inflammation. These observations suggest that Tec may represent a specific therapeutic target in acute gouty arthritis.

Acknowledgments

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Figures

Figure 1. MSU crystal stimulation induces Tec activation in human neutrophils in a Src-dependent manner. Neutrophils ($40x10^6$ cells/ml) were pre-incubated with 1 mM DFP for 10 min at room temperature. PP2 (10 μM) or its inactive analog PP3 (10 μM) were added for 10 min at 37°C. The neutrophils were then stimulated with 3 mg/ml MSU crystals and the stimulations were stopped at the indicated times. Cells were lysed in nondenaturing lysis buffer containing 0.6% CHAPS. The 16000 g supernatants were immunoprecipitated (IP) for Tec protein as described in Materials and Methods. (A) The Tec immunoprecipitates were revealed with an anti-phosphotyrosine (pY) or an anti-Tec antibody (Tec). WB, Western blot. (B) The Tec immunoprecipitates were resuspended in 100 μl kinase buffer containing 0.5 μg of Sam68-GST and transferred to 30°C for 10 min. Sam68-GST was then pulled-down from the supernatants of the kinase assays with 50 μl glutathione-Sepharose beads. The samples were revealed with an anti-phosphotyrosine (pY) or an anti-Sam68 antibody (Sam-68). The results shown are representative of at least three independent experiments.

Figure 2. MSU crystal stimulation induces an interaction between Tec and Lyn in human neutrophils. Neutrophils ($40x10^6$ cells/ml) were pre-incubated with 1 mM DFP for 10 min at room temperature. The cells were then stimulated with 3 mg/ml MSU crystals, and the stimulations were stopped on ice at the indicated times. The Lyn immunoprecipitations were performed as described in Materials and Methods and probed with an anti-Tec (Tec) or an anti-Lyn (Lyn) antibody. The immunoblots shown are representative of four independent experiments summarized on the diagram.

Figure 3. The kinase activity of Tec is necessary for MSU crystal-induced secretion of a chemotactic factor from human neutrophils. (A) Neutrophils ($40x10^6$ cells/ml) were pre-incubated with LFM-A13 (50 μM), LFM-A11 (50 μM), A771726 (50 μM) or an equal volume of Me_2SO for 15 min and then stimulated with 3 mg/ml MSU crystals for 15 min at 37°C. The supernatants were harvested and neutrophil chemotaxis was monitored as described in Materials and Methods. The results shown are the mean value of at least three independent experiments. (B) dPLB-985 cells were transfected with a Tec-specific siRNA (siRNA Tec) or with a negative control siRNA (siRNA ctrl) as described in Materials and Methods. 24h after nucleofection, cells were harvested and resuspended at 40 x 10^6 cells/ml in HBSS. Samples were boiled into 2X Laemmli's sample buffer before immunoblot analysis with anti-Tec (Tec), anti-Btk (Btk) and anti-Bmx (Bmx) antibodies. Densitometric analysis is summarized on the diagram. (C)

Transfected dPLB-985 cells ($40x10^6$ cells/ml) were stimulated with 3 mg/ml MSU crystals for 15 min at 37°C. The supernatants were harvested and neutrophil chemotaxis was monitored as described in Materials and Methods. The results shown are the mean value of three independent experiments.

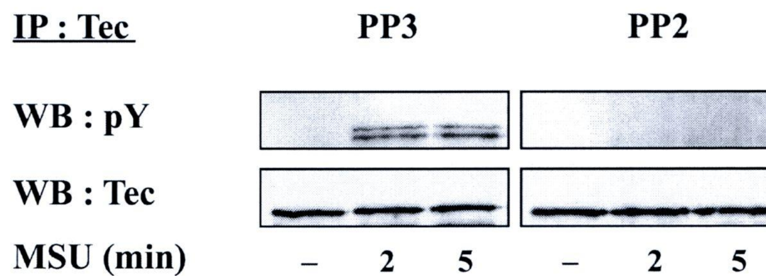
Figure 4. The kinase activity of Tec is necessary for MSU crystal-induced secretion of IL-8 from human neutrophils. (A) Neutrophils ($40x10^6$ cells/ml) were pre-incubated with LFM-A13 (50 μM), LFM-A11 (50 μM), A771726 (50 μM) or an equal volume of Me_2SO for 15 min and then stimulated with 3 mg/ml MSU crystals for 3h at 37°C. The stimulations were stopped by a quick spin (15 s, 6000 g), the supernatants were harvested and extracellular IL-8 was monitored as described in Materials and Methods. The results shown are the mean value of five independent experiments. (B) Transfected dPLB-985 cells ($40x10^6$ cells/ml) were stimulated with 3 mg/ml MSU crystals for 3h at 37°C and extracellular IL-8 was monitored as described in Materials and Methods. The results shown are the mean value of two independent experiments.

Figure 5. The kinase activity of Tec is necessary for MSU crystal-induced secretion of IL-1β from human neutrophils. (A) Neutrophils ($40x10^6$ cells/ml) were pre-incubated with LFM-A13 (50 μM), LFM-A11 (50 μM), A771726 (50 μM) or an equal volume of Me_2SO for 15 min and then stimulated with 3 mg/ml MSU crystals for 8h at 37°C. The stimulations were stopped by a quick spin (15 s, 6000 g), the supernatants were harvested and extracellular IL-1β was monitored as described in Materials and Methods. The results shown are the mean value of four independent experiments. (B) Transfected dPLB-985 cells ($40x10^6$ cells/ml) were stimulated with 3 mg/ml MSU crystals for 8h at 37°C and extracellular IL-1β was monitored as described in Materials and Methods. The results shown are the mean value of two independent experiments.

Figure 6. MSU crystal-induced tyrosine phosphorylation of Tec is colchicine-sensitive. Neutrophils ($40x10^6$ cells/ml) were pre-incubated with 1 mM DFP for 10 min at room temperature. Colchicine (10 μM) or its solvent, Me_2SO were added for 30 min at 37°C. The neutrophils were then stimulated with 3 mg/ml MSU crystals and the stimulations were stopped at the indicated times. Cells were lysed in nondenaturing lysis buffer containing 0.6% CHAPS. The 16000 g supernatants were immunoprecipitated (IP) for Tec protein as described in Materials and Methods. The Tec immunoprecipitates were revealed with an anti-phosphotyrosine (pY) or an anti-Tec antibody (Tec). The results shown are representative of three independent experiments summarized on the diagram.

Fig. 1

A.



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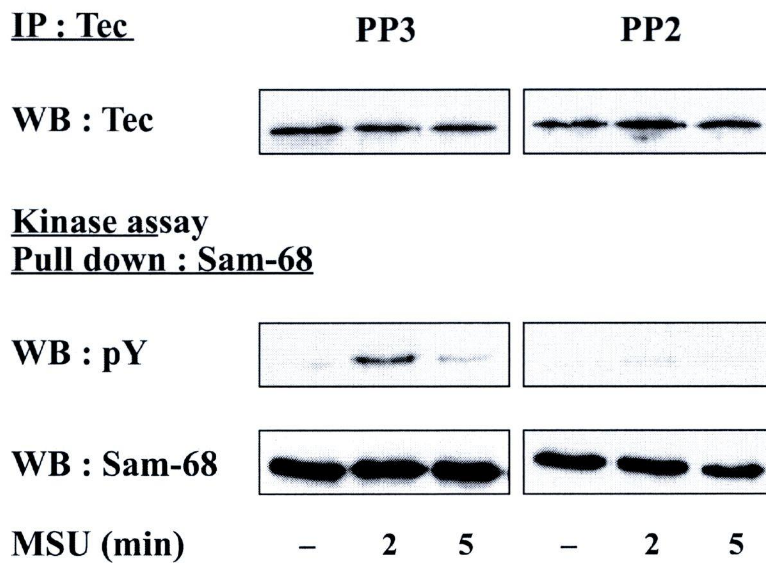
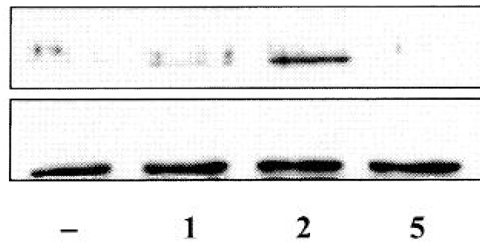


Fig. 2



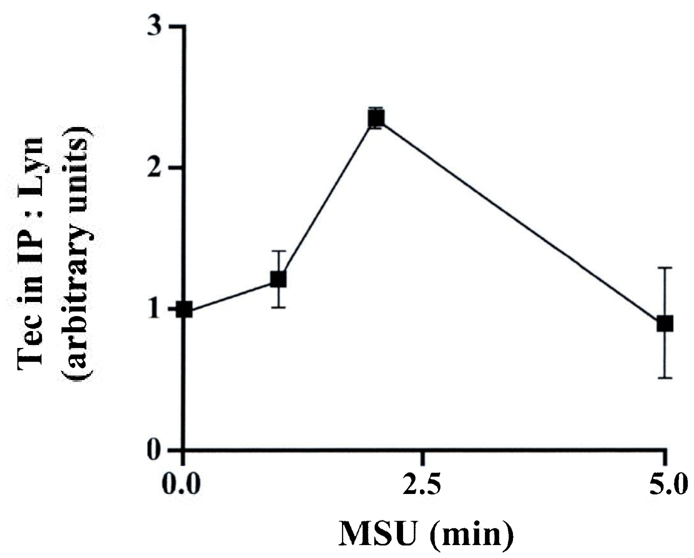
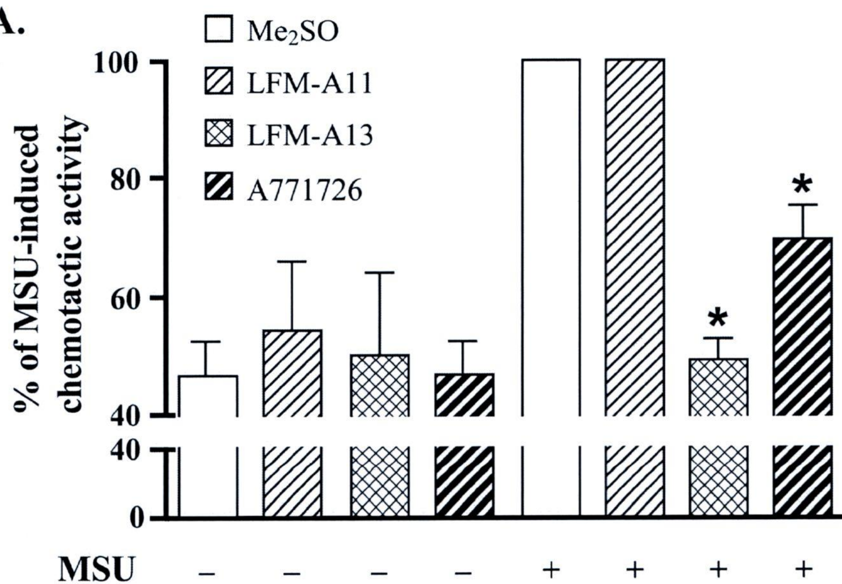
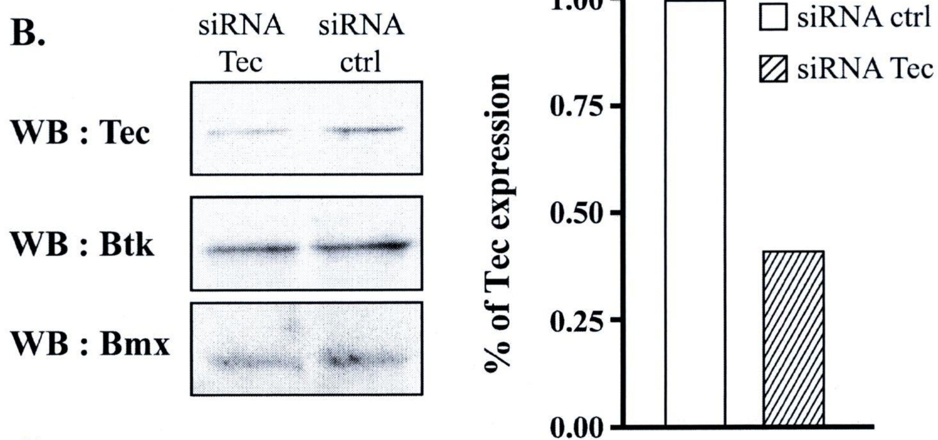


Fig. 3





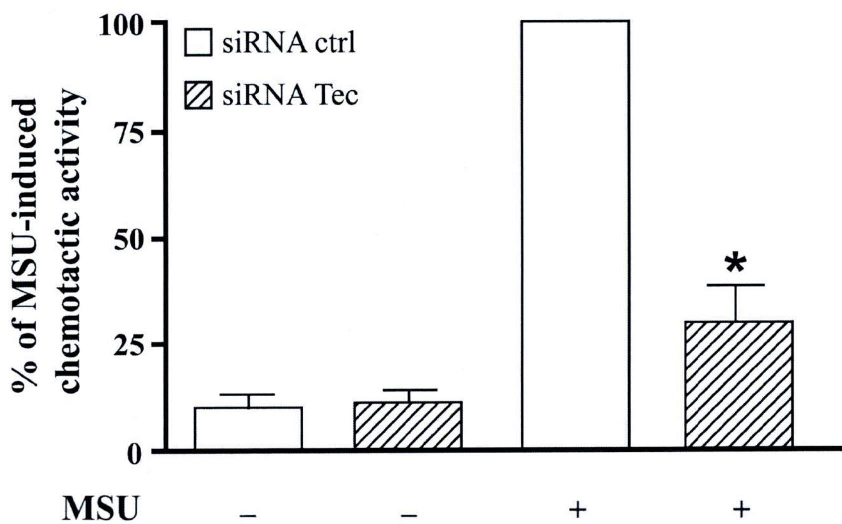
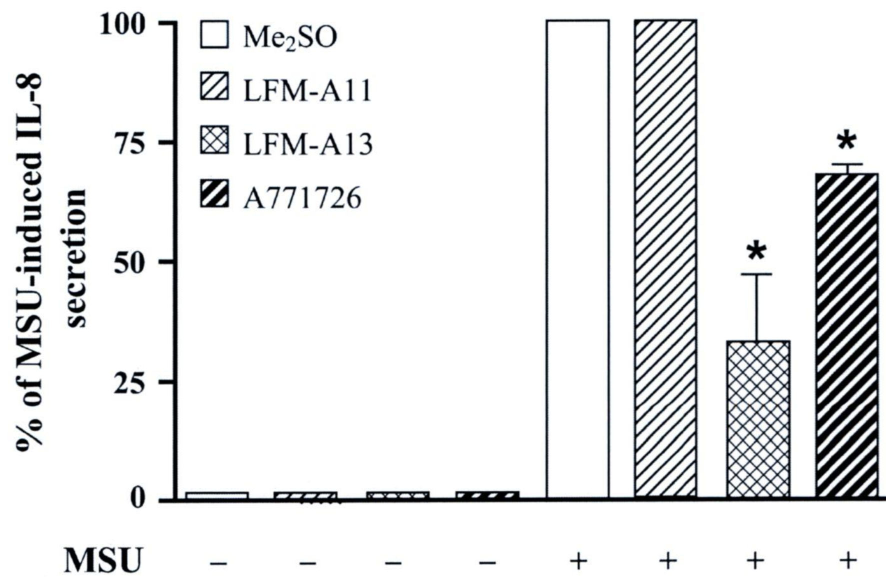


Fig. 4

A.



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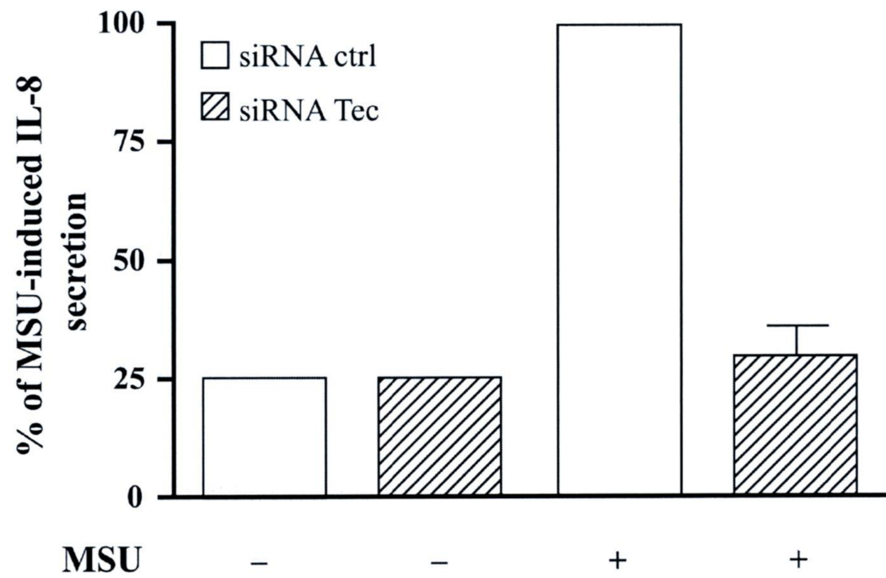
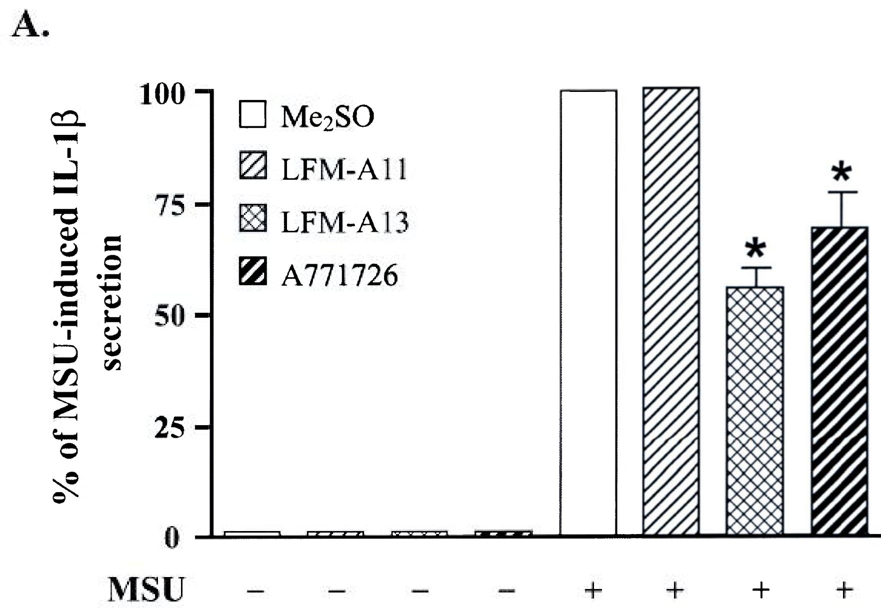


Fig. 5

A.



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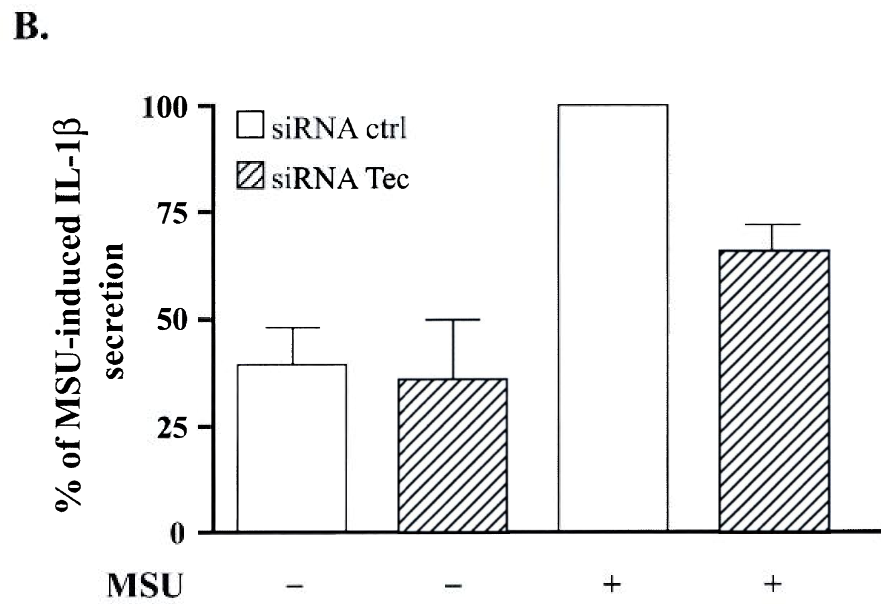
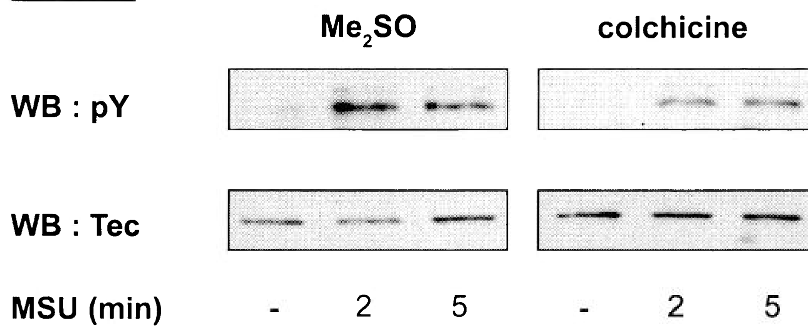
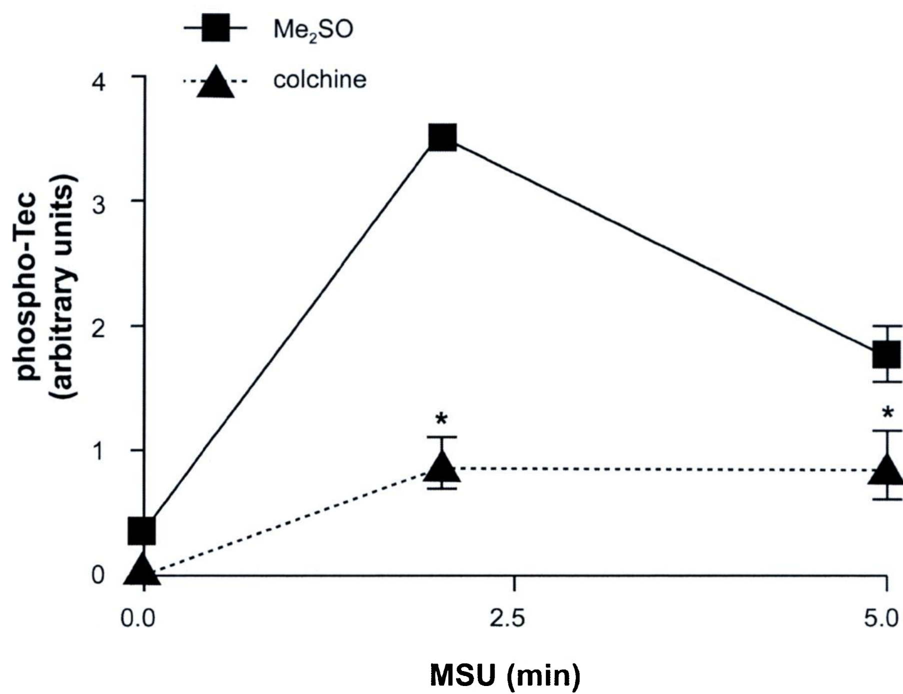


Fig. 6





Chapitre IV : Conclusion

Le dépôt des cristaux d'UMS dans les articulations des personnes souffrant d'une hyperuricémie provoque une puissante réaction inflammatoire connue sous le nom de goutte (Molloy and McCarthy 2004). Cette réaction est caractérisée par une infiltration massive des neutrophiles qui sont indispensables au déclenchement de l'inflammation goutteuse. Plusieurs observations expérimentales soutiennent cette hypothèse : l'injection de cristaux d'UMS provoque une réaction inflammatoire aigue, plus de 95% des cellules présentes dans un liquide synovial de goutte sont des neutrophiles (Agudelo and Schumacher 1973), il n'y a pas de réponse inflammatoire à la suite d'injections de cristaux d'UMS dans des souris déplétées en neutrophiles (Seegmiller and Howell 1962) et, finalement, des molécules qui inhibent les fonctions du neutrophile sont efficaces pour le traitement de la goutte (Phelps 1969). La stimulation des neutrophiles humains par les cristaux d'UMS entraîne la sécrétion de nombreuses cytokines (Hachicha, Naccache et al. 1995; Matsukawa, Yoshimura et al. 1998), d'eicosanoïdes (Rae, Davidson et al. 1982; Serhan, Lundberg et al. 1984; Poubelle, De Medicis et al. 1987) et de facteurs chimiotactiques restés inconnus (Spilberg, Mandell et al. 1974; Desaulniers, Marois et al. 2006) qui promeuvent le processus inflammatoire.

Un des premiers signes caractéristiques de la stimulation des neutrophiles par les cristaux d'UMS est l'augmentation du niveau global de phosphorylation sur résidus tyrosine (Roberge, Gaudry et al. 1993). Des études effectuées dans notre laboratoire ont établi une corrélation entre le profil de phosphorylation sur résidus tyrosine et les propriétés phlogogènes des cristaux d'UMS (Gaudry, Roberge et al. 1993). De plus, ce profil est différent des profils caractéristiques de la stimulation des neutrophiles humains par d'autres stimuli (solubles ou particulaires). Une autre spécificité du profil de phosphorylation sur résidus tyrosine engendré par les cristaux d'UMS est sa sensibilité à la colchicine, médicament utilisé pour le traitement de la goutte (Roberge, Gaudry et al. 1993; Roberge, Gaudry et al. 1996).

1. L'activation des PI3Ks par les cristaux d'UMS dans les neutrophiles humains dépend de la tyrosine kinase Syk

La tyrosine kinase Syk, initialement associée à la phagocytose (Kiefer, Brumell et al. 1998) et à la signalisation (Miller, Hong et al. 1999) dépendantes des récepteurs Fc et des intégrines, a été identifiée dans notre laboratoire comme jouant un rôle majeur dans les événements de signalisation engendrés par les cristaux d'UMS (Desaulniers,

Fernandes et al. 2001). Syk est nécessaire à l'activation des PI3Ks à la suite de la stimulation du récepteur des cellules B (Beitz, Fruman et al. 1999) ou des récepteurs Fcγ des neutrophiles et des macrophages (Okada, Maeda et al. 2000). De plus, la sous-unité régulatrice des PI3Ks, la protéine p85, est la principale protéine de liaison de Syk dans un système de double-hybride (Moon, Post et al. 2005). L'activité PI3 kinase des immunoprécipités anti-phosphotyrosine a déjà été rapportée comme étant augmentée dans les neutrophiles humains stimulés avec des cristaux d'UMS opsonisés avec du plasma (Jackson, Lauener et al. 1997).

Le principal objectif de cette première étude a été la caractérisation de la voie de transduction du signal qui aboutit à l'activation des PI3Ks et le rôle que cette famille de lipide kinases joue dans les réponses des neutrophiles humains aux cristaux d'UMS.

Les résultats que nous avons obtenus montrent que la stimulation des neutrophiles humains par les cristaux d'UMS entraîne une activation des PI3Ks de la classe Ia. Cette activation est dépendante de la tyrosine kinase Syk puisque le piceatannol, un inhibiteur spécifique de Syk, diminue significativement l'activité PI3 kinase des immunoprécipités anti-p85 provenant des neutrophiles stimulés ainsi que la phosphorylation d'AKT reconnue comme un indice d'activation des PI3Ks.

Les fractions cellulaires enrichies en cytosquelette et domaines membranaires résistants au détergent (DRMs) représentent des plateformes dynamiques de signalisation. La translocation des protéines de signalisation dans de telles fractions (insolubilisation) est un des signes caractéristiques de leur activation. Il a en effet, été montré dans notre laboratoire que les profils de phosphorylation sur résidus tyrosine et les activités enzymatiques des protéines de signalisation étaient concentrés dans les fractions insolubles dans du NP40 (détergent utilisé pour la caractérisation des DRMs) des neutrophiles stimulés (Gilbert, Rollet-Labelle et al. 2002). Nos résultats montrent que la stimulation des neutrophiles par les cristaux d'UMS entraîne une insolubilisation de p85 dans du NP40 qui corrèle avec l'activation des PI3Ks de la classe Ia. De plus, nous avons aussi observé que Syk et p85 forment un complexe dans les neutrophiles stimulés. La composition des DRMs formés à la suite de la stimulation des neutrophiles humains par les cristaux d'UMS reste à être déterminée. Les agents qui empêcheraient la formation des complexes de signalisation dans ces DRMs (en empêchant l'insolubilisation des protéines ou la formation des DRMs) pourraient donc représenter d'éventuelles cibles thérapeutiques pour la goutte.

Dans la deuxième partie de cette étude, nous avons analysé l'importance fonctionnelle de l'activation des PI3Ks dans les réponses des neutrophiles humains aux cristaux d'UMS. Nous avons ainsi montré que deux inhibiteurs des PI3Ks (wortmannine et LY294002) diminuent significativement l'activation de la PLD et la dégranulation, ce qui souligne le rôle important des PI3Ks dans ces réponses des neutrophiles humains qui sont, en partie, responsables de dommages tissulaires engendrés par les cristaux d'UMS.

2. Caractérisation et étude fonctionnelle de l'activation de la protéine tyrosine kinase Tec par les cristaux d'UMS dans le neutrophile humain

La famille des Tec kinases joue des rôles dans la réorganisation du cytosquelette d'actine (Yao, Janmey et al. 1999), la régulation de la transcription (Fowell, Shinkai et al. 1999), la survie (Uckun 1998) et la transformation cellulaires (Tsai, Su et al. 2000). Nous avons précédemment montré que la stimulation du récepteur CD16b active la protéine tyrosine kinase Tec (Fernandes, Lachance et al. 2005). Il a été aussi montré dans notre laboratoire que la reconnaissance des cristaux d'UMS se fait, en partie, par l'intermédiaire de ce récepteur (Barabe, Gilbert et al. 1998). Les membres de la famille Tec sont les seules tyrosine kinases connues qui possèdent un domaine PH qui permet leur association avec le PIP3, le principal produit des PI3Ks de la classe Ia (Okoh and Vihinen 1999). Ces résultats préliminaires ainsi que l'activation des PI3Ks dans les neutrophiles stimulés par les cristaux d'UMS rapportée précédemment (Popa-Nita, Rollet-Labelle et al. 2007) ont identifié la tyrosine kinase Tec comme un bon candidat pour l'étude des molécules participant à la transduction du signal amorcé par ces particules.

Le principal objectif de cette étude a donc été la caractérisation et l'étude fonctionnelle de l'activation de Tec par les cristaux d'UMS dans le neutrophile humain. Les résultats que nous avons obtenus montrent que la stimulation des neutrophiles par les cristaux d'UMS entraîne une augmentation du niveau de phosphorylation sur résidus tyrosine de Tec ainsi que de son activité kinase. L'activation de Tec est dépendante de la Src kinase Lyn qui forme un complexe avec Tec dans les neutrophiles stimulés. Ces observations sont en accord avec des études précédentes de notre laboratoire qui avaient montré que Lyn est activée à la suite de la stimulation des neutrophiles humains par les cristaux d'UMS (Gaudry, Gilbert et al. 1995). De plus, il avait déjà été rapporté que, dans

d'autres systèmes cellulaires, Lyn peut interagir avec Tec (Mano, Sato et al. 1994) et la phosphoryler (Mano, Yamashita et al. 1996).

Deux autres membres de la famille des kinases Tec (Btk et Bmx) sont exprimés dans le neutrophile humain (Lachance, Levasseur et al. 2002). Nous n'avons obtenu aucun indice d'activation de ces deux kinases à la suite de la stimulation des neutrophiles humains par les cristaux d'UMS. Ce résultat a été ensuite validé dans une lignée cellulaire (PLB-985) différenciée pour exprimer un phénotype « neutrophil-like » (Tucker, Lilly et al. 1987) où la diminution de l'expression de Btk et Bmx (en utilisant des siRNAs spécifiques) n'a eu aucun effet sur les réponses de ces cellules aux cristaux d'UMS alors que celle de Tec conduisait à une diminution des réponses aux cristaux d'UMS. L'absence de redondance entre les différents membres de la famille Tec avait déjà été mise en évidence dans le cas de la maladie XLA où les mutations délétères de Btk ne sont pas compensées par l'expression d'autres kinases Tec. En ce qui concerne les réponses pro-inflammatoires des neutrophiles humains aux cristaux d'UMS, ce résultat désigne la tyrosine kinase Tec comme une cible spécifique pour le traitement de l'arthrite goutteuse.

L'étude fonctionnelle effectuée en deuxième partie de ce projet a mis en évidence le rôle majeur que la tyrosine kinase Tec joue dans la sécrétion d'une activité chimiotactique ainsi que des cytokines comme IL-8 et IL-1β. Ces réponses des neutrophiles humains expliquent en partie le recrutement exclusif de ces cellules au site de l'inflammation goutteuse ainsi que la prolongation et l'exacerbation de la réponse immunitaire dans l'arthrite goutteuse. Un des inhibiteurs pharmacologiques utilisé pour diminuer l'activité kinase de Tec a été le A771726, la molécule active du leflunomide, médicament actuellement utilisé pour le traitement de l'arthrite rhumatoïde. Ses effets sur les réponses pro-inflammatoires des neutrophiles humains laissent entrevoir une éventuelle utilisation du A771726 en tant que traitement de l'arthrite goutteuse.

Finalement, nous avons aussi montré que la colchicine, médicament utilisé depuis des siècles pour le traitement de la goutte, diminue la phosphorylation sur résidu tyrosine de Tec, événement qui est nécessaire à son activation. Malgré le nombre d'années d'utilisation de la colchicine, son mécanisme d'action *in vivo* n'a pas encore été clairement caractérisé. La colchicine inhibe de nombreuses fonctions des neutrophiles telles que la chimiotaxie (Phelps 1969), l'adhésion, la phagocytose (Dallaverde, Fan et al. 1982) et la sécrétion de LTB_4 (Serhan, Lundberg et al. 1984), d'IL-8 (Matsukawa, Yoshimura et al. 1998) et d'IL-1β (Roberge, Grassi et al. 1991). Les mécanismes qui

contrôlent la dépendance de ces fonctions de l'intégrité des microtubules restent à être identifiés.

3. Modèle proposé

Les résultats obtenus pendant ce projet nous ont amenés à proposer un modèle de transduction du signal déclenché par les cristaux d'UMS qui comporte dans un premier temps l'activation de la famille des kinases Src suivie de l'activation de la PKC (translocation à la membrane plasmique et augmentation de l'activité kinase) (Figure supplémentaire 2, Annexe 1) qui régule celle de la tyrosine kinase Syk (Figure supplémentaire 3, Annexe 1). En interagissant avec leur sous-unité régulatrice, Syk régule l'activation des PI3Ks de la classe Ia (Popa-Nita, Rollet-Labelle et al. 2007) qui produisent du PIP3 à la suite de la stimulation des neutrophiles par les cristaux d'UMS. D'autre part, les kinases Src et plus particulièrement Lyn activent la tyrosine kinase Tec qui lie le PIP3 à la membrane plasmique (Popa-Nita, Marois et al. 2008). Nous avons, en effet, obtenu des résultats montrant une translocation de Tec à la membrane plasmique des neutrophiles stimulés par les cristaux d'UMS (Figure supplémentaire 4, Annexe 1). L'activation de cette voie de signalisation est nécessaire à des réponses précoces des neutrophiles humains telles que la dégranulation ou la sécrétion d'une activité chimiotactique ainsi qu'à des réponses tardives comme la sécrétion des cytokines IL-8 et IL-1β qui sont responsables en partie de l'inflammation goutteuse.

La diminution de l'activité phosphatase de SHP-1 observée à la suite de la stimulation des neutrophiles est vraisemblablement nécessaire à l'augmentation du profil de phosphorylation sur résidus tyrosine caractéristique de l'activation engendrée par les cristaux d'UMS. La phosphorylation sur la serine 591 de SHP-1 (et, donc, la régulation négative de son activité) semble dépendante de la PKC (Figure supplémentaire 5, Annexe 1). Il peut être envisagé qu'ensuite, une phosphorylation sur la tyrosine 536 de SHP-1 (et donc, une augmentation de son activité phosphatase) serait responsable de l'arrêt de la transduction du signal amorcé par les cristaux d'UMS. Des études supplémentaires sont requises pour valider cette hypothèse.

La spécificité thérapeutique de la colchicine pour l'arthrite goutteuse est la principale raison motivant l'étude de ses effets sur les voies de signalisation activées par les cristaux d'UMS dans les neutrophiles humains. L'hypothèse étant que la spécificité

thérapeutique de la colchicine pourrait s'expliquer par l'existence d'événements spécifiquement engendrés lors de l'inflammation causée par les cristaux d'UMS.

La colchicine inhibe plusieurs fonctions du neutrophile humain telles que la chimiotaxie (Phelps 1969), l'adhésion, la phagocytose (Dallaverde, Fan et al. 1982) et la sécrétion de LTB_4 (Serhan, Lundberg et al. 1984), d'IL-8 (Matsukawa, Yoshimura et al. 1998) et d'IL-1β (Roberge, Grassi et al. 1991). Alcaloïde tricyclique, la colchicine lie les monomères α et β de la tubuline pour entraîner un changement de conformation qui empêche ainsi la polymérisation des microtubules (Margolis and Wilson 1977). Les mécanismes dépendant des microtubules qui contrôlent les fonctions des neutrophiles responsables de l'inflammation goutteuse n'ont pas encore été identifiés.

Parmi les premières observations concernant ces mécanismes, l'inhibition du profil global de phosphorylation sur résidus tyrosine (Roberge, Gaudry et al. 1993; Roberge, Gaudry et al. 1996) et de la mobilisation du calcium intracellulaire (Naccache, Grimard et al. 1991) spécifiques de la stimulation par les cristaux d'UMS pourraient fournir quelques indices, d'autant plus que ces inhibitions n'ont pas été observées lors de la stimulation des neutrophiles par des agents chimiotactiques (Naccache, Grimard et al. 1991; Roberge, Gaudry et al. 1996). La mobilisation du calcium intracellulaire entraînée par les cristaux d'UMS est aussi inhibée par des inhibiteurs de tyrosine kinases (Burt, Jackson et al. 1993), ce qui suggère un rôle de ces enzymes dans cette voie de signalisation.

La tyrosine kinase spécifiquement responsable de ces événements n'a pas été encore identifiée mais il est connu que les membres de la famille des tyrosine kinases Src jouent des rôles clé dans la transduction du signal engendré par les cristaux d'UMS (Gaudry, Gilbert et al. 1995; Ryckman, Gilbert et al. 2004) (Popa-Nita, Marois et al. 2008). Les kinases Src peuvent phosphoryler la tubuline et il a été déjà montré dans d'autres types cellulaires qu'elles peuvent s'associer avec les microtubules (Matten, Aubry et al. 1990; Lee, Newman et al. 1998; Draberova, Draberova et al. 1999).

Il peut donc être envisagé qu'en inhibant la polymérisation des microtubules, la colchicine empêche l'activation des membres de la famille des kinases Src par les cristaux d'UMS et, subséquemment, l'activation des voies de signalisation contrôlées par ces enzymes. Des mesures d'activité kinase des différentes kinases Src sont requises afin de valider cette hypothèse.

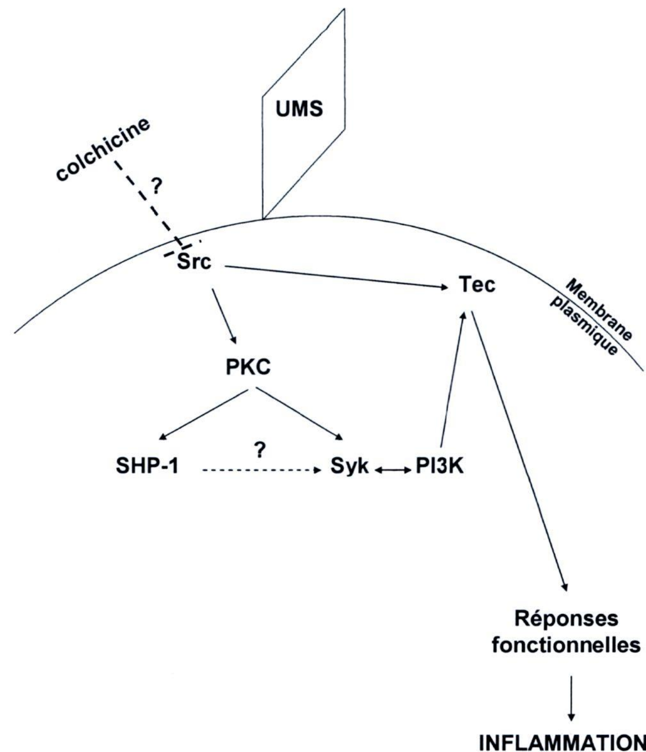


Figure 1 : Voies de signalisation activées par les cristaux d'UMS dans les neutrophiles humains. La stimulation des neutrophiles humains par les cristaux d'UMS comprend l'activation séquentielle des kinases Src, de Syk et des PI3Ks ainsi que la baisse de l'activité phosphatase de SHP-1. Le produit des PI3Ks, PIP3, sert d'ancre à la membrane plasmique pour Tec qui, phosphorylée par les Src (probablement Lyn) contrôle ensuite des réponses précoces et tardives qui sont en partie responsables de l'inflammation de l'arthrite goutteuse. Les effets de la colchicine dans le traitement de la goutte pourraient être expliqués au niveau moléculaire par une éventuelle inhibition de l'activation des kinases Src.

4. Perspectives

Bien qu'intensément étudiée et connue depuis longtemps, l'arthrite goutteuse reste la plus fréquente arthrite chez l'homme après l'âge de 40 ans avec une prévalence en constante augmentation (Eggebeen 2007). De plus, des associations entre la goutte et les

maladies cardiovasculaires, le diabète ou l'atteinte rénale chronique ont été récemment mises en évidence (Vazquez-Mellado, Alvarez Hernandez et al. 2004; Choi and Curhan 2007). Il est donc nécessaire de continuer l'étude au niveau moléculaire et cellulaire de cette pathologie afin d'améliorer les traitements actuels (qui peuvent souvent avoir de graves effets secondaires) ou d'évaluer de nouvelles thérapies. De plus, la caractérisation du rôle de l'alimentation ou des facteurs génétiques et environnementaux devrait compléter l'étude de l'arthrite goutteuse.

La stimulation des neutrophiles humains par des cristaux d'UMS peut aussi être considérée comme un modèle pour l'étude des disfonctionnements de ces cellules lors d'une très puissante réaction inflammatoire.

A court terme, les perspectives de ce projet de doctorat concernent l'étude des facteurs secrétés en réponse aux cristaux d'UMS qui activent indirectement les neutrophiles. Nous avons mis récemment en évidence la sécrétion d'un facteur chimiotactique dans les surnageants des neutrophiles humains stimulés par des cristaux d'UMS (Desaulniers, Marois et al. 2006). De nature protéique et avec une activité qui ne semble pas dépendre de la synthèse protéique *de novo*, ce(s) facteur(s) reste(nt) à ce jour inconnu(s). Nous avons montré dans notre laboratoire que les cristaux d'UMS provoquent la dégranulation du neutrophile humain (Popa-Nita, Rollet-Labelle et al. 2007). Il est donc probable que cette activité chimiotactique soit contenue dans les granules des neutrophiles. La séparation des différents types de granules suivie de l'identification du type qui contient l'activité chimiotactique et de son analyse en spectrométrie de masse peut être envisagée afin de caractériser plus en détail ce facteur qui serait en partie responsable de la prolongation et de l'exacerbation de l'inflammation goutteuse puisqu'une fois au site inflammatoire, les neutrophiles activés par les cristaux d'UMS sont ainsi capables de recruter et activer d'autres neutrophiles. L'identification de cette protéine (ou complexe protéique) avec des propriétés chimiotactiques pourrait ainsi représenter une nouvelle cible thérapeutique pour la goutte.

Il est fortement probable que les neutrophiles fraîchement isolés à partir du sang aient un phénotype différent de ceux ayant migré aux sites inflammatoires. De plus, il est connu qu'*in vivo*, les cristaux d'UMS absorbent à leur surface diverses protéines dont les propriétés physiques peuvent influencer leurs propriétés phlogistiques. A plus long terme, il serait donc important de mettre au point un protocole plus physiologique de stimulation des neutrophiles humains afin de prendre en compte un éventuel changement de phénotype des cellules ayant migré aux sites inflammatoires ainsi que la

dynamique d'absorption de protéines à la surface des cristaux. Des molécules telles que le TNF-α, l'IL-6, l'IL-1β et l'IL-8 sécrétées en grande concentration dans la cavité synoviale lors d'une crise de goutte (Di Giovine, Malawista et al. 1987; Guerne, Terkeltaub et al. 1989; di Giovine, Malawista et al. 1991; Terkeltaub, Zachariae et al. 1991) pourraient représenter, dans un premier temps, un bon choix d'agents « primants » pour les neutrophiles. Les surnageants de cellules (synoviocytes de type A ou B, neutrophiles) stimulées par les cristaux d'UMS représentent une alternative de *priming* qui nécessite plus de mise au point mais qui se rapproche un peu plus de la réalité physiologique de cette inflammation. La définition de la composition du liquide synovial caractéristique de chaque étape de l'inflammation goutteuse est nécessaire pour déterminer la meilleure méthode d'opsonisation des cristaux d'UMS en fonction de la nature de la question étudiée (déclenchement *vs* résolution de l'inflammation). Ainsi, les IgGs ou l'acide hyaluronique de faible poids moléculaire pourraient être utilisés pour opsoniser des cristaux représentatifs du début de la crise de goutte alors que recouvrir les cristaux d'UMS par des apolipoprotéines correspondrait plutôt à une étude portée sur la résolution de l'inflammation goutteuse.

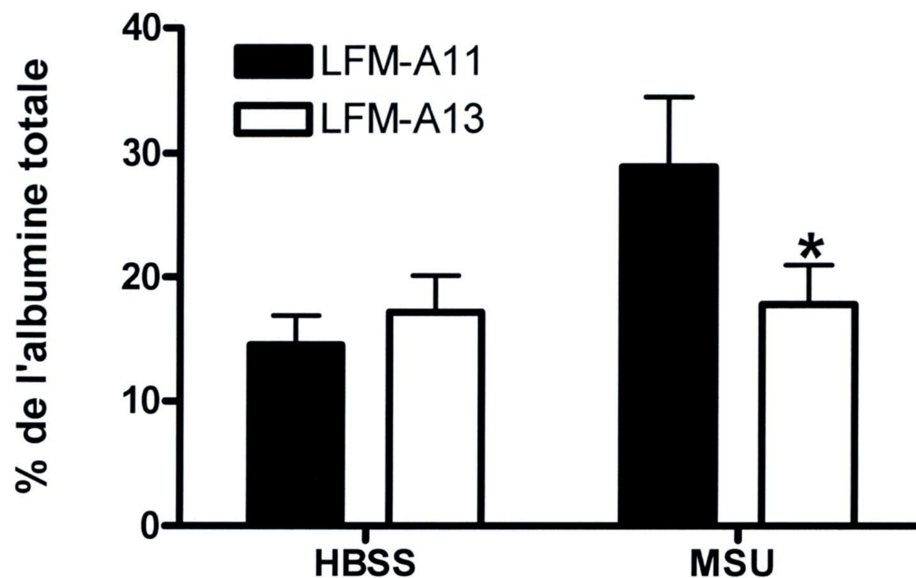
Les résultats obtenus pendant ce projet de recherche ainsi que ceux déjà rapportés dans la littérature serviront de référence pour comparer les réponses des neutrophiles pré-activés stimulés avec des cristaux opsonisés. L'utilisation des neutrophiles fraîchement isolés a permis la description détaillée de mécanismes moléculaires mis en place par la cellule lors d'une puissante réaction inflammatoire (comme celle engendrée par les cristaux d'UMS « nus »). Ces mécanismes seront sûrement contrôlés d'une manière plus complexe à la suite d'un *priming* des neutrophiles et/ou d'une opsonisation des cristaux d'UMS, mais il est peu probable que les voies de transduction du signal soient significativement modifiées. Cette assertion est soutenue par des études *in vivo* utilisant des modèles expérimentaux murins qui ont identifié principalement les mêmes mécanismes moléculaires responsables de l'inflammation goutteuse (kinases Src, kinases MAP, COX-2, PKC, IL-8, MRPs, LTB_4, *etc.*) (Brooks, Burton et al. 1987; Terkeltaub, Baird et al. 1998; Fam 2000; Ryckman, McColl et al. 2003).

Finalement, notre laboratoire est aussi intéressé par l'identification des molécules exprimées à la surface des neutrophiles humains responsables de la reconnaissance des cristaux d'UMS. Ces molécules incluent des récepteurs (ou complexes de récepteurs) spécifiques ou opportunistes de ces particules ainsi que des récepteurs pour les facteurs secondaires secrétés en réponse aux cristaux d'UMS (facteurs chimiotactiques,

nucléotides, formes réactives de l'oxygène) qui activent indirectement les neutrophiles. L'analyse des neutrophiles provenant des patients présentant des cristaux d'UMS dans leurs liquides synoviaux sans présenter des symptômes de goutte pourrait fournir des indices quant à l'identité des récepteurs de ces particules. La caractérisation de ces molécules apportera plus de renseignements en ce qui concerne les voies de signalisation activées par les cristaux d'UMS dans les neutrophiles humains et pourrait potentiellement fournir plusieurs cibles thérapeutiques pour la goutte.

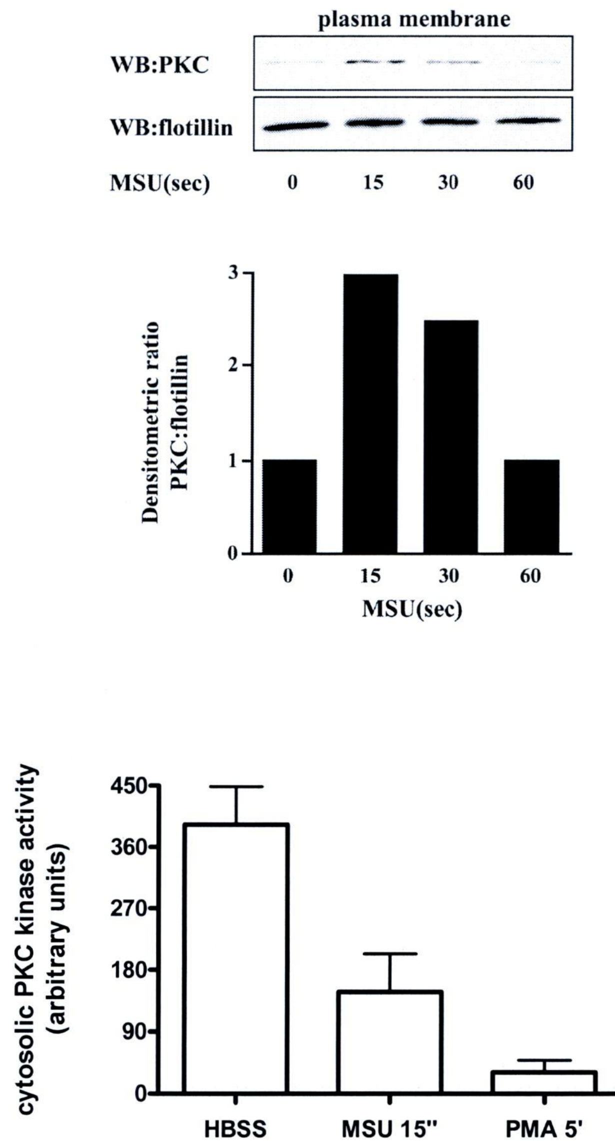
Annexe 1 : Figures supplémentaires

Figure supplémentaire 1 : La dégranulation des vésicules sécrétoires causée par les cristaux d'UMS est dépendante de l'activité de la tyrosine kinase Tec



À la suite de la stimulation par les cristaux d'UMS, la sécrétion d'albumine (contenue dans les vésicules sécrétoires des neutrophiles humains (Faurschou and Borregaard 2003)) a été corrélée avec la dégranulation des vésicules sécrétoires. Le LFM-A13, un inhibiteur de l'activité kinase de Tec, diminue significativement cette dégranulation. Ce résultat semble être en accord avec le rôle que Tec joue dans la réorganisation du cytosquelette d'actine (Jog, Rane et al. 2007).

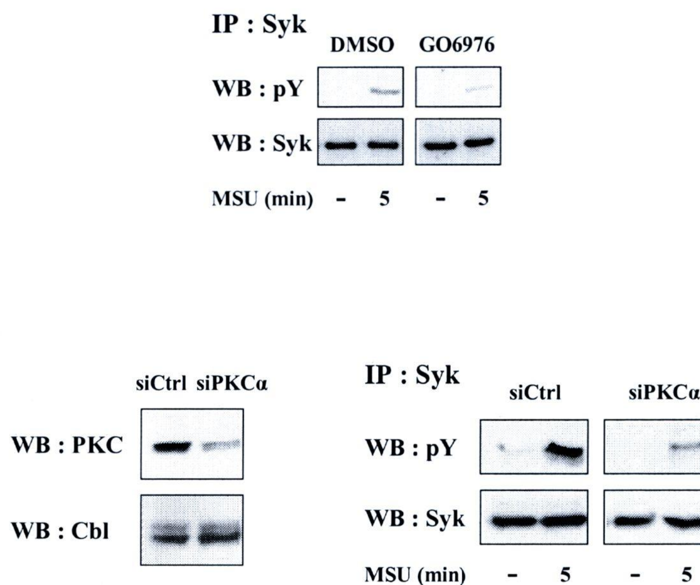
Figure supplémentaire 2 : La stimulation des neutrophiles humains par les cristaux d'UMS entraîne l'activation de PKC



La stimulation des neutrophiles humains par les cristaux d'UMS entraîne la translocation transitoire de PKC à la membrane plasmique, ce qui est un indice de son activation.

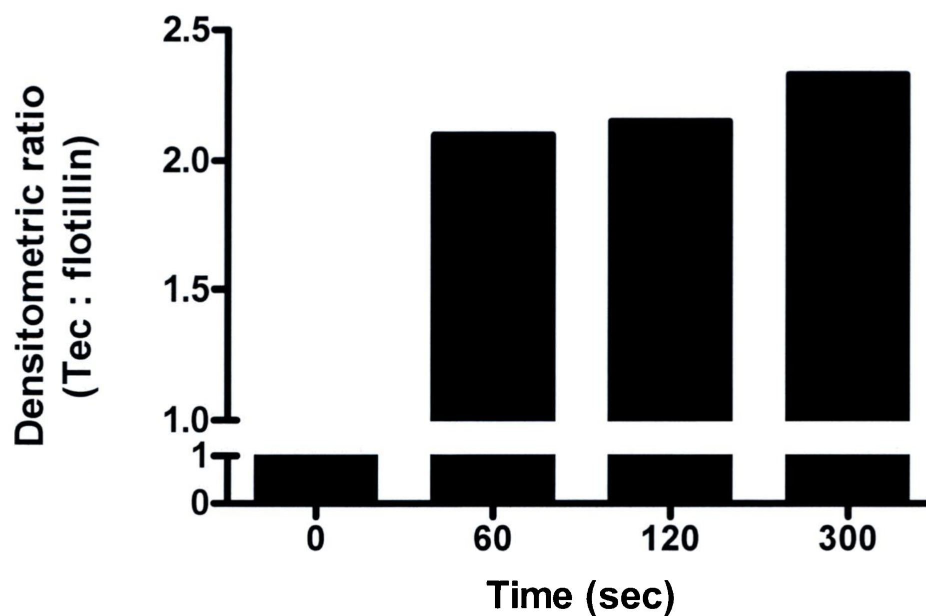
En accord avec cette translocation à la membrane plasmique, l'activité kinase de la PKC cytosolique est diminuée dans les neutrophiles stimulés par les cristaux d'UMS (à un niveau comparable à celui entraîné par la stimulation par le PMA (*phorbol myristoylacetate*).

Figure supplémentaire 3 : PKC contrôle l'activation de la tyrosine kinase Syk dans les neutrophiles stimulés par les cristaux d'UMS



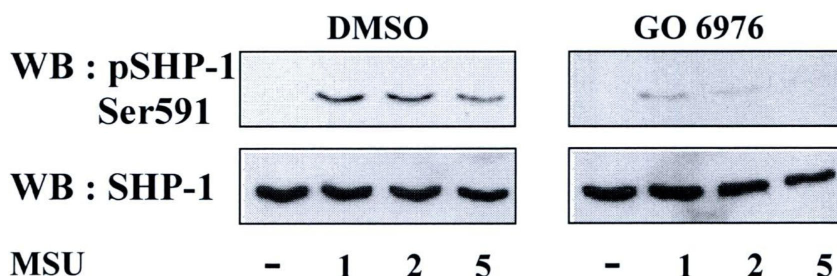
Le GO6976, un inhibiteur des PKCs classiques, diminue la phosphorylation sur résidus tyrosine de Syk dans les neutrophiles stimulés par les cristaux d'UMS. Les cellules PLB-985 différenciées pour exprimer un phénotype *neutrophil-like* ont été utilisées pour valider ce résultat. La transfection d'un siRNA spécifique pour l'isoforme α des PKCs diminue la phosphorylation sur résidus tyrosine de Syk dans les cellules PLB-985 stimulées par les cristaux d'UMS. Ce résultat identifie la PKCα comme la principale isoforme des PKC contrôlant l'activation de Syk dans les neutrophiles humains.

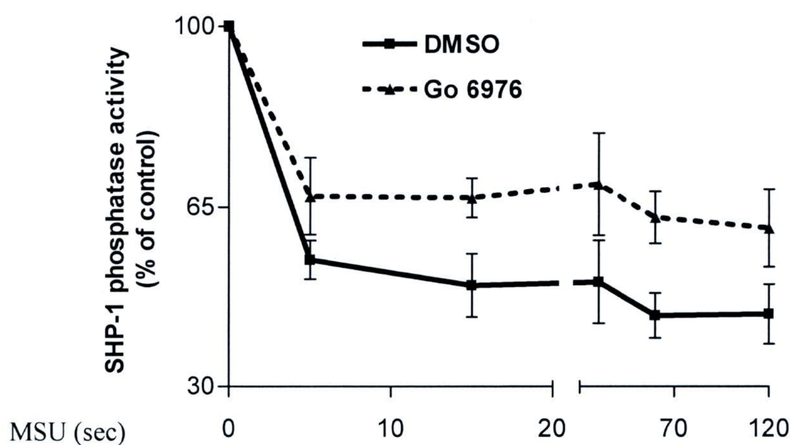
Figure supplémentaire 4 : La tyrosine kinase Tec transloque à la membrane plasmique des neutrophiles stimulés par les cristaux d'UMS



Les travaux menés pendant ce projet de recherche ont mis en évidence l'activation des PI3 kinases de classe Ia et de la tyrosine kinase Tec suite à la stimulation des neutrophiles humains par les cristaux d'UMS. Le principal produit des PI3 kinases de classe Ia, le PIP3 représente une ancre à la membrane plasmique pour des protéines contenant un domaine PH, telles que Tec. Le résultat présenté dans cette figure montre la translocation de Tec à la membrane plasmique des neutrophiles stimulés par les cristaux d'UMS.

Figure supplémentaire 5 : PKC contrôle la diminution de l'activité phosphatase de SHP-1 dans les neutrophiles stimulés par les cristaux d'UMS





MSU (sec)

La phosphorylation sur résidus serine de SHP-1 est un indice de la diminution de son activité phosphatase (Zhao, Shen et al. 1994). Cette diminution observée suite à la stimulation des neutrophiles humains est vraisemblablement nécessaire à l'augmentation du profil de phosphorylation sur résidus tyrosine caractéristique de l'activation engendrée par les cristaux d'UMS.

Les résultats présentés dans cette figure montrent que la phosphorylation sur résidus serine de SHP-1 et, donc, la régulation négative de son activité phosphatase sont dépendantes de PKC puisque le GO6976 les diminue significativement.

Annexe 2 : Characterization of an activation factor released from human neutrophils after stimulation by triclinic monosodium urate crystals

L'article présenté dans cette section a été publié dans Journal of Rheumatology, 2006, May ; 33(5) : 328-38

Characterization of an Activation Factor Released from Human Neutrophils After Stimulation by Triclinic Monosodium Urate Crystals

PHILIPPE DESAULNIERS, SÉBASTIEN MAROIS, GUILLAUME PARÉ, OANA POPA-NITA, CAROLINE GILBERT, and PAUL H. NACCACHE

ABSTRACT. ***Objective.*** To determine the presence and characterize the activity of a soluble activation factor rapidly released by human neutrophils after stimulation with monosodium urate (MSU) crystals.

Methods. Supernatants from human neutrophils stimulated by MSU crystals for 5 to 60 min were tested for their ability to stimulate a chemotactic response, induce a mobilization of calcium, and increase the tyrosine phosphorylation levels in naive neutrophils.

Results. Supernatant from neutrophils stimulated ≤ 15 min by MSU crystals was chemotactic for neutrophils, induced a mobilization of calcium, and increased the levels of tyrosine phosphorylation in fresh neutrophils. Generation of activity in the supernatant was independent of protein synthesis and was eliminated after digestion with trypsin. Leukotriene B_4 (LTB_4), platelet-activating factor (PAF), and formyl peptide receptor antagonists as well as neutralizing anti-interleukin 8 (IL-8) antibodies did not inhibit the chemotactic activity in the supernatant, although pertussis toxin did inhibit the mobilization of calcium observed in response to the supernatant. Stimulation of neutrophils with formyl-methionine-leucine-phenylalanine, IL-8, and LTB_4 inhibited subsequent mobilization of calcium by the supernatant.

Conclusion. There is rapid liberation of a potent activation signal from neutrophils after interaction with MSU crystals. This activation factor can further stimulate surrounding neutrophils and contribute to amplification of the inflammatory response induced by MSU crystals. (J Rheumatol 2006;33:928–38)

Key Indexing Terms:
NEUTROPHILS HUMAN GOUT CHEMOTAXIS
MONOSODIUM URATE CRYSTALS TYROSINE PHOSPHORYLATION

The polymorphonuclear neutrophil is the first leukocyte to accumulate in large numbers at the inflammatory site. Neutrophils can be recruited in response to multiple stimuli, endogenous as well as exogenous, including complement components (C5a), bacterial peptides [formyl-methionine-leucine-phenylalanine (fMLF)], chemokines [interleukin 8 (IL-8), growth-related oncogene-α (Gro-α), nerve action potential-2 (NAP-2)], lipid mediators [leukotriene B_4 (LTB_4), platelet-activating factor (PAF)], and members of the S100 family, also known as myeloid related proteins (MRP)[1-3]. These signals orient the neutrophil, increase its adhesion, and induce the expression of receptors and secretion of molecules to promote inflammation and/or postpone apoptosis[4-7].

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The neutrophil is by far the most abundant leukocyte recruited to the site of inflammation in gouty arthritis. Interaction of monosodium urate (MSU) microcrystals with cells in the joints leads to the production of inflammatory mediators such as IL-8[8], LTB_4 and LTC_4[9,10], PAF[11], Gro-α[12], and C5a[13], one or more of which can play a role in amplification of the recruitment of neutrophils. The accumulation and activation of neutrophils are associated with tissue damage caused by the release of neutrophil granules, reactive oxygen species, and proteolytic enzymes. Ingestion of the crystals by neutrophils also leads to the release of inflammatory mediators[14-16] that perpetuate the destructive cycle. The interaction of MSU crystals with neutrophils is thought to be mediated in part by the Fcγ receptor CD16b (with the participation of the CD11b/CD18 complex[17]) and leads to colchicine-dependent robust increases in tyrosine phosphorylation[18,19], mediated in part by the tyrosine kinase Syk[17], in the cytoplasmic concentration of free calcium[16], and in the activities of PI3 kinase[20] and phospholipases D[21] and A_2[22].

Despite the vigorous and rapid responses observed in suspensions of neutrophils exposed to MSU crystals, previous investigators reported that as little as 15% of neutrophils contained MSU crystals after 8 minutes, a fraction that increased to only 41% after 30 minutes[23], while other authors noted

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50% of the cells ingesting MSU crystals after incubations of up to 60 minutes[24]. This led us to examine whether the responses summarized above, which are mostly detected within seconds or at most minutes of the addition of MSU crystals, were due to the direct interaction of MSU crystals with neutrophils or instead to factors secreted by neutrophils in contact with the crystals.

We produced supernatants from MSU-stimulated cells (called SMC) and examined their effects on naive neutrophils. We noted significant activation of neutrophils by SMC that included increases in tyrosine phosphorylation, a mobilization of calcium, and induction of a chemotactic response. The basic characteristics of the activity of SMC differentiate it from lipid mediators, IL-8, and formylated peptides as well as from the previously reported crystal-induced chemotactic factor (CCF)[25-27]. Our data indicate that even very rapid responses of human neutrophils to MSU crystals may result from the additive effects of direct contact of MSU crystals with the cells and from the activity of SMC.

MATERIALS AND METHODS

Reagents. Ficoll-Paque was obtained from Wisent Canadian Laboratories (St-Bruno, Québec, Canada). The enhanced chemiluminescence (Renaissance) reagents used for immunoblotting were from DuPont Pharmaceuticals (Mississauga, ON, Canada). PP2 was purchased from Calbiochem (San Diego, CA, USA) and Biomol (Plymouth Meeting, PA, USA). Pertussis toxin was purchased from List Biologicals (Campbell, CA, USA). Dextran T-500 was purchased from Pharmacia (Baie d'Urfé, Québec, Canada). p-Nitrophenylphosphate, aprotinin, and leupeptin were purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). Cycloheximide and trypsin were obtained from Sigma-Aldrich (Oakville, ON, Canada). LTB_4 and PAF were generous gifts of Dr. Pierre Borgeat (Laval University, Québec, Canada). BN 50730 was a generous gift from the Institut Henri Beaufour (Paris, France). Cyclosporin H originated from Novartis Pharma AG (Basel, Switzerland). Triclinic MSU crystals were kindly provided by Drs. R. de Médicis and A. Lussier (University of Sherbrooke, Sherbrooke, Québec, Canada) and prepared as described[15]. The crystals used in this study were characterized by X-ray diffraction (Geigerflex D/max, Rigaku, Wakefield, MA, USA) and examined under phase and polarization microscopy and by scanning electron microscopy. Several distinct lots of crystals (sizes between 10 and 20 μM, specific areas between 0.7 and 2.4 m^2/g) were used with identical results (data not shown). CP 105.696 was provided by Pfizer Central Research (Groton, CT, USA).

Antibodies. Peroxidase-labeled anti-mouse (no. 115-095-072) antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). The anti-phosphotyrosine antibodies (no. UBI-05-321, clone 4G10) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Phosphospecific ($pTpY^{185/187}$) anti-phosphoERK1/2 (no. 44-680) antibodies were obtained from Biosource International (Camarillo, CA, USA). Anti-human IL-8 antibody (no. 500-M08) was purchased from Peprotech Canada Inc. (Ottawa, ON, Canada).

Neutrophil purification. Blood was obtained from the peripheral vein of healthy adults as described[28]. Neutrophils were obtained by means of 2% Dextran sedimentation followed by standard Ficoll gradient techniques. Contaminating erythrocytes were removed by hypotonic lysis, and purified granulocytes (> 95% neutrophils, < 5% eosinophils, < 0.1% monocytes) were resuspended in Hanks' balanced salt solution (HBSS) containing 1.6 mM calcium and no magnesium (pH 7.4) without serum. The isolation procedure was carried out under sterile conditions.

Production of SMC. Neutrophils (4×10^7 cells/ml in HBSS, 700 μl) were incubated in microcentrifuge tubes with MSU crystals (3 mg/ml) for 5–60 min at 37°C with agitation and then pelleted (6000 g for 15 s) and the supernatants were collected and filtered through a 0.22 μm filter. Special care was taken while collecting the supernatants to avoid contamination with neutrophils or MSU crystals. Control supernatants were also similarly prepared from unstimulated cells.

Tyrosine phosphorylation. Neutrophil suspensions (4×10^7 cells/ml) were either incubated at 37°C with MSU crystals (3 mg/ml) or resuspended in supernatants from MSU-stimulated cells (SMC). The reactions were stopped by the addition of cell aliquots to an equal volume (100 μl) of boiling 2× Laemmli sample buffer [1× is 62.5 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 5% ß-mercaptoethanol, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM paranitrophenylphosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.025% bromophenol blue] and boiled for 7 min. Samples were then subjected to 7.5–20% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Immunoblotting was performed using the 4G10 antiphosphotyrosine antibody at a final dilution of 1/4000 and revealed by the Renaissance Plus detection system as described[29].

Mobilization of intracellular calcium. Cells (10^7 cells/ml) were incubated 30 min at 37°C with 1 μM fura-2/AM (Molecular Probes, Eugene, OR, USA). The neutrophils were washed once in HBSS to remove the extracellular probe, resuspended at 5×10^6 cells/ml, and transferred to the thermoregulated (37°C) cuvette compartment of a spectrofluorimeter (SLM 8000; Aminco, Urbana, IL, USA). In experiments where activity of SMC was tested, cells were resuspended after loading with Fura-2AM in 1 ml of HBSS and then 1 ml of SMC was added. The fluorescence was monitored at excitation wavelength 340 nm and emission wavelength 510 nm. The internal calcium concentrations were calculated as described[30].

Measurement of neutrophil migration. Chemotaxis was measured as described[31]. Briefly, neutrophils were resuspended in RPMI-1640 and 10% fetal bovine serum (FBS) at 10^7 cells/ml and were preincubated with 5 μg/ml calcein-AM (Molecular Probes) at 37°C for 30 min in the dark with constant agitation. Cells were washed twice and resuspended in RPMI/FBS at 5×10^6 cells/ml at 37°C. Neutrophil migration was monitored using a 96-well chemoTX disposable chemotaxis system (NeuroProbe, Gaithersburg, MD, USA). The fluorescence of cells in the filters was measured with a microplate fluorescence reader (FL600; Bio-Tek Instruments, Winooski, VT, USA; excitation wavelength 485 nm, emission wavelength 530 nm). The fluorescence from known numbers of neutrophils was obtained by placing them into the bottom chamber. The results are expressed as the number of cells that penetrated the filters.

Microscopy. The neutrophils (2×10^7 cells/ml) were incubated with MSU crystals (1.5 mg/ml) for 30 min at 37°C before being observed with a polarized light microscope.

RESULTS

The interactions between neutrophils and MSU crystals were first monitored visually by optical microscopy. To this end, neutrophils were exposed to 1.5 mg/ml MSU crystals for 30 min. Aliquots of the cell suspensions were then deposited on microscope slides and examined. As illustrated in Figure 1, only a small percentage of the cells (indicated by arrows) were observed to interact directly with the crystals, despite the presence of multiple free-floating crystals and the relatively long incubation period. Neutrophils that have ingested MSU crystals can be identified by their stretched, elongated shape and the presence of MSU crystals within their membrane. It should be pointed out that the concentration of MSU crystals used in these experiments has been shown[16,18] to elicit near-

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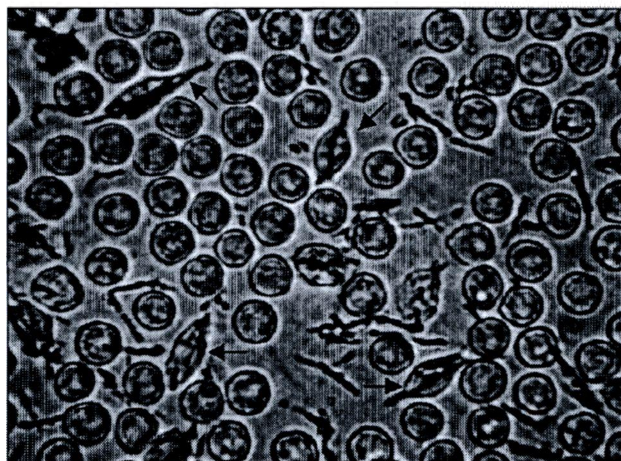

Figure 1. Visualization of neutrophil ingestion of MSU crystals. Cells (1.5×10^7 cells/ml) were stimulated with MSU crystals (1.5 mg/ml) at 37°C for 30 min. Cells were visualized under polarized light microscopy. Arrows indicate cells that have ingested MSU crystals.

maximal responses in calcium mobilization and tyrosine phosphorylation assays. These observations suggest that neutrophils exposed to MSU crystals produce activation mediators that stimulate surrounding cells.

To verify this hypothesis, we performed a chemotaxis assay using supernatants from neutrophils stimulated with MSU crystals (SMC). Neutrophils were incubated with MSU crystals for 15 to 60 min before being pelleted. The SMC were collected, filtered to eliminate free-floating cells and crystals, and then placed in the lower chamber of a ChemoTX plate for the chemotaxis assay carried out as described in Materials and Methods. There was significant chemotactic activity in response to SMC derived from incubation of neutrophils with MSU crystals for 15 min when compared to control supernatants obtained from unstimulated cells (Figure 2A). There was a slight increase of chemotactic potency in the SMC derived from incubation of neutrophils with MSU crystals for 30 to 60 min compared to SMC obtained from incubation of neutrophils with MSU crystals for 15 min. Diluting the SMC correlated with a reduced chemotactic activity (data not shown).

Calcium mobilization is involved in many signaling pathways and in the performance of multiple cellular functions. As such it is a hallmark of cell activation. Resting neutrophils loaded with Fura-2AM were stimulated with SMC obtained from incubation of neutrophils with MSU crystals for 15 to 60 min, and the concentrations of free intracellular calcium were then monitored as described in Materials and Methods. As shown in Figure 2B, SMC induced a rapid and transient mobilization of calcium in neutrophils. Generation of the calcium-mobilizing activity of SMC required as little as 15 min of incubation with MSU crystals and was not significantly increased by further incubation up to 60 min.

Preincubating cells with cycloheximide (20 μg/ml) for 30 min to block *de novo* protein synthesis before stimulating them with MSU crystals inhibited by only 20% the chemotactic response to supernatants stimulated with MSU crystals for 15 min (Figure 3A). The inhibitory effect of cycloheximide increased to 30% and 40% with supernatants from cells stimulated with MSU crystals for 30 to 60 min, respectively. It should be noted that the cycloheximide-resistant fraction of the chemotactic activity of the SMC remained constant over this time period.

We further characterized the activity of the SMC by examining whether it had the ability to affect the tyrosine phosphorylation pattern in human neutrophils. An increase in tyrosine phosphorylation in human neutrophils has been observed in response to many agonists and is closely associated with their activation status[18,32-35]. Supernatants from neutrophils stimulated by MSU crystals for as little as 5 min induced a rapid increase (within 30 s) in the pattern of tyrosine phosphorylation, with a prominent increase of the tyrosine phosphorylation of a 110–120 kDa band and minor increases in the 60–90 kDa region (Figure 3B). SMC derived from cycloheximide-treated cells was essentially as potent as SMC derived from untreated cells in its ability to stimulate a tyrosine phosphorylation response (Figure 3B). SMC also had the ability to stimulate the phosphorylation of Erk1/2 (Figure 3C).

The sensitivity of SMC to trypsin digestion was then tested. Since neutrophils contain antitrypsin activities in their granules and MSU crystals induce a significant degranulation response in neutrophils (data not shown), we boiled the SMC

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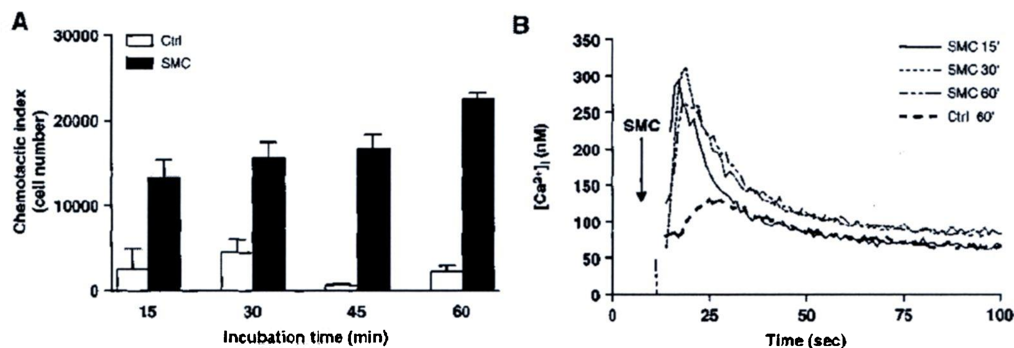

Figure 2. A. Chemotaxis and calcium mobilization induced by SMC from different incubation periods. Neutrophil migration was monitored with a ChemoTX disposable chemotaxis system. Polycarbonate filters were positioned on the plate, and neutrophils (30 μl, 60,000 cells/well) were placed on the filter and allowed to migrate in response to SMC from 15 to 60 min. Results are expressed as number of cells that penetrated the filters (n = 3), as described in Materials and Methods. B. Neutrophils (10^7 cells/ml) were loaded with 1 μM Fura-2/AM and stimulated at 5×10^6 cells/ml with 1 ml of SMC from 15–60 min incubation with MSU crystals. Data shown are representative of 3 independent experiments.

to inactivate the endogenous trypsin inhibitors, then incubated the SMC with or without trypsin (250 μg/ml) for 1 h at 37°C, before boiling the SMC again to denature the exogenous trypsin. The chemotactic activity of the SMC thus treated was then tested. The SMC treated with trypsin showed a drastically reduced chemotactic activity (data not shown). IL-8 chemotactic activity was also abrogated by trypsin digestion (data not shown).

We then submitted SMC to Centricon centrifugal filter separation using various cutoff sizes to get a rough indication of the molecular weight of the neutrophil-active factor(s). After centrifugal separation, we monitored the chemotactic activity of the flow-through and the excluded fractions. We found that the fraction above 30 kDa retained as much chemotactic activity as the starting material, while less than half the activity remained above 50 kDa and none above 100 kDa (data not shown).

Src kinases are known to be involved in phagocytosis as well as in chemotaxis[36–43]. The neutrophils incubated with MSU crystals in the presence of the potent Src kinase inhibitor PP2 retained a round shape and showed a marked reduction of MSU crystal ingestion (data not shown). Preincubating neutrophils with PP2 essentially abrogated the ability of SMC derived from these cells to induce a mobilization of calcium activity in naive neutrophils (Figure 4). It should be noted that the calcium-mobilizing activity of the SMC itself was insensitive to inhibition by PP2 (data not shown). Generation of neutrophil-stimulating activity in the SMC thus appears to be dependent on Src kinases.

The neutrophil secretes many chemotactic agents, including LTB_4, PAF, and IL-8. We next examined the potential contribution of the lipid mediators LTB_4 and PAF to the activity of the SMC. We used the LTB_4 and PAF receptor antagonists CP 105.696[44,45] and BN 50730[46], respectively. Preincubation of neutrophils with either of these 2 compounds (data not shown), or with both (Figure 5A) had no effect on the tyrosine phosphorylation response to the SMC. The activity of the receptor antagonists was verified by testing their effects on the responses to their respective lipid mediators (Figure 5B). We confirmed the lack of involvement of these 2 agonists in the activity of the SMC by observing that pyrrophenone, a cPLA2 inhibitor[47,48], had no effect on the tyrosine phosphorylation response induced by SMC and only marginally affected its chemotactic activity (data not shown). A blocking anti-IL-8 antibody was used next to determine the role of IL-8 in the activity of SMC. No significant inhibition of the chemotactic response to SMC was observed in the presence of anti-IL-8 antibodies, while the response to IL-8 was inhibited by this antibody (data not shown). The potential involvement of mitochondrial-derived formylated peptide or the granule protein cathepsin G, which has been identified as a novel chemotactic agonist that interacts with the formyl peptide receptor[49], was assessed by testing the inhibitory activity of cyclosporin H, a formyl peptide receptor-specific inhibitor[50]. We incubated neutrophils with cyclosporin H and found no inhibition of the chemotactic and mobilization of calcium responses to SMC, although the responses to fMLF were significantly decreased (Figures 5C, 5D, 5E, 5F).

Many chemotactic agents have receptors coupled to G-proteins[51]. We investigated the potential role of G-proteins in the response of human neutrophils to SMC by using pertussis toxin. The cells were incubated with 1 μg/ml pertussis toxin for 1 h, then their ability to respond to SMC, fMLF, and CD32a ligation was tested. Pertussis toxin drastically reduced the calcium responses to SMC as well as to fMLF, while it did not inhibit the calcium response to CD32a cross-linking (data not shown). These data indicate that pertussis toxin-sensitive G-proteins are involved in the mediation of responses to SMC.

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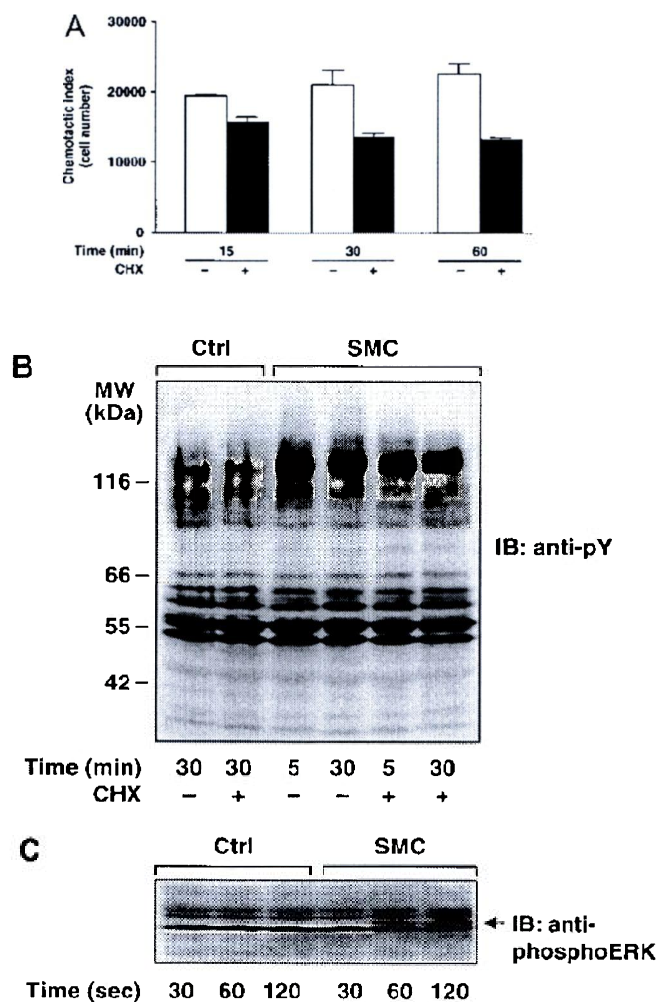

Figure 3. Production of SMC is independent of *de novo* protein synthesis. A. Cells (4×10^7 cells/ml) were preincubated with cycloheximide (CHX: 20 μg/ml) for 30 min before being stimulated with MSU crystals (3 mg/ml) for 15 to 60 min (as indicated). The SMC obtained were used in a chemotaxis assay as described in Materials and Methods (n = 3). B. Cells (4×10^7 cells/ml) were preincubated with CHX (20 μg/ml) for 30 min and stimulated with MSU crystals (3 mg/ml) for 5 to 30 min. The SMC obtained were used to stimulate naive neutrophils. Reactions were stopped after 30 s and samples were subjected to Western blot analysis (IB) with anti-phosphotyrosine (anti-pY) antibody. Data shown are representative of 3 independent experiments. C. Cells (4×10^7 cells/ml) were stimulated with MSU crystals (3 mg/ml) for 15 min. SMC obtained were used to stimulate naive cells. Reactions were stopped after 30, 60, or 120 s by transfer of a cellular aliquot to boiling sample buffer 2× and samples were subjected to Western blot analysis with anti-phosphoERK antibody. Control cells were stimulated with supernatant from unstimulated cells. Data shown are representative of 3 independent experiments.

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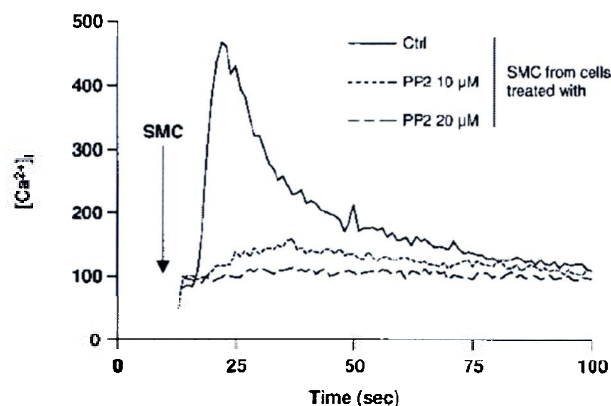

Figure 4. Calcium mobilization induced by SMC derived from PP2-treated neutrophils. Cells (10^7 cells/ml) were loaded with 1 μM Fura-2/AM as described in Materials and Methods and stimulated at 5×10^6 cells/ml with 1 ml of SMC derived from PP2 (10 and 20 μM) treated neutrophils stimulated with MSU crystals (3 mg/ml). Data shown are representative of 3 independent experiments.

Cross-desensitization experiments provided evidence that the responses of human neutrophils to SMC shared signaling pathways with those utilized by chemotactic factors. Neutrophils were first stimulated with IL-8 (10^{-7} M), LTB_4 (10^{-7} M), or fMLF (10^{-7} M) and 2 minutes later, with SMC. As can be seen in Figure 6, prior exposure to the chemotactic factors essentially abrogated the mobilization of calcium induced by SMC.

DISCUSSION

Although MSU crystals, the etiological agent of gout, are among the most potent proinflammatory stimuli, a complete account of the mechanisms underlying their phlogistic activity remains elusive. While direct effects of MSU crystals on, among others, neutrophils, monocytes/macrophages, fibroblasts, osteoblasts[52,53], and endothelial cells[54] have been described, significant gaps remain in our understanding of the inflammatory effects of MSU crystals. Our findings indicate that several of the previously reported effects of MSU crystals on human neutrophils may result, at least in part, from the indirect effects of a factor rapidly released from neutrophils exposed to MSU crystals that had previously escaped identification.

This investigation was prompted by our observation that only a small percentage of neutrophils ingested or adhered to MSU crystals within the timeframe in which most early responses to the crystals have been monitored, an observation in accord with the reported prevalence and kinetics of internalization of MSU crystals[23,24]. After stimulating neutrophils, we found that within a short time (5–15 min) a neutrophil-activating factor was clearly detectable in the supernatant of the cells. This activation factor induced an increase in tyrosine phosphorylation, stimulated the mobilization of calcium, and possessed chemotactic activity. It remains unknown whether the activities detected in the supernatants of MSU crystal-stimulated cells are due to one or more factors. Our observations favor the hypothesis of rapid (< 15 min) release of a prestored neutrophil-activating factor, followed in time (30–60 min) by that of a protein synthesis-dependent additional factor. The cycloheximide sensitivity of the increases in chemotactic activity of the SMC collected at 30 and 60 minutes, compared to SMC collected after 5–15 minutes of interaction between the cells and MSU crystals, supports this interpretation, although there was no corresponding increase in the ability of the 30–60-minute SMC to induce a mobilization of calcium or to stimulate a tyrosine phosphorylation response. Generation of SMC activity is dependent on active metabolic processes, as the Src kinase inhibitor PP2 inhibited the appearance of calcium-mobilizing activity in the SMC. On the other hand, its generation is insensitive to inhibition by colchicine (data not shown), although tyrosine phosphorylation induced by MSU crystals is inhibited by colchicine[19]. It is unclear whether the production of activity in SMC is a result of phagocytosis of MSU crystals or simply adherence to MSU crystals, although cytochalasin B (data not shown) and colchicine did not inhibit the production of SMC, which might indicate that adherence of MSU is sufficient.

Although presently unidentified, the activity detected in the supernatants of neutrophils exposed to MSU crystals shares several characteristics with classic chemotactic factors, including the pertussis toxin sensitivity of the responses to the SMC. These data correlate with indications that MSU crystals activate neutrophils through pertussis toxin-sensitive as well as insensitive pathways[55]. Calcium desensitization assays have revealed similarities and differences between the differ-

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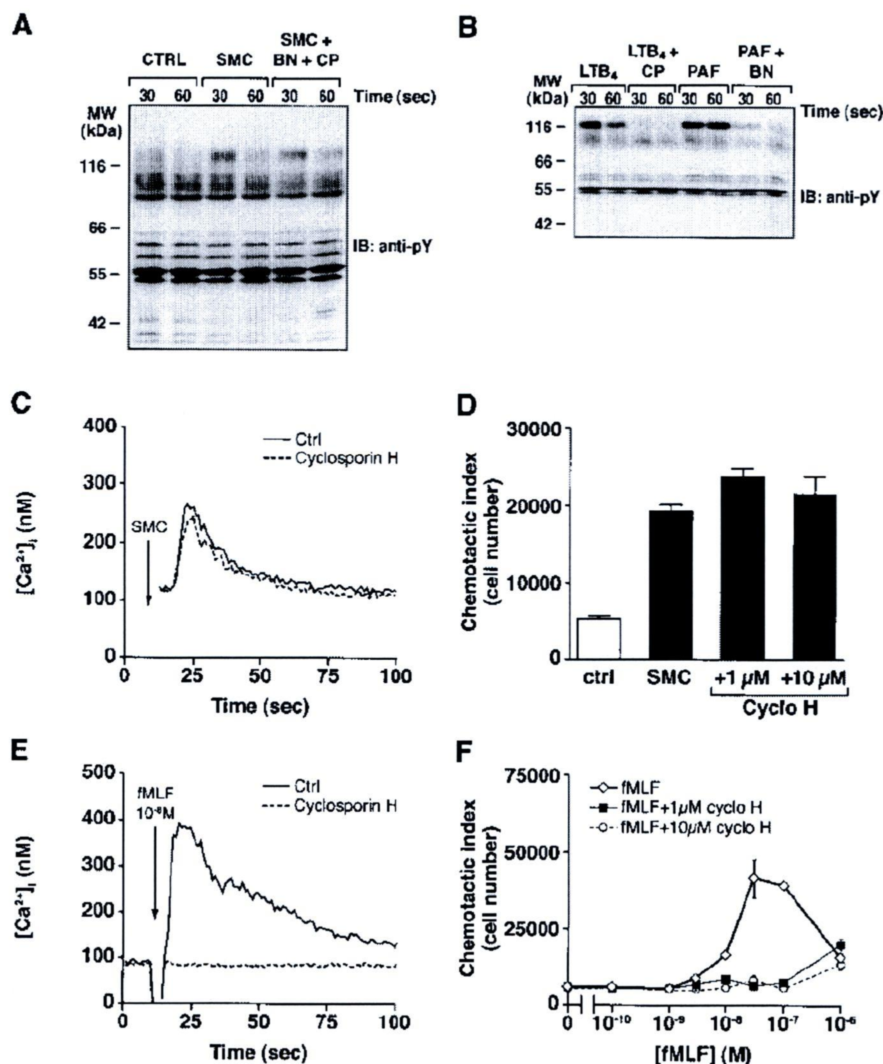

Figure 5. Effect of antagonists for LTB_4 (CP105.696) and PAF (BN 50730) receptors on the tyrosine phosphorylation induced by SMC: Cells were preincubat with CP (10^{-6} M) and BN (10^{-6} M) then resuspended in SMC (A), or stimulated with LTB_4 (10^{-7} M) or PAF (10^{-7} M) (B), as described in Materials and Method Samples were analyzed by Western blot (IB) with anti-phosphotyrosine antibody (anti-pY). Data shown are representative of 3 independent experiments. Effe of cyclosporin H (cyclo H) on chemotaxis and calcium mobilization induced by SMC: Cells were preincubated with cyclosporin H (1–10 μM) for 5 min, and mo itored for mobilization of calcium induced in response to SMC (C) or fMLF (10^{-8} M) (E), or the chemotactic responses to SMC (D) and fMLF (F). Cyclospor H was used at 10 μM in mobilization of calcium assays. HBSS was used as control in the chemotactic assay (D). Data shown are representative of 3 independe experiments.

ent agonists. fMLF and C5a desensitize the calcium responses to each other and to IL-8[56,57]. Desensitization of the calcium mobilization induced by SMC by preincubation with fMLF, IL-8, and LTB_4 indicates that chemotactic agents and SMC share common signaling pathways. This conclusion further supported by the pertussis toxin sensitivity and PP insensitivity of the calcium-mobilizing activities of chemc tactic factors and of SMC.

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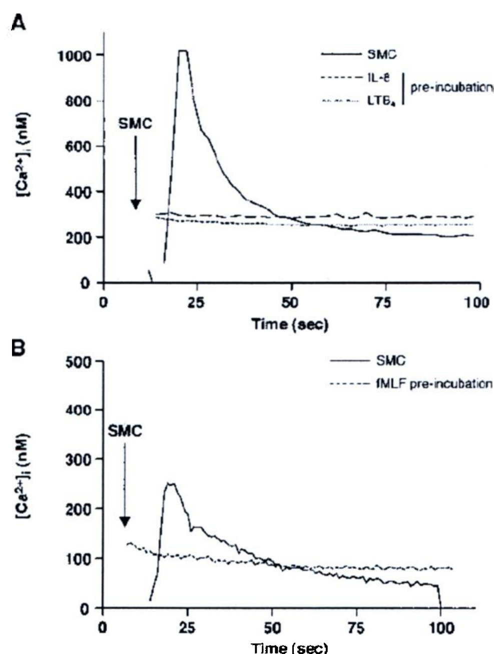

Figure 6. Cross-desensitization of the calcium mobilization response between IL-8, LTB_4, or fMLF and SMC. Neutrophils (10^7 cells/ml) were loaded with 1 μM Fura-2/AM as described in Materials and Methods, then stimulated with IL-8 (10^{-7} M) or LTB_4 (10^{-7} M) (A), or fMLF (10^{-7} M) (B) for 2 min before stimulation with 1 ml SMC (upper panel). Data shown are representative of 3 independent experiments.

Several inflammatory mediators are liberated by neutrophils in gouty arthritis. Lipid mediators[16,58], IL-1[14,15] and IL-8[59], oxygen radicals[60], S100A8/S100A9[61], and lysosomal enzymes[62] are all produced by neutrophils in response to MSU crystals. In gouty joints, there is also activation of complement[63]. Although many mediators have been identified in gouty arthritis, none of them, individually, is presently thought to be responsible for the bulk of the responses to MSU crystals. The lack of effect of the potent LTB_4 and PAF antagonists, of a cytosolic phospholipase A_2 inhibitor, and of an anti-IL-8 antibody on the activities of the SMC similarly indicates that the formation and secretion of these mediators do not account for the neutrophil-stimulating potential of the SMC.

It was also conceivable that the release of molecules such as formylated mitochondrial peptides following neutrophil lysis and necrosis may be responsible for the activity of SMC. However, previous studies have determined that very little neutrophil lysis was detected after interaction with MSU crystals[22,61]. Further, our data show that responses such as chemotaxis and calcium mobilization were not inhibited by incubation with cyclosporin H, an antagonist of the formyl peptide receptor[50], which eliminates endogenous formylated peptide signaling through the formyl peptide receptor as candidate for the activity in SMC. Preliminary data have shown that neutrophil degranulation can reproduce and desensitize the mobilization of calcium induced by the SMC. Cathepsin G, a granule protein, has previously been shown to be a chemoattractant for monocytes and to act through the formyl peptide receptor[49,64]. It could therefore have been responsible for the chemotactic activity of the SMC. However, cyclosporin H, which inhibits the activity of cathepsin G[49], did not affect the recruitment of neutrophils or the calcium mobilization induced by SMC. Studies are under way to characterize the role of granule components in the SMC.

The Centricon preparations are not reliable enough to give us a precise molecular weight, but they do nevertheless give some indications of the apparent size of the activation factor in the SMC. The chemotactic activity was mostly retained in the fractions above 30 kDa and below 50 kDa and none was found above 100 kDa. Lipid mediators, which have a very small molecular weight (< 1 kDa), are unlikely to be found above 30 kDa. Most obvious chemotactic agents have molecular weights averaging 10 kDa, including IL-8 (8–12 kDa, depending on its level of glycosylation) and S100 proteins. The activity in the SMC may derive from the association of multiple molecules into a complex that would not be separated under the native conditions used in our experiments.

A crystal chemotactic factor (CCF) with an estimated molecular weight of 15 kDa produced by neutrophils in response to MSU crystals has been described[25-27]. This factor is chemotactic for neutrophils and is produced after 1 hour[25-27], although shorter time periods were not discussed. CCF is not found preformed in neutrophils but is newly synthesized in response to MSU crystals[25]. Our own studies show some similarities between the activity observed and what has been associated with the CCF, although the activity was seen after only 5–15 minutes in the case of the SMC, instead of 1 hour as observed for CCF. The proposed molecular weight of CCF of 15 kDa does not correspond to our own indications of a molecular weight above 30 kDa. Further, the production of CCF was inhibited by cycloheximide and we found only a small inhibition of SMC production with cycloheximide within 15 minutes. Thus, it is unlikely that CCF accounts for a significant fraction of the activity of SMC.

The S100 family of proteins (also known as myeloid related proteins and calprotectin), particularly S100A8 and S100A9, are present in great quantities in neutrophils, are secreted in response to MSU crystals, and are chemotactic for neutrophils[3,61,65,66]. Human S100A12 showed strong chemotactic activity for monocytes and only weak chemotactic activity for neutrophils[67]. Several of our observations indicate that S100 proteins are not responsible for the neutrophil-activating properties of our SMC. The recovery of the activity of the SMC in fractions of Centricon separations larger than 30

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kDa argues against monomeric S100 proteins (with molecular weight < 10 kDa) being primarily responsible. The activity of the SMC was not inactivated by heat (data not shown), in contrast to that of recombinant S100A8/S100A9 proteins, which is lost upon heating[3]. The liberation of S100A8 and S100A9 induced by MSU crystals was inhibited by cytochalasin B[61], while generation of activity in the SMC was still observed in the presence of cytochalasin B (data not shown). Additionally, blocking antibodies for S100A8 and S100A9 did not inhibit the chemotactic responses or the mobilization of calcium observed in response to SMC (data not shown). Together, these results indicate that the activity observed in the SMC is unlikely to be due to S100A8, S100A9, or S100A8/S100A9.

Our study demonstrates that a chemotactic factor is released by neutrophils within 5 to 15 minutes of incubation with MSU crystals. This factor may be responsible for some of the neutrophil recruitment and activation in early phases of the inflammatory response in gout attacks. In addition to its chemotactic potential, the factor also elicits a mobilization of intracellular calcium and an increase in tyrosine phosphorylation in human neutrophils. These responses resemble those induced by classic chemotactic agents, a conclusion that is supported by the sensitivity of the responses to SMC to pertussis toxin. The liberation of an activation factor by neutrophils might serve to modulate neutrophil responses during the initial stage of inflammation, especially since this activation can be reproduced by inducing neutrophil degranulation. Special attention should be paid to determining the potential effects of SMC on inflammatory cells other than neutrophils. Further characterization and purification studies are under way to identify the factor and to determine its role in gout in particular and in inflammatory processes in general.

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Annexe 3 : Activation of human neutrophils by MSU crystals

Oana Popa-Nita and Paul Naccache

Cet article de review est en préparation pour être soumis à Trends in Immunology

As the primary phagocytic and microbe-killing cells of the innate immune system, neutrophils inform and shape immune responses, contribute to the repair of tissue as well as its breakdown, use killing mechanisms that enrich our concepts of specificity, and offer exciting opportunities for the treatment of neoplastic, auto-inflammatory and auto-immune disorders. Monosodium urate (MSU) crystals are among the most potent pro-inflammatory stimuli and an innate immune inflammatory response to the crystal surface is intimately involved in the pathology of gouty arthritis. The responses of human neutrophils to MSU crystals represent an integral part of this innate response and a key component of the acute inflammatory response associated with gout. Furthermore, uric acid has been shown to be an endogenous danger signal released from injured cells capable of stimulating the maturation of dendritic cells. Several extensive reviews of gout, its pathogenesis and clinical management have been published. The present review will focus on the current state of knowledge concerning the activation of human neutrophils by MSU crystals as recent data begin to draw a comprehensive picture of the events leading to the often excessive responses of neutrophils to these particulate agonists.

Preamble 1: Opsonization of MSU crystals

In vitro experiments with MSU crystals rely on their addition to cells and the monitoring of subsequent signalling events and functions. There is a longstanding, unresolved, question concerning the status of the crystals when they are added to cells which is made particularly acute as MSU crystals are known to adsorb on their surface various proteins: should the crystals be added naked or opsonized, and if the latter, opsonized with what? The answer to this question is unlikely to be simple or static since proteins coating MSU crystals change as inflammation evolves. IgGs (Ortiz-Bravo, Sieck et al. 1993), complement components (Terkeltaub, Tenner et al. 1983) or small polymeric size hyaluronate (Brandt 1974) coating MSU crystals may modify their interaction with target cells, facilitating their phagocytosis and, through the subsequent release of mediators, enhance the inflammatory response within the joint. As the inflammation subsides, Apolipoprotein B (Apo B) may displace these molecules competitively and thus contribute in part to the resolution of the acute gouty arthritis (Terkeltaub, Curtiss et al. 1984). Thus, a satisfactory resolution of the opsonisation question will require a detailed knowledge of the composition of the synovial fluids during an acute gouty crisis. In addition, the nature of the questions asked (e.g.,

initiation *vs* resolution of the inflammation) will dictate different experimental approaches and likely yield different answers.

Preamble 2: Priming of human neutrophils

Another question that is, for the moment, pushed aside and ignored, at least experimentally, concerns the state of the neutrophils that interact with MSU crystals. It is clear that, *in vivo*, the neutrophils that encounter MSU crystals are those that have migrated to the inflamed joint. These cells are likely to have a different phenotype than those in the circulation. It is important in this respect to point out that more and more evidence is accumulating in support of the plasticity of the neutrophils and of their ability, under the right circumstances, to change their functional characteristics (Colotta, Re et al. 1992; Fanger, Liu et al. 1997; Oehler, Majdic et al. 1998). The distinct pattern of responses of peripheral blood monocytes and of tissue macrophages to MSU crystals (Landis, Yagnik et al. 2002) provides a precedent for this line of reasoning. In vivo murine models for the study of MSU crystal-induced inflammation present the advantage of taking into account the priming of neutrophils although it should be mentioned that murine cells lack both of the Fcγ receptors expressed on human neutrophils (FcγRIIIB, FcγRIIA).

Molecular recognition of MSU crystals

The mechanisms underlying the recognition of MSU crystals by various immune cells and the role of these interactions in the development of the inflammatory response in gout have begun to be investigated. A number of complement components (including C1q, C1r and C1s) have been eluted from the surface of MSU crystals exposed to plasma (Terkeltaub, Tenner et al. 1983). Activation of complement pathways by MSU may contribute to the recruitment of neutrophils into the synovial cavity during an acute gouty attack. In accord with this hypothesis, complement activity was found to be increased in synovial fluid of acute gout (Pekin and Zvaifler 1964).

Several studies indicate that the activation of human neutrophils by MSU crystals is mediated to a significant extent by FcγRIIIB and the β2-integrin CD11b. Anti-FcγRIIIB antibodies inhibited the stimulation of tyrosine phosphorylation induced by MSU crystals, the mobilization of cytosolic Ca^{2+}, the activation of PLD, the production of superoxide anions (Barabe, Gilbert et al. 1998) as well as the release of the myeloid related proteins S100A8/A9 (Ryckman, Gilbert et al. 2004). Furthermore, an anti-CD11b antibody that interferes with the intra-membrane, lectin-type interaction

between FcγRIIIB and CD11b also inhibited the above responses to MSU crystals. In accord with these results, the interaction of naked MSU crystals with human platelets has also been shown to be mediated, at least in part, by their ability to interact with platelet membrane integrins (Jaques and Ginsberg 1982). The interactions of MSU crystals with FcγRIIIB/CD11b are likely to be opportunistic in nature. CD11b being, among others, a receptor for complement, it is intriguing to wonder whether it plays an even more pronounced role in the responses of human neutrophils to crystals coated with complement components. While these (opportunistic) interactions play a role in the responses of human neutrophils with MSU crystals, they do not provide a complete description of the molecular determinants involved in the recognition of the crystals by inflammatory cells. Only partial inhibition of the responses of human neutrophils to MSU crystals are obtained with the anti-FcγRIIIB or anti-CD11b antibodies. Furthermore, various other immune cells respond to MSU crystals even though they do not express one of these receptors (e.g. : monocytes and macrophages are devoid of FcγRIIIB). Finally, murine cells which lack both of these receptors also respond to MSU crystals.

Recent studies have shown that CD14 directly bound MSU crystals and was central for the capacity of ingested MSU crystals to induce IL-1β expression in bone marrow-derived murine macrophages (Scott, Ma et al. 2006). The pattern recognition molecule CD14 serves as a shared TLR2 and TLR4 adaptor molecule that increases TLR2- and TLR4-mediated functional responses to specific agonists, including peptidoglycan and LPS respectively (Fujihara, Muroi et al. 2003; Dziarski and Gupta 2005). Several lines of evidence indicate that MSU crystals induce NO release from bovine primary chondrocytes in a TLR-2-dependent manner (Liu-Bryan, Pritzker et al. 2005). In addition, it was also shown that the recognition of naked MSU crystals by TLR2 and TLR4 was critical for their capacities to induce phagocytosis and expression of pro-inflammatory cytokines *in vitro* in bone marrow-derived murine macrophages and acute inflammatory responses *in vivo* in mouse subcutaneous air pouches (Liu-Bryan, Scott et al. 2005). It is relevant in this respect to point out that CD11b/CD18 might also mediate MSU crystal-induced interactions between CD14 and TLR2 as in the case of phagocyte activation by certain bacterial products (Sendide, Reiner et al. 2005). It is thus conceivable that the role of CD14 in MSU crystal-induced inflammation is primarily *via* macromolecular complex formation with TLRs and integrins that are directly responsible for modulating cellular responsiveness to MSU crystals. It should be kept in

mind, however, that the applicability of these findings to the responses of human neutrophils to MSU crystals remains to be investigated. While these data provide intriguing evidence for the (opportunistic?) involvement of FcγRIIIB/CD11b and of the CD14/TLR2/TLR4 in the recognition of MSU crystals by human neutrophils, it is highly unlikely that they provide a complete catalogue of the surface molecular structures the latter interact with. Additional studies are clearly called for.

MSU crystal-induced neutrophil activation

The formation of MSU crystals into the joint cavity promotes an acute inflammation that include a massive recruitment of neutrophils migrating through activated endothelium. An essential role of neutrophils in acute gout is supported by the observations that experimental urate crystal-induced synovitis is markedly diminished when neutrophils are depleted by cytotoxic agents or by anti-neutrophil anti-serum (Phelps and McCarty 1966; Chang and Garalla 1968) and that therapeutic agents which modify neutrophil functions are dramatically effective in acute gout (Becker 1988). The contribution of neutrophils to the pathogenesis of acute gouty inflammation is believed to be partly caused by events that follow from the physical association of urate crystals and neutrophils (Terkeltaub and Ginsberg 1988). The results of early studies involving liposomes were interpreted as indicating that neutrophil activation by MSU crystals resulted from non-specific perturbation of the lipid bilayer of the cell membrane (Weissmann and Rita 1972). While this interpretation cannot be rigorously disproved, much evidence has since been obtained in support of the hypothesis that the interaction of MSU crystals with neutrophils shares many characteristics with that observed in response to classical neutrophil agonists. These include, among others, the observations of the activation of a variety of signalling pathways and the absence of loss of viability or non-specific permeabilisation of the plasma membrane (as evidenced by the lack of penetration of ethidium bromide or lactate dehydrogenase (LDH) release) as well as the evidence discussed above for an involvement of CD16b-CD11b/CD18 and/or TLRs in the mediation of the responses of neutrophils to MSU crystals.

Among the most characteristic responses of human neutrophils to MSU crystals is the stimulation of the tyrosine phosphorylation pathway. This is reflected in a robust pattern of tyrosine phosphorylation and associated with the stimulation of the activity of multiple tyrosine kinases including Lyn (Gaudry, Gilbert et al. 1995), Syk (Desaulniers, Fernandes et al. 2001) and Tec (Popa-Nita, Marois et al. 2008). Results from our

laboratory suggested, on the basis of the characteristics of the observed patterns of tyrosine phosphorylation, that this response was specific to MSU crystals and relevant to their phlogistic properties (Gaudry, Roberge et al. 1993).

The regulation of the levels of MSU crystal-induced tyrosine phosphorylation in human neutrophils is most likely the result of the coordinated actions of tyrosine kinases and phosphatases. Data from our laboratory showed that the activation of Src-related tyrosine kinase Lyn was one of the earliest steps in the MSU crystal-induced sequence of intracellular signaling events (Gaudry, Gilbert et al. 1995). The activation of Syk plays a central role in neutrophil activation as evidenced by the inhibitory effect of piceatannol, a Syk-specific inhibitor, on MSU crystal-induced mobilization of intracellular calcium, stimulation of phospholipase D (PLD) activation, the production of superoxide anions and the morphological changes in neutrophils characteristic of crystal internalization (Desaulniers, Fernandes et al. 2001). We have recently shown that Syk also mediates the MSU crystal-induced activation of class Ia PI3Ks (Popa-Nita, Rollet-Labelle et al. 2007). The PI3K activity associated with immunoprecipitates of the class Ia PI3K regulatory p85 subunit was increased in MSU crystal-stimulated neutrophils and this, as well as the MSU crystal-induced phosphorylation of Akt, was inhibited by piceatannol. These results are in accord with previous studies showing that the PI3K activity was increased in anti-phospho-tyrosine immunoprecipitates from opsonized MSU crystal-stimulated neutrophils (Jackson, Lauener et al. 1997). We also have shown that PI3Ks were involved in signal transduction pathways leading to the activation of PLD and the degranulation response induced by MSU crystals (Popa-Nita, Rollet-Labelle et al. 2007).

PI(3,4,5)P3, the major product of the action of PI3Ks, is thought to act as a membrane anchor recruiting regulatory proteins to the plasma membrane *via* their PH domains (Hannigan, Huang et al. 2004). In accordance with this model, studies from our laboratory indicate that MSU crystals activate the PH domain-containing tyrosine kinase Tec in human neutrophils in a Src-dependent manner. Furthermore, two distinct Tec inhibitors, LFM-A13 and A771726 significantly diminished the MSU crystal-induced secretion of IL-8 and IL-1β (Popa-Nita, Marois et al. 2008) which have been shown to play pivotal roles in gouty inflammation (Terkeltaub, Baird et al. 1998; Martinon, Petrilli et al. 2006).

Very little is known about the involvement of tyrosine phosphatases in the responses of human neutrophils. Ongoing studies in our laboratory indicate that the cytosolic SH2-

containing tyrosine phosphatase SHP-1 may be responsive to stimulation by MSU crystals in human neutrophils. Our preliminary data indicate that stimulation of human neutrophils by MSU crystals leads to a rapid protein kinase C-dependent phosphorylation of SHP-1 on S591 (Popa-Nita and Naccache, unpublished observations). The phosphorylation of SHP-1 on S591 is associated with a decreased tyrosine phosphatase activity which is likely to contribute to the enhancement of the tyrosine phosphorylation response to MSU crystals. Besides PI3K, phospholipase C (PLC)γ2 is also activated in neutrophils stimulated with MSU crystals and it is involved in signal transduction pathways leading to degranulation responses (Jackon, Tudan et al. 2000).

Phospholipase enzymes are the rate-limiting step in production of eicosanoids (Flower and Blackwell 1976) which have been implicated as important soluble mediators of inflammatory responses in gout (Rae, Davidson et al. 1982). Moreover, it has been reported that a positive regulator of phospholipase A2 (PLA_2) could be isolated from synovial fluid of patients with rheumatoid arthritis (Bomalaski, Fallon et al. 1990). In accordance with the key role of the interaction between human neutrophils and MSU crystals in promoting the gouty inflammation, it has also been shown that MSU crystals stimulated PLA_2 enzyme activities in human neutrophils (Bomalaski, Baker et al. 1990). The stimulation of the activity of PLA_2 leads, among others, to the synthesis of leukotriene B4 (Serhan, Lundberg et al. 1984; Poubelle, De Medicis et al. 1987), a potent chemo-attractant and of prostaglandin E2 (Gilbert, Poubelle et al. 2003), an eicosanoid with potential pro-inflammatory role.

Activation of protein kinase C (PKC) (Naccache, Grimard et al. 1991; Marcil, Harbour et al. 1999) and of Src family kinases (Gaudry, Gilbert et al. 1995) is among a variety of the known crystal-induced upstream signals potentially able to promote rapid activation of extracellular signal-regulated kinase 1 (Erk1) and 2 (Erk2) and MAPK activation has indeed been documented in certain cell types in the context of crystal-induced signal transduction. MSU crystals induced the activation of Erk1/Erk2, c-Jun N-terminal kinase (JNK) and p38 MAPK pathways in THP-1 monocytic cell lines. MSU crystals also induced the activation of nuclear factor kB (NF-kB), including the NF-kB complex c-Rel/RelA, and activator protein-1 (AP-1) in mononuclear phagocytes (Liu, O'Connell et al. 2000). It should be pointed out that the effects of MSU crystals on transcription factors in human neutrophils remain to be investigated.

Assembly and activation of the inflammasome is an essential process in innate immune defence (Ogura, Sutterwala et al. 2006). The inflammasome is a cytosolic, multiprotein platform that allows activation of caspase-1 which then cleaves the precursor of interleukin-1β (IL-1β) into the active form, the secretion of which leads to a potent inflammatory response (Martinon, Burns et al. 2002). Upon activation, the inflammasome is formed by a member of the NALP protein family, such as NALP1, NALP2 or NALP3, and the adapter protein ASC that connects the NALPs with caspase-1 (Tschopp, Martinon et al. 2003). Recently, it has been shown that, in differentiated monocytic THP-1 cell lines incubated with MSU crystals, the NALP3 inflammasome was activated in a colchicine-dependent manner (Martinon, Petrilli et al. 2006). This observation was validated in an *in vivo* model of crystal-induced peritonitis in inflammasome-deficient mice in which an impaired neutrophil influx was monitored. Further studies are required in order to document the MSU crystal-induced activation of the NALP3 inflammasome in human neutrophils, the major cell type recruited in the the synovial cavity during an acute gouty attack.

Functional responses of human neutrophils to MSU crystals

The activation of human neutrophils by MSU crystals leads to the production and secretion of pro-inflammatory products such as lysosomal enzymes (Ginsberg, Kozin et al. 1977), oxygen-derived free radicals (Abramson, Hoffstein et al. 1982), eicosanoids (Serhan, Lundberg et al. 1984), myeloid-related proteins (MRPs) S100A8/A9 (Ryckman, Gilbert et al. 2004), interleukin-1 (IL-1) (Roberge, de Medicis et al. 1994), IL-8 (Hachicha, Naccache et al. 1995), unidentified factors (crystal-induced chemotactic factor (Spilberg, Mandell et al. 1974) and others (Desaulniers, Marois et al. 2006)) and immediate production of prostaglandin E2 (PGE2) (Gilbert, Poubelle et al. 2003).

Very few studies describe the ingestion of MSU crystals by human neutrophils. However, it has been shown that small MSU crystals (averaging 2 µm in length) were ingested more avidly that large MSU crystals (averaging 15 µm in length) by human neutrophils (Schumacher, Fishbein et al. 1975). Upon ingestion by synovial fluid neutrophils, MSU crystals lied within phagosomes. After fusion of lysosomal membrane, there was a crystal-membrane interaction that resulted in disruption and lysis of the phagolysosomal membrane, internal release of lysosomal contents and cell death (Shirahama and Cohen 1974). This mechanism of crystal-induced cytolysis has been termed "rupture from within" hypothesis. On the other hand, the lifetime of

neutrophils which did not ingest any crystal was shown to be prolonged by the direct contact with MSU crystals which inhibited their TNF-α-induced or spontaneous apoptosis (Tudan, Jackson et al. 2000). These two events (release of lysosomal contents and delayed apoptosis) are likely to be responsible, at least in part, of the damage occurring in the tissues surrounding the inflamed gouty joint.

Myeloid related proteins, S100A8 and S100A9, are small, calcium-binding proteins highly expressed in the neutrophil cytosol which play a key role in the inflammatory process (Roth, Vogl et al. 2003). S100A8/A9 were shown to be essential for neutrophil recruitment during MSU crystal-induced inflammatory reaction (Ryckman, McColl et al. 2003) and high levels of S100A8/A9 detected in synovial fluids of patients with gout further indicated a role for these molecules in the pathogenesis of gouty arthritis. Moreover, it was also reported that MSU crystals can induce the release of S100A8/A9 from neutrophils, and the tyrosine kinases of Src and Syk family were involved in mediating this effect (Ryckman, Gilbert et al. 2004).

Early studies suggested that the interaction of neutrophils with urate crystals leads to the appearance of a chemotactic factor in the lysosomal fraction of the cell (Spilberg, Mandell et al. 1974). The secretion of this chemotactic factor of approximately 8.4 kDa appeared dependent on new protein synthesis. On the other hand, recent studies from our laboratory determined the presence and characterized the activity of a soluble chemotactic factor rapidly (within 15 min) released by human neutrophils after stimulation with MSU crystals (Desaulniers, Marois et al. 2006). Generation of this activation factor with molecular weight comprised between 30 and 50 kDa was independent of *de novo* protein synthesis.

Early vasodilatation, erythema and pain are characteristic of joints with acute gouty arthritis, and these manifestations are likely mediated, at least in part, by kinins, complement-derived peptides, histamine and vasoactive prostaglandins (PGs). Prostaglandins are found in the synovial fluid and the presence of PGE_2 in crystal-induced inflammation has been documented (Wigley, Fine et al. 1983). However, PGE_2 may also have antiinflammatory effects (Weissmann, Smolen et al. 1980). Results from our laboratory established that the stimulation of human neutrophils by MSU crystals leads to a very rapid synthesis of PGE_2 which was not dependent on *de novo* synthesis of COX-2 (Gilbert, Poubelle et al. 2003). Further studies should be undertaken in order to establish the role of PGE_2 in the pathology of gouty arthritis.

Previous studies from our laboratory have shown the ability of MSU crystals to induce IL-1 synthesis and release by human neutrophils (Roberge, Grassi et al. 1991). The IL-1 secretion was partly inhibited by colchicine, a clinically specific and effective drug in the treatment of acute gout. This argues, once again, in favour of the critical role of neutrophil/MSU crystals interaction in amplifying the acute gouty arthritis condition since IL-1 was recently proven to be essential in mediating the MSU crystal-induced inflammatory responses (Chen, Shi et al. 2006).

IL-8, a potent neutrophil chemoattractant, plays a central role in many forms of neutrophil-mediated inflammation (reviewed in (Mukaida 2003)). Functionally activated IL-8 was shown to be generally abundant in the synovial fluid of patients with acute gout (Terkeltaub, Zachariae et al. 1991). Furthermore, a critical role of IL-8 in neutrophil ingress in experimental models of acute gouty arthritis *in vivo* has been also documented (Terkeltaub, Baird et al. 1998). In our laboratory, MSU crystals were previously shown to increase the secretion of IL-8 by human neutrophils in a dose- and time-dependent manner (Hachicha, Naccache et al. 1995). This observation suggests that not only neutrophils recruited to the synovial cavity during a gouty attack will be activated when exposed to inflammatory microcrystals, but also that neutrophils will significantly contribute to their own recruitment because of their ability to release high levels of IL-8.

Spontaneous resolution of acute gouty arthritis

One of the characteristic features of acute gouty arthritis is its self-limiting course, since this arthritis generally subsides spontaneously after 1 week, even in the absence of any treatment (Schumacher 1996). A number of possible explanations for this spontaneous improvement have been postulated. They include crystal coating with protective proteins such as apolipoproteins B and E; monocyte-macrophage switch resulting in production of antiinflammatory cytokines such as transforming growth factor (TGF)-β, IL-10, and peroxisome proliferator-activated receptor (PPAR)-γ and clearance of apoptotic cells (reviewed in (Choi, Mount et al. 2005)).

TGF-β is a major anti-inflammatory cytokine with the ability to block endothelial cell activation, leukocyte adhesion and recruitment, to reduce pro-inflammatory cytokine production, and to increase IL-1ra production (Landis, Yagnik et al. 2002). Degranulation is an important property of neutrophils that fulfils two vital roles in innate immunity. It releases the microbicidal contents of neutrophil granules and adds

adhesion molecules to the surface of the plasma membrane (Sengelov, Follin et al. 1995; Videm and Strand 2004). On the other hand, tissue-degrading enzymes and inflammatory cytokines are also secreted, exacerbating conditions such as arthritis (Faurschou and Borregaard 2003). TGF-β might act to moderate the ability of neutrophils to provoke inflammation within tissues since it has recently been shown that TGF-β inhibited the human neutrophil degranulation irrespective of whether neutrophils were stimulated with ligands of TLR-2, TLR-4 or FPR (the G-protein-coupled receptor for formylated peptides) (Shen, Smith et al. 2007).

It has long been recognized that hyperuricaemia does not inevitably lead to acute gout. In patients with gout, MSU crystals are readily detectable in asymptomatic joints and during the inter-critical phase. In contrast with joints of patients with acute gout, which have intense neutrophilic infiltration in the synovium, asymptomatic joints of patients with gout have a predominantly mononuclear cell infiltrate (Louthrenoo, Sieck et al. 1991). Moreover, crystals can be detected within the cytoplasm of mononuclear cells in the absence of symptomatic gout (Pascual and Jovani 1995). These observations suggest that cells of the monocyte/macrophage lineage might play a beneficial role in maintaining asymptomatic hyperuricaemia. A beneficial role of monocyte/macrophages, however, is difficult to reconcile with the fact that monocytes, when challenged with urate crystals, secrete tumour necrosis factor α (TNF-α) (di Giovine, Malawista et al. 1991), interleukin 1β (IL-1β) (Di Giovine, Malawista et al. 1987), IL-6 (Guerne, Terkeltaub et al. 1989) and IL-8 (Terkeltaub, Zachariae et al. 1991). This activates expression of endothelial cell adhesion molecules (E-selectin, intracellular adhesion molecule 1, vascular cell adhesion molecule 1), which leads to secondary neutrophil recruitment to sites of crystal deposition (Chapman, Yarwood et al. 1997). But, on the other hand, a study of mouse monocyte/macrophage cell lines showed that incompletely differentiated monocytic cell lines synthesized TNF-α and activated endothelial cells upon challenge with urate crystals, whereas well-differentiated macrophage cell lines did not (Yagnik, Hillyer et al. 2000). Studies using an *in vitro* model of acute gout showed that human macrophages, obtained by differentiation of peripheral blood monocytes for a minimum of 3-5 days in the presence of autologous serum, did not secrete TNF-α, IL-1β, IL-6 or any other factors capable of inducing endothelial cell E-selectin expression or promoting secondary neutrophil recruitment. In contrast, freshly isolated monocytes from the same donor challenged with MSU crystals secreted these pro-inflammatory cytokines, induced E-selectin expression, and promoted rolling and

adhesion of neutrophils on HUVECs (Landis, Yagnik et al. 2002). These data strongly suggest that the process of macrophage differentiation may break the cycle of inflammation triggered by MSU crystals, preventing pro-inflammatory cytokine synthesis, endothelial cell activation, and secondary neutrophil recruitment.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily and acts as a ligand-dependent transcription factor by forming heterodimers with the retinoid X receptor (RXR) (Kersten, Desvergne et al. 2000). PPARγ is primarily found in adipose tissue and is well characterized as a regulator of various genes related to lipid and glucose metabolism. It has also been showed that this receptor was expressed in a wide variety of other cell types including monocytes and macrophages. Increased expression of PPARγ has been documented at sites of inflammation in arthritis (Kawahito, Kondo et al. 2000) and it was suggested that PPARγ might function as a negative regulator of inflammatory responses since its ligands were capable of reducing the expression of genes for cytokines (TNF-α, IL-6 and IL-1β), iNOS, gelatinase B, scavenger receptor A and COX-2 in activated macrophages (Ricote, Huang et al. 1999). Recent studies showed that MSU crystals could potently stimulate the expression of a functional PPARγ in monocytes from a mouse air-pouch model of MSU crystal-induced inflammation suggesting that PPARγ may also contribute to the spontaneous resolution of MSU crystal-induced acute inflammation (Akahoshi, Namai et al. 2003).

Among the molecules that could favour the resolution of acute gouty arthritis, natural and synthetic melanocortins were suggested to be endogenous down-regulators of the inflammatory process (Lipton and Catania 1997). ACTH1-39 is the prototype of melanocortins which are naturally occurring hormones derived from a larger precursor molecule known as the pro-opiomelanocortin gene product (O'Donohue and Dorsa 1982). A controlled clinical trial showed the efficacy of the intramuscular administration of ACTH1-39 for the treatment of human gout (Ritter, Kerr et al. 1994). More precisely, it has been also showed in a murine experimental model of gouty arthritis that the systemic administration of ACTH1-39 produced a dose-dependent reduction of several parameters of MSU crystal-induced joint inflammation by activating the melanocortin type 3 receptor (MC3-R) expressed on resident knee joint macrophages (Getting, Christian et al. 2002). The central role of the seven transmembrane G-protein-coupled MC3-R in regulating the anti-inflammatory activity of melanocortins was confirmed by recent studies using a selective MC3-R ligand and

MC3-R deficient mice (Getting, Lam et al. 2006) therefore justifying potential innovative antiinflammatory drug discovery research centered on MC3-R.

Human neutrophils derived from acutely inflamed joints and undergoing morphological and chromatin fragmentation changes of programmed cell death are recognized by macrophages at a stage when their cell membrane still appears structurally and functionally intact (Savill, Wyllie et al. 1989). Clearance of apoptotic neutrophils by macrophages contributes to resolution of inflammation in two ways. First, it prevents the release of cytolytic intracellular molecules that can exacerbate the inflammatory response and tissue injury (Savill, Wyllie et al. 1989). Second, it triggers the release of anti-inflammatory cytokines such as TGF-β and IL-10 (Landis, Yagnik et al. 2002).

Role of microtubules. Opportunities for the identification of the specificity of the responses of human neutrophils to MSU crystals.

Colchicine, originally isolated from the autumn crocus, was first used to treat rheumatic complaints and especially gout as early as 500 BC. Colchicine is a tricyclic alkaloid that binds to both α and β monomers of tubulin. Following this interaction, colchicine induces a conformational change which prevents curved tubulin from adopting the straight structure needed for the assembly with other tubulin monomers in order to form whole microtubules, thereby destabilizing the polymerization of microtubules (Margolis and Wilson 1977). Its present medicinal use is mainly in the treatment of gout. It is also used in a limited number of other clinical settings including Familial Mediterranean Fever (Zemer, Pras et al. 1976), Behçet's disease (Matsumura and Mizushima 1975), amyloidosis (Franklin 1975), scleroderma (Housset 1964) and recurrent pericarditis (Markel, Imazio et al. 2008). Furthermore, colchicine is also being investigated for its potential use as an anti-cancer drug due to its anti-mitotic effects (Bane, Ravindra et al. 2007). The partial clinical specificity of colchicine in the treatment of gout has prompted investigations of its effects on the signalling pathways activated by MSU crystals in human neutrophils. The expectation was that the clinical specificity may translate into the identification of events or pathways critically implicated in crystal-induced inflammation.

Colchicine affects many neutrophil functions including motility (Phelps 1969), adhesion, phagocytosis (Dallaverde, Fan et al. 1982) and release of LTB_4 (Serhan, Lundberg et al. 1984), IL-8 (Matsukawa, Yoshimura et al. 1998), and IL-1β (Roberge, Grassi et al. 1991). The mechanisms by which neutrophil functions are modulated by

microtubules remain to be defined. Colchicine has been shown to inhibit the synthesis of certain membrane lipids (Pike, Kredich et al. 1980). Specific arrangements of membrane lipids may be required for the activation of signaling pathways and for certain functions. Perturbations of this organization may also alter cytoskeletal arrangements. It may thus be hypothetised that neutrophils might require a specific relationship between lipids in the membrane and membrane-associated and cytosolic enzymes for optimal responses to MSU crystals. While these effects of colchicine may be related to its anti-phlogistic activities, they did not, by themselves, provide direct hints as to the specific intracellular event(s) it affected.

A first hint that the above expectation could be substantiated came from studies in which the effects of colchicine on the tyrosine phosphorylation responses of human neutrophils to MSU crystals was examined. As previously reported, MSU crystals augment the levels of protein tyrosine phosphorylation in neutrophils in a very characteristic fashion (Gaudry, Roberge et al. 1993). Apart from its unique morphological signature, the profile of tyrosine phosphorylation detected in crystal-stimulated neutrophils differs from that of all other neutrophil stimuli tested, soluble or particulate, in that it was inbibited by colchicine and other microtubule disruptive molecules (Roberge, Gaudry et al. 1993; Roberge, Gaudry et al. 1996). Furthermore, we have also observed that the mobilization of calcium induced by MSU crystals in human neutrophils, as opposed to that induced by chemotactic factors, was also exquisitely sensitive to inhibition by colchicine (Naccache, Grimard et al. 1991).

The MSU crystal stimulated mobilization of the intracellular calcium is also sensitive to tyrosine kinase inhibitors (Burt, Jackson et al. 1993) suggesting that it is likely to be mediated by PLCγ. Indeed, it has already been shown that PLCγ plays a central role in signal transduction pathways leading to respiratory burst and degranulation responses in neutrophils activated by inflammatory crystals and that $PLC_{\gamma2}$ may be the isoform involved in these pathways (Jackon, Tudan et al. 2000). In agreement with these results, we have observed that $PLC_{\gamma2}$ is tyrosine phosphorylated in response to MSU crystals in human crystals (unpublished observations). The specific tyrosine kinases involved remain to be identified although the implication of Src family kinases is highly probable. Src kinases are central to the signaling pathways activated by MSU crystals in human neutrophils as shown by the significant inhibition of neutrophil responses when cells are treated with PP2 (a Src kinase family inhibitor) prior to stimulation (Ryckman, Gilbert et al. 2004)(Popa-Nita, Marois et al. 2008)(our unpublished observations). On

the other hand, Src kinases are well-known for their ability to phosphorylate tubulin and associations between Src kinases and microtubules have already been reported in other cellular systems (Matten, Aubry et al. 1990; Lee, Newman et al. 1998; Draberova, Draberova et al. 1999). By inhibiting the polymerization of microtubules, colchicine might thus prevent the MSU crystal-induced activation of members of the Src family. Taken together, these various observations suggest that one of the earlier steps that follows the interaction of human neutrophils with MSU crystals implicates a microtubule-associated Src kinase family member that plays a key role in phosphorylating $PLC_{\gamma 2}$, a critical event in the initiation of the inflammatory program thereby initiated. The implication of the above observations remain to be experimentally pursued.

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