Innate cell recognition of Lipopolysaccharide and other bacterial molecules

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Innate Cell Recognition of Lipopolysaccharide and Other Bacterial Molecules

THÈSE

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LIST OF ABBREVIATIONS

18W	18-wheeler
aa	amino acid
ACTH	adrenocorticotropic hormone
ARDS	acute respiratory distress syndrome
BLAST	basic local alignment search tool
BPI	bactericidal/permeability-increasing protein
CD	leukocyte cluster differentiation antigen
cDNA	complementary DNA
CETP	cholesterol ester transfer protein
CHO	chinese hamster ovary
CR	complement receptor
DAF	decay accelerating factor
EC	endothelial cell
ELAM-1	endothelial-leukocyte adhesion molecule-1
ERK	extracellular signal-regulated kinase
ESCIT	evolutionarily conserved signaling intermediate in Toll pathways
EST	Expressed-Sequence Tag
FACS	flow analysis cytometry sorting
GCK	germinal center kinase
GDF	growth differentiation factor
GM-CSF	granulocyte macrophage-colony stimulating factor
GPI	glycosylphosphatidylinositol
HDL	high density lipoproteins
HEK	human embryonic kidney
HRP	horseradish peroxidase
HSP	heat shock protein
HUVEC	human umbilical vein endothelial cell
ICAM-1	intracellular adhesion molecule-1
IFN	interferon
IKK	inhibitor-κB kinase
IL	interleukin

IRAK	IL-1 receptor-associated kinase
I-κB	inhibitor-kappa B
JNK	c-Jun N-terminal kinase
Kd	dissociation constant
kDa	kilodalton
Kdo	3-deoxy-D-manno-octulosonic acids
LAM	lipoarabinomannan
LAP	LPS/LTA associated protein
LBP	lipopolysaccharide-binding protein
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LRR	leucine rich repeats
LTA	lipoteichoic acids
M	molaire
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
mCD14	membrane CD14
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage-colony stimulating factor
MDP	muramyl dipeptide
MEK	MAPK ERK kinase
MEKK	MAPK ERK kinase kinase
mRNA	messenger RNA
NF-κB	nuclear factor-kappa B
NIK	NF-κB inducing kinase
NK	natural killer
NO	nitric oxide
PAF	platelet activating factor
PAK	p-21 activated kinase
PDGF	platelet-derived growth factor
PI3K	phosphatidyl inositol 3 kinase
PKC	protein kinase C

PKR	protein kinase RNA-regulated
PLTP	phospholipid transfer protein
PNH	paroxysmal nocturnal hemoglobinuria
SAPK	stress-activated protein kinase
sCD14	soluble CD14
SIRS	systemic inflammatory response syndrome
SR	scavenger receptor
STEM	surface tubules for entry into macrophage
TAK1	transforming growth factor β-associated kinase 1
TIR domain	Toll/IL-1 receptor signaling domain
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF-α receptor-associated factor
VCAM-1	vascular cell adhesion molecule-1
VLDL	multivalent β-very-low-density lipoprotein

1 RESUME EN FRANÇAIS

L'immunité innée est un système immunitaire ancestral qui constitue une première ligne de défense lorsque des microbes ou des particules étrangères pénètrent dans l'organisme. Afin de neutraliser et d'éliminer ces « envahisseurs », deux principales stratégies ont été développées chez les mammifères : une voie soluble et une voie utilisant certains récepteurs exprimés à la membrane des monocytes/macrophages, des neutrophiles, des cellules endothéliales et autres.

La première voie implique des protéines solubles comme les protéines de liaison du mannose, des lipopolysaccharides, les protéines amyloïde sérique et C-réactive, les protéines du complément ainsi que certaines enzymes (lysozymes). Ces molécules sont spécialisées dans la reconnaissance de divers carbohydrates et lipides présents dans les microorganismes. Cette capacité de reconnaissance rend ces protéines efficaces pour attaquer et tuer les organismes pathogènes. Ce processus de neutralisation et de destruction se déroule de manière directe en induisant l'opsonisation de ces microbes par les cellules phagocytaires ; ou indirecte, en les présentant à d'autres cellules.

La seconde voie est caractérisée par des récepteurs (CD14, scavengers, mannose) présents à la surface de diverses cellules, capables de détecter spécifiquement les molécules étrangères. Après reconnaissance, les récepteurs internalisent ces molécules ou microbes à l'intérieur des cellules, qui les détruisent et les éliminent de l'organisme. En plus de cette fonction de clairance, certains récepteurs comme le CD14 peuvent médier l'activation des cellules après avoir lié des ligands bactériens. En effet, l'endotoxine ou LPS (lipopolysaccharide) détachée de la membrane externe des bactéries Gram-négatif a été proposé comme étant l'un des activateurs cellulaires les plus puissants au travers du récepteur CD14. La molécule de LPS se compose principalement d'une chaine polysaccharidique et d'une partie lipidique. La chaine polysaccharidique constitue la partie immunogénique de la molécule et peut varier en longueur, ce qui caractérise le sérotype de chaque bactérie Gram-négatif. La partie lipidique nommée « lipid A » correspond à la portion cytotoxique du LPS reconnue par la LBP, une protéine de la phase aigue présente dans le plasma, ainsi que par le récepteur CD14. Ce dernier est ancré par une ancre glycosylphosphatidyl inositol (GPI) au niveau du feuillet externe de la double couche lipidique de la membrane plasmique. Il y a plus

de 10 ans, le CD14 a été découvert comme étant un récepteur capable de médier les effets du LPS sur les cellules de mammifères. Le transfert du LPS sur le CD14 s'effectue par le biais de la LBP capable de monomériser le LPS à partir d'agrégats de molécules LPS. Après la liaison du LPS au CD14, les cellules sont activées et le LPS est internalisé dans les cellules afin d'être détoxifié, puis éliminé. Il existe aussi une forme soluble du CD14 (sCD14) présente dans le plasma et les fluides biologiques. Ce CD14 soluble est également capable de lier le LPS avec l'aide de la LBP. Ainsi, le complexe LPS-sCD14 peut activer des cellules dépourvues de CD14 membranaire en se liant probablement à un co-récepteur présent à la surface de la cellule. La principale voie activée est celle de NF-κB. Ce facteur de transcription est retenu dans le cytoplasme par un inhibiteur naturel le I-κB qui se lie au NF-κB. Lorsque la voie des I-κB kinases est activée, I-κB est phosphorylé et dégradé par le système ubiquitine/protéasome. Le NF-κB libéré peut ainsi se diriger à l'intérieur du noyau et se fixer sur les sites de liaison NF-κB des promoteurs de gènes codant pour des médiateurs impliqués dans le processus inflammatoire. Dans les monocytes/macrophages, la voie des « mitogen-activated protein kinase » (MAPK) est également activée par le LPS. Cette voie de signalisation intracellulaire inclut les voies des kinases « extracellular signal-regulated kinase» (ERK), « c-Jun N-terminal kinase » (JNK) et p38. Cette activation cellulaire mène à la libération de médiateurs biologiques (cytokines, chemokines,etc...) qui vont initier la réaction inflammatoire. Une telle réaction est essentielle pour activer le système de défense qui peut ainsi neutraliser et détruire les bactéries et les molécules bactériennes comme le LPS et procéder à la cicatrisation des tissus. Cependant, la production de ces médiateurs proinflammatoires doit être étroitement contrôlée et doit rester locale. En effet, une surproduction de médiateurs inflammatoires dans la circulation sanguine pourrait entrainer une réaction inflammatoire systémique, un phénomène typique du choc induit par l'endotoxine ou encore appelé « choc septique ». Les causes d'un tel dérèglement restent pour le moment inconnu, mais pourrait impliquer des composantes génétiques, biochimiques, physiologiques et environnementales qui prédisposeraient certaines personnes à ce type de choc. Chaque année le syndrome septique provoque la mort de 200'000 personnes seulement aux Etats-Unis. Actuellement, les traitements disponibles pour le choc septique restent limités et sont principalement des mesures empêchant l'aggravation des symptômes et des traitements de soutien des organes défaillants. Le LPS a été caractérisé comme étant une molécule puissante capable de reproduire un syndrome similaire à celui du sepsis après injection à des animaux. Sa reconnaissance par les cellules immunitaires a été depuis largement étudiée. Une

meilleure compréhension des mécanismes cellulaires et moléculaires sous-jacents à la pathologie du sepsis pourrait mener à la découverte de nouvelles cibles thérapeutiques. Il s'avère qu'une des étapes critiques de la pathogénèse du syndrome septique est la reconnaissance des bactéries ou des produits bactériens tels que le LPS par les cellules de l'hôte. C'est pourquoi nous avons souhaité dans cette thèse approfondir la compréhension des mécanismes intimes qui interviennent lors de l'intéraction de l'endotoxine avec la cellule humaine. Nous nous sommes également intéressés à une autre molécule de bactéries Gram-négatif : la toxine aérolysine d'*Aeromonas hydrophila*, dont la liaison avec une ancre GPI de récepteurs de surface comme le CD14 est le point de départ d'effets cellulaires délétères irréversibles.

Comme mentionné précédemment, après avoir lié le CD14, le LPS est internalisé par les monocytes/macrophages, une étape importante pour la détoxification et l'élimination du LPS de l'organisme. Durant plusieurs années, l'internalisation du LPS a fait l'objet de nombreuses investigations. Cependant des questions sont restées en suspend à savoir par quelle voie le LPS est internalisé, si le CD14 internalise le LPS ou alors si ces deux molécules internalisent indépendemment. Dans cette thèse, nous avons tenté de répondre à ces interrogations. La question du rôle éventuel de l'ancre GPI du CD14 dans la localisation du récepteur à la membrane, dans l'activation des cellules induite par LPS, et dans l'internalisation du LPS a également été abordée. Afin de mener ces investigations, nous avons utilisé une lignée cellulaire humaine de promonocytes nommée THP-1 transfectée avec un plasmide contenant le gène sauvage du CD14 (wtCD14), ou un gène codant pour une forme transmembranaire chimérique du CD14 (tmCD14). Utilisant les propriétés de flotabilité différentielle des gradients de sucrose permettant la séparation de domaines membranaires dont la composition lipidique varie, nous avons pu montrer que les deux formes (wt et tm) de CD14 se retrouvaient dans différents domaines membranaires. Le tmCD14 a été localisé dans des domaines membranaires où se retrouvaient d'autres protéines transmembranaires, tandis que le wtCD14 se trouvait concentrer dans des domaines membranaires riches en lipides de type sphyngomyéline et cholesterol (« lipid rafts »). L'ancre GPI du CD14 semble donc importante pour cibler la localisation du récepteur dans des zones biochimiquement spécifiques de la membrane. Une analyse ultrastructurelle a pourtant permis de déterminer que les deux formes de CD14 étaient préférentiellement concentrées au niveau de microvillosités ou replis de la membrane plasmique. Ces études de microscopie électronique ont également indiqué que le CD14 était internalisé par la voie de la macropinocytose, un processus ressemblant

étroitement à celui de la phagocytose qui permet « l'ingestion » de bactéries opsonisées par la cellule. Cette voie d'endocytose du CD14 est apparue être différente de la voie classique d'internalisation des récepteurs comme celle des vésicules recouvertes de clathrine (clathrin-coated pits). Cette voie d'internalisation du CD14 nous a semblé d'autant plus surprenante qu'il était plutôt attendu que le CD14 internaliserait par la voie des cavéoles. Il est reconnu dans la littérature que beaucoup de protéines ancrées par des GPI sont concentrées dans des domaines riches en lipides eux-mêmes fortement associés à des stuctures membranaires telles que les cavéoles. De plus, nous avons pu montrer que ni la cinétique d'internalisation du CD14, ni la voie par laquelle il a été internalisé n'étaient modulées par la présence du ligand LPS ou par le type d'ancrage du CD14 à la membrane. Par conséquent, le CD14 semble être internalisé de façon constitutive en une vingtaine de minutes. A 37°C, les replis membranaires enrichis en CD14 ont fusionné pour former de larges vésicules, qui se sont aplatis. Puis, ces vacuoles plates se sont condensées pour donner des structures de type endosome, puis phago-lysosome. A chaque étape décrite précédemment, les grains d'or qui marquaient la présence de récepteurs CD14 se sont retrouvés associés à ces structures internes de la cellule. L'internalisation du LPS radiomarqué s'est effectuée parallèlement à celle du CD14. Les études de microscopie confocale ont confirmé la co-localisation du LPS et du CD14 à la surface de la cellule et dans les compartiments endosomaux. De plus, l'utilisation de cytochalasine D, un agent empêchant la polymérisation de l'actine et par conséquent inhibiteur spécifique de la macropinocytose, a bloqué l'internalisation du LPS et du CD14. Cependant, l'activation cellulaire n'était pas inhibée, ce qui tend à indiquer que les processus d'internalisation et d'activation cellulaire induits par le LPS, ne sont pas directement couplés. Des études similaires d'autres équipes ont confirmé ce dernier point, ainsi que l'implication du CD14 dans l'internalisation du LPS, deux sujets soumis à de nombreuses controverses. En effet, certains auteurs ont publié que le LPS et le CD14 internalisent de façon séparée et que l'activation cellulaire s'effectue après l'endocytose du LPS. D'autre part, il est possible que le LPS n'internalise pas uniquement par macropinocytose, mais également par d'autres voies. Kitchens et al. ont montré que le LPS pouvait être endocyté par la voie d'invaginations non-recouvertes d'un manteau qui dirigeraient ensuite le LPS dans des compartiments autres que les endosomes contenant la transferrine. Une petite fraction du LPS entrerait également *via* des vésicules recouvertes de clathrine.

La réponse au LPS n'implique pas seulement les cellules d'origine myéloide comme les monocytes et les macrophages, mais également les cellules endothéliales et épithéliales. Les

cellules épithéliales ne servent pas uniquement de barrière physique, mais elles participent aussi activement au maintien, au renouvellement et à la défense des surfaces de l'organisme. Les cellules épithéliales intestinales sont en permanence en contact direct avec des bactéries et des produits bactériens. Le foie constitue également un haut lieu d'interaction avec les bactéries lors d'infections. Par conséquent, ces types de cellules épithéliales ont dû développer des stratégies de défense et de reconnaissance permettant la discrimination entre les bactéries pathogènes et les bactéries appartenant à la flore de l'hôte. En effet, la reconnaissance de microorganismes potentiellement pathogènes ou de molécules bactériennes comme le LPS par ces cellules est fondamentale pour initier la réponse de défense de l'organisme. Nous avons donc souhaité étudier la réponse au LPS de certaines lignées de cellules épithéliales comme les HepG2 (foie), les T84 (colon) et les SW620 (colon). En premier lieu, nous avons investigué l'expression protéique du CD14 et la production de CD14 soluble par ces cellules. Il a été montré que beaucoup de cellules épithéliales répondent au LPS d'une façon dépendante du CD14 soluble. Pour mieux comprendre l'activation de ces cellules par le LPS, nous avons étudié ces cellules dans différentes conditions. Ces conditions correspondent aux cellules stimulées par LPS ou d'autres agents pour comparer les réponses, en présence de sérum (FCS= apport de LBP et de CD14 soluble) ou de LBP recombinante (pas d'apport de CD14 soluble). Ces cellules épithéliales testées dans les conditions décrites précédemment étaient transfectées ou non par le wtCD14 (ancré par le GPI). En plus de l'activation cellulaire par LPS, nous avons investigué l'effet de cette transfection du wtCD14 sur l'internalisation du LPS par ces cellules. En utilisant des techniques de RT-PCR et de Western blot, nous avons déterminé les niveaux d'expression du CD14 au niveau de l'ARN messager (m) et de la protéine elle-même dans les cellules transfectées ou non. Nous avons pu déterminer que les cellules SW620 non transfectées exprimaient le CD14 sous forme d'ARNm, de protéine et à un moindre niveau produisaient du CD14 soluble. Les cellules T84 et HepG2 semblaient exprimer le CD14 que très faiblement. Dans les cellules HepG2, le CD14 cellulaire n'a pas été détecté et très peu de CD14 soluble a été produit. Après transfection avec le gène du wtCD14, les trois types cellulaires CD14(+)-SW620, CD14(+)-T84 et CD14(+)-HepG2 ont exprimé de manière importante le CD14 aux niveaux ARNm, cellulaire et soluble. Le CD14 soluble détecté pourrait provenir du CD14 membranaire libéré dans le milieu de culture après un clivage enzymatique. Afin d'étudier la réponse au LPS de ces cellules épithéliales, nous avons dans un premier temps mesuré la production d'IL-8 dans les surnageants par ELISA. Nous avons montré qu'en présence de sérum, (apport exogène de CD14 soluble et de LBP) les cellules CD14(+)-SW620 ainsi que les cellules rsv-SW620

(cellules contrôles transfectées avec le vecteur vide) ont produit des doses d'IL-8 croissantes en fonction de l'augmentation de la concentration de LPS. En présence de LBP uniquement (pas de CD14 soluble disponible), seules les CD14(+)-SW620 ont répondu au LPS, suggérant que le CD14 présent à la membrane a joué son rôle de récepteur au LPS et a permis l'activation des cellules comme pour les monocytes/macrophages. En revanche, dans les mêmes conditions de stimulation, les deux autres types cellulaires HepG2 et T84, transfectées avec le wtCD14 ou le vecteur vide, n'ont pas produit d'IL-8 après stimulation avec le LPS. Pourtant, ces cellules tout comme les SW620 ont été capables de produire de l'IL-8 en réponse à d'autres agents stimulateurs de la voie NF-κB tels que le phorbol 12-myristate 13-acetate (PMA), le TNF-α ou l'IL-1β. Afin de confirmer cette non-réponse des cellules HepG2 et T84 au LPS, nous avons investigué l'activation cellulaire au niveau du facteur de transcription NF-κB. Après stimulation avec le LPS, aucun NF-κB n'a été détecté dans les extraits nucléaires de cellules CD14(+)-HepG2 et CD14(+)-T84 alors que cela a été le cas pour les cellules de la lignée myéloide CD14(+)-THP-1 utilisées comme cellules contrôles. En revanche, le NF-κB a été activé dans ces cellules épithéliales stimulées avec d'autres agents comme le TNF-α et le PMA. Le PMA stimule la voie du NF-κB en activant directement la protéine kinase C qui va mener à la phosphorylation du I-κB et par conséquent à sa dégradation. Afin d'activer le facteur de transcription NF-κB, le TNF-α et l'IL-1 stimulent des voies de signalisation très similaires à celles induites par LPS. Les différences ne peuvent résider que dans des molécules membranaires exprimées différentiellement entre les différents types cellulaires, et activées très proximalement après contact avec l'endotoxine. Ainsi, la non-réponse au LPS des cellules épithéliales HepG2 et T84 en présence de CD14 soluble ou après surexpression du CD14 dans ces cellules suggère que le CD14, bien que nécessaire, n'est pas suffisant pour induire un signal. Cette observation ainsi que les données obtenues au cours de notre étude nous ont amené à penser que ces cellules étaient déficientes en une ou plusieurs molécule(s) impliquées de façon très proximales dans la voie de signalisation du LPS. Cette molécule manquante pourrait bien être une protéine transmembranaire responsable de la transmission du signal induit par LPS depuis l'extérieur vers l'intérieur de la cellule. En raison du type d'ancrage du CD14 (lipide non-transmembranaire), il a souvent été questionné comment le CD14, après avoir lié le LPS, pouvait transmettre le signal vers l'intérieur de la cellule. Plusieurs hypothèses ont été émises à l'issue de certains travaux. Cependant, l'hypothèse qui tend à revenir le plus souvent est celle d'une protéine additionnelle transmembranaire impliquée dans la signalisation du LPS.

Récemment, cette dernière hypothèse s'est vérifiée avec la découverte du « Toll-like receptor 4 » (TLR4) humain, une protéine transmembraire impliquée dans la réponse des cellules au LPS. Des observations chez la mouche drosophile des mécanismes de défense anti-bactériens et anti-fongiques induits par Toll et 18-wheeler respectivement, deux récepteurs transmembranaires, sont à l'origine de cette découverte. Ce sont des techniques de bioinformatique telle que la génomique comparative qui ont permis de faire le lien entre la mouche et l'homme, pourtant éloignés par des millions d'années d'évolution. Depuis, dix membres de cette nouvelle famille de « Toll-like receptors » ont été clonés. Ces récepteurs semblent révolutionner l'idée d'une non-spécificité dans la reconnaissance des molécules étrangères par le système immunitaire inné. En effet, il a été montré que le TLR2 reconnaissait plus spécifiquement les molécules issues de bactéries Gram-positif, mais également certains sérotypes de LPS (TLR2 a été initialement décrit comme étant le récepteur au LPS). Le TLR3 serait spécialisé dans la reconnaissance des ARN double brins viraux. Le TLR5 reconnaît la flagelline provenant des flagelles bactériens. Le TLR9 est spécialisé dans la reconnaissance de l'ADN bactérien et viral (motifs CpG). La fonction de certains membres de la famille TLR, comme le TLR10, n'est pas encore connue précisément. Cependant, il apparaît que l'hétérodimérisation de certains TLRs (TLR2/TLR1 ou TLR2/TLR6) augmente la spécificité en permettant à la cellule de discriminer différentes lipoprotéines. Il a été également montré que le TLR4 doit s'associer à une molécule accessoire, le MD-2 pour être fonctionnel. Cette protéine ne possède aucune ancre transmembranaire et la manière dont elle se lie au TLR4 reste encore indéterminée. Ce complexe MD-2/TLR4 est capable de reconnaître et de lier le LPS. Les TLRs, comme le CD14, possèdent des domaines riches en leucine au niveau de la partie extracellulaire, ce qui pourrait favoriser des interactions protéine-protéine. Il est possible d'imaginer et même très probable que le CD14 lie le LPS et que le récepteur TLR4/MD-2 reconnait et interagit avec ces deux molécules pour former un hétérocomplexe, point de départ du signal LPS vers l'intérieur de la cellule.

Les types cellulaires les plus importants dans la reconnaissance et l'élimination des bactéries ou des produits bactériens tels que le LPS sont les macrophages et les cellules dendritiques. Ces cellules peuvent également induire et orienter la réponse immunitaire secondaire en sécrétant des cytokines spécifiques et en présentant un antigène aux lymphocytes. Le macrophage et la cellule dendritique dérivent d'un précurseur commun, le monoblaste/promonocyte, lui-même issu d'une cellule myéloïde souche. Au cours des étapes

de différenciation, les cellules myéloides peuvent acquérir ou perdre des fonctions diverses et spécifiques selon la variation d'expression de molécules intracellulaires, de récepteurs de surface et de leur localisation tissulaire. Dans le contexte de notre sujet, il a été observé que les cellules myéloides répondent différemment au LPS en fonction de leur état de différenciation. De nombreuses études illustrent cette observation. Par exemple, les promonocytes acquièrent la réponse au LPS après différenciation en monocytes. En revanche, les cellules dendritiques perdent la réponse au LPS après maturation. Avec la découverte récente des TLRs et de la molécule MD-2, nous étions intéressés d'investiguer si les variations dans les réponses au LPS pouvaient être éventuellement attribuées à une expression différentielle du CD14 et des TLRs dans les cellules myéloides à différents stades de différenciation. Dans un premier temps, pour mener cette étude nous avons utilisé une lignée de cellules promonocytaires THP-1 transfectées avec le gène sauvage du CD14 (CD14(+)THP-1). Ces cellules qui expriment à la base un taux élevé de CD14 (dû à la transfection), TLR2 et TLR4 au niveau de la surface membranaire, ont été traitées 72 heures avec différents agents de différenciation : la 1,25 $(OH)_2$ vitamine D_3 (D3), l'acide rétinoïque (AR) ou l'interféron γ (IFNγ). Seul l'IFNγ était capable d'augmenter l'expression du TLR4 alors que les niveaux d'expression des autres molécules de surface n'étaient pas significativement modifiés par le processus de différenciation. Aucune correlation entre l'expression des TLRs et la magnitude de la réponse des CD14(+)THP-1 au LPS n'a pu être établie à l'issue du dosage de cytokines sécrétées telles que l'IL-6 et l'IL-8. Utilisant des anticorps monoclonaux spécifiques, nous avons pu montré que les cellules CD14(+)THP-1 non-différenciées répondaient au LPS d'une manière prédominante TLR2-dépendante. Par contre, les cellules différenciées perdaient partiellement cette dépendance TLR2, et répondaient au LPS d'avantage *via* TLR4. En bloquant les deux récepteurs, le signal induit par LPS était complètement abrogé, suggérant que TLR2 et TLR4 étaient impliqués dans la réponse au LPS. Cette acquisition de la réponse au LPS *via* TLR4 pourrait être corrélée à l'apparition de l'expression d'ARNm du MD-2 dans les cellules différenciées. Ces résultats suggèrent que le MD-2 nouvellement exprimé, rendrait le TLR4 fonctionnel, ce qui expliquerait cette modification dans la TLR-dépendance de la réponse au LPS dans les cellules CD14(+)THP-1 différenciées. Ayant réalisé des expériences similaires avec les cellules du sang complet ou avec des macrophages alvéolaires, nous avons observé une réponse au LPS exclusivement dépendante du TLR4. Pourtant, TLR4 apparaît être faiblement exprimé à la surface des monocytes et des macrophages alvéolaires. Les monocytes expriment fortement le CD14 et un peu moins le TLR2, tandis qu'environ dix fois moins de TLR2 et

CD14 sont présents à la surface des macrophages alvéolaires. Cependant, ces deux types cellulaires expriment l'ARNm du MD-2 à un niveau relativement plus important que celui obtenu dans les CD14(+)THP-1 différenciées. Avec ces résultats, il est possible d'imaginer que l'association des deux molécules MD-2 et TLR4 rendrait le récepteur TLR4 très performant pour transmettre le signal induit par LPS et par conséquent ceci ne nécessiterait pas une importante expression de surface du TLR4.

Dans un deuxième temps, nous avons mené une étude sur les cellules dendritiques (DCs). Cette cellule établit un lien clé entre l'immunité innée et adaptative. Durant une infection tissulaire, les DCs détectent la présence d'antigènes microbiens, participent à l'inflammation locale, migrent vers les organes lymphoïdes et se transforment en cellules professionnelles de la présentation de l'antigène au lymphocyte. Utilisant un modèle de DCs cultivées *in vitro* et dérivées de monocytes humains, nous avons analysé les niveaux d'expression de certains TLRs, du MD-2 et du CD14 ainsi que la réponse de ces cellules au LPS. Nous avons montré que la réponse des DCs au LPS variait de façon importante durant le processus de différenciation et de maturation de ces cellules. Les niveaux de réponse cellulaire au LPS étaient étroitement corrélés aux niveaux d'expression du CD14, TLR4 et MD-2, tous étant des composants clés du récepteur au LPS. En effet, les DCs immatures dérivées des monocytes sanguins maintiennent leur sensibilité au LPS et expriment encore le CD14 et le TLR4 à la surface de leur membrane, même si comparée aux monocytes cette expression est diminuée. A l'opposé, les DCs qui deviennent matures après un traitement avec du LPS ou des cytokines pro-inflammatoires comme l'IL-1β et le TNF-α, perdent l'expression de surface du CD14 et du TLR4, ainsi que la capacité de répondre au LPS. Utilisant des anticorps spécifiques, nous avons déterminé que les DCs immatures répondent au LPS en partie *via* le TLR4. Le TLR2 ne semble pas impliqué dans l'activation des DCs par LPS. Par conséquent, ces résultats suggèrent qu'une ou plusieurs autres molécules de surface pourraient jouer un rôle dans la transmission du signal LPS dans les DCs immatures. Il est intéressant de noter que nous n'avons détecté la présence d'ARNm du TLR3 qu'exclusivement dans les DCs immatures et à un plus faible niveau dans les DCs matures. Tous ces résultats suggèrent que le « pattern » et les niveaux d'expression des récepteurs microbiens dans les DCs gouvernent leur fonction effectrice telle que la capture et le « processing » de l'antigène *versus* la présentation de l'antigène.

Au cours de cette thèse, nous avons pu constater le rôle primordial du CD14 dans la reconnaissance et l'internalisation du LPS ainsi que sa participation à l'activation cellulaire par LPS. A de nombreuses reprises, la question de la fonction de l'ancre GPI par laquelle le CD14 est inséré dans la membrane a été soulevée. Hormis son rôle dans la localisation du CD14 dans les domaines riches en lipides, aucune fonction particulière de cette ancre n'a été trouvée dans l'activation cellulaire induite par LPS. Il est possible que le système d'ancrage GPI soit important pour d'autres fonctions de signalisation. Il a été montré que de nombreuses toxines comme la toxine α de *Clostridium speticum*, la leishporine de *Leishmania amazonensis* ou la leukotoxine de *Pasteurella haemolytica* sont capables de médier des actions mortelles en se liant à l'ancre GPI de protéines de surface. Nous nous sommes intéressés à l'une de ces toxines, l'aérolysine sécrétée par *Aeromonas hydrophila,* une bactérie Gram-négatif présente dans les environnements aqueux et à l'origine de maladies gastrointestinales, de pneumonie, de méningite et également de sepsis chez l'humain. L'aérolysine qui se lie à des ancres GPI, heptamérise pour former un pore dans la membrane plasmique. Par conséquent, la cellule perforée gonfle en se remplissant d'eau et éclate sous la pression. L'aérolysine ainsi que certaines toxines comme la toxine α, la leukotoxine possèdent des similarités importantes dans leur séquence et leur mode d'action. Il a été montré que la leukotoxine, une toxine formant également des pores, induit l'élévation de calcium intracellulaire et active le facteur de transcription NF-κB dans les leukocytes et les plaquettes de ruminants. A l'issue de cette activation, des cytokines pro-inflammatoires sont produites par les cellules. Il a été ultérieurement montré que l'activation du NF-κB est dépendante de l'élévation de calcium induite par la leukotoxine. Tout comme la leukotoxine, l'aérolysine augmente le calcium intracellulaire des granulocytes. Dans notre étude, nous étions intéressés d'investiguer si l'aérolysine peut également activer le NF-κB et induire la sécrétion de protéines pro-inflammatoires qui pourraient être impliquées dans l'inflammation observée lors des infections avec l'*Aeromonas hydrophila*. En utilisant le récepteur CD14 ancré par le GPI, nous souhaitions étudier un nouveau mécanisme d'action d'une molécule de bactérie Gram-négatif autre que le LPS. Pour réaliser une partie de l'étude, nous avons utilisé la lignée de cellules promonocytaires "THP-1" transfectées de façon stable avec un plasmide encodant une protéine fluorescente verte (GFP) sous le contrôle du promoteur de l'interleukine-8 (pIL-8/GFP-THP-1). Afin d'augmenter l'expression de surface du CD14 ancré par le GPI, les cellules ont été traitées 72h avec de la 1,25 $(OH)_2$ vitamine D_3. Après exposition avec l'aérolysine des cellules pIL-8/GFP-THP-1 différenciées, nous avons montré que ces cellules

sécrétaient de l'IL-8 de façon dépendante de la concentration d'aérolysine et du temps de traitement. Des observations similaires ont été effectuées avec la protéine GFP produite au niveau intra-cytoplasmique de ces cellules, indiquant que le promoteur de l'IL-8 placé en amont de la séquence codant pour la GFP a été activé par des facteurs de transcription. Utilisant la technique classique de la « E-selectin luciferase reporter assay », nous avons déterminé que le traitement de cellules HEK 293 avec l'aérolysine est associé à l'activation des cellules de façon dépendante du NF-κB. De plus, cette activation cellulaire est considérablement augmentée par la surexpression (après transfection) à la surface de protéines ancrées par le GPI telles que le CD14, le CD55 et une version GPI de la GFP construite par biologie moléculaire. Ces protéines qui possèdent l'ancre GPI ont toutes servi de récepteurs à la toxine. Afin d'exclure la possibilité que l'activation cellulaire observée soit dûe au choc osmotique induit par l'aérolysine, nous avons incubé les cellules dans un milieu de plus en plus hypo-osmolaire. Aucune activation n'a été observée, mettant ainsi hors de cause le gonflement des cellules lors du choc osmotique induit par l'aérolysine. La liaison de l'aérolysine à l'ancre GPI semble donc être une étape critique pour l'induction d'un signal pro-inflammatoire par les cellules. Il est intéressant de noter que le cross-linking du CD14 avec des anticorps provoque l'apparition d'un flux de calcium intra-cytoplasmique, phénomène n'ayant jamais été observé lors d'une situation physiologique comme par exemple avec le LPS. En revanche, cette mobilisation de calcium ne se produit pas lors du cross-linking du CD14 dont la région GPI a été remplacée par une partie transmembranaire. Suite à toutes ces observations concernant l'aérolysine et l'ancre GPI, il est tentant d'imaginer que l'héptamérisation de l'aérolysine ait induit un cross-linking du récepteur sur lequel la toxine est liée et que les ancres GPI intéragissant les unes avec les autres génèrent un signal permettant l'augmentation de calcium. En utilisant des inhibiteurs spécifiques, il serait intéressant de déterminer si certaines protéines tyrosine kinases ou phospholipase C sont impliquées dans cette élévation du calcium intracellulaire comme il a été montré dans d'autres études. De plus, l'hypothèse d'un lien entre l'augmentation de calcium et l'activation du NF-κB que nous avons observé, est émise et reste à être démontrée. Toutes les observations effectuées lors de cette étude sont importantes au regard du rôle pathogénique éventuel de l'aérolysine en tant que molécule pro-inflammatoire en plus de son rôle de molécule cytotoxique. La question d'un nouveau mécanisme cellulaire qui engendrerait un signal pro-inflammatoire est également soulevée à l'issue de cette étude sur l'aérolysine.

Cette thèse apporte de nouvelles connaissances dans le domaine de l'immunité innée. La reconnaissance des molécules bactériennes comme le LPS par les cellules de l'immunité primaire est une étape critique pour le déroulement des évènements de défense et dans certains cas peut être à l'origine de la pathogénèse du sepsis. Une meilleure compréhension de ce phénomène permettrait de soulever petit à petit l'interrogation qui porte sur l'origine de cette pathologie en déterminant les cibles thérapeutiques les plus pertinentes pour le développement de nouveaux médicaments ou traitements. Il est également important de comprendre les mécanismes qui régissent l'internalisation et la détoxification du LPS, évènements critiques de l'élimination de cette molécule hautement activatrice. Dans cette thèse, nous avons déterminé que le récepteur CD14 après avoir lié le LPS, l'internalise par la macropinocytose, une voie d'endocytose qui ressemble étroitement à la phagocytose. L'ancrage GPI du CD14 ne semble pas influencer la voie par laquelle le CD14 et le LPS sont internalisés ainsi que leur cinétique d'internalisation. En revanche, ce type d'ancrage localise le CD14 à la membrane dans des domaines spécifiques riches en lipides. Inhibant spécifiquement l'internalisation du LPS et du CD14, il nous est apparu que l'activation cellulaire n'était pas bloquée, suggérant que l'internalisation du LPS et l'activation des cellules induite par LPS sont des processus régulés différemment. L'étude menée avec les cellules épithéliales également impliquées dans la défense de l'organisme, nous a permis de montrer que le CD14 exprimé à la surface de certains types de cellules n'était pas suffisant pour induire une activation lors d'un traitement avec LPS. La conclusion de cette étude a soulevé l'hypothèse qu'une ou plusieurs molécule(s) très proximale(s) à la membrane devaient être impliquée(s) dans la signalisation du LPS, et qu'elles étaient absentes dans ces types cellulaires. Cette hypothèse s'est révélé être exacte par la suite avec la découverte fascinante des récepteurs « Toll » et notemment avec l'identification du TLR4 humain, une protéine transmembranaire directement impliquée dans la voie de signalisation du LPS. Depuis environ 3 ans, quasi chaque membre de la famille des récepteurs Toll a trouvé comme ligand une molécule ou une famille de molécules microbiennes. Des études ont depuis montré que la réponse au LPS de différents type de cellules pouvait varier selon leur état de différenciation. En analysant le « pattern » d'expression de ces nouveaux récepteurs au niveau de cellules myéloides à différents stades de différenciation et parallèlement la réponse de ces cellules au LPS, nous avons souhaité déterminer si ces récepteurs étaient impliqués dans les variations de réponse à l'endotoxine. Nos résultats ont révèlé que les promonocytes différenciés en monocytes/macrophages avaient acquis une réponse au LPS de plus en plus dépendante du récepteur TLR4 et que cette observation pourrait être corrélée avec l'apparition

de l'expression du MD-2, une molécule accessoire indispensable au bon fonctionnement de TLR4. Des expériences menées avec des cellules primaires telles que les monocytes sanguins et les macrophages alvéolaires humains ont indiqué que ces cellules répondaient au LPS de façon totalement TLR4-dépendante et exprimaient le MD-2. En ce qui concerne la différenciation des monocytes circulants en cellules dendritiques immatures, puis matures, il nous est apparu que les cellules perdaient leur capacité de réponse au LPS après maturation et que cette perte corrélait avec la disparition de l'expression de surface du TLR4 et du CD14. Cette observation est en accord avec le fait que la cellule dendritique immature en présence d'une molécule étrangère telle que le LPS est capable de s'activer et de la « phagocyter », alors qu'après maturation cette cellule perd ces fonctions, mais acquiert la capacité de présenter l'antigène aux lymphocytes. En résumé, notre étude montre que la modulation de l'expression du CD14, du MD-2 et de certains TLRs comme le TLR4, au cours de la différenciation des cellules myéloïdes semble être un facteur déterminant de la variation de réponse de ces cellules au LPS. Suite à la découverte de ces nouvelles molécules, les TLRs, de nombreuses interrogations sont soulevées à savoir : i) si comme le CD14, les TLRs sont présents à la surface dans des domaines riches en lipides ou bien s'ils sont recrutés dans ces domaines lorsque le ligand bactérien est lié au CD14 afin d'agir avec le complexe. Comme récemment montré par Triantafilou et al., cette dernière hypothèse semble se vérifier pour le TLR4 recruté dans ces microdomaines, en présence du LPS [5] ; ii) si d'autre(s) molécule(s) interviennent comme chaine(s) supplémentaire(s) du complexe récepteur au LPS, comme il a été suggéré dans une étude récente ; iii) si le TLR4/MD-2 participent à l'internalisation du LPS ; iiii) si le « pattern » d'expression des TLRs au niveau des monocytes et d'autres cellules se modifie durant les différentes étapes de la pathologie du sepsis comparé à un état non pathologique ; iiiii) si des mutations éventuelles au niveau des gènes codant pour les TLRs pourraient rendre des individus plus susceptibles à développer un sepsis. Il a été en effet récemment montré que des polymorphismes de TLR4, mais pas de CD14, seraient associés à un risque accru aux infections induites par les bactéries Gram-négatif [6].

Dans toutes nos études, il est apparu que le CD14 était une molécule pivot dans la reconnaissance du LPS, son internalisation et l'activation cellulaire probablement en établissant un lien essentiel entre le ligand LPS et le complexe TLR4/MD-2. Ces processus constituent un mécanisme important d'interaction des cellules de l'hôte avec une molécule bactérienne comme LPS et pourraient être à l'origine de certaines dérégulations menant à une pathologie telle que le sepsis. Dans cette thèse, nous étions intéressés d'étudier un mécanisme d'action différent d'une autre molécule bactérienne : l'aérolysine de *Aeromonas hydrophila*

également à l'origine de pathologies septiques et gastrointestinales. Cette toxine bactérienne entre en contact avec les cellules en se liant à l'ancre GPI des protéines de surface comme le CD14. Utilisant des cellules qui expriment le CD14 ancré par un GPI, nous avons investigué l'action de l'aérolysine au niveau des cellules. Nous avons montré pour la première fois que l'aérolysine liée à l'ancre GPI induisait un signal pro-inflammatoire *via* l'activation du facteur de transcription NF-κB, ce qui conduisait à la production de cytokines comme l'IL-8. Cependant, nous avons déterminé que cette activation cellulaire observée n'est pas dûe au gonflement des cellules induit par le choc osmotique résultant de la formation de pores à la membrane après l'heptamérisation de l'aérolysine. Les perspectives de cette étude soulèvent de nombreuses questions concernant la génèse de ce signal pro-inflammatoire induit par l'aérolysine, à savoir: i) si en plus de son rôle de localisation de protéines dans des domaines spécifiques de la membrane, l'ancre GPI éventuellement en se « cross-linkant » lors de l'heptamérisation de la toxine pourrait être capable d'activer la voie de signalisation intracellulaire du NF-κB et peut-être d'autres voies; ii) si l'aérolysine après avoir liée l'ancre GPI entre en contact avec ou est transférée à une autre molécule qui pourrait être impliquée dans l'induction du signal ; iii) si l'insertion dans la membrane du canal formé après l'heptamérisation de l'aérolysine ne pourrait pas provoquer une perturbation de la membrane plasmique qui serait à l'origine de l'activation. Il a été monté que les domaines lipidiques dans lesquels sont ancrées les protéines GPI agissent comme des plateformes pour concentrer l'aérolysine à la surface cellulaire. Cependant, le rôle de ces domaines lipidiques dans l'activation des cellules par l'aérolysine reste à être déterminé [7]. Concernant les molécules impliquées dans la voie de signalisation, la question d'un lien éventuel entre l'activation du NF-κB que nous avons observée et l'augmentation de calcium intracellulaire mesurée dans les granulocytes lors d'un traitement avec l'aérolysine, est également posée. Ainsi, cette toxine bactérienne, en plus d'induire des effets cytolytiques délétères, semble déclencher une réponse immunitaire primaire qui pourrait s'additionner à celle induite par l'endotoxine présente dans la bactérie. Comme pour le LPS, une meilleure compréhension des mécanismes d'action de l'aérolysine permettront de trouver de nouvelles solutions thérapeutiques à ces pathologies sévères voire mortelles.

2 GENERAL INTRODUCTION

2.1 Innate immunity

The non-adaptive or innate immunity is defined as immune mechanisms that are “hard-wired” in the genome and can be modified by evolution, but not by the experiences of the individual such as that observed in “adaptive” immunity, which requires gene rearrangements. When microorganisms invade human or other mammal tissues, immediate host immune defenses occur to fight against these non-self “invaders”. These reactions involve elements of the non-adaptive immunity that are “ready-to-go” and do not require an induction by signal-response mediators. These “actors” of innate immunity are essential to rapidly distinguish microbes from non-self, and to activate host defenses. Different strategies are used to neutralize and eliminate microbes.

2.1.1 Soluble pathways

Soluble proteins present in the plasma and body fluids recognize and attack pathogenic organisms by detecting surface expressed carbohydrate or lipid moieties that are usually not found in the normal host. These proteins carry out specific functions, recognizing molecular targets that are found in a wide range of different microorganisms. This strategy enables the innate immune system to immediately recognize many pathogens as chemically foreign even if they have never been encountered before. It also helps to minimize the risk that these proteins might inadvertently attack host tissues (table 1).

Soluble mediators	Acute Phase Protein	Complement activation	Microbicidal activity	Induce opsonization	Induce phagocytosis	Microbial structures recognized	Ligands presentation to
Complement			yes	yes			
CRP	yes	yes			yes	Microbial polysaccharides	
SAP					yes	*Extracellular matrix proteins *Microbial cell wall carbohydrates	
MBP		yes			yes	* microbial cell wall saccharides *C1q receptor	
C3	yes					OH-groups of carbohydrates and proteins	*CD21 *CD35
LBP	yes					*Lipid A of soluble LPS *LPS at the surface of Gram-negative bacteria *Mycobacterial lipoarabinanmannan (LAM) *Pseudomonas mannuronic acid polymers	*Membrane CD14 *Soluble CD14

Table 1: Mediators involved in soluble pathway of host defenses.
CRP: C-reactive protein; **SAP**: Serum amiloid protein; **MBP**: Mannose binding protein; **C3**: Complement factor 3; **LBP**: Lipoplysaccharide-binding protein; **BPI**: bactericidal/permeability-increasing protein; sCD14: soluble CD14. All these molecules are produced by the liver.

These soluble proteins usually:

- can display a direct microbicidal activity
- opsonize microbes or foreign particles which are phagocytosed by macrophages
- present efficiently these molecules to receptors present at the surface of effector cells

Some of these soluble mediators are:

Enzymes that can directly injure or kill microbial pathogens. A prototypical example of such an enzyme is the lysozyme. It attacks the peptidoglycan of the weakened bacterial cell wall and renders the bacteria vulnerable to osmotic lysis [8].

The Mannose-binding protein (MBP) a lectin that binds mannose sugar residues commonly found at the exposed ends of glycoprotein or glycolipid side chains from bacterial outer membranes, viruses and parasites [9].

The serum amyloid protein P and the C-reactive protein which bind a subset of carbohydrates and lipids dysplayed by many types of bacteria [10, 11]. In contrast to enzymes, binding of these latter proteins has little direct effect on pathogen but greatly increases opsonization, thus enhancing the capture and the destruction by specialized hosts cells [12].

The lipopolysaccharide (LPS)-binding protein (LBP) and the soluble form of leukocyte cluster differentiation antigen 14 (CD14), two human serum proteins, are specialized in the binding of LPS, a unique bacterial surface molecule found in the outer membrane of Gram-negative bacteria. The complexes LPS-soluble CD14 (sCD14) are then recognized by receptors on the surface of endothelial cells (ECs), neutrophils, monocytes/macrophages and other human cell types, which facilitates the binding and destruction of Gram-negative bacteria. At the same time, binding of LPS to cell surface receptors activates cells to secrete various cytokines responsible for triggering a wide array of immunologic responses that will be described later in this document [13].

The complement system represents a group of serum proteins called “complement factors” which display an elaborate and important type of antimicrobial defense [14]. Most of these proteins have proteinase activities and their activation occurs through a cascade. When the first protein is proteolytically cleaved, it gets activated and catalyzes further cleavage and

activation of a different protein of the complement and so on. When complement becomes activated on the surface of the pathogen, it leads to several distinct types of host protective effects:

- makes pores in the membrane of the pathogen.
- opsonizes bacteria that are internalised and killed by host cells.
- chemoattracts leukocytes.

Throughout life, most of these soluble proteins are expressed in the serum in relatively small amounts under normal conditions. But, they can increase as much as 1000-fold during serious infections, as part of a coordinated protective reaction called *the acute-phase response*. The liver is responsible for the synthesis of more than a dozen serum proteins that participate in antimicrobial defense. This synthesis occurs when hepatocytes are exposed to cytokines such as interleukin (IL)-6, IL-1 or tumor necrosis factor alpha (TNF-α) released locally or into the bloodstream by other host cells [15]. Interestingly, one of the most potent inducers of these cytokines and hence the acute phase response is the bacterial molecule, LPS.

2.1.2 Receptor pathways

A newly recognized set of receptors present on effector cells such as monocytes/macrophages, neutrophils and ECs have evolved to recognize pathogen-associated molecular patterns (PAMPs), a wide variety of conserved surface microbial structures described in the Table 1. This recognition leads to the initiation of an innate immune response and/or clearance of bacteria and their products.

Scavenger receptors (SRs) and the mannose receptor are prototypical examples of "clearance" receptor for bacteria and bacterial products [16, 17]. Scavenger receptors display affinities for cell wall components of both Gram-negative and Gram-positive bacteria such as polysaccharides as well as fordextrans, polyribonucleotides, acetylated and oxidized low-density lipoprotein (LDL) [18, 19]. All these ligands have in commun a polyanionic moiety. The mannose receptor also binds exogenous sugar-bearing particles [20-22]. These receptors are very efficient to "detoxify" the extracellular milieu, but ligation of "foreign molecules" does not activate host cells.

The CD14 receptor exhibits properties similar to those of the receptors described above. CD14 is a pattern recognition receptor since it binds ligands originating from various microbial pathogens [23], is able to internalise some of these molecules, and has been shown to participate in bacteria phagocytosis [24]. In addition to these characteristics, CD14, expressed mainly on myeloid cells, mediates the activation of these effector cells [25]. Mediators such as cytokines, chemokines produced by effector cells are messengers, which allow the communication between cells and thus inform the environment of the presence of non-self "invaders". Ligation of bacterial products with CD14 leads to an "inflammatory reaction" which allows the host organism to rapidly fight against microbes and further orientate the adaptive immune response [26, 27].

2.2 Inflammation

In the second century AD, the Roman encyclopaedist Aulus Celsus described the four cardinal signs of inflammation: rubor (redness), calor (heat), tumor (swelling) and dolor (pain). This description of inflammation was the result of a direct observation without understanding of the underlying mechanisms which lead to this state. Many types of mechanical, chemical or microbial insult of a solid tissue are followed by an inflammatory reaction. Inflammation is in essence a protective response, to get rid of the initial cause of cell injury (e.g. microbes, toxins) as well as the consequences of such injury (e.g. necrotic cells and tissues). Without inflammation, infections would go unchecked, wounds would never heal, and injured organs might remain permanent festering sores. Inflammation and repair may also be potentially harmful. Systemic inflammation has led to a massive increase in microvascular permeability, and a movement of fluid and leukocytes from the blood into the extravascular space, which helps to localize and eliminate altered cells, foreign particles, microorganisms or antigens.

2.2.1 The environment where a local inflammatory reaction occurs

The inflammatory response occurs in the vascularized connective tissue, including plasma, circulating cells, blood vessels, and cellular as well as extracellular constituents of connective tissue. The circulating cells include neutrophils, monocytes, eosinophils,

lymphocytes, basophils and platelets [28]. The connective tissue cells are the mast cells, which surround blood vessels; the connective tissue fibroblasts; and occasional resident macrophages and lymphocytes [29]. The extracellular matrix consists of the structural fibrous proteins (collagen, elastin), adhesive glycoproteins (fibronectin, laminin, nonfibrillar collagen, tenascin and others), and proteoglycans [30]. The basement membrane is a specialized component of the extracellular matrix consisting of adhesive glycoproteins, elastin and proteoglycans [31].

2.2.2 Leukocytes involved in the host defense

Different types of leukocytes are involved in the host defense, but three cell types have prominent roles. Neutrophils and monocytes-macrophages, both phagocytes act primarily by engulfing, killing and digesting bacteria, as well as cellular debris [32]. Lymphocytes (γδ cells and NF lymphocytes) cooperate with phagocytic cells, but are more involved in the adaptive arm of the immunity [33].

- Neutrophils:

The polymorphonuclear neutrophil known as the main "warrior" in the host defense to bacteria is a terminally differentiated leukocyte. It is characterized by a multilobular nucleus, by the presence in the cytoplasm of storage granules containing bactericidal agents and lysosomal enzymes important to kill and digest engulfed microorganisms [34]. Enzymes are produced at different stages during neutrophil differentiation in the bone marrow. The types of enzymes that can be found in granules are myeloperoxidases (catalyse the hypochlorous acid formation by combining hydrogen peroxide with chloride ions), oxidases (convert molecular oxygen into highly reactive oxygen species, which spontaneously dismutates to form hydrogen peroxide), lysosomal enzymes (digest internalised particles present in lysosomes), lactoferrin (inhibits bacterial growth by chelating iron), gelatinases (digest basal membrane of vessels during diapedesis), defensins (antimicrobial peptides which kill by permeabilizing bacterial and fungal cell membranes), nitric oxide (NO), a soluble, highly labile, free radical gas converted to other products, such as peroxynitrite, which are highly toxic to bacteria, yeasts, viruses and other pathogens) [35-41].

Neutrophils represent roughly 2/3 of circulating white blood cells. If these cells do not encounter any stimulus, they are programmed to die by apoptosis in 12-24 hours. At inflammatory foci, neutrophils adhere to vessels in a process called "margination" which takes place in 3 phases:

1) selectin-mediated phase:

P-selectin and E-selectin on the surface of activated ECs bind to neutrophil surface mucins which bear carbohydrate side chains [42, 43]. L-selectin constitutively expressed on neutrophils binds to its target mucins on activated ECs. This adhesive contact between neutrophils and the blood vessel is not very strong allowing neutrophils to roll along the endothelium pushed by the force of the blood flow [44].

2) chemoattractant-mediated phase:

Chemotactic factors are produced by stimulated endothelium but also by cells in the injured tissue. Some of them such as chemokines (soluble proteins which adhere to ECs and extracellular matrix) diffuse from this region into the blood forming a gradient of concentrations through the tissues with its peak at the site of injury. Thus, chemotactic factors signal the presence of an infected region to neutrophils and induce changes in the conformation of integrins on the leukocyte surface, enabling them to bind to specific glycoprotein ligands on the endothelium [45-47].

3) integrin-mediated phase:

The previous phase leads to a stable long lasting molecular contact that prevents further movement of the neutrophil and cause it to flatten out at the surface of the endothelium. Once attached, the neutrophil can transmigrate into the extracellular tissue and move to the infected region (transmigration), a phenomenon that is favored by a gradient of chemotactic molecules such as IL-8, ENA-78 and GRO-alpha. The latter mediators will also activate neutrophils and enhance natural defense mechanisms such as the production of enzymes, oxygen radicals and phagocytosis [48].

At the site of infection, neutrophils start the recognition of "non-self" bacteria and cellular debris, and the process of phagocytosis. Macromolecules are taken up into the cell through a receptor-mediated endocytosis mechanism, and phagocytosis for larger particles such as bacteria [34]. To enhance phagocytosis, certain particles need to be covered by opsonins. Phagocytosed particles are localized in phagosomes which then fuse with lysosomal granules emptying their contents into the lumen. Then, killing and digestion of

microorganisms with antibacterial agents and multiple enzymes (see details of action above) occur [49].

Neutrophils are very effective at killing bacteria, at engulfing and digesting cellular debris as well as exogenous particules. However, a defense system based solely on neutrophils would have significant limitations. Neutrophils can for example not eradicate dangerous agents such as proteinaceous toxins, viral particules and other types of potentially injurious agents, because these molecules generally do not bind to the neutrophil surface. Neutrophils have only a limited repertoire of possible responses (phagocytosis and intracellular or extracellular degranulation) [50].

- Monocytes-Macrophages :

The monocyte, a large circulating white blood cell (12-20 μm) with a kidney-shaped nuclei and a fairly abundant cytoplasm, is produced by the bone marrow and is then released into the blood circulation. The monocyte has a large number of lysosomes which contain a similar array of enzymes as those found in the neutrophil (see above). Circulating monocytes represent only 1 to 6 % of all nucleated blood cells. These cells circulate for one day and then migrate into nearly all tissues and serosal cavities, where they reside permanently. They become tissue macrophages and act as “sentinel” cells, sensing the environment (timelife: 2-4 months). They may be called with more specific names according to the tissue where they have migrated. The largest macrophage reservoir is the hepatic Kuppfer cells, for example. The mechanisms by which monocytic cells select their tissue residence under normal conditions remain largely unknown [51].

Blood monocytes are attracted to injured or infected sites through steps identical to those described for neutrophils. Through their large array of receptors, monocytes sense their environment and can readily detect chemotactic factors and microbial molecules. In response to the latter and to other factors present in the local microenvironment, monocytes undergo morphological and functional changes to become tissue macrophages. Tissue macrophages also sense their environment through an extensive array of receptors on their surface and by using pinocytosis. The different receptors found at the surface of macrophages which are involved in self/non-self recognition and uptake of particles are: the mannose receptor, CD14, scavenger receptors, receptors for complement, for immunoglobins and for opsonins which coat particules and allow their phagocytosis by a “zippering” mechanism [16, 52-54].

The nature of stimuli recognized by these receptors which can result in macrophage activation, can be quite heterogenous. Known stimuli are microorganisms (bacteria, virus, yeast), various particules, bacterial LPS and other soluble bacterial molecules, host tissue breakdown products, complement fractions, enzymes from the blood coagulation, and various cytokines. Activated macrophages increase their metabolic rate, mobility and phagocytic capacities which render them more efficient at killing and digesting pathogenic microorganisms. After activation, many new proteins such as the inducible form of the NO-synthase are synthesized. This latter enzyme increases the production of NO which plays an important role in microbicidal functions [55]. The macrophage also secretes a large variety of biologically active substances into the surrounding environment such as enzymes, cytokines, complement components, coagulation factors, reactive oxygen species, etc. To date about one hundred of these products have been identified. Some products are generated specifically upon the stimulus, whereas other are synthesized as part of a generalized response. The role of some of these secreted factors are to attract other nearby tissue macrophages and blood monocytes.

The macrophage is one of the pivotal cell type of innate immunity not only because of its “sentinel” role, but also because it acts as an important link between innate and acquired immunity. Indeed, macrophages control lymphocyte functions in two ways: 1) by secreting TNF-α, IL-1 and IL-18, pro-inflammatory cytokines which control lymphocyte proliferation, differentiation and effector function; and 2) by processing and presenting antigen to lymphocyte [56]. Through these two types of regulatory interactions with lymphocytes, the macrophage plays a crucial role in initiating and coordinating nearly all types of acquired immune responses [33].

2.2.3 Cytokines and chemokines

Cytokines are molecules of cellular communication that influence the growth and activities of other cell types during an inflammatory situation [57, 58]. In order to facilitate the understanding of this manuscript, the prominent cytokines and chemokines involved in the inflammatory and immune response to pathogens that will be discussed later, are described here.

TNF-α and IL-1 are major pro-inflammatory cytokines typically secreted by macrophages stimulated with endotoxin, immune complexes, toxins and physical injury. Typical target cells include endothelial and epithelial cells, leukocytes and fibroblasts, and are responsible for inducing systemic acute-phase reactions. By acting on endothelium, they induce the synthesis of adhesion molecules and mediators such as "secondary" cytokines, chemokines, growth factors, ecosanoids and NO. TNF-α causes the aggregation and the priming of neutrophils, leading to an increased response of these cells to other mediators. TNF-α also stimulates neutrophils to release proteolytic enzymes, thus contributing to tissue damage in certains situations. The acute-phase response induced by both cytokines leads to fever, loss of appetite, the production of slow-wave sleep, the release of neutrophils into the circulation, the release of adrenocorticotropic hormone (ACTH) and corticosteroids, and particularly with regard to TNF-α, the hemodynamic effects of septic shock: hypotension, decreased vascular resistance, increased heart rate and decreased blood pH [59, 60].

IL-8 is a chemokine secreted by activated macrophages, ECs, and other cell types in response to TNF-α, IL-1 or directly in response to bacterial products [61, 62]. IL-8 is responsible for the activation and the chemotaxis of neutrophils. It has limited activity on monocytes which are more responsive to chimiotactic signals triggered by other chemokines such as the monocyte chemotactic protein-1 (MCP-1) [63].

IL-6 is secreted by leukocytes, endothelial, epithelial and fibroblastic cells after a bacterial stimulation or in response to TNF-α and IL-1. This cytokine activates hepatocytes which produce acute phase proteins during an inflammatory reaction. Like TNF-α, plasma IL-6 is also considered as a prognostic marker in sepsis.

The granulocyte macrophage-colony stimulating factor (GM-CSF) is a glycoprotein of 144 aa secreted by endothelial and fibroblastic cells, activated T lymphocytes and monocytes. GM-CSF amplifies the inflammatory response by increasing the production of IL-1 and TNF-α by macrophages. It can also activate neutrophils and eosinophils. Moreover, this cytokine has a role as a growth factor by maintaining the survival and by stimulating the proliferation and the differentiation of myeloid progenitors (neutrophils, monocytes, eosinophils) [64].

2.2.4 Endotoxin as a model activator of innate immunity: from acute inflammation to Gram-negative septic shock

After the description of the principal actors involved in the inflammatory reaction in the previous section and to remain focused on our topic of interest, we will limit here our description to the scenario of an inflammatory process induced by Gram-negative bacterial microorganisms. The entry of Gram-negative bacteria in tissue is followed by a rapid dilatation of small blood vessels in and around the infected tissue. This vasodilatation of the capillaries, arterioles or venules occurs within a few minutes and leads to a local increase of the blood flow. During this process, ECs lining some of these vessels actively retract away from one another to create temporary microscopic pores [65]. This results in an increased permeability of the post-capillary wall, allowing protein-rich fluid from the plasma to diffuse through the gaps into the extracellular space of the surrounding tissue [66, 67]. Two processes induce the vasodilatation in the injured site: 1) a spinal reflex which causes relaxation of arteriolar smooth muscles [68, 69]; 2) vasoactive mediators such as histamine, prostaglandins produced in part by mast cells present in connective tissues [70]. This local release of vasoactive mediators and other substances produce swelling, redness, heat and pain at the involved site. These local effects constitute the pattern of host response known as acute inflammation, a rapid phenomenon which will last minutes, several hours, or a few days. The increased local blood supply helps to improve delivery of oxygen, nutrients, platelets, and clotting factors to the infected region, which in turn helps to stabilize damaged tissues and facilitates wound healing and repair. Moreover, the vascular fluid that leaks into the tissues can immediately dilute or dissolve bacteria present in the tissue. This fluid also carries with it antimicrobial proteins from the serum, thereby helping to deliver non-specific defensive factors rapidly to the site of infection.

Some of these serum proteins such as the lipopolysaccharide-binding protein (LBP) opsonize Gram-negative bacteria through their interaction with LPS, a major component of the outer membrane of these bacteria [71]. Soluble LPS is also readily complexed by LBP [72]. When tissue macrophages present locally encounter minute amounts of LPS, it is detected by the receptor CD14, a central component of the innate immunity. The interaction of LPS and CD14 results in the activation of tissue macrophages which produce pro-inflammatory cytokines such as TNF-α and IL-1 [73]. Then, LPS, together with TNF-α and

IL-1 diffuse in the neighbouring tissue where they in turn activate the ECs lining capillaries. These cells express high levels of certain surface adhesion molecules such as E-selectin and P-selectin, as well as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [74]. These newly expressed adhesion molecules markedly increase the affinity of ECs for leukocytes, an essential step in attracting and capturing these cells to the site of infection. Granulocytes such as neutrophils and monocytes bound to ECs then transmigrate to the site of infection guided by chemokine gradients [75]. There, their role is to engulf as many bacteria as they can to kill them and to prevent their multiplication. This process is accompanied by the synthesis of cytokines which alert surrounding cells of the presence of pathogenic invaders. This is an essential, first line of defense, but has to be strictly controled. Once the inflammatory reaction has succeeded in neutralizing the injurious stimulus, it should end with restoration of the site of acute inflammation to normal. This resolution is the usual outcome when the infection is limited or short-lived, and when there has been little tissue destruction and the damaged parenchymal cells can regenerate. Resolution involves neutralization or spontaneous decay of the chemical mediators, with subsequent return of normal vascular permeability, cessation of leukocytic infiltration (death largely by apoptosis of neutrophils) and finally removal of edema fluid and protein leukocytes, foreign agents, and necrotic debris from the site. Lymphatics and phagocytes play a definite role in these events [76]. In case of substantial tissue destruction, healing occurs by connective tissue replacement produced by activated fibroblasts [77]. All the events described above correspond to a required and normal situation to fight against bacteria. In some situations, acute inflammation becomes uncontroled and pathologic. An overactivation of the system may result in the release of excessive amounts of pro-inflammatory mediators. Localized, this may lead to chronic inflammation when the acute inflammatory response cannot be resolved, owing either the persistance of the bacteria or to some interference in the normal process of healing. But if this overactivation is systemic, a global activation of ECs may ensue leading to leakness of the capillaries. This set the stage for a catastrophic drop in blood pressure, multi-organ dysfunction and other symptoms of septic shock [78]. A better understanding of this pathology in its intimate mechanisms is the central interest of this manuscript.

2.3 Sepsis

Despite recent progress in antibiotics and critical care therapy, human sepsis is still associated with a high mortality rate. In the USA, the incidence of severe sepsis and septic shock has been estimated to be 750,000 cases per year, with mortality rates ranging from 30% to 50% [79]. In the most severe patients with septic shock, mortality rates raise to 60-70%. Sepsis triggered by Gram-negative bacteria represents 45-60 % of bacterial infections. It is now admitted that bacteria themselves have little toxic effects in comparison with other infectious processes, but the host immune response induced to fight against bacteria seems "disproportionate". Different stages in "the sepsis syndrome" are correlated with the degree of severity of the pathology and are defined by a combination of clinical signs and symptoms. SIRS is described as a systemic inflammatory response syndrome, which usually precedes bacterial infection. It corresponds to a condition in which the patient is "activated" with systemic inflammatory signs, but not yet infected. Systemic inflammation may be caused by different conditions, such as multiple trauma or pancreatitis, which are predisposing factors for the development of sepsis. Sepsis is defined as a SIRS with a documented infection. Septic patients display several physiological disturbances such as fever or hypothermia, tachycardia, tachypnoea, leukocytosis or leukopenia [80]. Patients with severe sepsis are defined as sepsis patients with one or more organ dysfunction. Patients with septic shock are defined as severe sepsis patients with refractory hypotension leading to septic shock [80]. Twenty to 50% of patients with sepsis eventually evolve into a septic shock [81, 82].

The concept of septic shock as being a consequence of a systemic "over-inflammation" induced by infection was essentially based on animal models of endotoxinemia or systemic infections with injections of important amounts of bacterial products or bacteria. The idea was that multiple organ failure resulted from the systemic spreading of pro-inflammatory mediators from the infected organ. In animal models, important concentrations of circulating pro-inflammatory cytokines such as TNF-α or IL-1β have been measured. Importantly, different therapeutic agents blocking these cytokines were tested and significantly decreased lethality [83-85]. Meanwhile, no beneficial effects of treatments with cytokine inhibitors were observed in septic models with a local inflammation when the insult was a local infection (as opposed to systemic infection), such as peritonitis or pneumonia [86-88]. To cite a few, steroidal and non-steroidal anti-inflammatory molecules, monoclonal antibodies against bacterial endotoxin, TNF receptor I and II, antagonists of IL-1 receptor or platelet activating factor (PAF) failed in the treatment of sepsis [89-94, 95, Dhainaut, 1998 #426]. One of the reasons for these successive failures was that the "pro-inflammatory" concept leading to

sepsis may have been wrong. New concepts based on several clinical studies have taken into account the "notion of compartimentalization" of inflammation, and not only the concentration of cytokines in blood, but also their bioactivity (measured with bioassays), which better reflects the "degree of inflammation" in the systemic compartment [96]. It appeared that in infected tissues the inflammation is important and necessary to trigger cellular reactions, which aim at preventing the expansion of infection, and then induce healing and remodeling of injured tissues. At the same time, in the blood compartment, high concentrations of TNF-α and IL-1β were measured, but no inflammatory bioactivities could be detected [97]. Not only no pro-inflammatory but important anti-inflammatory activities were measured in plasma from septic patients, due to high amounts of anti-inflammatory molecules such as IL-4, IL-10 (inactivate leukocytes), IL-1 receptor antagonist and soluble TNF receptor I and II [98]. It is likely that this anti-inflammatory reaction is induced by a small amount of pro-inflammatory cytokines or bacterial products, which reached the blood compartment after diffusion from the infected tissue through vessels of increased permeability. It is probably deleterious for the organism to have a circulating pro-inflammatory state, and the aim of a dominant systemic anti-inflammatory response might be to concentrate inflammatory cells to the injured organs [96].

Whatever the exact sequence of events during sepsis, every thing starts with the recognition of bacterial molecules by molecules and cells from the host. This manuscript will focus on how bacterial products and especially LPS (or endotoxin), the most potent inducer of pro-inflammatory mediators in Gram-negative sepsis, interacts and activates monocytes/macrophages, endothelial and epithelial cells, cells of the innate immunity. The ultimate goal of this behaviour is the recognition of key bacterial and host molecules that could be targeted in the therapy of sepsis.

2.4 Principal actor-molecules involved in endotoxin-induced cell activation and internalization

2.4.1 Gram-negative bacteria

Gram-negative bacteria, with a few exceptions, share fundamental properties of cell-wall assembly: the presence of a structurally and functionally complex outer membrane, an inner cytoplasmic membrane, and an interjecting thin layer of peptidoglycan [99]. The bilayered outer membrane is organized asymmetrically. In the outer layer, pointing to the outside, LPS (or endotoxin) is the dominating constituent, whereas the inner layer is composed of proteins and phospholipids [100].

2.4.2 LPS

LPS (or endotoxin) is exclusively synthesized by Gram-negative bacteria. This molecule is essential for the growth, the integrity and the shape of bacteria [101]. LPS from different Gram-negative bacteria share a common architecture in that they all consist of a polysaccharide chain directed out from the surface, covalently linked to a lipid component, named lipid A [101]. This latter moiety carries the endotoxic feature of the molecule and is anchored into the hydrophobic phase of the outer membrane. Lipid A is composed of a disaccharide carrying two phosphoryl groups and four hydroxy-fatty acids on two of which are linked two secondary acyl groups. When the secondary acyl chains are removed by the enzyme acyloxyacyl hydrolase in intracellular acidic compartments of mammalian host, the lipid A looses its endotoxic effects.

The polysaccharide chain, the immunogenic moiety of the molecule, is linked to the disaccharide of the lipid A and is characterized by the core oligosaccharide and the O-specific chain. The core is composed of 3-deoxy-D-manno-octulosonic acids (Kdo), heptoses and hexopyranoses. The O-specific chain is made of several repeating units of two to eight distinct glycosyl residues which lead to the polysaccharide chain formation of variable lengths and define the bacteria species. Molecular weights of LPS range from 2-3 kDa for “rough” LPS (Re595 *S. minnesota* mutant) corresponding to the minimal structure found in growing bacteria and 20 kDa for LPS from “smooth” Gram-negative bacteria, harbouring long polysaccharidic chains

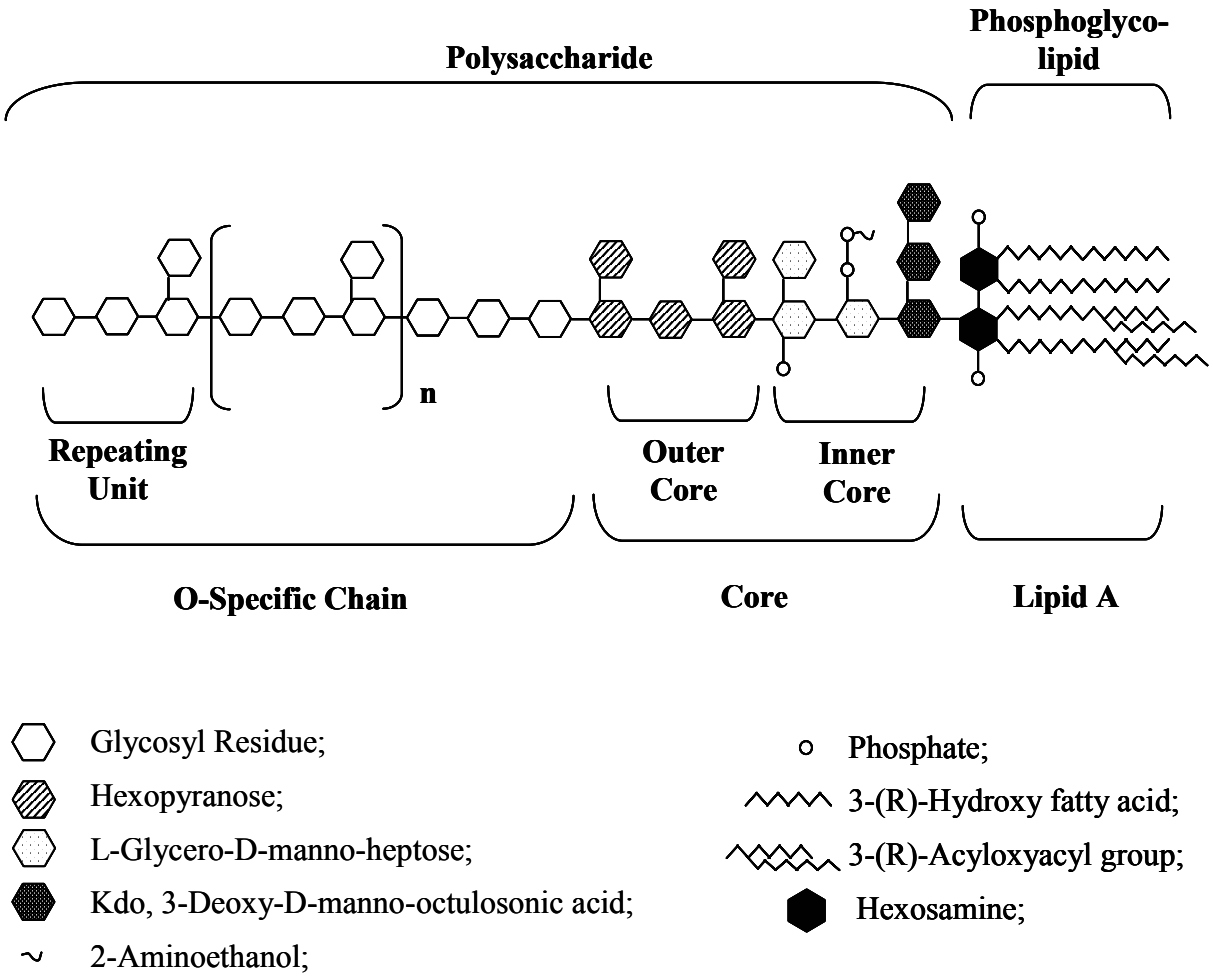

Figure 1: Schematic structure of enterobacterial wild-type (Smooth-form) LPS

The interest for biological effects of LPS came from the observation that striking parallels existed between the biological activities of LPS in animals injected with LPS and those observed in patients with Gram-negative sepsis [102, 103]. A most remarkable feature of LPS is its capacity to elicit the nearly entire spectrum of host effector molecules. With regard to its effects in the mammalian body, LPS exibits two features: i) a highly diversified pattern of immediate pathophysiological activities including fever, hypotension, changes in leukocytes counts, and disseminated intravascular coagulation, ii) a more delayed effects, such as the induction of antibodies being specific for defined LPS structures but also for other antigens [104]. LPS is known to interact with both humoral and cellular systems of the host. LPS binds to specific proteins such as LBP, high- and low-density lipoproteins.

2.4.3 LBP

LBP is a 60 kDa glycoprotein present in normal plasma at concentration of 3 to 7 μg/mL. During inflammation or sepsis, serum levels of this acute phase protein can raise up to 50 to 100-fold [105, 106]. The mature protein contains four cysteines, five potential glycosylation sites and appears to be highly conserved since for example human and rabbit LBP share 69 % sequence identity [72]. LBP is principally produced by the liver, but also in smaller amounts by the lung or other tissues [107]. Tobias et al. identified this protein in human serum in 1985 [108] and showed that the specificity of LBP was directed towards the hydrophobic lipid A portion of LPS [109]. Its affinity was measured to be 10^{-9} M for both smooth and rough forms of LPS [110] with a 1:1 LPS-LBP stoichiometry [111]. The principal function of LBP is to dissociate LPS aggregates into LPS monomers and deliver it catalytically to the receptor CD14. A mutant form of LBP made of residues 1-197 showed that LBP still binds LPS, but fails to transfer it to CD14 [112]. Thus, the LPS binding activity is contained within the amino-terminal half of LBP and the CD14 interaction site resides in the carboxyl-terminal half of LBP. Other studies based on site directed-mutagenesis and peptide blocking allowed to determine that the LPS-binding domain is located in the amino acid (aa) portion 89-97 of LBP [113]. LBP facilitates the interaction of LPS with CD14 by two basic processes: i) LBP acts as an opsonin for LPS-bearing particles enhancing the interaction with CD14 [71], and ii) LBP enables cells to respond to extremely low LPS concentrations *via* a CD14-dependent way [72]. LBP seems to also play a role in LPS internalization as shown by Gegner et al. with the identification of LBP antibodies that can prevent LPS endocytosis, but not cell activation [114].

LBP belongs to a family of lipid transferases structurally similar proteins that include bactericidal/permeability-increasing protein (BPI), cholesterol ester transfer protein (CETP), and phospholipid transfer protein (PLTP) [115-117]. With its capacity to transfer lipids, LBP mediates LPS detoxification by involving other proteins than CD14. LBP catalyses movement of LPS monomers to lipoproteins such as HDL, LDL and VLDL present in plasma [118-120]. These lipoproteins by retaining LPS prevent binding of LPS to CD14 and thus neutralize noxious effects of LPS [121]. Moreover, this process was demonstrated to increase LPS clearance [122]. This, in consequence, leads to diminished production of pro-inflammatory cytokines [123]. Animal studies with infusion of lipoproteins or hyperlipoproteinemia in

mice, demonstrated their "protective" role against endotoxemia or severe Gram-negative infection [124, 125].

2.4.4 CD14

2.4.4.1 Discovery of CD14 and its expression in tissues

CD14, first described as a leukocyte cluster differentiation (CD) antigen, is a 55 kDa glycoprotein expressed at the surface of monocytes, macrophages and to a lower extend on neutrophils. Human monocytes express ~ 10^5 CD14 molecules whereas expression on neutrophils is much lower (~ 3.10^3 receptors/cell) [126]. Its expression on B cells has been reported [127, 128], however, it is generally accepted that normal B cells are CD14-negative. CD14 was originally identified as a myeloid differentiation antigen, present on mature cells but absent on myeloid precursors. Its expression has also been correlated with various forms of malignant myeloid leukemias, and has been used in establishing diagnosis.

In situ hybridization experiments revealed extramyeloid expression of CD14 messenger (m)RNA in many tissues such as uterus, adipose tissue, lung [129], tracheal epithelial cells [130] and hepatocytes from LPS-treated [131]. This expression was either constitutive or inducible in response to inflammatory stimuli.

In 1988, CD14 complementary (c) DNA was cloned and mapped to chromosome 5 into a region (5q 23-31) that encodes for other growth factors and receptors such as IL-3, GM-CSF, macrophage-colony stimulating factor (M-CSF), platelet-derived growth factor (PDGF) receptor, and the β-adrenergic receptor. The CD14 gene was located within the "critical region" that is frequently deleted in certain myeloid leukemias [132]. The gene contains two exons separated by one 88-bp intron. The first exon only codes for the first aa methionine. The genomic structure and the mRNA analysis do not provide any evidence of exons coded domains or alternative splicing mechanisms.

The primary sequence of CD14 in different species revealed a considerable amount of conservation. Human CD14 shares about 65 % similarity with the orthologs in mouse, rat, rabbit and bovine [105, 133-135].

2.4.4.2 Biochemical properties of CD14

The human protein consists of 375 aa residues from which a 19 aa signal peptide is cleaved in the endoplasmic reticulum [136]. The sequence ends with a 21 aa hydrophobic domain and lacks the characteristic basic residues of a stop transfer domain. CD14 has been demonstrated to be anchored into the cell membrane surface by a glycosylphosphatidyl-inositol moiety. CD14 can be cleaved with a phosphatidyl-inositol specific phospholipase C treatment. CD14 has four potential N-linked glycosylation sites and in addition bears O-linked carbohydrates. Carbohydrates account for 20 % of the total molecular weight of the protein [137]. No functional properties have been associated with the sugars. The human CD14 also contains 10 leucine-rich repeats located between residues 86 and 329 [138]. These repeats are supposed to mediate protein-protein interactions, however no clear funtion has been assigned to these domains in CD14.

The key role of CD14 in the binding and in the increase of cell responsiveness to LPS was recognized in 1990 for the first time [139]. Recognition of LPS by CD14 requires LBP, an acute-phase serum reactant (described above) [110, 139, 140]. LPS-LBP complexes bound to CD14 leads to the formation of a large ternary complex as shown by Gegner et al. [114]. CD14 is also present in plasma as a soluble protein at concentrations of 1-2,5 μg/mL [141]. In several clinical situations such as psoriasis [142], sarcoidosis [143], systemic lupus erythematosus [144], HIV infection [145], patients suffering from polytrauma and burns [146], septic patients with multi-organ failure [147], acute respiratory distress syndrome (ARDS) [148], plasma sCD14 concentration can increase. This protein can be considered as a marker of chronic and acute inflammation and viewed as an acute phase protein, although its role in these diseases is not yet understood. Pugin et al. have first described the essential role of sCD14 in LPS-induced activation of some non-CD14 bearing cells such as ECs and in some epithelial cells [149]. The mechanism by which sCD14 activates these cells will be further detailed in next sections. The protease-induced shedding of membrane CD14 (mCD14) from the surface of activated monocytes was first reported to be the source of

sCD14 [150]. Later, Bufler et al. provided evidence that sCD14 may also be produced by a protease-independent mechanism [151]. Indeed, the generation of a low-molecular-mass form of sCD14 was described as a process involving endocytosis followed by exocytosis [152]. Another way to produce sCD14 is when the synthesis of a glycosylphosphatidylinositol (GPI) anchor may be leaky and therefore a fraction of the sCD14 is directly secreted; or when minor forms of a lower molecular weight are not completely glycosylated [137].

To better understand interactions between LPS and CD14, different types of experiments were performed to map epitopes of CD14 essential for LPS binding and LPS-induced cell activation. This information will be detailed in further sections. CD14 does not only bind soluble LPS but also whole Gram-negative bacteria and a variety of ligands derived from different biological sources. This raises the question of whether these molecules interact with the same binding site on CD14. It was shown that the integrity of region 39-44 was important for recognition of both Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* bacteria [153, 154]. Kusunoki et al. found that deletion of residues 57-64, which eliminates LPS binding also abrogates CD14-dependent responses to *Staphiloccocus aureus* molecules [155]. Finally, by using monoclonal antibodies Dziarski et al. demonstrated that conformational binding sites on CD14 for soluble peptidoglycan and LPS are partially identical and partially different [156]. Together, these data suggest that the domain by which different molecules are recognized by CD14 is at least similar if not identical. However, the common pattern shared by all these ligands and how it fits into the binding site of CD14 has not yet been defined. Further studies using X-ray crystallography or NMR techniques are required to finally solve this problem.

2.4.4.3 Other ligands of CD14

The search for CD14 ligands other than LPS has revealed that CD14 can bind diverse structures derived from both microbes and animal hosts. Beta 1-4-linked polyuronic acids from bacteria, fungi, and oxidized cellulose was described as the first non-LPS ligands [157]. The notion of “pattern recognition receptor” for CD14 was first mentioned by Pugin et al. when they showed that CD14 recognized soluble peptidoglycan from Gram-positive cell walls, and mycobacterial lipoarabinomannan [23]. The evidence for the involvement of CD14 in mediating cell activation in response to bacterial agonists other than LPS came from the

following observations: 1) the presence of mCD14 enhanced CD14-bearing cell responses to Gram-positive cell walls and mycobacterial lipoarabinomannan by 30- to 300-fold; 2) cell responses could be inhibited with anti-CD14 antibodies; 3) Gram-positive cell walls activated non-CD14 bearing cells in a sCD14-dependent manner; and 4) direct binding of these new ligands to sCD14 could be demonstrated [23]. As for LPS, LBP also enhanced cellular responses to lipoarabinomannan (LAM) [158]. Various groups have now confirmed these results and identified additional putative CD14 ligands originating from bacteria and yeasts as shown in the table 1.

Soluble peptidoglycan, the basic structural component of bacterial cell walls, has been shown to bind and activate cells through CD14 [159]. Studies with synthetic muramyl dipeptides (MDP) have shown that these peptidoglycan subunits are the minimal structures required for binding to CD14 [159]. Dziarski et al. found that soluble peptidoglycan binds to sCD14 and mCD14, whereas MDP monomers do not [156]. MDP binds to CD14 only when the MDP is polymerized, aggregated, or immobilized on a solid support. Although CD14 appears to have a broad ligand binding specificity for certain microbial polymers, its inability to bind a variety of other polymers such as dextrans and heparin indicates that it is not a general receptor for polysaccharides and others biological polymers [156].

Lipoteichoic acids (LTA) from Gram-positive bacteria have also been reported to stimulate cell reponses by inducing NO and IL-12 production by a mechanism that is enhanced by CD14 [160, 161]. However, other investigators claimed that highly purified LTA binds sCD14 but has no stimulatory activity.

Isolated membrane lipoproteins and synthetic lipopeptides based on the structures of lipoproteins stimulate nuclear factor-kappaB (NF-κB) and cytokine production in phagocytes and ECs by mechanisms that are enhanced by mCD14 and sCD14, respectively [162].

Recent studies show that the ligand-binding specificity of CD14 is not restricted to “non-self” microbial structures. Indeed, it has been shown that both sCD14 and LBP can act as transfer proteins for certain host-derived phospholipids [163, 164]. Phosphatidylinositides and phosphatidylserine bind to mCD14 in a LBP-dependent manner. mCD14 has also be involved in the binding and the phagocytosis of apoptotic bodies [165]. During cell apoptosis, phosphatidylserine is externalised and can be recognized by mCD14. In contrast to microbial

molecules, self structures that are recognized by CD14 and LBP are non-stimulatory. This raises the possibility that CD14 and LBP have physiologic roles apart from microbial recognition.

2.5 General problematic

This thesis is composed of three chapters. Each chapter is introduced and a specific detailed problematic is raised. As previously mentioned, an important step in the pathogenesis of sepsis is the recognition of bacterial molecules and particularly in the context of this thesis, endotoxin by innate immune cells and the LPS response of these cells. In order to better understand these phenomena, we were interested in studying LPS interactions with the cell surface of myeloid and epithelial cells. Studies of the chapter 1 consisted in investigating the mechanisms by which LPS-induced cell activation and internalization. Furthermore, the question of a possible role of CD14 in such mechanisms was raised. In several studies, LPS response has been shown to be modulated in myeloid cells at different steps of differentiation. Subsequently, we hypothized in the chapter 2, that CD14 and new receptors of innate immunity: Toll-like receptors (introduced in this chapter) could be involved in such LPS response modulation. Throughout this thesis, the role of the GPI-anchor of CD14 was raised in processes of cell activation, LPS internalization, but also in cellular mechanisms mediated by another bacterial molecule, the aerolysin from *Aeromonas hydrophila* introduced in the chapter 3 and responsible also for septic and gastrointestinal pathologies. In order to perform all these studies, biochemical, cellular and molecular approaches were used.

3 CHAPTER I : LPS-CD14 induced-cell activation and internalization

3.1 Introduction

3.1.1 Endotoxin-induced cell activation

Mechanisms of activation of CD14-bearing cells and non CD14-bearing cells by LPS will be detailed in the following sections.

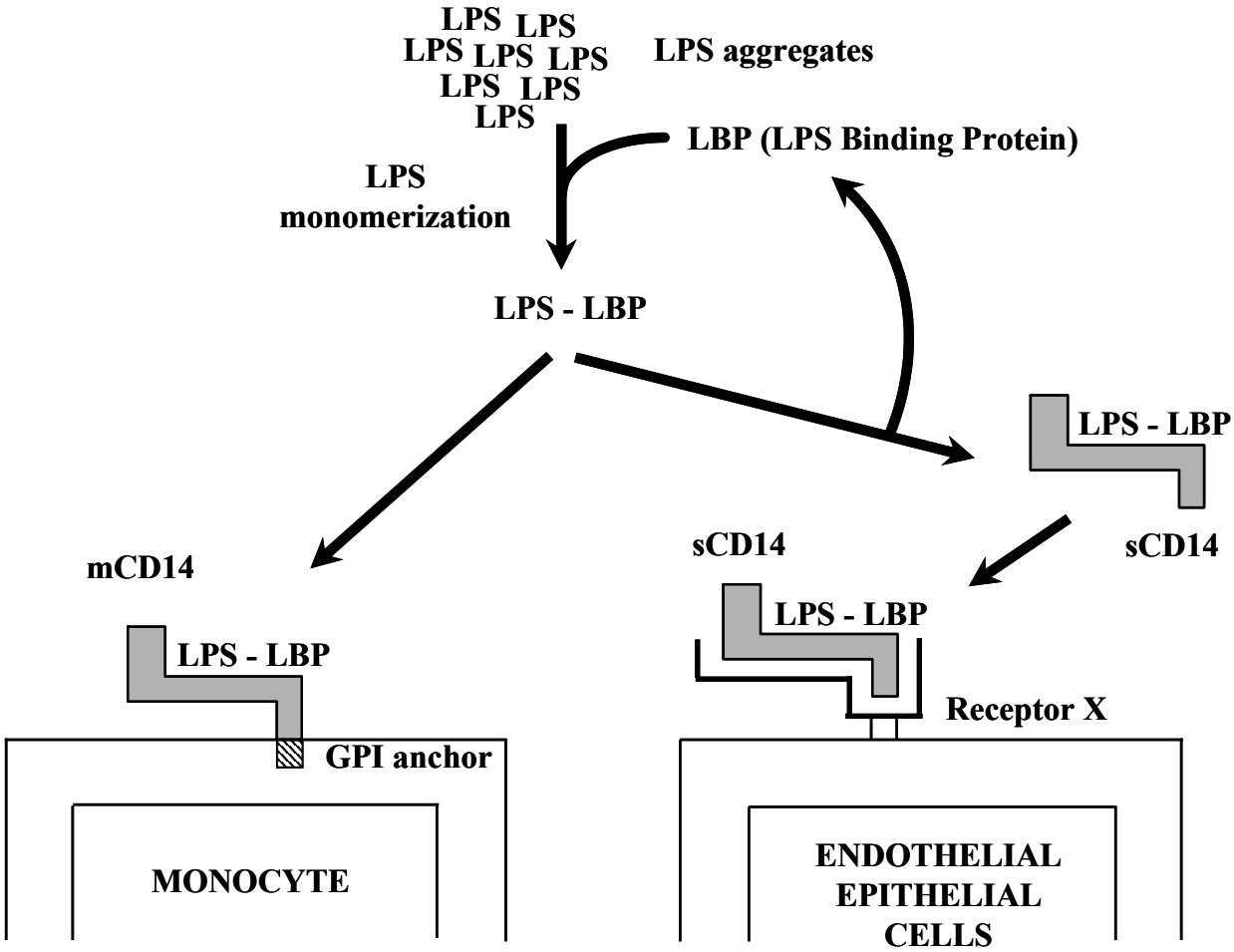

Figure 2: Schematic view of the activation of CD14-bearing cells (monocyte/macrophage) and non CD14-bearing cells (endothelial and some epithelial cells) by LPS.

3.1.2 LPS stimulation of CD14-bearing cells

The GPI-anchored mCD14 is present at the surface of myeloid cells such as monocytes, macrophages, neutrophils and basophils. The first evidence that mCD14 was a receptor for LPS and had a pivotal role in LPS-induced cell activation, came from studies by Wright et al. Antibody-blocking experiments demonstrated that CD14 interacted with LPS complexed with LBP and that this complex activated cells [139]. Crosslinking experiments performed by Tobias et al. clearly confirmed that LPS directly associated with mCD14 in macrophages and with sCD14 in the serum [54, 149]. Investigations from various groups further demonstrated the pivotal role of mCD14 in mediating cellular activation by LPS. Lee et al. showed that 70Z/3 pre-B cells poorly responsive to LPS increased their sensitivity to this bacterial molecule by two orders of magnitude after transfection with the gene encoding for the membrane form of CD14 [166]. Importantly, it was shown that in patients with paroxysmal nocturnal hemoglobinuria (PNH), which hematopoietic cells are devoid of GPI-linked proteins including CD14, macrophages did not respond to LPS. This response was restored only when sCD14 was added to cells [167, 168]. It was also shown that CD14 knock-out mice were highly resistant to shock induced either by LPS or living Gram-negative bacteria [169]. The ability of CD14-deficient mice peripheral blood mononuclear cells to respond to LPS was partially restored through the addition of recombinant sCD14 to this assay [169]. In contrast, overexpression of human CD14 in transgenic mice rendered these mice hypersensitive to LPS, as evidenced by their increased susceptibility to endotoxic shock [170]. Taken together, these results indicate that mCD14 clearly plays a central role in LPS-induced cell activation and that myeloid cells can also respond to LPS through a sCD14-dependent pathway which acts independently of mCD14. With respect to mCD14-bearing cells, sCD14 appears to play a dual role as either an activator or inhibitor of LPS-mediated activation. At high concentrations, sCD14 dampen the responses of CD14-bearing cells to LPS. For example, recombinant sCD14 could inhibit the oxidative burst in isolated human monocytes [171], the TNF-α release in whole blood [172], the priming of neutrophils [173] and could protect mice from LPS-induced lethality [174]. Using various concentrations of LPS, sCD14 and mCD14, Haziot et al. showed that LPS-induced signal transduction mediated by mCD14 was more efficient than that mediated by sCD14 [169].

The mechanism by which LPS activates myeloid cells is not perfectly understood, even if several clues have been discovered. Like other membrane phospholipids, LPS is a

amphiphilic molecule that exists in an aggregated form in aqueous environments such as tissue culture media or plasma. LBP acts as an opsonin or a lipid transfer protein, catalytically transferring LPS monomers from aggregates including bacterial membranes to acceptor molecule such as CD14 [111, 114, 140]. By dramatically accelerating the binding of LPS monomers to CD14, LBP enhances the sensitivity of cells to LPS [175]. Additional studies have shown that LBP transfers LPS not only to cell surface CD14 but also to lipoproteins such as high density lipoproteins (HDL), and it may facilitate transfer of LPS from CD14 into cell membranes by lipid exchange [175, 176]. CD14 is anchored by a GPI that does not cross the membrane lipid bilayer. Consequently, it was questioned how the signal could transduce through the plasma membrane when the ligand LPS binds to CD14. Several hypothesis were raised: 1) when the ternary complex CD14-LPS/LBP is formed, it could associate with (an)other(s) transmembrane protein(s) that could play the role of signal transducer molecule, 2) LPS is inserted into the phospholipid bilayer either by membrane or soluble CD14 and this results in a packing geometry that perturbs the membrane and subsequently triggers cellular activation [176, 177], and 3) CD14 engaged by its ligand interacts in the membrane lipid bilayer via its GPI tail with a protein, and this interaction leads to transmembrane signaling.

The second hypothesis may somewhat be supported by a study by Joseph et al. These authors proposed that after integration of LPS into the membrane it mimics the second messenger function of ceramide and stimulates cells by interaction with a ceramide-activated kinase [178]. This second scenario may also be indirectly supported by the observed effects of certain pharmacological agents on LPS-mediated signaling [177, 179]. Whether LBP and CD14 actually transfer LPS into the phospholipid bilayer of an intact cell, and whether this action would result in cellular activation, has yet to be established. The last hypothesis was addressed by converting GPI-anchored CD14 to a transmembrane form by constructing mutants of CD14 [180]. This conversion of CD14 would uncouple the receptor-mediated signal in the third model, but would not affect signal transduction in the first hypothesis. CD14 mutant was constructed to contain either a sequence coding for the C-terminal transmembrane peptide portion of tissue factor or part of the decay accelerating factor (DAF) receptor containing the GPI-attachment site of DAF. 70Z/3 cells transfected with these different CD14 constructions responded normally to LPS, indicating that the anchoring system was irrelevant for cell activation [180]. Several studies supported the first hypothesis. Cells devoid of CD14 have little or no response to LPS, except at high doses (micromoles instead of nanomoles) [181]. This suggested a CD14-independent cell activation and the

involvement of protein(s) other than CD14. C3H/HeJ or C57BL/10ScCr mice are non-responsive to LPS, however they normally express CD14 [182, 183], raising the hypothesis of another key component required for LPS sensibility. Moreover, it has been shown that the anti-human CD14 monoclonal antibody, 18E12 was capable of blocking LPS-induced cell activation without preventing LPS binding to CD14 [180]. Finally, the addition of sCD14 to CD14-negative cells from PNH patients restored cell sensitivity to LPS. It is therefore likely that this antibody binds to an epitope of CD14 important for an interaction between CD14 and the putative transducing molecule. Consequently, CD14 was thought to be an important ligand-binding unit of a multiple chain receptor complex [184]. Furthermore, it is known that lipid IVa, a precursor of LPS lacking two acyl chains, acts as an LPS antagonist for human cells and as an LPS agonist for mouse cells [185]. Although both of these actions are mediated by CD14, the heterologous expression of mouse or human CD14 does not alter cellular responses to lipid IVa which remain species specific [185]. These findings demonstrate that the species specific effect must be mediated by a cell-signaling component, other than CD14, that effectively discriminates between LPS and lipid IVa. Altogether, these results are evidence for at least one accessory molecule for CD14. Several candidates have been presented as putative accessory molecules of CD14 and will be further described.

3.1.3 LPS stimulation of non CD14-bearing cells

ECs contribute significantly to the pathophysiologic events leading to Gram-negative sepsis. In fact, the vascular endothelium itself actively participates in organ inflammation by expressing chemokines, cytokines and adhesion molecules. The ability of LPS to directly activate ECs has been recognized for almost two decades. Early studies revealed that human ECs cultured *in vitro* display increased procoagulant activity, expression of tissue factor adhesiveness in response to LPS [186, 187]. Subsequently, it was shown that a variety of cell surface adhesion molecules are strongly expressed in response to LPS including endothelial-leukocyte adhesion molecule-1 (ELAM-1), ICAM-1 and VCAM [188-190]. In addition, ECs were found to release the cytokines IL-1, IL-8, IL-6 as well as to activate NF-κB upon treatment with LPS [191, 192]. In contrast to the responses observed using human cells, a pronounced LPS-mediated cytotoxic effect was observed on bovine EC morphology and metabolism leading to increased permeability of the endothelial layer [193]. Importantly, a number of studies revealed that this injurious effect of LPS required the presence of serum

and could be completely blocked by an anti-CD14 monoclonal antibody [194]. Meanwhile, ECs were found not to express any measurable cell surface mCD14, which could explain this finding. The factor in serum responsible for mediating responses of ECs to LPS was found to be the soluble form of the receptor CD14 [149, 195]. This discovery was demonstrated by the loss of responsiveness through immunodepletion of sCD14 from normal serum and by the acquisition of LPS responsiveness through the addition of recombinant sCD14 to serum-free media [149, 196, 197]. Thus, an exceptionally unusual feature of CD14 is its ability to mediate LPS-induced activation of cells as a membrane receptor and as a soluble co-factor depending on the cell types.

Interestingly, the ability of sCD14 to mediate LPS-induced cellular activation is not restricted to ECs. LPS can also activate cells from epithelial origin by inducing IL-8 synthesis in colonic epithelial cell lines (SW620, HT29), IL-6 in epithelial-like astrocytoma cell line (U373), bronchial or tracheal epithelial cells [130, 149, 198], gingival fibroblasts, human vascular smooth muscle cells and peripheral blood dendritic cells [199-201].

This response is greatly enhanced by the co-factor LBP [149]. In the absence of LBP, the binding of LPS by sCD14 is kinetically a low process. Biochemical and biophysical studies have revealed that LBP catalyses the formation of sCD14-LPS complexes [140, 202]; first, by binding to LPS monomers from aggregates and second, by delivering LPS to sCD14 present in the plasma. Then, the complex sCD14-LPS would bind to an as yet unknown receptor present on non CD14-bearing cells. This hypothesis is supported by studies of several groups who have directly examined the physical association between sCD14 and whole cells. One of these groups found a specific cell surface binding of radiolabeled sCD14 to human umbilical vein endothelial cells (HUVECs) in the absence of LPS [203]. It was also shown that sCD14 associates with several cell types in a LPS-dependent manner. This process is slow to reach the equilibrium and requires high concentrations of LPS [204]. One important drawback of this latter work is that the iodinated sCD14 used in these experiments was not able anymore to activate cells in the presence of LPS. When sCD14 was radiolabeled with another technique and shown to be functional, sCD14 was not able to bind to any cell type neither in a LPS-dependent nor in a LPS-independent manner [205]. Altogether, these results indicate that a putative receptor for sCD14 is either of low cell-surface abundance or has a low affinity for sCD14.

3.1.4 Intracellular signaling pathways

LPS stimulation of monocytes results in the activation, translocation and binding of numerous transcription factors to promoters of LPS-inducible genes. Typically, these genes encode for inflammatory mediators such as cytokines (TNF-α, IL-1, GM-CSF…) and chemokines (IL-8, MCP-1), but also receptors (tissue factor, IL-2 receptor α), transcription factors (p50, c-Rel, Egr-1…), inhibitors (I-κB) and others such as the inducible NO-synthase. LPS-responsive *cis*-acting DNA promoter elements have been characterized in the 5' flanking region of many of these genes [reviewed by 206]. Many transcription factors involved in LPS signaling are activated by dimerization and by phosphorylation through different intracellular pathways. To date, the I-κB kinase (IKK)/NF-κB pathway as well as mitogen-activated protein kinase (MAPK) pathways, which include the extracellular signal-regulated kinase (ERK)-, the c-Jun N-terminal kinase (JNK)- and the p38-pathway were shown to be implicated in this stimulation process. Most studies have used dominant-negative mutants, knock-out mice or specific inhibitors of intracellular molecules to study these signaling pathways.

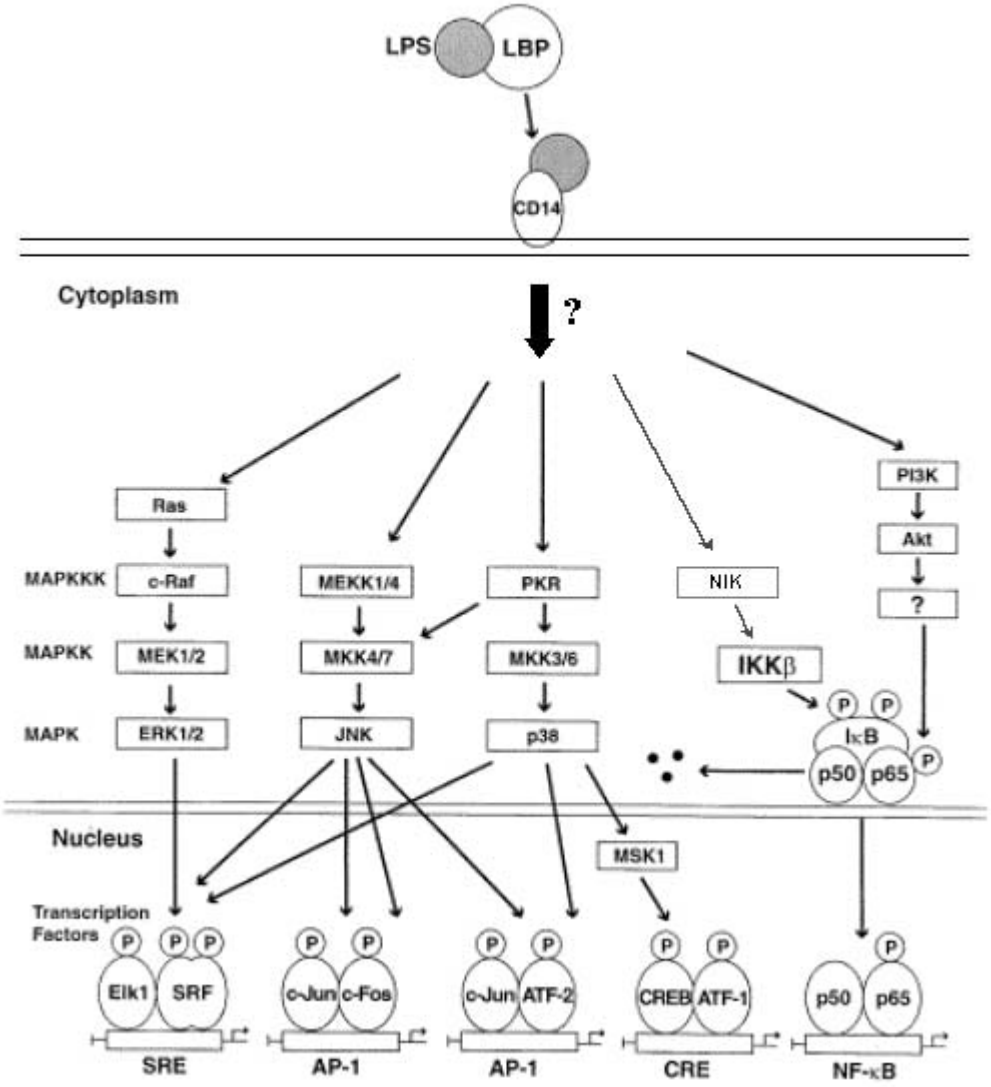

Figure 3: LPS activation of different signaling pathways and transcription factors in monocytes [adapted from 2].

In epithelial and ECs, LPS-induced signaling pathways are less well known than those unraveled in monocytes, but in virtually all cells, the transcription factor NF-κB is activated. The pattern of signaling pathways activated by LPS depends on tissue or organ cells originate from. This may serve as the basis for a differential response of tissues or organs depending on the microbial stimuli and the function of organs.

3.1.4.1 The IKK/NF-κB pathway

It has now been well established that phylogenetically conserved signaling mechanisms in innate immunity providing an immediate cellular reaction to LPS utilize the NF-κB pathway in the first-line of defense. The NF-κB pathway is not only restricted to innate immunity, but plays important roles in the adaptive immune system, in the protection against apoptosis and in the cell cycle [207, 208]. NF-κB family members contain a conserved DNA binding and dimerization domain called the Rel homology domain. Mammalian cells contain five NF-κB subunits: RelA (p65), c-Rel, RelB, p50 and p52, which form various hetero- and homo-dimers. The p50 and p52 subunits which lack transactivation domains, are produced by processing of precursor molecules of 105 kDa and 100 kDa, respectively. NF-κB has been shown in numerous studies to be activated by LPS in monocytic cells [209]. Intracellular signaling pathways mediating this activation have remained unknown for many years. Recently, second messengers were identified as mediators of NF-κB activation in response to LPS. In "resting" situation, the p50-p65 dimer is retained in the cytoplasm by its natural inhibitor, inhibitor-kappa B (I-κB). After LPS stimulation, NF-κB translocates into the nucleus after I-κB is phosphorylated by the two kinases IKKα and β, and degraded by the ubiquitin-proteasome pathway [210]. These kinases form a large multiprotein complex with a scaffold protein called NEMO (IKKγ) [211]. Some studies have suggested that IKKβ rather than IKKα was required for LPS activation of NF-κB in THP-1 monocytic cells [212]. The IKKs themselves might be activated through phosphorylation by an upstream kinase(s). Candidates for this kinase include NF-κB inducing kinase (NIK) and MAPK ERK kinase kinase 1 (MEKK1). Both kinases can activate NF-κB through phosphorylation and activation of the IKKs [213-215].

It appears that to be fully activated, NF-κB needs to translocate into the nucleus, but also needs to be transactivated. Transactivation requires p65 phosphorylation. The p65 subunit of the dimer is phosphorylated by the phosphatidyl inositol 3 kinase (PI3K)-Akt signaling pathway [216]. By activating the DNA polymerase, this phosphorylation enhanced p65-mediated transcription without affecting I-κB degradation, nuclear translocation or the ability of NF-κB to bind to DNA. To date, the kinases which directly phosphorylate the transactivation domains of p65 and c-Rel have not been identified.

3.1.4.2 The MAPK pathways

Tyrosine phosphorylation of proteins including the src tyrosine kinase family members $p53/56^{lyn}$, $p58/64^{c\text{-}fgr}$ is rapidly induced after LPS stimulation of monocytes/macrophages [217, 218]. LPS also activates isoforms of protein kinase C (PKC) as well as G-proteins (Gi and transducin) whose activity can be blocked by pertussis toxin in a mouse macrophage line [219]. These and other signaling molecules may play a role in the LPS-induced activation of the MAPK cascades composed of a MAPK, MAPK kinase, (MAPKK) and a MAPKK kinase (MAPKKK) [206].

3.1.4.2.1 The ERK pathway

In monocytic cells, ERK1/2 were shown to be phosphorylared on their TEY motif after LPS treatment. It has been suggested that LPS stimulation of tyrosine kinases led to the activation of the MAPK ERK kinase (MEK)-ERK1/2 pathway in a Raf1-dependent manner [220]. It has been shown that Ras and c-Raf, two upstream signaling molecules were implicated in MEK MAPK pathway in LPS induction of TNF-α expression [221]. The use of specific inhibitors of MEK in monocytes, revealed a reduction of LPS induction of several inflammatory cytokines (IL-1, IL-8, TNF-α), prostaglandin E_2 [222] and of the tissue factor gene as recently shown [unpublished data reviewed by 2]. One of the downstream targets of the MEK-ERK1/2 is Elk1 [223-225]. After phosphorylation of its C-terminal domain, this transcription factor changes its conformation. This promotes its assembly with other factors to form an active ternary complex ready for inducing transcription.

3.1.4.2.2 The JNK pathway

Another MAP kinase pathway rapidly activated by LPS is the JNK pathway as demonstrated in monocytic THP-1 cells and RAW 264.7 cells [226]. Upstream protein kinases such as m p21-activated kinase (PAK) 3, hPAK1 and germinal center kinase (GCK) trigger a phosphorylation cascade which leads further downstream to the activation of MAPKK kinases: MEKK1, MEKK4 and MUK/DLK/ZPK [227-232]. Both stimulated MAPK

kinases MKK4 and MKK7 are then direct activators for JNK, also known as the stress-activated protein kinase (SAPK) 1 [233, 234]. Two isoforms of JNK (54 and 46 kDa) are known and they contain a typical TPY motif in their kinase domain that is required for activation [235]. These JNK proteins act on various transcription factors as downstream targets. They can phosphorylate c-Jun, ATF-2 and Elk-1, which heterodimerize and then regulate the expression of various inflammatory genes by binding to specific responding elements of their promoters [236].

3.1.4.2.3 The p38 pathway

p38 was first recognized as a novel MAPK activated by LPS in 70Z/3 cells by the group of Ulevitch [237]. LPS treatment of monocytes/macrophages is rapidly followed by the activation of upstream signaling molecules such as Cdc42, PAK and Rac1 which in turn activate the p38 pathway [227, 238, 239]. Then, MAPKKKs including protein kinase RNA-regulated (PKR), TAK1 and others, MAPKKs such as MKK3, MKK6 and possibly MKK4 are activated through kinase phosphorylation cascades [240-243]. These latter activated kinases phosphorylate p38, which itself can phosphorylate several transcription factors (ATF-2, Elk-1, CHOP, MEF2C, Sap1a), and downstream kinases [244-247]. Subsequently, these enzymes trigger the phosphorylation of other transcription factors such as CREB and ATF-1 [225]. The implication of p38 in the LPS signaling has been further demonstrated by the use of highly specific inhibitors of p38α,β belonging to pyridinyl imidazole compounds such as SB203580 [248]. The inhibitory effect of these molecules is attributed to binding of the drug to the ATP binding pocket in the kinase domain [249].

3.1.5 Binding, internalization and clearance of endotoxin

As stated above, LPS not only binds to cell surface to specific receptors resulting in the activation of cells, but is also rapidly internalised by the cell for detoxification [114, 250, 251]. Numerous techniques have been used to study LPS binding, uptake and distribution in various cell types. Direct methods include LPS labeled with radioactive isotopes such as ^{125}I, ^{131}I, ^{3}H, ^{51}Cr, ^{14}C [252-254] and LPS directly linked to markers such as horseradish peroxidase (HRP), fluorescent molecules, uranyl acetate, biotin or colloidal gold particles. Indirect methods involve specific antibodies against polysaccharide regions or lipid A of LPS and a secondary antibody linked to a marker probes [255]. Experiments using electron microscopy, fluorescence and confocal microscopy, optical microscopy, flow analysis cytometry sorting (FACS) and radioactivity could be performed with "tagged" LPS or antibodies raised against LPS.

To prevent misunderstanding in the next sections, the following terms are defined as : 1) LPS binding corresponds to the quantity of ligand attached to the cell surface ; 2) LPS internalization or endocytosis takes into account the amount of ligand that goes into cells, but not the LPS bound at the plasma membrane ; 3) LPS uptake takes into account both LPS bound and internalised.

3.1.5.1 Binding of LPS

Binding of LPS to plasma membranes is the essentiel step for interaction between endotoxin and cells. The LPS-cell interaction triggers a cascade of cellular activities including uptake of LPS and synthesis of cytokines by macrophages [72]. Endotoxin may bind to the plasma membrane as molecules, aggregates (micelles) and complexed with LBP [109, 250, 256]. The interaction of endotoxin with the cell surface occurs either "specifically" by binding to receptors, "non-specifically" by interacting with membrane constituents other than "specific" receptors or through its insertion into the lipid bilayer [256, 257].

3.1.5.1.1 Specific binding of LPS

3.1.5.1.1.1 Binding of LPS to CD14

Pioneer work on LPS binding to CD14 was performed by Couturier et al. using ^{3}H-labeled LPS. It was shown that in the presence of serum, LPS could bind to monocytes in a dose-dependent, saturable, and displaceable fashion. This specific binding of LPS was demonstrated to occur through the CD14 molecule. This was evidenced by the ability of monoclonal antibodies (mAb) and of F(ab')2 fragments of mAb directed against specific epitopes of CD14 to inhibit the binding of ^{3}H-LPS to cells, and by the lack of LPS binding to CD14-deficient cells from patients with PNH [258]. One year later, Heumann et al. found similar results by using fluorescein-labeled LPS and the MY4 anti-CD14 mAb and by enzymatically removing CD14 from monocyte surface. In addition, the LBP-dependent binding of LPS to CD14 was further established by using serum depleted of LBP with anti-LBP antibodies [259]. A more formal proof that CD14 was a receptor for LPS came from studies by Tobias et al. Crosslinking of LPS to cell membranes expressing high levels of CD14 (THP-1 cells differentiated with the 1,25 $(OH)_2$ vitamin D3), identified only one band corresponding to mCD14. This was determined by a characteristic molecular weight on SDS PAGE, and by immunoprecipitation with CD14 mAbs. Labeling of mCD14 was totally dependent on the addition of LBP, and could be inhibited by an excess of « cold » LPS and by anti-CD14 mAbs blocking LPS-CD14 binding (28C5, 3C10), but not by the anti-CD18 mAb IB4 [260]. Kinetics studies indicated that LPS binding to mCD14 on monocytes occured within 1 minute and that few minutes of exposure to physiological concentrations of LPS were sufficient to activate monocytes [261]. Studies using ^{3}H-labeled LPS binding to human CD14 expressed on Chinese hamster ovary cells and on a human monocytic cell line (THP-1), indicated that LPS binding occurred within 10 min, and was relatively unaffected by temperature over the range of 4-37 °C. Quantitative binding assays were performed at 10 or 37 °C, using cells depleted of ATP to prevent LPS internalization. At 10°C, the apparent dissociation constant of human CD14 for LPS was $2.7.10^{-8}$ M in CHO cells and $4.9.10^{-8}$ M in THP-1. At this temperature, the molar ratio of LPS bound per CD14 was 20:1, and this ratio was 8:1 at 37°C very different from an expected 1:1 stochiometry [262]. It was hypothesized that the leucine-rich repeats of CD14 might represent multiple binding sites for LPS molecules or that CD14 bound small aggregates [262]. This idea of mutiple LPS binding sites

was also raised by Kitchens et al. In their studies, it was demonstrated that in addition to its ability to compete with LPS for binding LBP and CD14, deacylated LPS potently antagonized LPS-induced cell activation at an unknown site distal to the LPS binding site of CD14 [263]. Troelstra et al. confirmed that LPS binding to monocytes was enhanced by serum or LBP for LPS concentrations up to 100 ng/mL, and that LPS binding was completely CD14 dependent. For LPS concentrations exceeding 100 ng/mL, binding was still partially CD14 dependent, but became serum and LBP-independent. Moreover, they showed that association of LPS with monocytes was shown to be totally saturable [264]. The non-CD14-dependence of LPS when used at high concentrations was confirmed by several groups [265, 266].

Studies using mutants of soluble CD14 have demonstrated that the N-terminal half (152 aa) of the molecule was sufficient for binding of LPS and for promoting cell responses [267, 268]. Results from experiments using protease protection and CD14 mutagenesis suggested that amino-acids containing regions 9-13 and 57-64 are critical for LPS binding and may contribute to a three-dimensional structure corresponding to the LPS-binding domain of human CD14 [267, 269]. A common feature of many LPS-binding molecules including LBP, BPI protein, the Limulus anti-LPS factor and polymyxin B is the presence of positively charged amino acids which are thought to interact with negatively charged phosphate groups contained in the lipid A moiety of LPS [113, 270-273]. Four basic positively charged amino acids are present in the region 67-75 of human CD14, and this raised the question whether this region may also participate in LPS binding. Although a peptide derived from this region was able to interact with LPS, it appeared that the basic region between residues 67-75 was not essential for the recognition of LPS by human CD14 (Menzel and Stelter, unpublished observations). Other studies showed that residues 7-10 of the receptor were necessary for LPS-induced cellular activation but not for LPS binding [267]. A second region between aa 91-101 had also a role in the signaling of LPS. It was finally demonstrated that the integrity of region 39-44 was crucial for serum-dependent binding of LPS to human CD14, suggesting that LBP could interact with CD14 in this region. An alanine substitution in this region abrogated LPS and *E. coli* binding to CD14 [153]. Taken together, these studies suggested that LPS binds CD14 *via* different epitopes contained within the first 152 aa of the protein. Interestingly, different LPS (from *E. coli* versus *P. gingivalis*) may have distinct binding sites on CD14. For example, a point mutation at E47 still enabled binding of *E. coli* LPS, but abrogated *P. gingivalis* LPS binding to CD14 [274]. The most recent study on the subject brought evidence that several different hydrophilic regions of the amino-terminal region of

CD14 were involved in LPS binding. Epitope-mapping studies revealed that these hydrophilic regions are located on one side of the protein surface. These studies suggest that CD14 uses a charged surface in a manner similar to the macrophage scavenger receptor to "capture" LPS ligands and to present them to other components of the innate host defense system [275].

3.1.5.1.1.2 LPS-binding receptors other than CD14

Several reports have suggested the existence of LPS receptors other than CD14. Some of these proteins interacting with LPS have been considered as having a role in LPS signal transduction. A specific receptor for LPS should be specific, show saturable and reversible binding, and high affinity for the ligand [276]. According to this definition, various receptors for lipid A, LPS, LPS-LBP complex or LPS-sCD14 complex were proposed in different cell types including monocytic cells, epithelial and endothelial cells.

A 80 kDa protein able to bind different serotypes of LPS in the presence of sCD14 and LBP, was isolated from the human monocytic cell line Mono-Mac-6 [277]. This 80 kDa protein has been identified as the DAF/CD55, a GPI-anchored protein present on a large array of cell types. The significance of these observations remains to be established [278].

Using biochemical approaches, two proteins of molecular weight 70 kDa and 80 kDa interacting with LPS and peptidoglycan on the surface of cells expressing or not CD14 were identified and designated as LPS/LTA associated proteins (LAP)-1 and LAP-2 proteins, respectively. LPS binding to LAP-1 and -2 was shown to be dependent on serum. It was also found that after 1 hour, LPS bound to two new LAP proteins termed as LAP3 and LAP4 [279]. Immunoaffinity chromatography and peptide mass fingerprinting techniques identified LAP1, 2, 3 and 4 proteins as the heat shock protein (HSP70), the HSP90α, the chemokine receptor CXCR4 and the growth differentiation factor (GDF) 5, respectively [279]. Further work is needed to determine whether these proteins play a role in LPS-induced cell activation.

Another receptor candidate for sCD14-LPS is the 216 kDa protein structure which specifically binds iodinated sCD14 only when LPS is present [204]. This 216 kDa structure was detected on monocytes, endothelial and epithelial cells. However, the primary structure of the 216 kDa protein has not been determined yet.

Other candidates showing affinity for LPS include proteins of 18 kDa on 70Z/3 pre-B cell line; of 38-40 kDa on mouse lymphocytes, macrophages, splenocytes, J774.1 and 70Z/3 cells; and of 95-96 kDa, which have been identified by a variety of experimental approaches [280-283].

One of the well-characterized receptors displaying affinity for LPS is the leukocyte integrin CD18 [284]. All three members of the CD18 family (CD11a/CD18, CD11b/CD18 and CD11c/CD18) are capable of binding LPS and participate in the phagocytosis of bacteria. However, in patients with leukocyte adhesion deficiency (LAD)-1 (point mutation in CD18 gene), leukocytes were still able to produce cytokines and superoxide anion in response to LPS, strongly suggesting that CD18 was not essential for mediating cellular response to LPS [285, 286]. Interestingly, both the CD11b/CD18 protein complex and the complement receptor (CR) 3, have been recently shown to associate with CD14 in the presence of serum or LBP on the surface of neutrophils [287]. In chinese hamster ovary (CHO) cells, CR3 was shown to facilitate LPS-induced NF-κB translocation presumably by presenting LPS to downstream signal transducer [288].

Scavenger receptors (SR) are a family of cell surface glycoproteins able to bind modified lipoproteins (mLDLs) such as oxidised and acetylated LDLs [289]. Analysis of macrophage SR function quickly highlighted their broad ligand binding capacities. Among various ligands interacting with SRs, pathogens such as Gram-negative bacteria were found to bind to SRs through recognition of LPS [18, 290]. These receptors fall into a broad family of transmembrane multidomain proteins classified into six subgroups based on their proposed tertiary structure [291]. *In vitro* studies demonstrated the ability of the SR of the class A (SR-A) to bind LPS and suggested a role of this SR in LPS clearance [18]. Supporting this, Haworth et al. showed that SR-A deficient mice were more sensitive to LPS than littermates. Indeed, these mice have a higher mortality than controls and produce more pro-inflammatory mediators like IL-6 and TNF-α. The underlying mechanism by which SR-A protects against endotoxic shock has not been determined, though this mechanism may include SR-A-mediated removal of excess LPS from the circulation, making it unavailable to CD14 [292].

3.1.5.1.2 Non-specific binding of LPS

LPS, an amphiphilic molecule with a lipid moiety, can easily binds to various cytomembranes in a non-receptor mediated (non-specific) way [250]. This process occurs *via* the high affinity of lipid A for the plasma membrane phospholipids [256]. Both smooth LPS and rough LPS were shown to attach to human monocytes in perpendicular or parallel orientation [250]. This non-specific binding often leads to "solubilization" of LPS lipid into the plasma membrane [293]. Insertion of lipid A into bilayers of the plasma membrane causes membrane perturbation and results under particular experimental conditions in membrane disruption [250]. More recently, Schromm et al. showed that LBP known to disaggregate LPS aggregates, can also transport LPS monomers directly into the host cell membrane [266]. Interestingly, sCD14 is also capable of transfering LPS to membrane of cells such as monocytes or neutrophils. It appeared that LPS preferentially interacts with particular classes of lipid such as phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and sphingomyelin [176]. Another study revealed that monomeric LPS was shuttled from mCD14 into the plasma membrane and trafficked within the cell independently of mCD14 [294]. It has been suggested that the insertion of endotoxin molecules into the lipid matrix may also be a prerequisite for the activation of host cells, but would not be sufficient [295]. Some authors have suggested that there may be a link between LPS internalization and cell activation, although only scarce evidence exists to support this theory [177, 296]. It has also been supported that LPS inserted into membranes could directly interact with DAF (CD55), the purinergic receptors P2X7 and ion channels [278, 297, 298].

3.1.5.2 Uptake / internalization and clearance

3.1.5.2.1 Uptake / internalization of LPS

When Gram-negative bacteria invade the body, they release a significant amount of LPS in the surrounding milieu. Then, how the body gets rid of this highly potent inflammatory inducer? In the next sections, different endocytosis pathways in mammalian cells will be decribed. These pathways represent possible mechanisms of LPS internalization.

3.1.5.2.1.1 Different internalization / endocytosis pathways in mammalian cells

Mammalian cells have evolved with a variety of mechanisms to internalise small molecules, macromolecules and particles, and target them to specific intracellular organelles. Collectively, these processes are called "endocytosis", a term proposed by de Duve in 1963 [299]. The ultrastructural analysis of endocytosis enabled to shed light onto different endocytosis pathways that can be distinguished by the size and the morphology of endocytic vesicles, the association of these vesicles with different molecular machineries, their regulation modes, and their functions. Under its broad definition, endocytosis includes various methods of uptake of extracellular material by cells, including phagocytosis (or "cell eating"), (macro)pinocytosis (or "cell drinking"), clathrin-dependent receptor-mediated endocytosis and clathrin–independent endocytosis [1]. In order to further better define the mechanisms by which LPS is internalised, the following section is devoted to the different internalization pathways.

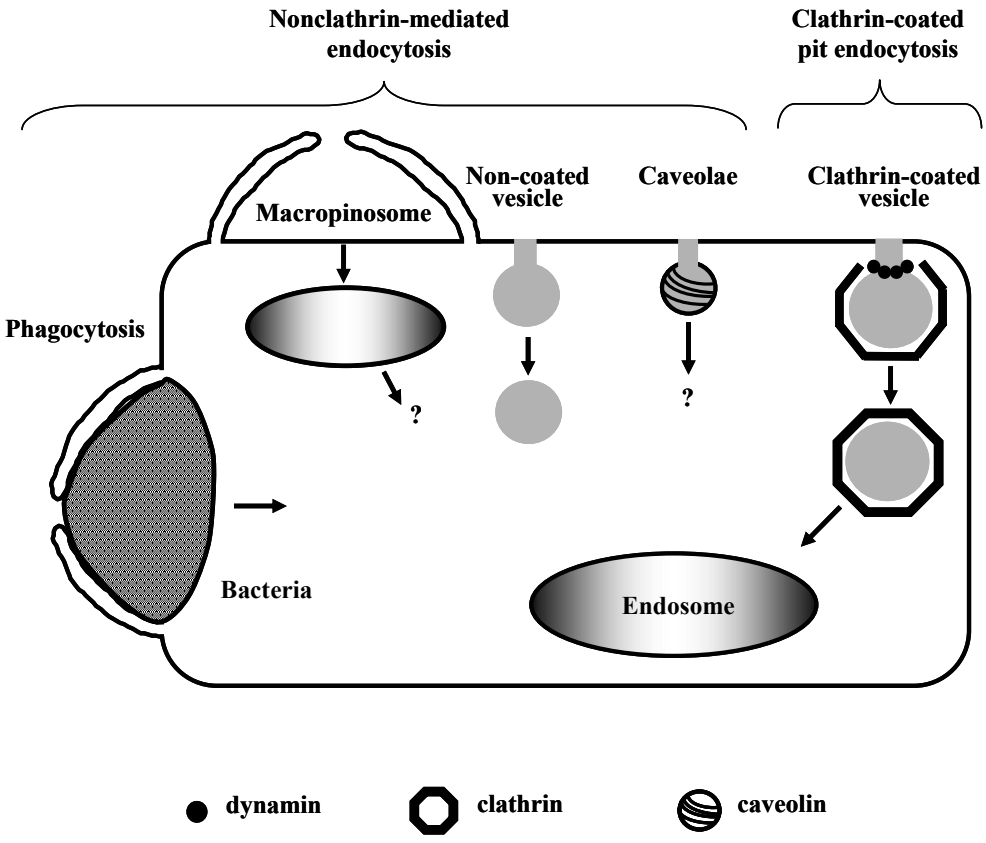

Figure 4: Different endocytosis pathways in mammalian cells. From Lamaze, Current Opinion Cell Biol (1995) [1].

3.1.5.2.1.1.1 Clathrin-coated pit endocytosis

Receptor-mediated endocytosis *via* coated pits is a shared pathway used for the internalization of a variety of ligand-receptor complexes, a very efficient way to clear molecules from the cell surface. The coated pits and vesicles were first described in mosquito oocytes in 1964 [300]. Clathrin-coated pits are ~110-150 nm invaginated plasma membrane structures, and are covered by a coat that corresponds to a basketlike structure essentially made of clathrin [301, 302]. Some receptors are constitutively concentrated in coated pits, but others are recruited to these structures after ligand binding [303-306]. Specific targeting of most receptors to coated pits results from interactions between recognition (internalization) motifs in their cytoplasmic domains with intracellular proteins (assembly or adaptor proteins) contained in the clathrin coat [307, 308]. The coat proteins assemble in the cell surface as planar structures that later gain curvature, forming invaginated pits [309]. This process of vesicle budding is ATP-dependent [310]. After coated pits are pinched off from the plasma membrane, their traffic between endocytic compartments can be directed by many of the "internalization motifs" [311].

3.1.5.2.1.1.2 Nonclathrin-mediated endocytosis

3.1.5.2.1.1.2.1 Phagocytosis

Large particles and microorganisms (typically > 0,5 μm) are internalised in a receptor- and actin-dependent, and clathrin-independent mechanism [312]. Particles that are endocytosed by this mechanism may be directly recognized by receptors on the cell surface, or may be recognized after opsonization by "opsonins", which are frequently proteins from the complement and immunoglobins [313]. Phagocytosis involves the extension of pseudopods around the particle to be engulfed, with a sequential recruitment of cell surface receptors to interact with proteins or opsonins on the surface of the particle. As a result, pseudopods extend only as far as its surface has ligand to bind the receptors, in a way analogous to the teeth of a zipper [314, 315]. The extension of pseudopods requires a

reorganization of the actin cytoskeleton underlying the region of plasma membrane that contacts the particle, and this occurs as a response to intracellular signals following receptors cross-linking on the cell surface [316]. The two advancing sleeves of the pseudopods finally fuse, engulfing the particle and forming the phagosome which then matures into a phagolysosome, where degradation of the ingested particle starts [317]. During periods of high phagocytic activity, up to 40% of the plasma membrane may be internalised in 15 min [318]. In mammals, phagocytosis is carried out primarily by the so-called "professional phagocytes", which include neutrophils, monocytes and macrophages as well as other cells of myelogenic lineage such as microglia in the brain [319, 320].

3.1.5.2.1.1.2.2 Non-clathrin coated pits, smooth invaginations, and surface tubules for entry into macrophages (STEMs)

The biochemical and functional properties of noncoated invaginations (average diameter ~95 nm) are not well understood. Indeed, the best evidence for non-clathrin coated pit endocytosis comes from the observation that bulk membrane internalization and the uptake of fluid-phase markers continue even after cells have been specifically treated to inhibit clathrin-mediated internalization [321-325]. A novel type of non-clathrin coated pit endocytosis *via* STEMs has been observed in macrophages [326]. It was shown that these surface-connected invaginations of ~250 nm in diameter and 1 μm or more in length can sequester large multivalent β-very-low-density lipoprotein (VLDL) particles. These latter are delivered from the STEMs to late endosomes and lysosomes [327].

3.1.5.2.1.1.2.3 Caveolae

Caveolae are uniform omega- or flask-shaped membrane invaginations that are considerably smaller than clathrin-coated pits (50-80 nm diameter) [328]. The cytoplasmic surface of caveolae is often decorated with a coat composed of delicate filaments arranged in striations, consisting in part of the membrane protein VIP21/caveolin [329-331]. Caveolae contain high concentrations of certain lipids such as cholesterol, sphingomyelin, and glycolipids, as well as GPI-anchored proteins and various signaling molecules such as members of the Src-family kinases and GTP-binding proteins [332-336]. Caveolae are present

in a variety of cell types (smooth muscle cells, fibroblasts, adipocytes, ECs and many epithelial cells) and are thought to be involved in potocytosis, endocytosis, transcytosis, and signal transduction [337-340]. Potocytosis is proposed to be an alternate mechanism for the concentration and delivery of small molecules directly to the cytoplasm, bypassing the classical endosomal/lysosomal pathway. Transcytosis is a process that aimed at transporting selected (macro)molecules through cells.

3.1.5.2.1.1.2.4 Macropinocytosis

Macropinocytosis is another type of nonclathrin-mediated endocytic pathway [341]. This process is characterized by macropinosomes which are formed primarily from ruffles that are themselves made by bands of outwardly directed actin polymerization that result in planar folds or circular, cup-shaped extensions of the cytoplasm. These ruffles often close off to form vesicles of heterogeneous size, which are sometimes as large as 5 μm in diameter. In addition to the ingestion of large amounts of plasma membrane, this route accounts for the non-selective uptake of large volumes of fluid. Macropinocytosis closely resembles phagocytosis, involving actin-assisted membrane extensions that ultimately fuse and pinch off as intracellular vesicles. However, pseudopod extension in macropinocytosis is independent of the "zippering" mechanism of phagocytosis, suggesting that the two processes are regulated differently [317].

3.1.5.2.2 Clearance and detoxification of LPS

To be neutralized, LPS has to be internalised in order to be detoxified inside the cell. Hepatic Kuppfer cells as well as hepatocytes were shown to play a major role in such functions *in vivo* and *in vitro* [252, 342]. Lung macrophages were also involved in the elimination of endotoxin [343]. In macrophages, lipid A was shown to be converted to a less toxic precursor-like molecule *via* removal of non-hydroxylated fatty acids by acyloxyacyl hydrolases, which work best at acid pH [344, 345]. This enzymatic reaction termed as deacylation, occurs over several hours and can be inhibited by agents that reduce lysosomal (endosomal) acidification, suggesting that LPS moves at least transiently into an acidic intracellular compartment [251]. Deacylated LPS (or lipid IVa) has been shown by Kitchens

et al. to lack cell-activating properties [346]. The ingested LPS is also modified by enriching the lipid content, by shortening the length of O-antigen, and by dephosphorylation of the lipid A moiety [254, 347]. All of these molecular modifications are believed to be implicated in LPS detoxification. Detoxified LPS may then be excreted by exocytosis or retained by macrophages in larger micelles [254].

3.1.6 Problematic

As previously mentioned LPS activates cells *via* CD14 and is further internalised into cells. The fate of LPS after its binding to the plasma membrane has raised the interest of many researchers. Indeed, LPS endocytosis leads to the detoxification and clearance of endotoxin, an important step to neutralize this potent pro-inflammatory molecule. This function is principally ensured by monocytes/macrophages. Before CD14 was characterized as a receptor for LPS, several microscopic studies had been carried out with labeled antibodies against LPS or labeled LPS to follow the bacterial molecule. Kang et al. observed that LPS inserted into the plasma membrane of macrophages, perturbed and then disrupted the membrane. This disruption facilitated massive migration of LPS bilayers into the cytoplasm by passive diffusion. LPS entering cells *via* this mechanism often appears as large aggregates in the cytoplasm without a surrounding limiting membrane. Kang et al. proposed that massive ingestion of LPS and destruction of cytomembranes may cause the death of cells [250]. It is important to note that these experiments were done with very high concentrations of LPS (~100 μg/mL), which hardly reflect "physiologic" situations in which macrophages usually encounter nanograms or picograms of LPS. Other mechanisms for LPS uptake by monocytes and macrophages are absorptive pinocytosis and phagocytosis. In addition to human monocytes, phagocytosis of biotin-LPS was also observed in human natural killer (NK) cells and platelets [255, 348]. Human monocytes are able to ingest bulks of LPS by phagocytosis at multiple sites when they are exposed to extremely high doses of LPS (50-100 μg/mL). Following LPS endocytosis, LPS have been localized in pinocytic vesicles, phagocytic vacuoles and phagosomes in human macrophages. The ingested LPS has also been found in association with the endoplasmic reticulum, the outer nuclear membrane, mitochondria, the Golgi apparatus, and also free in the cytoplasm and nucleoplasm of human monocytes. The phagocytic vacuoles and phagosomes containing LPS often display peroxidase and acid

phosphatase activities [250]. McGivney et al. have shown that LPS enhances lysosmal enzyme activity possibly by direct action on the subcellular constituents [349].

Electron microscopic studies on CD14 internalization were performed with the anti-CD14 RoMo-1 monoclonal antibody early after its identification. Endocytosed CD14 followed a pathway similar to that of receptor-mediated endocytosis, a process known for a variety of ligands such as cell nutrients, growth factors, hormones and others [350]. Despite these studies on LPS and CD14 internalization, many questions remain opened. In the first part of this chapter, we have investigated the question of LPS and CD14 internalization pathway in monocytic cells. We were first interested in the localization of CD14 at the cell membrane, that could bring a hint in the determination of internalization pathways. Moreover, since CD14 has been characterized to be the receptor for LPS, the question of its participation in LPS internalization arose, and if so, whether both molecules were associated and trafficked together within the cell. By transforming the GPI anchor of CD14 into a transmembrane tail, we explored the possible role of the GPI anchor of CD14 in the targeting of CD14 at the cell membrane, in LPS-induced cell activation, and also in LPS internalization. In addition to these interrogations, we raised the question of a possible relationship between LPS-induced cell activation and LPS internalization by using specific inhibitors of internalization pathways.

Myeloid cells are not the only cells interacting with microorganisms. Endothelial and some epithelial cells such as intestinal and liver cells are in direct contact with bacteria and bacterial products. These cells might have develop different immune strategies to protect themselves and their environment. For the professional phagocytic cells classically considered to be the primary mediators of innate immunity, distinction between commensal and pathogenic microbes is not particularly important. Indeed, any microbe that breaches the outer host defensive barriers by a pathogen-specific or opportunistic mechanism to reach these phagocytes is likely to be identified hazardous to its host. However, epithelial cells that line mucosal surfaces being on the front line of host defense, also play an active role in innate immunity particularly by secreting chemokines and other immune mediators in response to pathogenic microbes. Since some mucosal surfaces such as the intestinal epithelium are normally densely colonized by a wide variety of microbes, the ability to distinguish the occasional pathogen from the sea of commensals presents an important challenge. These epithelial cells may have develop a powerful recognition strategy that would discriminate

commensal bacteria from pathogens. The liver is also an important site of host-microbe interaction when infections occur. In addition, hepatocytes and Kupffer cells clear bacterial molecules such as LPS from the blood streaming through the liver. To interact with and discriminate between pathogenic molecules, intestinal cells as well as hepatocytes may have develop a powerful strategy of recognition. CD14 is a pattern recognition receptor able to bind an array of microbial molecules such as LPS. Human epithelial cells were shown to respond to LPS *via* a soluble CD14-dependent manner [149]. However, the expression and release of CD14 by these so-called CD14-negative cells have not been studied in detail. In the second part of this chapter, we investigated CD14 expression at molecular and cellular levels in different colonic and hepatocytic cell lines. Moreover, to better understand interactions between LPS and epithelial cells, the role of CD14 in LPS-induced cell activation and LPS internalization was also questioned in these cells.

3.2 Part I: «CD14-dependent endotoxin internalization *via* a macropinocytic pathway»

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CD14-dependent Endotoxin Internalization via a Macropinocytic Pathway*

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Gram-negative bacterial endotoxin (a lipopolysaccharide (LPS)) specifically binds to CD14, a glycosylphosphatidyl inositol (GPI)-anchored surface myeloid glycoprotein. This interaction leads to cell activation, but it also promotes LPS internalization and detoxification. In this work, we investigated the route of LPS and CD14 internalization and the relevance of CD14 GPI anchor in the endocytic pathway. In promonocytic THP-1 cells transfected with a GPI or a chimeric integral form of CD14, we showed by differential buoyancy in sucrose density gradients that these two forms of CD14 were sorted to different plasma membrane subdomains. However, both forms of CD14 associated preferentially with the same surface microfilament-enriched microvilli or ruffles. Electron microscopic studies indicated that CD14 internalized via macropinocytosis, a process resembling that of phagocytosis, different from "classical" receptor-mediated endocytic pathways, such as clathrin-coated pits or caveolae. With cell warming, the CD14-enriched ruffles fused and formed large vesicles. Later, these vacuoles made stacks and condensed into phago-lysosomes. CD14 was specifically associated with all of these structures. Radiolabeled LPS internalization paralleled CD14 internalization. Confocal microscopic studies confirmed the co-localization of LPS and CD14 both at the cell surface and in endosomal compartments. The microfilament-disrupting, macropinocytosis blocking agent cytochalasin D inhibited LPS and CD14 internalization but did not prevent LPS-dependent activation, indicating that these two processes are dissociated.

Clearance of bacteria and bacterial molecules, predominantly performed by macrophages, is an important feature of bacterial immunity and requires specific recognition and endocytic pathways (1, 2). A prototypic example of a bacterial molecule with profound effects on mammalian cells is the Gram-negative lipopolysaccharide (LPS),[1] or endotoxin (3). Various macrophage cell surface molecules can bind LPS (4), including scavenger receptors (5) and CD14 (6). However, in CD14-positive cells, LPS internalization is almost completely dependent on the presence of CD14 and is not inhibited by scavenger receptor ligands (7). CD14, a 55-kDa glycoprotein, has been recognized as a surface myeloid differentiation marker (8) and the LPS receptor (9). Engagement of CD14 by complexes of LPS and LPS-binding protein, as well as other bacterial ligands leads to cell activation (9, 10). The mechanism of transmembrane LPS signal transduction remains to be unraveled (11). CD14 also exists in a soluble form, and complexes of LPS and soluble CD14 activate CD14-negative cells, such as endothelial and epithelial cells (12).

Apart from cell activation, membrane-bound CD14 plays other important roles: it mediates LPS internalization (7, 13, 14), as well as Gram-negative bacteria and mycobacteria phagocytosis (15, 16). LPS internalization by myeloid cells is a critical step for its detoxification. After endocytosis, LPS is biologically deactivated by a specific enzyme (acyloxyacyl hydrolase) present in phagocytic granules (17).

Glycosylphosphatidyl-inositol (GPI)-linked molecules are sorted to plasma membrane subdomains, rich in sphyngomyelin, cholesterol, receptors, and other signaling molecules (18–20). In epithelial cells, these plasma membrane domains correspond morphologically to noncoated pits and vesicles, known as caveolae (19). Caveolae have been implicated in GPI-linked receptor-mediated endocytosis (19, 21, 22). CD14 is naturally attached to macrophage and polymorphonuclear neutrophil plasma membranes via a GPI moiety (23, 24). CD14 association with particular membrane subdomains, routes of LPS and CD14 internalization remain to be elucidated.

In this study, we found that LPS and CD14 internalized via a macropinocytosis independently of "classical" receptor-mediated endocytic pathways, such as clathrin-coated pits or caveolae. The modification of the GPI-anchored CD14 into an integral protein did not significantly affect rates and pathways of receptor-ligand internalization, and CD14 endocytosis did not seem to be regulated by its ligand, LPS. Finally, cytochalasin D inhibited LPS endocytosis but did not prevent LPS-dependent cell activation.

MATERIALS AND METHODS

Cells—Wild type CD14 cDNA or a chimeric cDNA construct of CD14 attached to the transmembrane portion of human tissue factor were used to transfect a human promonocytic, CD14-negative, THP-1 cell line, as described elsewhere (15, 25). Stable transfectants expressed either a GPI-anchored CD14 (wtCD14-THP1 cells) or a transmembrane form of CD14 (tmCD14-THP1 cells). Cells transfected with the empty vector (pRc/RSV) served as control cells (25). Transfectants were cultured in suspension in RPMI 1640 medium containing penicillin and gentamicin antibiotics, 10% fetal bovine serum, and 0.5 mg/ml G418 (Geneticin®), all from Life Technologies, Inc. CD14 expression in wtCD14-THP1 and tmCD14-THP1 cells was 2×10^6 and 2×10^5 CD14 molecules/cell, respectively (25).

Membrane Targeting of CD14—Membrane targeting of CD14 was assayed using sucrose density gradient according to published methods

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[1] The abbreviations used are: LPS, lipopolysaccharide; GPI, glycosylphosphatidylinositol; FITC, fluorescein isothiocyanate; wt, wild type; tm, transmembrane; IL, interleukin.

(26). Briefly, 2×10^7 wtCD14-THP1 and tmCD14-THP1 cells were lysed for 20 min at 4 °C in a lysis buffer containing anti-proteases (TNE (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA) plus 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 200,000 IU/ml aprotinin), and 1% Triton X-100 or 1% Triton X-100 1% plus 60 mM octylglucoside (26). Cell lysates were then homogenized (8 strokes, Dounce homogenizer), and 2 ml of an 80% sucrose solution was added to 2 ml of cell lysates. A 7.5-ml 5–30% linear sucrose gradient was layered onto the 40% sucrose/cell lysate mixture, and tubes were ultracentrifuged (Beckman SW41 rotor) at 39,000 rpm at 4 °C for 18 h. One-ml fractions were collected from the bottom of the tubes. Proteins in each fractions were precipitated in 10% trichloroacetic acid, resuspended in 2× SDS-polyacrylamide gel electrophoresis sample buffer containing 5% β-mercaptoethanol, boiled for 5 min, and separated using a 12% acrylamide SDS gel. Proteins were then electrotransferred onto a nitrocellulose membrane. CD14 was detected by Western blot using a goat anti-human CD14 antibody (from R. J. Ulevitch, The Scripps Research Institute, La Jolla), protein G-horseradish peroxidase (Bio-Rad), and ECL (Amersham Pharmacia Biotech). In some experiments, cell membrane proteins were detected by dot-blot (27): proteins contained in 2–20 μl of the sucrose density gradient fractions (non-trichloroacetic acid-precipitated) were diluted in 200-μl TNE buffer and transferred onto nitrocellulose by filtration (Bio-Rad DotBlot™ apparatus). Membrane proteins were detected by Western blot using specific monoclonal antibodies, a secondary goat anti-mouse IgG-HRP conjugate (Santa Cruz Biotechnologies, Santa Cruz, CA), and the ECL detection system (27). Murine monoclonal antibodies to human membrane proteins used in this dot-blot assay were 28C5 (anti-CD14, a gift from D. Leturcq, RW Johnson Pharmacological Institute, San Diego), MEM 154 (anti-CD16), MEM 48 (anti-CD18), MEM 102 (anti-CD48), MEM 118 (anti-CD55), and MEM 43/5 (anti-CD59). Antibodies of the MEM series were characterized and donated by V. Horejsi, Prague, Czech Republic (28). In other experiments, GPI-rich membrane subdomains were isolated without detergent, using a combination of mechanical homogenization of cells, membrane sonication, successive Percoll™ (Amersham Pharmacia Biotech) and Optiprep™ (Nycomed Pharma, Oslo, Norway) gradients, as described elsewhere (29), and assayed by dot-blot for CD14 (see above).

Immunoelectron Microscopy—THP-1 transfectants were sequentially incubated for 2 h at 4 °C with 0.3 mg/ml of biotinylated 63D3 monoclonal antibody (a noninhibitory anti-CD14 monoclonal antibody, not interfering with LPS binding site) (ATCC, Rockville, MD) and for 1 h at 4 °C with a 1:10 dilution of streptavidin coupled to 10 nm colloidal gold particles (ANAWA, Wageningen, The Netherlands) in phosphate-buffered saline/bovine serum albumin 1% buffer. After two washes in 4 °C phosphate-buffered saline, cells were warmed and kept 5 or 20 min in a 37 °C water bath in the presence or in the absence of 2 μg/ml *Escherichia coli* K12 LCD25 LPS (List Biological Laboratories Inc., Campbell, CA)/10% fetal bovine serum. Cells were then fixed for 30 min at 20 °C with 2.5% glutaraldehyde diluted in pH 7.4 phosphate buffer, dehydrated, and processed for electron microscopy as described elsewhere (30). Thin sections were examined in a Philips electron microscope 301, and gold particles were quantitatively analyzed. For each time point studied, 450–800 gold particles from more than 50 cells were analyzed from cells expressing either wt- or tmCD14. Gold particles were scored in terms of their association with cellular structures, such as microvilli or membrane ruffles, the inner leaflet of macropinosomes (vesicular structures), endocytic vesicles (clathrin-coated pits/vesicles), and lysosome-like organelles. The percentage of membrane surface represented by ruffles or microvilli was determined on randomly photographed pictures of wtCD14-THP1 cells (231.4 ± 11 μm of plasma membrane length on each micrography; $n = 57$) and tmCD14-THP1 cells (256.7 ± 12.6 μm; $n = 50$) at an initial magnification of × 7200 as described previously (31).

LPS Internalization—One hundred ng/ml [^{3}H]LPS (biosynthetically tritiated *E. coli* K12 LCD25 LPS, List Biological Laboratories) (32), preincubated for 15 min at 37 °C with fetal bovine serum, were added to the cells for various times. In some experiments, the microfilament-disrupting agent cytochalasin D (Sigma) (3 μM), or the phosphoinositide-3 kinase inhibitor wortmannin (Calbiochem, San Diego, CA) (1 μM) were added to the cells 30 min prior to the addition of LPS (33, 34). After various times of LPS incubation, cells were washed and treated at 4 °C with 200 μg/ml Pronase (Boehringer Mannheim) for 1 h (35, 36). Supernatants and cells were separated by centrifugation and transferred into scintillation liquid. Radioactivity was measured in a scintillation counter to determine cell-bound and internalized (Pronase-resistant, cell-associated) counts. Internalization was defined as the percentage of counts resistant to Pronase relative to the total counts (cell-associated counts before the Pronase treatment). Cell viability during the experiment was tested using the Live/Dead™ Eukolight viability kit (Molecular Probes, Leiden, The Netherlands), according to the manufacturer's protocol.

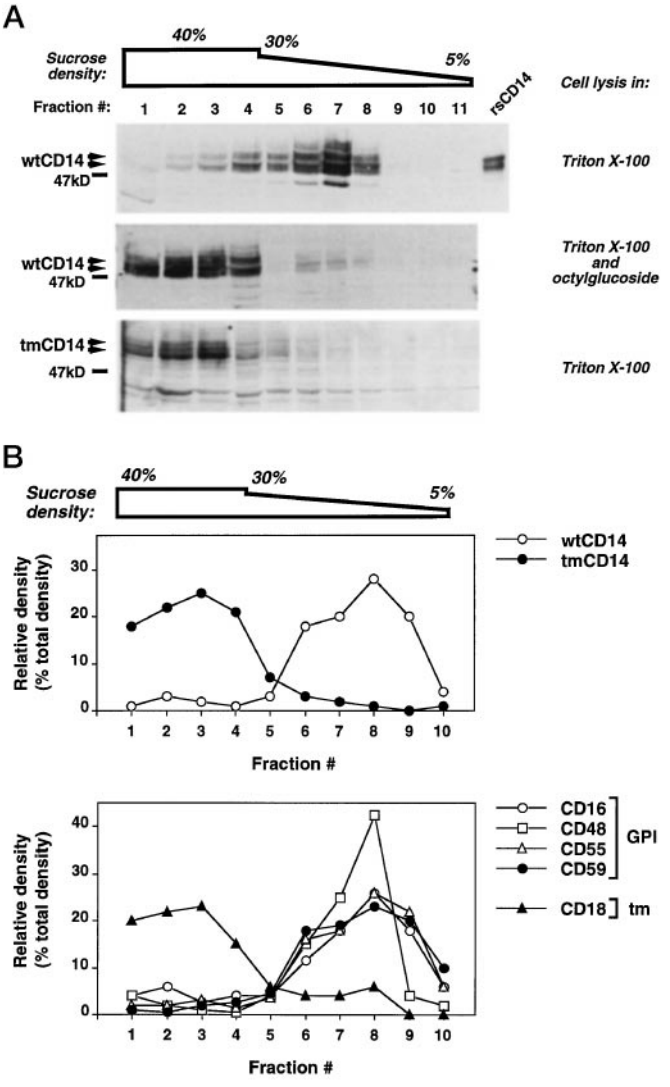

FIG. 1. *A*, distribution of wt- and tmCD14 in sucrose density gradient of THP-1 cell lysates. CD14 was detected in fractions by Western blot. Recombinant soluble CD14 (*rsCD14*) was used as a positive control. *B*, comparison of the distribution of wt- and tmCD14 with various other GPI and transmembrane proteins in sucrose density gradient of THP-1 cell lysates.

TABLE I
Association of wt- and tm-CD14 with plasma membrane structures using morphometrical studies of electron microscopy and immunogold labeling of CD14

	Membrane surface found as microvilli or ruffles	Gold particles associated with microvilli or ruffles	CD14 enrichment on microvilli or ruffles[a]
	%	%	
wtCD14-THP1 cells	37 ± 2.2	54.9 ± 0.8	1.48 × ± 0.07
tmCD14-THP1 cells	37.7 ± 2.1	45.9 ± 0.4	1.22 × ± 0.06

[a] CD14 enrichment on microvilli or ruffles was calculated as the ratio of the percentage of gold particles associated with microvilli or ruffles over the percentage of membrane surface found as microvilli or ruffles.

Confocal Laser Microscopy—wtCD14- and tmCD14-THP1 cells preincubated for 30 min in the presence or in the absence of 3 μM cytochalasin D (Sigma) were labeled with 60 μg/ml biotinylated 63D3 anti-CD14 monoclonal antibody and 30 μg/ml streptavidin-Texas red (Molecular Probes) at 4 °C for 45 min. After washing, 1 μg/ml fluoresceinated (FITC) LPS (donated by P. S. Tobias, The Scripps Research

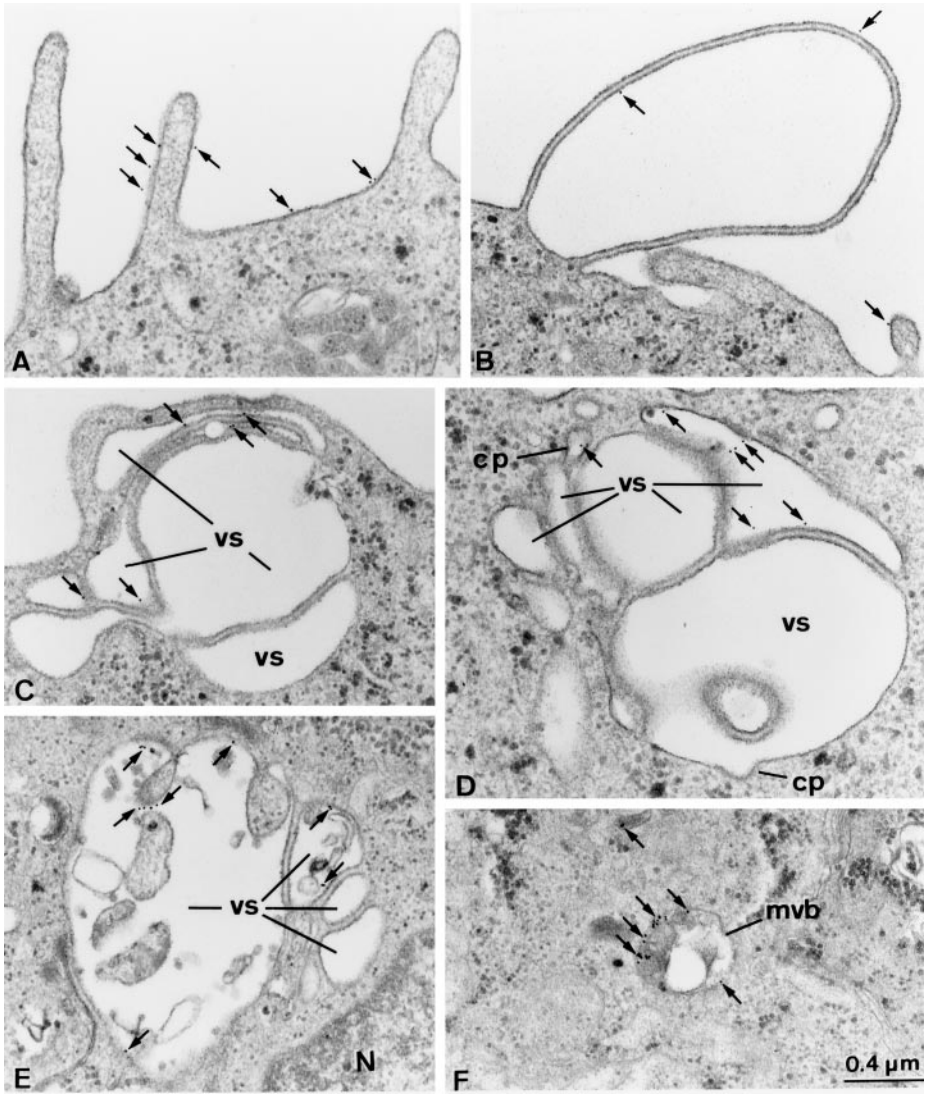

FIG. 2. **Representative electron micrography showing the association of wtCD14 (gold particles) with macropinocytic structures in transfected THP-1 cells.** CD14-associated gold particles are indicated by *arrows*. At 4 °C, the bulk of CD14-associated gold particles was found on cell surface protrusions (likely membrane ruffles or microvilli) (*A*). When the temperature was shifted to 37 °C to allow endocytic process to occur, membrane ruffles merged (*B*) and formed large vesicular structures (*vs*) organized in stacks (*C* and *D*). CD14-associated gold particles were found enclosed in these vesicular structures after 5 min at 37 °C (*C* and *D*). Clathrin-coated pits (*cp*) were observed budding off from these cisternae, but no significant labeling was found associated with these structures (*D*). After 20 min at 37 °C, CD14-associated gold particles were retrieved in vesicular structures deeper in the cytoplasm near the nucleus (*N*) or in lysosome-like structures such as multivesicular bodies (*mvb*) (*E* and *F*), which suggests that gold-containing cisternae stacks have matured into lysosome-like structures. Similar cell structures and pattern of gold labeling was observed in tmCD14-THP1 cells. *Arrows* indicate immunogold-labeled CD14 particles.

Institute, La Jolla, CA) in 10% normal human serum was added, and cells were put at 37 °C for 1 or 30 min. Cells were then chilled, washed, and fixed in Tris/glycerol/polyvinyl alcohol mounting solution. Specimens were examined with a Zeiss confocal laser fluorescence inverted microscope (LSM 410, Carl Zeiss, Oberkochen, Germany) using simultaneous lasers with excitation wavelengths of 543 and 488 nm for red and green, respectively, and detection using red and green narrow band filters. Cells were observed through an oil plan-neofluar × 63/1.3 objective. The best plan of section was optimized through optical sectioning.

Cell Activation—Cytoplasmic protein tyrosine phosphorylation by LPS (200 ng/ml) in the presence or absence of 3 μM cytochalasin D (1 h pretreatment) was assayed using a 4G10 phosphotyrosine Western blot (Upstate Biotechnology), as described elsewhere (25). In similar experiments, LPS-induced nuclear factor-κB activation was assessed using classical electromobility shift assay of nuclear proteins (25). Interleukin-8 was measured by enzyme-linked immunosorbent assay (Endogen, Cambridge, MA) in conditioned supernatants from THP-1 transfectants treated for 7 h with various concentrations of LPS, with or without 3 μM cytochalasin D (25).

RESULTS

Membrane Targeting of CD14 and Surface Events—Using sucrose density fractionation of Triton X-100 cell extracts, we observed that the GPI form of CD14 (wtCD14) and the transmembrane CD14 chimera (tmCD14) were targeted to different membrane subdomains, as indicated by their differential buoyancy in the sucrose gradient. The Triton X-100-extracted wtCD14 floated in low sucrose density fractions, previously recognized as GPI-rich domains, whereas tmCD14 remained in the 40% sucrose solution (Fig. 1*A*). Octylglucoside known to disrupt GPI-rich domains solubilized wtCD14, which was recovered in the 40% sucrose density fractions (Fig. 1*A*), whereas the buoyancy of tmCD14 was not modified by this detergent. In Triton X-100 lysis buffer, wtCD14 co-localized in low sucrose density fractions with other GPI-anchored proteins (CD16, CD48, CD55, and CD59), but tmCD14 remained in the high sucrose density fractions, along with the transmembrane CD18 protein (Fig. 1*B*). Nondetergent isolation of low-density GPI-rich domains (29) showed that CD14 was enriched at least 100 times in these domains as compared with tmCD14, as determined by serial dilutions of GPI-rich domains of wt- and tmCD14-THP1 cells in the dot-blot assay (data not shown). This latter experiment indicated that the separation by buoyancy of wt- and tmCD14 was not an artifact due to the presence of detergents. Taken together, these experiments demonstrated that the GPI anchoring was responsible for targeting CD14 to GPI-rich, lipid-rich plasma membrane subdomains, whereas tmCD14 was sorted into other membrane domains, where most integral proteins are found. LPS treatment did not modify the distribution pattern of wt- or tmCD14 in the sucrose density fractions (not shown).

CD14 from 1,25-$(OH)_2$ vitamin D_3-differentiated THP-1 cells (CD14-positive THP-1 cells) (6), human monocytes, and epithelial cells (COS and HepG2) transfected with GPI-CD14 was

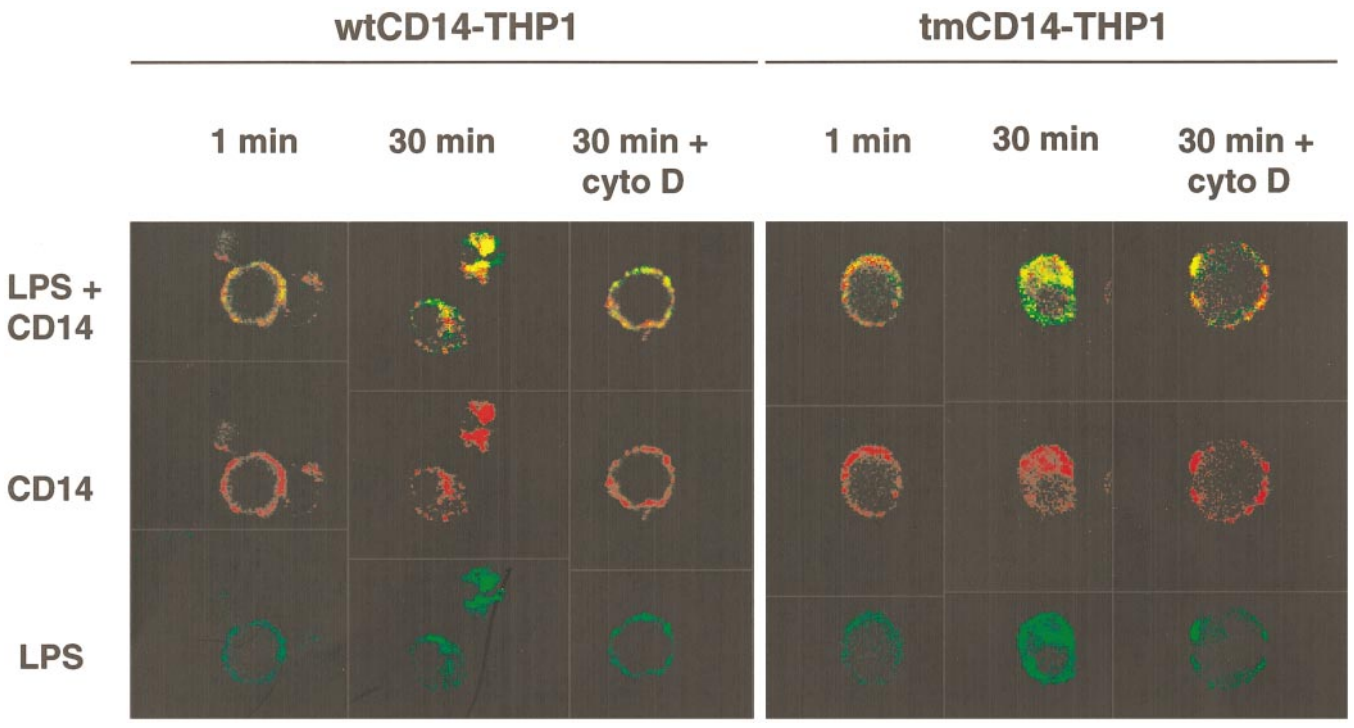

FIG. 3. **Representative confocal microscopy images of wt- and tmCD14-THP1 cells incubated with 1 μg/ml FITC-LPS (*green*) and Texas red-antibody labeled CD14 (*red*).** After labeling at 4 °C, cells were warmed at 37 °C for 1 and 30 min in the presence or in the absence of the microfilament-disrupting agent cytochalasin D. It is shown here that LPS and CD14 co-localized (*yellow*) at the plasma membrane and in endosomal compartments in both wt- and tmCD14 transfectants. LPS and CD14 internalization was blocked by cytochalasin D.

found in the same sucrose density fractions as wtCD14 originating from transfected THP-1 cells (not shown), indicating that GPI-CD14 targeting to lipid-rich domains was independent of the cell type tested.

We next determined wt- and tmCD14 association with plasma membrane structures using electron microscopy and immunogold labeling of CD14. The THP-1 cell plasma membrane was found to be very convoluted, with 37% of the membrane surface found as microvilli or ruffles (Table I). CD14 immunogold labeling at 4 °C indicated that wtCD14, and to a lesser extent tmCD14, associated preferentially with membrane microvilli or ruffles, with enrichment factors of CD14 on these structures of 1.48× and 1.22×, respectively (Table I and Fig. 2). Very little, if any, wt- or tmCD14 localized in clathrin-coated pits. We did not observe clear caveolar structures in THP-1 cells. No immunogold CD14 labeling was observed in RSV-control THP1 cells.

LPS and CD14 Internalization—Co-localization of LPS and CD14 at the plasma membrane level was assessed by confocal laser microscopy and dual LPS and CD14 fluorescent labeling. After 1 min of incubation at 37 °C, Texas red-immunolabeled CD14 and FITC-LPS co-localized exclusively at the membrane surface in both THP-1 transfectants (Fig. 3). No fluorescent labeling was detectable in control RSV cells. We next addressed whether LPS and CD14 co-localized during the process of endocytosis using confocal laser microscopy. After 30 min of cell warming, both LPS and CD14 internalized and were found to be co-localized in intracellular endocytic compartments (Fig. 3). LPS internalization patterns and rates were assessed using a classical internalization protocol. Cells were put at 37 °C in the presence of [^{3}H]LPS and serum for various times and then chilled and treated with Pronase. Cell-associated counts after Pronase treatment were considered internalized. Association of [^{3}H]LPS with THP1 transfectants was entirely dependent on the presence of CD14, because RSV control cells did not show significant [^{3}H]LPS binding. In addition, in experiment not shown here, anti-CD14 monoclonal antibodies specifically blocking [^{3}H]LPS binding to CD14 (28C5 and 3C10, ATCC) prevented [^{3}H]LPS binding and internalization. In wt- and tmCD14-THP1 cells, [^{3}H]LPS binding (defined as ^{3}H counts released by Pronase) was rapid and plateaued after 5–10 min (not shown). [^{3}H]LPS was internalized with similar rates (~2% per min) in both THP1 transfectants. A plateau of internalization (maximal internalization of ~45%) was reached after 30–60 min in wtCD14-THP1 cells (Fig. 4). In tmCD14-THP1 cells, although initial internalization rates were quite similar to those measured in wtCD14, internalization did not quite reach a plateau after 120 min (~70% internalization at 120 min; Fig. 4).

Rates and routes of wt- and tmCD14 internalization were studied using immunoelectron microscopy. After immunolabeling of CD14 at 4 °C, THP1 transfectants were warmed at 37 °C for various times. Gold-labeled CD14, mainly present at the surface of microvilli or ruffles, localized in macropinocytic vesicles after 5 min, forming large endocytosed vacuoles organizing in stacks (Figs. 2 and 5). After 20 min, CD14 was found deeper in the cytoplasm in denser endosome-like or lysosome-like structures (Figs. 2 and 5). These pictures are typical of macropinocytosis. CD14 internalization route was identical in wt- and tmCD14-THP1 cells. Very similar rates of CD14 internalization were found in both THP-1 transfectants (Figs. 4 and 5). Only tmCD14 internalized more and was found more associated with endosome-like or lysosome-like structures than wtCD14-THP1 cells (75 *versus* 45% internalization at 20 min, respectively) (Figs. 4 and 5). These findings are in accordance with what was found in the LPS internalization assay, with similar rates of LPS and CD14 internalization in both assays (Fig. 4). All of this strongly suggested a common pathway of LPS and CD14 internalization. In experiments not shown here, LPS treatment of THP-1 transfectants did not significantly influence rates or routes of CD14 internalization.

Effect of Macropinocytosis Inhibitors on LPS and CD14 In-

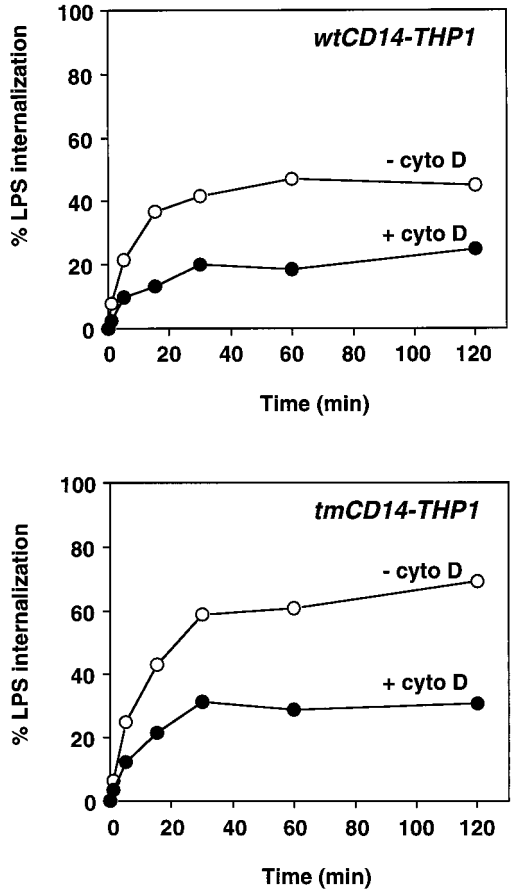

FIG. 4. **Kinetics of LPS internalization (100 ng/ml ^{3}H-LPS) in wtCD14-THP1 cells (*upper panel*) and tmCD14-THP1 cells (*lower panel*) (2 × 10^6 cells/experiment), in the presence (*closed circles*) or in the absence (*open circles*) of cytochalasin D.** Percentage of LPS internalization was defined as the Pronase-resistant cell associated counts relative to the cell-associated counts before Pronase treatment. Data presented here are the mean of three independent experiments performed in duplicate.

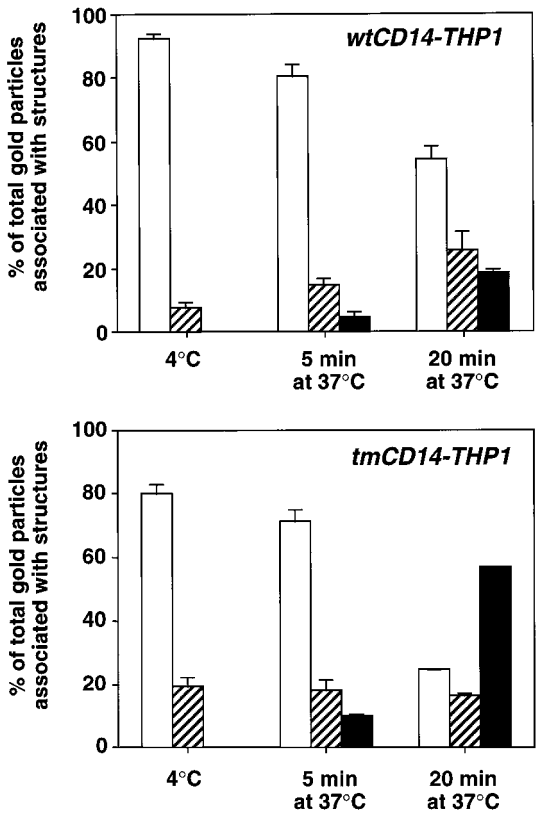

FIG. 5. **Cellular localization and kinetics of CD14 internalization in wt- and tmCD14-THP1 cells at 37 °C, derived from electron microscopic studies.** Gold-labeled CD14 was associated with the following structures: plasma membrane (*open bars*), stacks and vacuolar structures (*hatched bars*), and multivesicular bodies or lysosome-like structures (*filled bars*). It is shown here that with time, CD14 progressively moves from the plasma membrane to deeper cytoplasmic compartments in both wt and tm-CD14 transfectants. The kinetic of tmCD14 sorting to lysosomal-like structures was more rapid that for wtCD14. Results are expressed as mean ± range of two experiments.

ternalization—The actin filament disrupting agent cytochalasin D markedly decreased [^{3}H]LPS internalization in both wtCD14- and tmCD14-THP1 cells, with a maximum effect observed at 3 μM cytochalasin D concentration (Fig. 4). In confocal microscopy, cytochalasin D clearly prevented FITC-LPS and Texas red-CD14 internalization (Fig. 3). Phosphoinositide-3 kinase has been implicated in macropinocytosis (34). In experiments not shown here, the phosphoinositide-3 kinase inhibitor wortmannin decreased [^{3}H]LPS internalization by 30%. Importantly, these inhibitors had no effect on cell viability at the concentrations tested.

LPS Activation of Cells—As previously shown (25), LPS treatment of wt- and tmCD14-THP1 cells induced rapid phosphorylation of p38 mitogen-activated protein kinase, NF-κB activation, and IL-8 secretion (Fig. 6). Although cytochalasin D had a profound inhibitory effect on LPS internalization, it modified neither LPS-induced p38 mitogen-activated protein kinase phosphorylation nor NF-κB activation. Importantly, LPS internalization and activation was assayed within the same time frame, *i.e.* 30–45 min (Fig. 6). IL-8 secretion occurring 7 h after LPS treatment was markedly increased by the treatment of cytochalasin D. These results strongly suggest that LPS internalization (blocked by cytochalasin D) and LPS activation (unaffected or increased by cytochalasin D) of cells are separate events.

DISCUSSION

Herein, we show that LPS is internalized via a CD14-dependent macropinocytic process resembling that of phagocytosis. This endocytotic pathway has been proposed as an efficient route for nonselective endocytosis of solute macromolecules, as well as for invasion by some pathogenic bacteria, such as *Salmonella thyphimurium* (37, 38). Macropinocytosis has also been described for receptor-mediated endocytosis (22, 38), such as complement receptors CR1 (30) and CR3 (39), involved in phagocytosis by human neutrophils. Macropinocytosis requires the development of cytoplasmic projections or membrane ruffles in a microfilament-dependent mechanism (40). After ruffles form, they merge and close into large intracellular vesicles, termed macropinosomes. These endocytic vesicles then organize in stacks, interact with other intracytoplasmic compartments, and evolve into dense phagolysosome-like structures. In our work, immunogold-labeled CD14 was preferentially localized on membrane ruffles or microvilli. Upon cell warming, CD14 was found specifically associated with all the macropinosomal and the phagolysosomal structures described above. Interestingly, before CD14 was recognized as the LPS receptor, Kang *et al.* (41) described in electron microscopic studies that LPS endocytosed through a phagocytotic, pinocytotic mechanism in human monocytes, very similar to the process described in the present work. Similar findings were reported with murine macrophages (42). These authors showed that LPS-gold particles were found in deep labyrinthic invaginations of the plasma membrane and with coated pits budding off

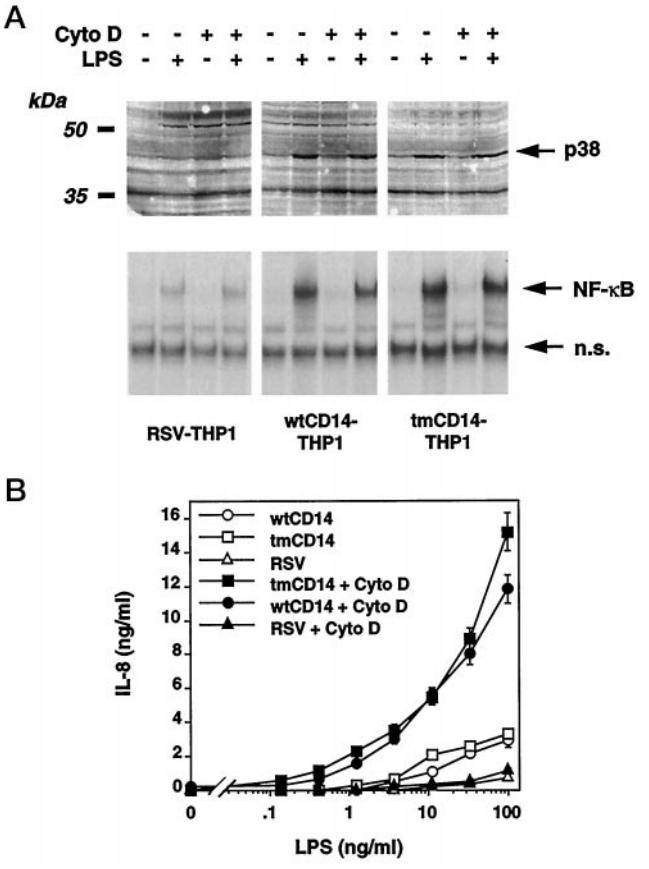

FIG. 6. **Effect of cytochalasin D on LPS-induced p38 mitogen-activated protein kinase phosphorylation, NF-κB activation, and IL-8 secretion.** *A*, upper gel, phosphotyrosine Western blot of cytoplasmic lysates of THP-1 transfectants treated with LPS, cytochalasin D (*Cyto D*), or both for 30 min. The phosphorylated p38 band is indicated by an arrow. Lower gel, electromobility shift assay of nuclear proteins from THP-1 transfectants treated as above for 45 min. The complex of activated NF-κB protein and the ^{32}P-radiolabeled NF-κB DNA oligoprobe is indicated by an *arrow*; *n.s.*, nonspecific band. *B*, IL-8 secretion by wtCD14-THP1 cells (*circles*), tmCD14-THP1 cells (*squares*), and RSV cells (*triangles*). Cells were treated with LPS in the presence (*filled symbols*) or in the absence (*open symbols*) of cytochalasin D for 7 h. Data shown here are a representative experiment, and points are means ± S.D. of triplicates.

these invaginations. We did not find CD14-gold particles in coated pits or vesicles. To reconcile these data, one might argue that CD14 passes LPS onto a second molecule internalized via coated pits. Interestingly, cross-linked CD55, another GPI-anchored receptor, internalized via a similar pathway, and was found to be associated with the same endocytic compartments (35). LPS internalization has also been studied by confocal microscopy by two different groups: in monocytes (13) and in neutrophils (43). These authors found that fluorescent LPS was rapidly internalized (within 5 min), in a CD14-dependent manner. In our work, we showed that FITC-LPS and Texas red-CD14 co-localized both at the cell membrane and in endosomal compartments.

Macropinocytosis requires actin polymerization, and this process is sensitive to cytochalasins (44). In confocal microscopy experiments, we found that both LPS and CD14 endocytosis were abrogated by cytochalasin treatment of cells (Fig. 3). In a protease-based internalization assay, cytochalasin D markedly decreased LPS internalization (Fig. 4). The incomplete inhibition of LPS internalization by cytochalasin D in our Pronase assay could possibly indicate a transfer of LPS into a protease-resistant compartment (insertion of LPS into the plasma membrane, for example). This could explain why we did not observe LPS internalization in cytochalasin D-treated cells by confocal microscopy, yet some LPS remained cell associated after protease application. Wortmannin, a phosphoinositide-3 kinase inhibitor known to inhibit macropinocytosis (33, 34) and CD14-dependent phagocytosis of *E. coli* (15) decreased [^{3}H]LPS internalization by 30% but had a less pronounced effect than cytochalasin D. LPS treatment of CD14-positive cells did not influence the rate or the route of CD14 endocytosis, as if this CD14-dependent uptake was not regulated by the ligand.

Cytochalasin D had a profound inhibitory effect on LPS internalization, probably by blocking the microfilament formation necessary for macropinocytosis. In contrast, this substance did not block LPS activation of cells (p38 mitogen-activated protein kinase phosphorylation and NF-κB activation) within the same time frame, indicating a dissociation between LPS internalization and activation, as suggested by others (5, 7, 14). Cytochalasin D enhanced LPS-induced IL-8 production by THP-1 transfectants. This could be explained either by a sensitization of cells due to the cellular deformation induced by a microfilament disrupting agent (45), by a posttranslational effect of the drug, or by a prolonged time for ligand-receptor interactions at the surface of the cell. In the latter situation, our findings may indicate that LPS internalization could be a process aimed at down-regulating the activating effects of LPS.

We postulate that CD14 molecules present on macrophage membrane projections sense the extracellular milieu and bind LPS when they encounter it. Part of the LPS may be transferred to a protease-resistant membrane molecule/structure or simply be inserted into the lipid bilayer. Bound LPS and CD14 are then internalized in a ligand unregulated manner by macropinocytosis, a process not related to cell activation.

It also makes sense that the LPS internalization pathway resembles that of bacterial phagocytosis, because LPS is the most abundant ligand at the surface of Gram-negative bacteria. Cells of the innate immunity system may well have evolved with identical mechanisms for engulfment of bacteria and bacterial products (15). It was recently recognized that CD14 was the receptor for bacterial ligands other than LPS, originating from Gram-positive bacteria, mycobacteria, and yeasts (10). It was proposed that CD14 was a polyspecific receptor for nonself, conserved bacterial molecules, mediating innate immune responses upon ligand binding (10). Whether or not these non-LPS ligands internalize in a CD14-dependent manner, and by routes similar to that of LPS, remains to be determined.

The role of CD14 GPI anchoring has always puzzled investigators. In the present work we showed that GPI anchoring sorted CD14 to lipid-rich plasma membrane subdomains, where other GPI-anchored receptors are found. Modifying the anchoring system into a transmembrane-type protein sorted CD14 into "integral protein subdomains." It has been postulated that GPI-anchored molecules specifically associated with caveolae, or non-clathrin-coated pits (19). We did not find caveolar structures in promonocytic THP-1 cells, and clearly, gold-labeled CD14 was not associated with such structures. This is congruent with a previous report by Wang *et al.* (46). These authors found that tritiated LPS associated with low-density, lipid-rich THP-1 membrane fragments in the absence of visualized caveolae (46). Similar findings were reported for lymphocytes (47). This strongly suggests that GPI lipid-rich membrane domains are entities distinct from the morphological structure known as caveolae (46, 47). A transmembrane chimeric CD14 construct was found to be targeted to different subdomains, as assessed by detergent solubility and differential buoyancy of sonicated membrane extracts. Neither the different anchoring systems nor their membrane localization affected the rate and the route of the ligand-receptor complex

endocytosis. No difference in rates of LPS-binding and LPS-dependent function was found in wt- *versus* tmCD14 transfectants (Fig. 6) (25, 48). The absence of association of tmCD14 with clathrin-coated pits and vesicles during the process of LPS internalization was somewhat surprising. This might suggest that tmCD14 associated early in its biosynthesis with another molecule responsible to keep it outside of coated pits. This contrasts with another system where a transmembrane form of the natural GPI-anchored folate receptor was targeted to clathrin-coated pits (21). The only difference observed between the two CD14 anchoring systems was the calcium mobilization induced by anti-CD14 cross-linking, present only in GPI but not in transmembrane CD14 cells (25), findings of unknown relevance.

In conclusion, our data demonstrate that in monocytic cells, LPS and its receptor CD14 internalize through macropinocytosis, a pathway independent of coated pits or caveolae. Macropinocytosis resembles that of phagocytosis in several aspects, in particular in its requirement for intact microfilaments and formation of large vesicles. The natural GPI anchoring system of CD14 seems irrelevant in the internalization of the complex ligand-receptor, and LPS does not regulate the internalization of its receptor CD14. LPS internalization and cell activation are dissociated events.

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Note Added in Proof—Data published by Kitchens and Munford (49) after this manuscript was submitted indicate that LPS is internalized by a constitutive mechanism, compatible with our findings of a macropinocytic pathway.

REFERENCES

1. Janeway, C. A. J. (1992) *Immunol. Today* **13,** 11–16
2. Fearon, D. T., and Locksley, R. M. (1996) *Science* **272,** 50–54
3. Raetz, C. R., Ulevitch, R. J., Wright, S. D., Sibley, C. H., Ding, A., and Nathan, C. F. (1991) *FASEB J.* **5,** 2652–2660
4. Wright, S. D. (1991) *Curr. Opin. Immunol.* **3,** 83–90
5. Hampton, R. Y., Golenbock, D. T., Penman, M., Krieger, M., and Raetz, C. R. H. (1991) *Nature* **352,** 342–344
6. Tobias, P. S., Soldau, K., Kline, L., Lee, J. D., Kato, K., Martin, T. P., and Ulevitch, R. J. (1993) *J. Immunol.* **150,** 3011–3021
7. Gegner, J. A., Ulevitch, R. J., and Tobias, P. S. (1995) *J. Biol. Chem.* **270,** 5320–5325
8. Goyert, S. M., Ferrero, E., Rettig, W. J., Yenamandra, A. K., Obata, F., and Le Beau, M. M. (1988) *Science* **239,** 497–500
9. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) *Science* **249,** 1431–1433
10. Pugin, J., Heumann, D., Tomasz, A., Kravchenko, V. V., Akamatsu, Y., Nishijima, M., Glauser, M. P., Tobias, P. S., and Ulevitch, R. J. (1994) *Immunity* **1,** 509–516
11. Ulevitch, R. J., and Tobias, P. S. (1994) *Curr. Opin. Immunol.* **6,** 125–130
12. Pugin, J., Schurer-Maly, C. C., Leturcq, D., Moriarty, A., Ulevitch, R. J., and Tobias, P. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2744–2748
13. Gallay, P., Jongeneel, C. V., Barras, C., Burnier, M., Baumgartner, J.-D., Glauser, M. P., and Heumann, D. (1993) *J. Immunol.* **150,** 5086–5093
14. Kitchens, R. L., Ulevitch, R. J., and Munford, R. S. (1992) *J. Exp. Med.* **176,** 485–494
15. Schiff, D. E., Kline, L., Soldau, K., Lee, J. D., Pugin, J., Tobias, P. S., and Ulevitch, R. J. (1997) *J. Leukocyte Biol.* **62,** 786–794
16. Peterson, P. K., Gekker, G., Hu, S., Sheng, W. S., Anderson, W. R., Ulevitch, R. J., Tobias, P. S., Gustafson, K. V., Molitor, T. W., and Chao, C. C. (1995) *Infect. Immun.* **63,** 1598–1602
17. Munford, R. S., and Hall, C. L. (1986) *Science* **234,** 203–205
18. Lisanti, M. P., Scherer, P. E., Tang, Z., and Sargiacomo, M. (1994) *Trends Cell Biol.* **4,** 231–235
19. Anderson, R. W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10909–10913
20. Chun, M., Liyanage, U. K., Lisanti, M. P., and Lodish, H. F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11728–11732
21. Ritter, T. E., Fajardo, O., H., M., Anderson, R. G. W., and Lacey, S. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3824–3828
22. Lamaze, C., and Schmid, S. L. (1995) *Curr. Concepts Cell Biol.* **7,** 573–580
23. Haziot, A., Chen, S., Ferrero, E., Low, M. G., Silber, R., and Goyert, S. M. (1988) J. *Immunol.* **141,** 547–552
24. Simmons, D. L., Tan, S., Tenen, D. G., Nicholson-Weller, A., and Seed, B. (1989) *Blood* **73,** 284–289
25. Pugin, J., Kravchenko, V. V., Lee, J.-D., Kline, L., Ulevitch, R. J., and Tobias, P. S. (1998) *Infect. Immun.* **66,** 1174–1180
26. Brown, D. A., and Rose, J. K. (1992) *Cell* **68,** 533–544
27. Ilangumaran, S., Arni, S., Chicheportiche, Y., Briol, A., and Hoessli, D. C. (1996) *Anal. Biochem.* **235,** 49–56
28. Horejsi, V., Angelisova, P., Bazil, V., Kristofova, H., Stoyanov, S., Stefanova, I., Hausner, P., Vosecky, M., and Hilgert, I. (1988) *Folia Biol. (Prague)* **34,** 23–34
29. Smart, E. J., Ying, Y.-S., Mineo, C., and Anderson, R. G. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10104–10108
30. Carpentier, J. L., Lew, D. P., Paccaud, J. P., Roy, G., Lacopetta, B., Kazatchkine, M., Stendhal, O., and Pozzan, T. (1991) *Cell Regul.* **2,** 41–55
31. Carpentier, J. L., Paccaud, J. P., Backer, J., Gilbert, A., Orci, L., and Kahn, C. R. (1993) *J. Cell Biol.* **122,** 1243–1252
32. Munford, R. S., DeVeaux, L. C., Cronan, J. E., and Rick, P. D. (1992) *J. Immunol. Methods* **148,** 115–120
33. Grassmé, H. U. C., Ireland, R. M., and van Putten, J. P. M. (1996) *Infect. Immun.* **64,** 1621–1630
34. Araki, N., Johnson, M. T., and Swanson, J. A. (1996) *J. Cell Biol.* **135,** 1249–1260
35. Tausk, F., Fey, M., and Gigli, I. (1989) *J. Immunol.* **143,** 3295–3302
36. Bamezai, A., Goldmacher, V., Reiser, H., and Rock, K. L. (1989) *J. Immunol.* **143,** 3107–3112
37. Francis, C. L., Ryan, T. A., Jones, B. D., Smith, S. J., and Falkow, S. (1993) *Nature* **364,** 639–642
38. Swanson, J. A., and Watts, C. (1995) *Trends Cell Biol.* **5,** 424–428
39. Detmers, P. A., Wright, S. D., Olsen, E., Kimball, B., and Cohn, Z. A. (1987) *J. Cell Biol.* **105,** 1137–1145
40. O'Shea, J. J., Brown, E. J., Gaither, T. A., Kakahashi, T., and Frank, M. M. (1985) *J. Immunol.* **135,** 1325–1330
41. Kang, Y. H., Dwivedi, R. S., and Lee, C. H. (1990) *J. Leukocyte Biol.* **48,** 316–332
42. Kriegsmann, J., Gay, S., and Bräuer, R. (1993) *Cell. Mol. Biol.* **39,** 791–800
43. Detmers, P. A., Thieblemont, N., Vasselon, T., Pironkova, R., Miller, D. S., and Wright, S. D. (1996) *J. Immunol.* **157,** 5589–5596
44. Allison, A. C., Davies, P., and de Petris, S. (1971) *Nat. New Biol.* **232,** 153–155
45. Shibata, Y., Nakamura, H., Kato, S., and Tomoike, H. (1996) *J. Immunol.* **156,** 772–777
46. Wang, P. Y., Kitchens, R. L., and Munford, R. S. (1995) *J. Inflamm.* **47(3),** 126–137
47. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1994) *J. Biol. Chem.* **269,** 30745–30748
48. Lee, J. D., Kravchenko, V., Kirkland, T. N., Han, J., Mackman, N., Moriarty, A., Leturcq, D., Tobias, P. S., and Ulevitch, R. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9930–9934
49. Kitchens, R. L., and Munford, R. S. (1998) *J. Immunol.* **160,** 1920–1928

3.3 Part II: «CD14 expression in epithelial cells is not sufficient for LPS-induced cell activation»

CD14 expression in epithelial cells is not sufficient for LPS-induced cell activation

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Abstract

Human epithelial cells were shown to respond to lipopolysaccharides (LPS) *via* soluble CD14. However, the expression and release of CD14 by these so-called CD14-negative cells have not been studied in detail. Liver and intestinal epithelial cells are in close contact with bacteria and bacterial products such as LPS, and might be involved in innate immune host defense. Using three human intestinal and liver epithelial cell lines, SW620, T84 and HepG2, we studied CD14 expression as well as LPS-response and –endocytosis in these cells. Colonic-T84 and hepatocytic-HepG2 appeared to poorly expressed CD14 gene and protein, whereas CD14 expression was found to be significantly higher in colonic-SW620 cells. These latter cells could be activated by LPS in the presence of serum, which contained soluble CD14. In contrast , T84 and HepG2 cells did not respond at all to LPS in same conditions. Following this observation, we investigated the effect of wild type human CD14 gene transfection on these cells. All cells highly expressed CD14 at molecular and cellular levels. CD14(+)-SW620 cells were still responsive to LPS. To the opposite, CD14(+)-T84 and CD14(+)-HepG2 cells did not produce any IL-8 under LPS stimulation. This result was confirmed at the NF-κB level. However, all cells were activated under stimulation with other agonists such as TNF-α or phorbol 12-myristate 13-acetate (PMA), which stimulated the NF-κB pathway. Although some of cells were non-responding to LPS, all cells could internalise LPS with similar rates. In conclusion, our study demonstrated that some epithelial cells could naturally express the CD14 gene and protein. HepG2 and T84 cells appeared to be resistant to LPS stimulation. Following the CD14 overexpression, both cell types did not restore their LPS sensitivity, suggesting that these cells are deficient in (a) molecule(s) of the proximal LPS-dependent signaling pathway. Despite this observation, all cells were able to internalise LPS, suggesting that LPS internalization and LPS signaling are separate processes.

Introduction

Gram-negative bacterial endotoxin specifically binds to CD14, a glycosylphosphatidyl inositol-anchored surface glycoprotein of myeloid cells. Binding of LPS to membrane CD14 of monocyte / macrophage induces secretion of various pro- and anti-inflammatory mediators, and also promotes LPS internalization and deacylation (1-4). CD14 also exists in a soluble form present in plasma and biological fluids (5). Endothelial cells and some epithelial cell lines have been shown to also respond to LPS *via* a soluble CD14-dependent manner (6). Indeed, epithelial cells not only serve as a physical barrier, but they also actively participate in the maintenance, renewal and defense of their surfaces (7). Some studies raised the idea that these cells may also play important roles in host responses to LPS during sepsis (8, 9). Epithelial cells can be activated by an indirect pathway *via* cytokines produced by LPS-stimulated myeloid cells or as previously mentioned, directly by LPS *via* the soluble CD14 (10-13). Activation of epithelial cells occurs *via* the production of cytokines such as IL-6 and TNF-α, chemokines such as IL-8 and MCP-1, complement regulatory proteins, and in some cases *via* the expression of antibiotic peptide genes (10, 11, 14, 15). Actually, host recognition of pathogenic microorganisms, molecules such as LPS or the complex LPS/sCD14 requires probably specific receptors in these cells and is fundamental to induce a defense response. CD14 expression in epithelial cells remains unclear. Although, epithelial cells are often considered as CD14 negative-bearing cells, some authors discovered CD14 gene and protein expression in epithelial cells from trachea, lung, uterus and intestine (15-17). Some epithelial cells such as intestinal cells are directly in contact with bacteria and bacterial products such as LPS. Likewise, hepatocytes in close proximity with blood due to their role in molecules metabolisation, can encounter LPS during bacterial infections. These cells might have develop some specific characteristics to protect themselves and organs against bacteria and LPS

pathogenicity. They might also be able to discriminate between harmful pathogens and antigens which are beneficial, such as food proteins or commensal bacteria present in gut. Little is known about interaction, response and internalization of LPS in these cells. Using different human colonic (SW620, T84) and liver (HepG2) epithelial cell lines, we first determined the synthesis of CD14 and studied LPS responsiveness in these cells. We found that little cellular and soluble CD14 was produced by T84 and HepG2 cells, but that CD14 was significantly higher expressed in SW620 cells. Interestingly, these latter cells could be activated by LPS in the presence of serum, whereas, in same conditions, T84 and HepG2 cells were unresponsive. Subsequently, we investigated the effect of human CD14 gene transfection of these cells on LPS-responsiveness and -internalization. Despite the high expression of CD14, CD14(+)-HepG2 and CD14(+)-T84 cells still remained unresponsive to LPS. Furthermore, these latter cells, as well as CD14(+)-SW620 cells, could however internalise endotoxin with similar rates.

Materials and methods

Cell culture and transfections.

Human epithelial SW620 and T84 (colorectal adenocarcinoma cells), HepG2 (hepatocellular carcinoma cells) cell lines (ATCC) and promonocytic THP-1 cell line were transfected with a plasmid encoding for the wild type human CD14 gene or with the empty vector pRc RSV. Human CD14 protein expressed were anchored by a glycosylphosphatidyl inositol at the membrane surface of cell lines (CD14(+)-SW620, -T84-, -HepG2). or the empty vector pRc RSV (rsv) served as control cells. Transfected T84, HepG2 and SW620 cell lines were maintained in DMEM and THP-1 cells in RPMI 1640 (Gibco). Both medium contained penicillin and gentamicin antibiotics, 10% fetal bovine serum and 0.5-1 mg/mL G418 (Geneticin ®) all from Life Technologies, Inc. PBMC were obtained from buffy coat by centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech). For all experiments, CD14(+)- and rsv-THP-1 cells were used as a positive and negative control respectively.

CD14 mRNA expression determined by RT-PCR.

Total mRNA was extracted from cells using a RNeasy mini kit (Qiagen). To remove any DNA contamination, the samples were treated with 2 μg/mL RNase free DNase I (Boehringer, Mannheim) for 1 h at 37°C. Samples were purified and the RNA concentration of each sample was determined. One μg of RNA was reverse-transcribed for 1 hour at 37°C with Superscript reverse transcriptase (RT, 200 U/μl; Life Technologies, Inc.). The cDNA products were diluted in 100 μl of water. RT-PCR was performed with 10 μl of RT product, 0.2 μM specific primers for CD14 mRNA, 80 μl of reaction mix (10 mM Tris pH=8, 50 mM

KCl, 2.5 mM $MgCl_2$ and 200 µM for each dNTP) and 2.5 U Taq polymerase (Core kit, Qiagen). PCR was performed for 35 or 40 cycles at 95°C (60 s, melting), 62°C (60 s, annealing), and 72°C (90 s, extension ; addition of 2 s of extension time per cycle). Fifteen µl of PCR samples were run on a 1.8 % agarose gel, visualised by UV light and photographed. The expected size of this PCR product corresponding to CD14 was 1,140 bp.

Detection by Western blot of soluble CD14 in conditioned supernatants or cellular CD14 of lysed cells.

i) *Soluble CD14*. At 80 % of confluence, cells were washed and maintained 24 hours in serum free DMEM or RPMI 1640. Supernatants were centrifuged to remove any cells remaining. Further, 15 mL of supernatant were concentrated to 0.2 mL in ultrafree centrifugal filter (Millipore).

ii) *Cellular CD14*. Cells were detached with PBS containing 5 mM EDTA, washed twice in TBS and treated with 500 µL of lysis buffer (20 mM TBS / EDTA 2 mM ; 10 % glycerol ; 1% triton X-100, anti-proteases : 1 mM PMSF, 1 µg/mL leupeptin, 200 U/mL aprotinin ; and 60 mM octylglucoside) for 1 hour at 4°C. Lysates were centrifuged at 13,000 rpm for 10 min and supernatants were kept at 4°C.

Immunoprecipitation was performed with two different anti-human CD14 monoclonal antibodies (10 µg/mL) for 4 h at 4°C. 75 ul of protein G agarose beads (Sigma) were added and the tubes were shaken overnight. Beads were centrifuged and washed five times with TBS, resuspended in SDS-PAGE sample buffer (0.5 M Tris-HCl pH 6.8 ; 10% glycerol ; 10% SDS ; bromophenol blue) and boiled for 5 min. After removal of beads by centrifugation, 50

μL of supernatant was subjected to SDS-PAGE followed by an electrotransfer procedure to a nitrocellulose membrane. Human CD14 was detected with biotinylated polyclonal antibodies, G protein-Horseradish peroxidase conjugate (Bio-Rad) and enhanced chemiluminescence (ECL ; Amersham Life Science). A film was exposed different times and analysed by scanning densitometry.

Interleukin-8 production.

Twenty four hours prior the experiment, SW620, T84 and HepG2 transfectants were trypsinised, washed and distributed in microtiter plate (110,000 cells/well) in DMEM or RPMI 1640 medium supplemented with 5% FBS / antibiotics without geneticin. The following day, cells were washed and incubated (15 hours ; 37°C) in RPMI 1640 medium containing 0.5 mg/mL human serum albumin (HSA) and various concentrations of phorbol 12-myristate 13-acetate (PMA) or LPS (O111:B4) in the presence of either lipopolysaccharide binding protein (LBP ; 2.5%) or FBS (5%). Interleukin-8 was measured by ELISA (Endogen, Cambridge, MA) in conditioned supernatants.

NF-kappa B activation.

Cells were cultured without geneticin 24 hours prior the experiment. Cells were washed and maintained in RPMI 1640 medium with 3% fetal bovine serum for 3 hours at 37°C in the incubator. Cells were then incubated in RPMI 1640 medium / 3% FBS containing 1 μg/mL *E. Coli* O111:B4 or K12 LCD 25 LPS, 10 ng/mL recombinant TNF-α or 2 μg/mL PMA for 30 or 60 minutes. After the stimulation, cells were rapidly chilled on ice, washed twice with cold PBS pH 7.3, mechanically removed and transferred into Eppendorf tubes. Nuclear extracts

were then prepared as described elsewhere (18). Nuclear proteins were used for electrophoretic mobility shift assay (EMSA). Nuclear proteins (8 to 10 μg) were added to 12 μL of a binding buffer containing 5 mM Hepes pH 8.5, 5 mM $MgCl_2$, 50 mM DTT, 0.4 mg/mL poly(dI-dC), 0.1 mg/mL sonicated double-stranded salmon sperm DNA, 10% glycerol and 20-50 fmoles of ^{32}P-labeled NF-κB oligonucleotide probe (30,000-50,000 cpm) (Promega, 5'-AGTTGAGGGGACTTTCCAGG-3') and incubated for 10 minutes at room temperature. Samples were migrated on a non-denaturing 5% acrylamide gel made in Tris-glycine-EDTA buffer. The gel was further transferred onto Whatman paper, dried and subjected to autoradiography.

^{3}H-LPS internalization.

Twenty ng/mL ^{3}H-LPS (biosynthetically tritiated E. Coli K12 LCD25 LPS, List Biological Laboratories), preincubated 5 min at 37°C with 2% LBP, were added to the CD14(+)-cells for various times (0 to 2 hours). Cells were centrifuged and washed with ice-cold RPMI 1640 medium. Cells were then treated with 0.02% proteinase K (Boehringer Mannheim GmbH, Germany) for 1 hour at 4°C. Supernatants and cells were seperated by centrifugation, and transferred into scintillation liquid. Radioactivity was measured in a scintillation counter to determine cell-bound and internalised (proteinase resistant, cell associated) counts. Internalization was defined as the percentage of counts resistant to proteinase K relative to the total counts (cell-associated counts before the proteinase K treatment).

Results

RT-PCR.

RT-PCR technique was performed in order to determine the mRNA expression of CD14 in human epithelial cell lines. To validate the RT-PCR, three control were done. First, amplification of total RNA without reverse transcription was achieved to insure the absence of contaminating genomic DNA (not shown). Second, water was used as substrate in PCR to investigate the presence of any contaminating DNA. Third, the hybridization specificity of primers for human CD14 gene was verified by amplifiying cDNA originating from mouse 3T3 fibroblast cell line that does not express any CD14 mRNA. In all cases, no amplification of DNA was seen (fig.1). cDNA of PBMC was used as a positive control to amplify CD14 cDNA after the reverse transcription of mRNA. On the gel, we observed a band corresponding to a fragment of 1140 bp expected size for the CD14 cDNA. At the same level, intense and slight band were present on the gel for CD14- and rsv-transfected cells respectively (fig.1). For rsv-SW620 cells, the band was more intense than those for other rsv-transfected cells.

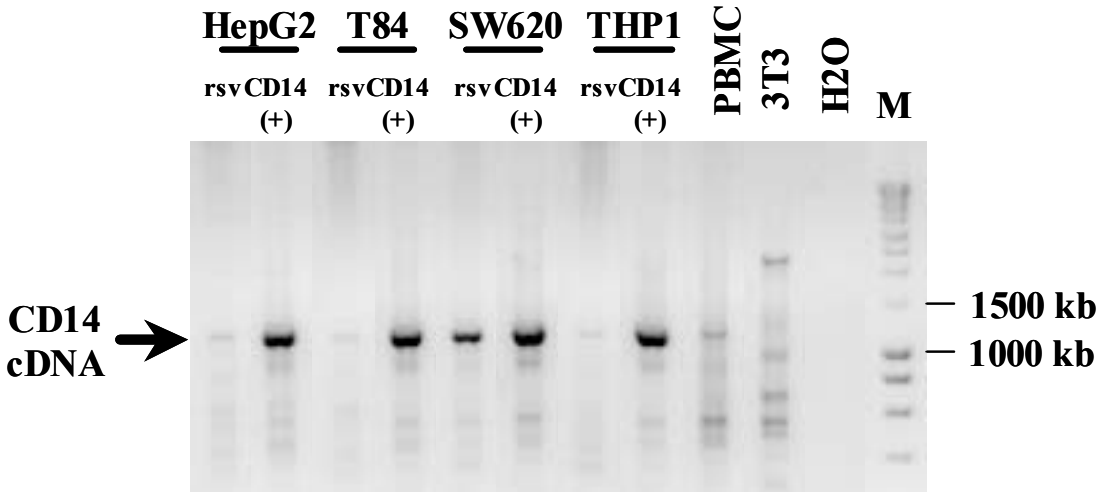

Figure 1. Detection by RT-PCR of human CD14 mRNA in different cell lines. Epithelial cell lines HepG2, T84, SW620 and the monocytic cell line THP-1 were transfected with a plasmid encoding for the CD14 gene (CD14(+)) or with the empty vector (rsv). The size of the CD14 cDNA transcript was 1140 bp as estimated with DNA markers (M). CD14 cDNA of peripheral blood monocytic cells (PBMC) was used as a positive control. H_2O and cDNA of 3T3 mouse fibroblasts served as negative controls.

Western blot of CD14.

i) Cellular CD14. Using Western immunoblotting technique, we determined the presence of CD14 in SW620, T84, HepG2, THP-1 cell lines. The CD14 protein expression was significantly higher in SW620 cells than in other cells. Recombinant human CD14 was used as positive control and was observed migrating at roughly 55 kDa in all experiences. In all cell types transfected with cDNA encoding for the glycosylphosphatidyl inositol anchored CD14, we observed a strong signal of immunoreactive protein (fig.2). A more intense signal was detected in THP-1 and SW620 cells. Cells transfected with the CD14 cDNA produced immunoreactive CD14. Cells transfected with the empty vector (rsv-SW620, -T84, -HepG2, -THP-1) also produced CD14, however to a lesser extent (fig.2).

ii) Soluble CD14 (sCD14). The presence of sCD14 was determined by immunoblotting technique in conditioned supernatants of cells cultured 24 h in serum free medium. Positive control was ascertained by using recombinant human CD14 as in figure 2. Bands corresponding to immunoreactive sCD14 generated by CD14(+)-transfected cells were intense. The band corresponding to sCD14 originated from rsv-HepG2 cells was less intense (fig.2).

Measurement of soluble CD14 by ELISA nevertheless revealed the presence of sCD14 in supernatants of all cell types, but to different concentrations according to rsv- (low concentrations) and CD14(+)-transfected cells (high concentrations) (Data not shown). The fact that no sCD14 was detected by Western blot in T84 and SW620 rsv-transfected cells could be explained by a greater dilution of sCD14 in supernatants. This could be due to volume of culture medium/number of cells ratio greater than that obtained with rsv-HepG2 cells.

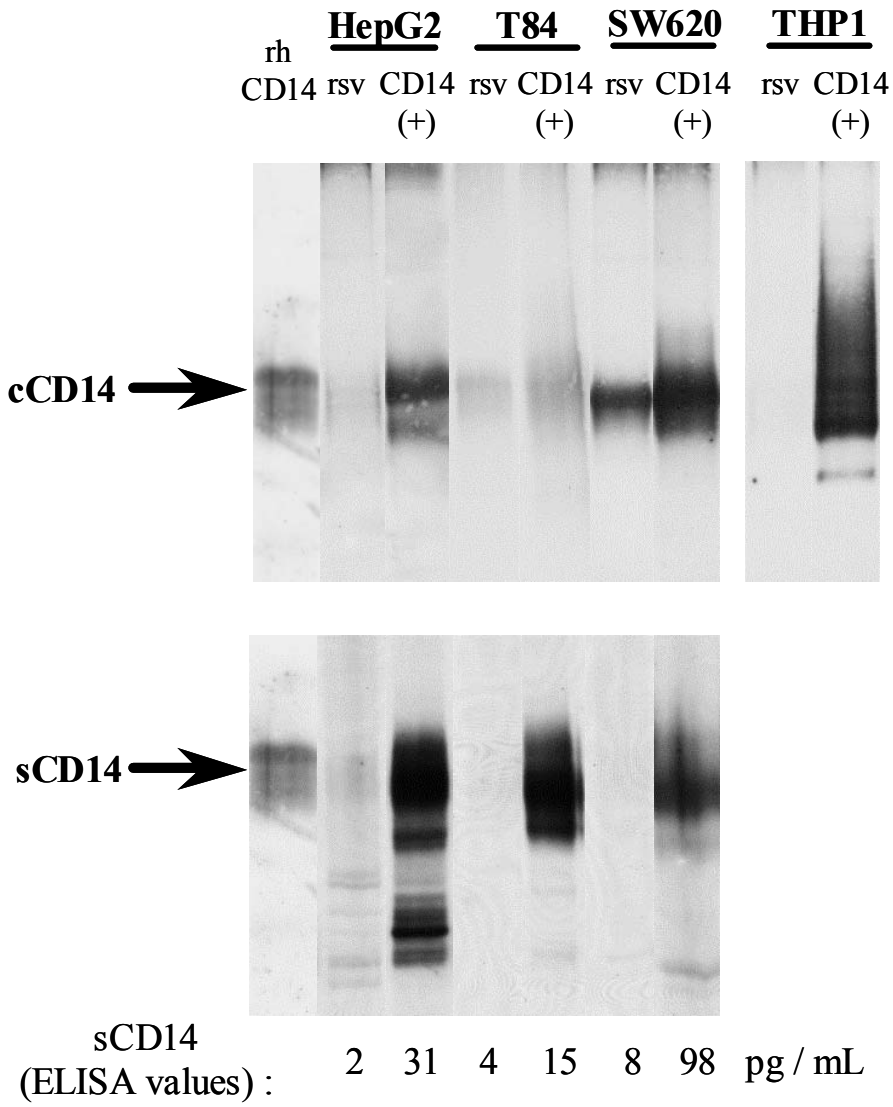

Figure 2. Detection by Western blot of soluble (s) or cellular (c) CD14 in different epithelial cells. Different cell types were transfected with the empty vector (rsv) or the plasmid encoding for the CD14 gene (CD14(+)). Recombinant human (rh) CD14 as well as transfected THP-1 cells were used as a positive control. The rhCD14 size was determined to be 55 kDa. Bands corresponding to immunoreactive sCD14 and cCD14 of different cell types were found to migrate at the same level than to that of the recombinant human CD14.

Interleukin-8 production.

In order to test the responsiveness of these epithelial cells, we incubated cells with various concentrations of LPS or PMA in the presence of either 5% FCS or LBP. After 15 h stimulation, IL-8 was measured in conditioned supernatants by ELISA. HepG2, T84 and SW620 cell types produced large amounts of IL-8 in a dose-dependent manner in response to PMA stimulation. After LPS treatment, only SW620 cells released IL-8 in a dose-dependent manner. In the presence of 5% FBS (containing soluble CD14), CD14(+)-SW620 and rsv-SW620 cells synthesized equivalent amounts of IL-8 (fig.3). In contrast, rsv-SW620 cells produced very little amount of IL-8 compared to CD14(+)-SW620 cells in the presence of LBP. Surprisingly, no IL-8 was secreted by HepG2- and T84-transfected cells after LPS stimulation in the presence of either FBS or LBP (fig.3). Similar results were found for these cells, even at LPS concentration of 10 μg/mL (data not shown). All cells stimulated with TNF-α (0 to 10 ng/mL) and IL-1β (0 to 2 ng/mL) produced IL-8, except T84 cells which appeared to be resistant to the IL-1 treatment (data not shown).

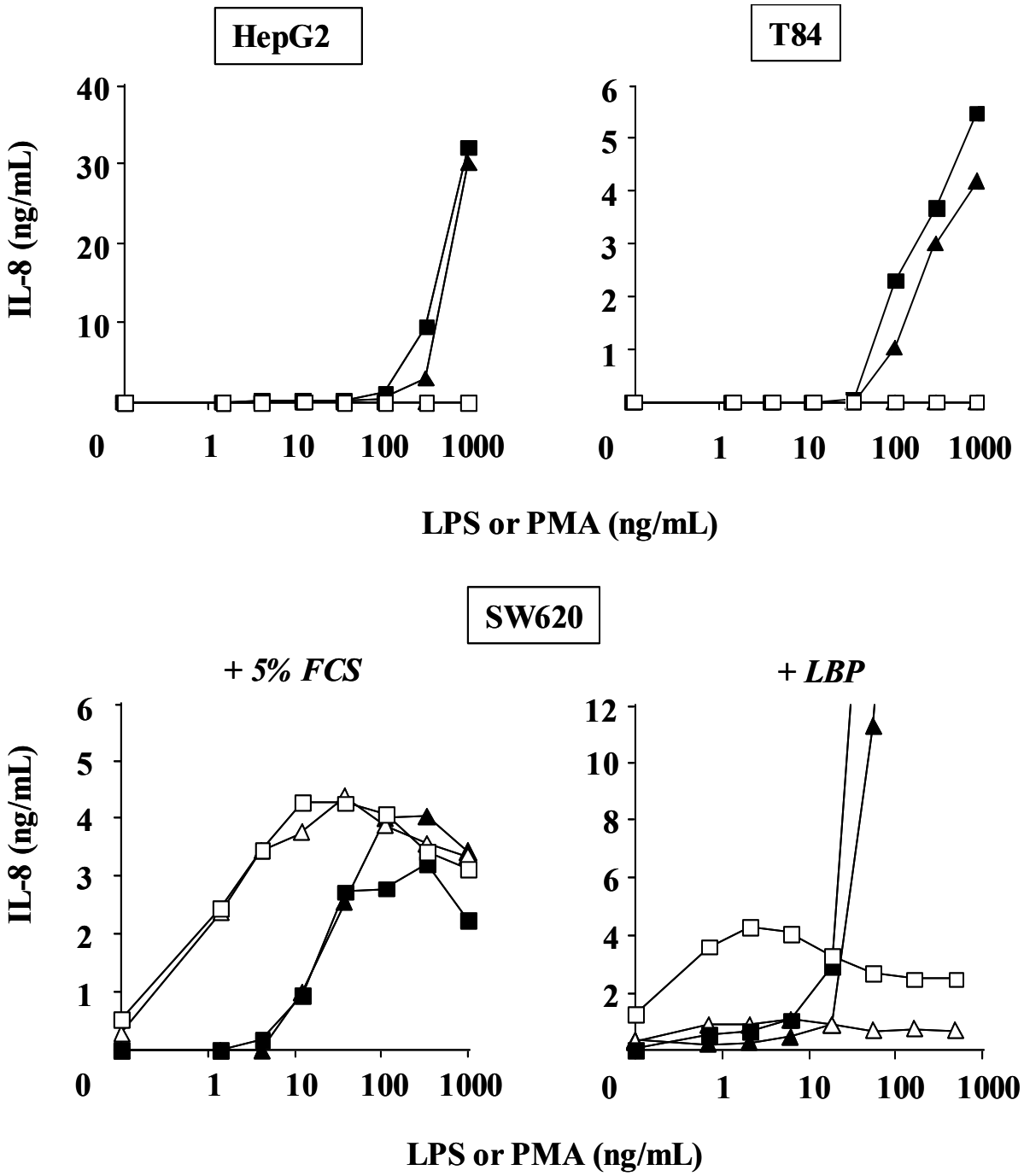

Figure 3. Effect of LPS or PMA treatment on IL-8 production in different epithelial cells. IL-8 secretion was assessed by ELISA in conditioned supernatants of transfected epithelial cell lines treated with LPS (open symbols) or PMA (closed symbols) in the presence of either FBS or LBP for 15h. CD14(+) expressing cells were represented by squares and rsv cells (transfected with the empty vector) by triangles.

NF-κB activation.

To confirm the non-responsiveness of HepG2 and T84 cell lines to LPS, we investigated whether nuclear factor (NF) kappa-B was activated. After stimulation with LPS, TNF-α or PMA, CD14(+)- and rsv-transfected cells were lysed. Nuclear proteins were extracted and an electrophoretic mobility shift assay (EMSA) was performed with the nuclear extracts (fig.4). In both cell types, a band corresponding to activated NF-κB was visualised on the gel after TNF-α and PMA stimulations for rsv- and CD14(+)-transfected cells. In contrast, no NF-κB activation was observed after LPS stimulation in these epithelial cells. In contrast, NF-κB was activated after LPS stimulation in CD14(+)-THP-1 cells used as a positive control (fig.4). In any case, cells treated with medium only, used as a negative control, did not produce any NF-κB activity. Therefore, LPS did not activate NF-κB in CD14(+)-HepG2 and -T84 cells.

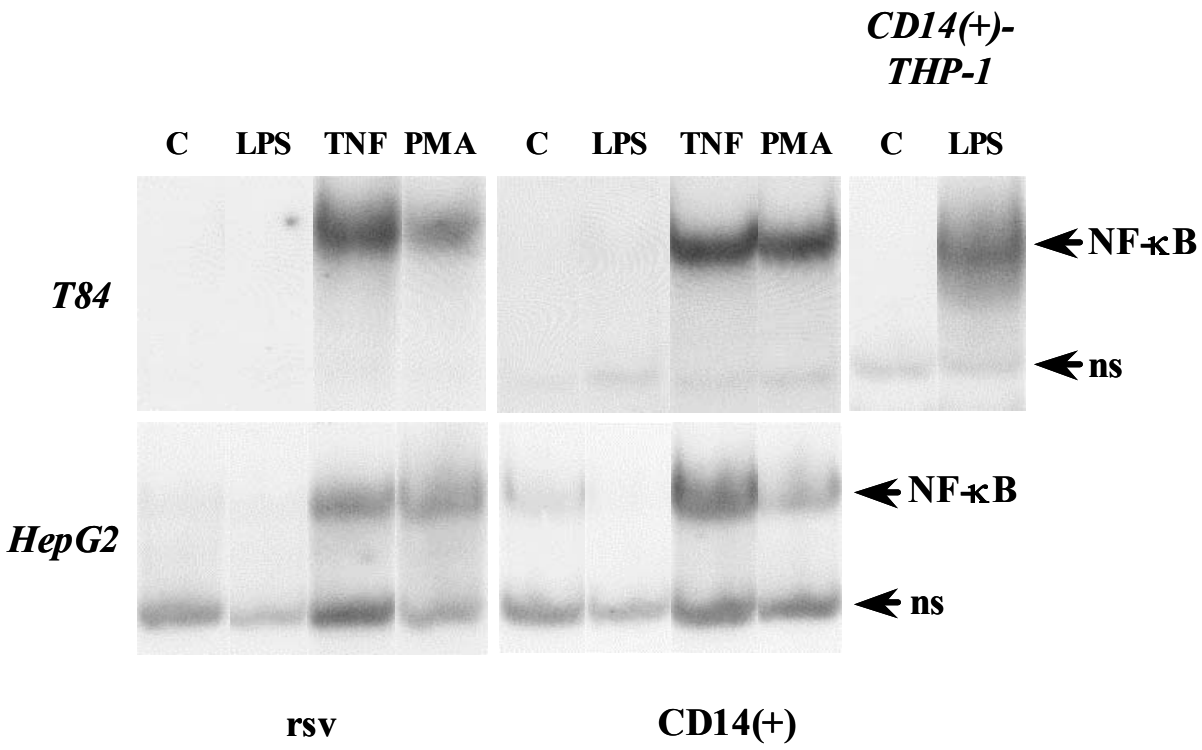

Figure 4. Effect of several inflammatory mediators on NF-κB activation in T84 and HepG2 cells measured by electromobility shift assay. Cells were either transfected with the empty vector (rsv) or a plasmid encoding for the CD14 gene (CD14(+)) and treated with LPS, TNFα or PMA. Nuclear proteins from each preparation were isolated 60 min following cell stimulation and added to a mixture containing a ^{32}P-radiolabeled DNA oligoprobe specific for the NF-κB binding site. Binding of NF-κB to DNA oligoprobe reduces the electromobility of the DNA oligoprobe in the gel compared to that of an unbound DNA oligoprobe. Bands corresponding to activated NF-κB proteins complexed with ^{32}P-radiolabeled DNA oligoprobe are detected by autoradiography and indicated by an arrow NF-κB ; n.s., non-specific band. CD14(+)-THP-1 cells treated with LPS were used as a positive control.

LPS internalization in epithelial cell lines.

After studying LPS-responsiveness of various human epithelial cells, we performed a protease-based internalization assay to investigate whether these cells could internalise LPS. At 37°C, LPS was rapidly internalised in the first 5 minutes with similar rates for CD14(+)-transfected epithelial cell types (fig.5). LPS internalization reached a plateau at 30-40 min after incubation of cells with LPS at 37°C. By using an anti-CD14 monoclonal antibody 3C10 with CD14(+)-transfected epithelial cells, LPS internalization was partially inhibited (20-30%) (Data not shown).

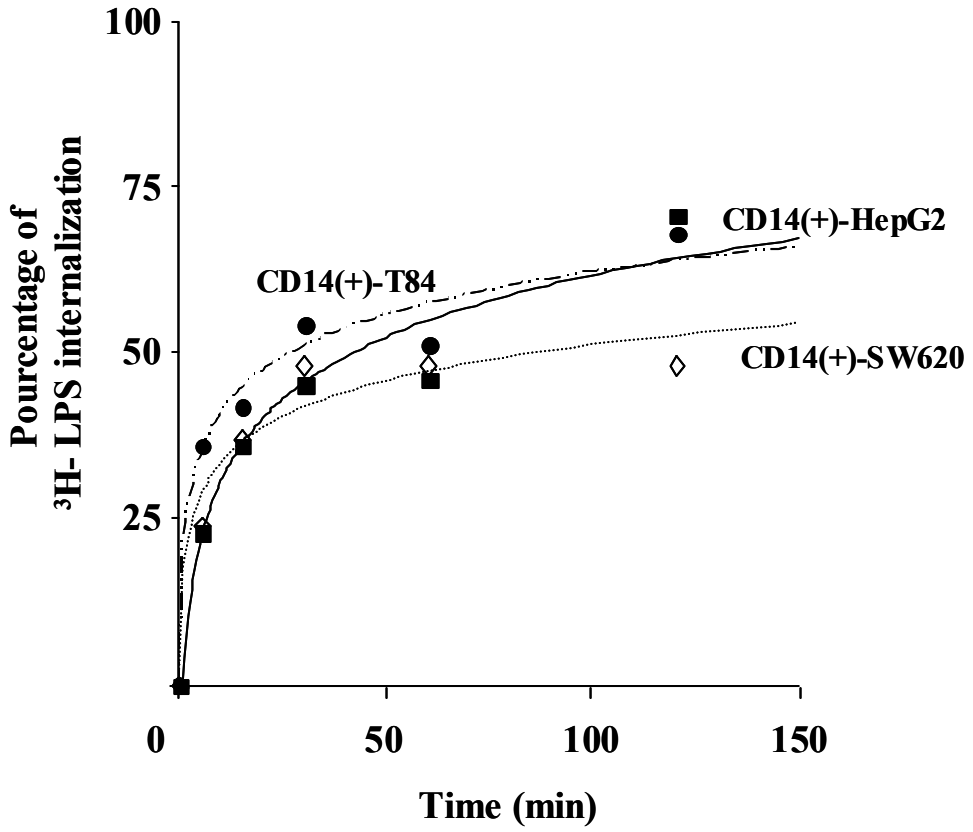

Figure 5. Kinetics of LPS internalization in different CD14-transfected epithelial cells. CD14(+)-HepG2 (closed square), CD14(+)-T84 (closed circle), CD14(+)-SW620 (open diamond) transfected epithelial cells were incubated with ^{3}H-LPS (20 ng/mL) at 37°C. Percentage of internalization was defined as the proteinase K-resistant cell associated counts relative to the cell-associated counts before proteinase K treatment. A logarithmic regression was calculated with data of each epithelial cell type. Logarithmic regression curves are represented by a continuous line for CD14(+)-HepG2, a dotted line for CD14(+)-SW620 and a pecked line for CD14(+)-T84.

Discussion

In this study, we investigated CD14 expression, LPS-responsiveness and -endocytosis in intestinal and liver cells. Epithelial cells are considered as CD14-negative cells. Our data demonstrated that some of these cells actually express CD14 at a transcriptomic and proteomic levels, but with different degree of expression depending on the cell types. Indeed, HepG2 and T84 cells were found to express little CD14. A study by Nanbo et al. supported our results obtained with HepG2 cells (19). In contrast, Wang et al. reported that HepG2 cells do not express CD14 (20). This discrepancy might be explained by a variation in CD14 expression found in different HepG2 cell lines, with a very low CD14 expression to the limit of detection as suggested by Nanbo et al. We showed that intestinal SW620 cells expressed significantly higher CD14 compared to HepG2 and T84 cells. In the presence of serum containing soluble CD14, SW620 cells produced IL-8 after LPS treatment. Similar results using these cells were obtained by Schuerer-Maly et al. (21). Moreover, Pugin et al. had previously demonstrated that the LPS activation of SW620 cells was dependent on soluble CD14 (6). Despite CD14 expression, LPS stimulation in these cells, in the presence of LBP alone induced a weaker response compared to that obtained with the serum. Such a difference could be explained either by an insufficient expression of CD14 at the cell surface, or by differential efficiencies of LPS/LBP and LPS/sCD14 complexes to bind the cell surface of SW620 cells. Similarly to Schuerer-Maly et al., we found that T84 cells were not responsive to LPS in the presence of either serum or LBP (21). We found that HepG2 cells behaved in a very similar way as T84 cells, and could not be activated by LPS under similar conditions. In contrast, other investigators reported that HepG2 cells could be activated by LPS i) by producing LBP and up-regulating their own CD14 expression (19); ii) by inducing the expression of diverse pro-inflammatory factors such as TNF-α and IL-6 (22). To explain this

discrepancy, we could suggest that the low level of CD14 expression or a gene mutation that could have occurred in our HepG2 cell line might have altered the LPS response of these cells.

By transfecting human CD14 gene in epithelial cells, we were interested in investigating whether the increase of CD14 expression in these cells could restore the LPS responsiveness of LPS-resistant cells. Interestingly, CD14(+)-SW620 cells had the same degree of LPS response in the presence of serum compared to that of "CD14-negative" cells, but greatly increased their response to LPS in the presence of LBP alone. This result suggested that CD14(+)-SW620 cells used CD14 present at the cell membrane when no soluble CD14 was available. In contrast, the expression of CD14 by transfection in T84 and HepG2 cells was not sufficient to restore LPS responsiveness. However, both cell types could be activated at the NF-κB level and produced IL-8 after stimulation with other agonists such as TNF-α or PMA. Similar results were obtained with IL-1β in HepG2 cells, but not in T84 cells which were not activated by IL-1. PMA was shown to stimulate the NF-κB pathway directly through the activation of protein kinase C (23). Whereas the starting point of TNF-α or IL-1-induced NF-κB cascade activation corresponds to specific receptors localised at the cell membrane, as CD14 for LPS (24, 25). The divergence of results obtained with LPS and those obtained with IL-1 or TNF-α led us to suggest that at least some of the most proximal components of the TNF-α, IL-1 and LPS signaling cascades were unique to each pathway. This would indicate that LPS-unresponsive cells, CD14(+)-HepG2 and -T84, could be deficient in (a) molecule(s) of the proximal LPS-dependent signaling pathway. The non-responsiveness of CD14(+)-T84 to LPS and IL-1 rather suggests that either i) T84 cells are devoid of IL-1 receptor in addition to this "lacking LPS-signaling molecule", or ii) LPS- and IL-1-induced NF-κB pathway have in common a missing or non-functional signaling molecule.

While CD14 clearly initiates the process of LPS-induced cellular activation, a mechanistic description of the molecular events that occur subsequent to LPS binding to CD14 remains elusive. CD14 is a GPI-linked protein with no known signaling motifs. Thus, it does not seem likely that CD14 actually effects a transmembrane signaling event. Strong evidence supports a model in which complexes of CD14 and LPS signal cells through interactions with a distinct signal-transducing molecule(s) (26-30). Eventual candidates that could be proposed for this role, would be i) the newly-discovered transmembrane protein TLR2 described to confers responsiveness to bacterial LPS (31, 32) or ii) TLR4, involved in the defectiveness of LPS response in C3H/HeJ and C57BL/10ScCr mice following a deleterious mutation in the TLR4 gene (33).

As shown in our study, rsv-HepG2 and -T84 cells also do not respond to LPS *via* the soluble CD14 receptor. Several studies on the role of sCD14 in the activation of endothelial and epithelial cells have suggested that a CD14/LPS complex might form a ternary complex with an as yet unidentified signal-transducing molecule with transmembrane and cytoplasmic signaling domains (6, 34, 35). Observations on LPS-unresponsiveness of rsv-HepG2 and -T84 cells as well as the other results raised the idea that a molecule involved in the proximal LPS signaling cascade would miss or would not be functional anymore. We hypothesized that this essential molecule could correspond to the “receptor” of the sCD14/LPS complex mentioned above.

Internalization of bacteria in epithelial cells is extensively studied since this mechanism can be the starting point of bacteria invasion beyond the epithelial barrier (36). However, little is known about endocytosis of LPS in epithelial cells. LPS internalization is an important process that leads to LPS detoxification by the deacylation of lipid A, the cytotoxic part of the

LPS molecule (3). LPS endocytosis has been in major part investigated in monocytic cells. In these latter cells, LPS has been shown to be internalised by CD14-dependent and independent pathways (37, 38). Using our CD14-overexpressed transfected epithelial cell lines, we were interested to investigate LPS internalization. All cell types were able to internalise LPS despite the unresponsiveness of some of these cell types to LPS. This observation suggested that LPS signaling and LPS endocytosis were separate processes, as concluded by other investigators (37, 39, 40). By using an anti-CD14 monoclonal antibody 3C10 with CD14(+)-transfected epithelial cells, LPS internalization was partially inhibited (20-30%) (Data not shown). This result supported the concept that part of LPS internalization is CD14 dependent and part of it is not. One mechanism that could support the latter is the transcytosis of LPS from the apical surface of polarized intestinal epithelial cells to the basolateral pole as described by Beatty et al. (41). Another possibility would be that LPS is transferred from CD14 to another membrane component which would endocytose endotoxin molecules into cells.

In this study, we demonstrated that some epithelial cell lines can express, at different levels, CD14 gene and protein. The epithelial T84 and HepG2 cells were found to be unresponsive to LPS. In both cell types, overexpression of CD14 did not restore responsiveness to LPS. This suggested that these cells were deficient in (a) molecules(s) of the proximal LPS recognition/signaling pathway, such as a transmembrane signaling molecule, for example. In contrast, all CD14-transfected cells were able to internalise LPS, thus suggesting that LPS internalization and signaling were separate processes.

References

1. Munford, R. S. 1986. Deacylation of bacterial endotoxins by neutrophils and macrophages: early observations and hypotheses. *Methods Find Exp Clin Pharmacol 8:63*.
2. Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science 249:1431*.
3. Luchi, M., and R. S. Munford. 1993. Binding, internalization, and deacylation of bacterial lipopolysaccharide by human neutrophils. *J Immunol 151:959*.
4. Henderson, B., S. Poole, and M. Wilson. 1996. Microbial/host interactions in health and disease: who controls the cytokine network? *Immunopharmacology 35:1*.
5. Grunwald, U., C. Kruger, J. Westermann, A. Lukowsky, M. Ehlers, and C. Schutt. 1992. An enzyme-linked immunosorbent assay for the quantification of solubilized CD14 in biological fluids. *J Immunol Methods 155:225*.
6. Pugin, J., C. C. Schurer-Maly, D. Leturcq, A. Moriarty, R. J. Ulevitch, and P. S. Tobias. 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc Natl Acad Sci U S A 90:2744*.
7. Van Leeuwen, P. A., M. A. Boermeester, A. P. Houdijk, C. C. Ferwerda, M. A. Cuesta, S. Meyer, and R. I. Wesdorp. 1994. Clinical significance of translocation. *Gut 35:S28*.
8. Kang, Y. H., M. C. Falk, T. B. Bentley, and C. H. Lee. 1995. Distribution and role of lipopolysaccharide in the pathogenesis of acute renal proximal tubule injury. *Shock 4:441*.
9. Landmann, R., A. M. Reber, S. Sansano, and W. Zimmerli. 1996. Function of soluble CD14 in serum from patients with septic shock. *J Infect Dis 173:661*.
10. Rothman, B. L., A. W. Despins, and D. L. Kreutzer. 1990. Cytokine regulation of C3 and C5 production by the human type II pneumocyte cell line, A549. *J Immunol 145:592*.
11. Standiford, T. J., S. L. Kunkel, S. H. Phan, B. J. Rollins, and R. M. Strieter. 1991. Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. *J Biol Chem 266:9912*.

12. Schmouder, R. L., R. M. Strieter, R. C. Wiggins, S. W. Chensue, and S. L. Kunkel. 1992. In vitro and in vivo interleukin-8 production in human renal cortical epithelia. *Kidney Int 41:191*.
13. Hedges, S., M. Svensson, and C. Svanborg. 1992. Interleukin-6 response of epithelial cell lines to bacterial stimulation in vitro. *Infect Immun 60:1295*.
14. Eckmann, L., H. C. Jung, C. Schurer-Maly, A. Panja, E. Morzycka-Wroblewska, and M. F. Kagnoff. 1993. Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8. *Gastroenterology 105:1689*.
15. Diamond, G., J. P. Russell, and C. L. Bevins. 1996. Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. *Proc Natl Acad Sci U S A 93:5156*.
16. Fearns, C., V. V. Kravchenko, R. J. Ulevitch, and D. J. Loskutoff. 1995. Murine CD14 gene expression in vivo: extramyeloid synthesis and regulation by lipopolysaccharide. *J Exp Med 181:857*.
17. Martin-Villa, J. M., S. Ferre-Lopez, J. C. Lopez-Suarez, A. Corell, M. Perez-Blas, and A. Arnaiz-Villena. 1997. Cell surface phenotype and ultramicroscopic analysis of purified human enterocytes: a possible antigen-presenting cell in the intestine. *Tissue Antigens 50:586*.
18. Lee, J. D., K. Kato, P. S. Tobias, T. N. Kirkland, and R. J. Ulevitch. 1992. Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. *J Exp Med 175:1697*.
19. Nanbo, A., H. Nishimura, T. Muta, and S. Nagasawa. 1999. Lipopolysaccharide stimulates HepG2 human hepatoma cells in the presence of lipopolysaccharide-binding protein via CD14. *Eur J Biochem 260:183*.
20. Wang, S. Y., L. Y. Chen, T. F. Tsai, T. S. Su, K. B. Choo, and C. K. Ho. 1996. Constitutive production of colony-stimulating factors by human hepatoma cell lines: possible correlation with cell differentiation. *Exp Hematol 24:437*.
21. Schuerer-Maly, C. C., L. Eckmann, M. F. Kagnoff, M. T. Falco, and F. E. Maly. 1994. Colonic epithelial cell lines as a source of interleukin-8: stimulation by inflammatory cytokines and bacterial lipopolysaccharide. *Immunology 81:85*.
22. Gutierrez-Ruiz, M. C., S. C. Quiroz, V. Souza, L. Bucio, E. Hernandez, I. P. Olivares, L. Llorente, F. Vargas-Vorackova, and D. Kershenobich. 1999. Cytokines, growth

factors, and oxidative stress in HepG2 cells treated with ethanol, acetaldehyde, and LPS. *Toxicology 134:197*.

23. Bomsztyk, K., J. W. Rooney, T. Iwasaki, N. A. Rachie, S. K. Dower, and C. H. Sibley. 1991. Evidence that interleukin-1 and phorbol esters activate NF-kappa B by different pathways: role of protein kinase C. *Cell Regul 2:329*.
24. Kruppa, G., B. Thoma, T. Machleidt, K. Wiegmann, and M. Kronke. 1992. Inhibition of tumor necrosis factor (TNF)-mediated NF-kappa B activation by selective blockade of the human 55-kDa TNF receptor. *J Immunol 148:3152*.
25. Auron, P. E. 1998. The interleukin 1 receptor: ligand interactions and signal transduction. *Cytokine Growth Factor Rev 9:221*.
26. Duchow, J., A. Marchant, A. Crusiaux, C. Husson, C. Alonso-Vega, D. De Groote, P. Neve, and M. Goldman. 1993. Impaired phagocyte responses to lipopolysaccharide in paroxysmal nocturnal hemoglobinuria. *Infect Immun 61:4280*.
27. Sultzer, B. M., R. Castagna, J. Bandekar, and P. Wong. 1993. Lipopolysaccharide nonresponder cells: the C3H/HeJ defect. *Immunobiology 187:257*.
28. Lee, J. D., V. Kravchenko, T. N. Kirkland, J. Han, N. Mackman, A. Moriarty, D. Leturcq, P. S. Tobias, and R. J. Ulevitch. 1993. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. *Proc Natl Acad Sci U S A 90:9930*.
29. Ulevitch, R. J., and P. S. Tobias. 1994. Recognition of endotoxin by cells leading to transmembrane signaling. *Curr Opin Immunol 6:125*.
30. Delude, R. L., R. Savedra, Jr., H. Zhao, R. Thieringer, S. Yamamoto, M. J. Fenton, and D. T. Golenbock. 1995. CD14 enhances cellular responses to endotoxin without imparting ligand-specific recognition. *Proc Natl Acad Sci U S A 92:9288*.
31. Kirschning, C. J., H. Wesche, T. Merrill Ayres, and M. Rothe. 1998. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J Exp Med 188:2091*.
32. Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature 395:284*.
33. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science 282:2085*.

34. Frey, E. A., D. S. Miller, T. G. Jahr, A. Sundan, V. Bazil, T. Espevik, B. B. Finlay, and S. D. Wright. 1992. Soluble CD14 participates in the response of cells to lipopolysaccharide. *J Exp Med 176:1665*.

35. Pugin, J., R. J. Ulevitch, and P. S. Tobias. 1995. Activation of endothelial cells by endotoxin: direct versus indirect pathways and the role of CD14. *Prog Clin Biol Res 392:369*.

36. Meyer, D. H., K. P. Mintz, and P. M. Fives-Taylor. 1997. Models of invasion of enteric and periodontal pathogens into epithelial cells: a comparative analysis. *Crit Rev Oral Biol Med 8:389*.

37. Poussin, C., M. Foti, J. L. Carpentier, and J. Pugin. 1998. CD14-dependent endotoxin internalization via a macropinocytic pathway. *J Biol Chem 273:20285*.

38. Vasselon, T., E. Hailman, R. Thieringer, and P. A. Detmers. 1999. Internalization of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14. *J Exp Med 190:509*.

39. Kitchens, R. L., R. J. Ulevitch, and R. S. Munford. 1992. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J Exp Med 176:485*.

40. Kitchens, R. L., and R. S. Munford. 1998. CD14-dependent internalization of bacterial lipopolysaccharide (LPS) is strongly influenced by LPS aggregation but not by cellular responses to LPS. *J Immunol 160:1920*.

41. Beatty, W. L., and P. J. Sansonetti. 1997. Role of lipopolysaccharide in signaling to subepithelial polymorphonuclear leukocytes. *Infect Immun 65:4395*.

4 CHAPTER II : Response to endotoxin during cell differentiation: involvement of newly discovered Toll-like receptors

4.1 Introduction

4.1.1 Discovery of Toll-like receptors (TLRs)

The "Toll story" begins in the fruit fly *Drosophila*, which like all insects is very resistant to microbial infections despite the lack of an adaptive immune system. The protein Toll (which means "great" in German) was first described as a type I transmembrane protein that controls the dorso-ventral patterning of the fly [351]. Its homolog 18-Wheeler (18W) is also required for morphogenesis [352]. Aside from their role in embryogenesis, Toll and 18W, which belong to a recently extended Toll protein family (Toll-3 to -8) [353] are also involved in the fly immunity. When activated through Toll and 18W, cells from the fat body generate an anti-microbial response with the secretion of anti-fungal and anti-bacterial peptides such as attacin and drosomycin [354, 355]. An essential step in the activation of such responses in Drosophila seems to be the activation of a proteolytic cascade that produces peptidic ligands such as Spätzle, a ligand for Toll. Common features are found in the different Toll members. The extracellular regions contain multiple leucine-rich repeats (LRR) and carboxy-terminal cysteine-rich domains. The cytoplasmic tails display domains that share striking homology with the mammalian type 1 IL-1 receptor [356]. Moreover, signaling through Toll parallels the signaling pathway induced by the IL-1 receptor in mammalian cells: IL-1 receptor signals through the NF-κB/I-κB pathway and similarly Toll through intracellular proteins Dorsal and Cactus, homologous to NF-κB and I-κB, respectively [357, 358]. Remarkably, the intracellular DRgN protein of plant or the N-protein encoded by the tobacco-virus-resistance gene, are also similar to Toll in that it contains both a Toll signaling domain and a LRR domain [359]. Thus, it appears that the immune response mediated by Toll and its homologs represents an ancient host defense mechanism conserved throughout the evolution [358]. Using the sequence profiles of the Toll/IL-1 receptor signaling domains (TIR domain), basic local alignment search tool (BLAST) searches were performed and allowed to identify a matching sequence in the expressed sequence tags (ESTs) database of the National Center for Biotechnology Information (NCBI). The first human Toll (hToll), later termed as Toll-like receptor (TLR) 4 was cloned in 1997 [360]. A chimeric mutant of hToll (CD4 ectodomain fused to the human Toll intracellular domain) was found to constitutively activate

the NF-κB pathway and to induce the pro-inflammatory IL-1, IL-8 and the co-stimulatory B7.1 genes [360].

In 1998, five new human Toll-like receptors were discovered and named TLR1 to TLR5 [4, 361]. Subsequently, a sixth TLR (TLR6) was cloned and characterized in human and mouse [362]. Recently, sequences of four new TLRs have been registered in Genbank, but for the moment only little information on TLR7 to 10 exits. RP105, a murine B-cell protein, also belongs to this large family of TLRs and was actually cloned before these receptors were known. TLRs are mapped to different chromosomes and their primary structure remains well conserved. As for *Drosophila* Toll, they are type I transmembrane proteins, with highly homologous regions in the extracellular portion (LRRs), and in the intracellular part (TIR domain). Interestingly, similar LRR are also found in CD14. The TIR similarity suggests that TLR proteins may activate cells through similar signaling pathways. This intracytoplasmic tail plays a pivotal role to transduce signal since mutations detected in this portion result in a complete loss of function [183, 363, 364].

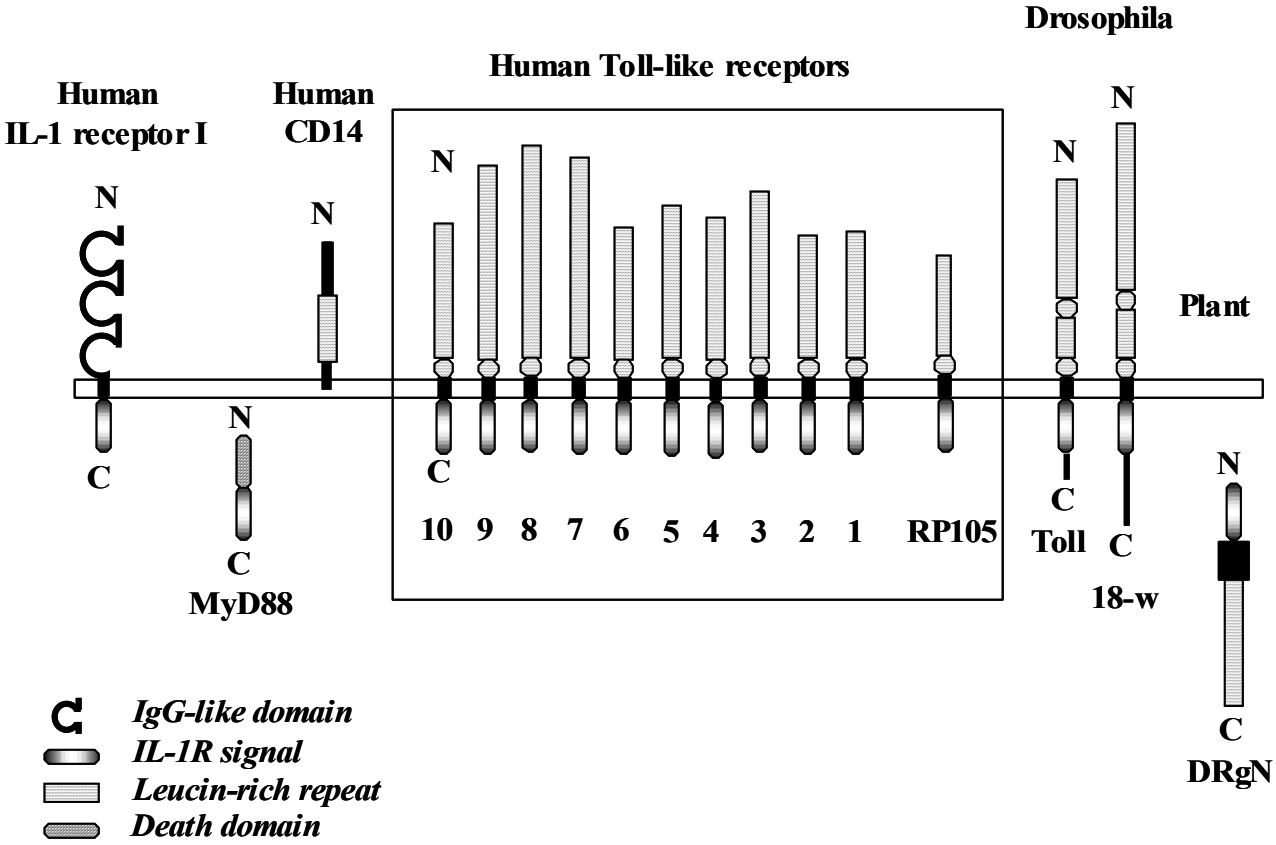

Figure 5: Structure of Toll-like receptors from human, drosophila and plant compared with the structure of the human IL-1 receptor, human CD14 and the intracellular signaling molecule MyD88 (Adapted from [3, 4]).

4.1.2 Ligands for TLRs

Janeway and co-workers had hypothesized that the innate immune system can sense invading pathogens by virtue of nonclonal pattern recognition receptors that interact with microbial structures and deliver a danger signal to the host cells [365, 366]. There is increasing evidence that TLR proteins may serve this purpose. TLR4 was reported to function as the signal transducer for LPS-dependent activation of cells. Details on the involvement of these receptors in LPS signaling will be further discussed. Recent data suggest that TLR2 and TLR4 can recognize a variety of microbial products. Experiments using cells from a natural knock-out chinese hamster for TLR2, specific monoclonal antibodies to TLR2, and cells transfected with the TLR2 cDNA, showed that the receptor TLR2 recognized components from various microbial pathogens which can induce cell activation in a TLR2-dependent manner. These microbial molecules include peptidoglycan and lipoteichoic acid from Gram-positive bacteria [367-369], lipoarabinnomannan from mycobacteria [370, 371] and zymosan from fungi [372]. Lipoproteins from pathogens such as Gram-negative bacteria, Gram-positive bacteria, mycobacteria and spirochetes are also TLR2 ligands [373-378]. Some of these lipoproteins and lipopeptides have been shown to be potent stimulators by inducing NF-kB activation and cytokines production in monocytes/macrophages [373, 375, 379]. It was observed that some of these molecules could induce cell apoptosis [373]. It was recently published that TLR2 needed to associate with TLR6 to discriminate between different microbial lipopeptides or to detect the specific pattern of peptidoglycan or a modulin secreted from *staphylococcus aureus* [380, 381]. In addition to LPS, live *Mycobacterium tuberculosis* can activate cells *via* TLR4, but independently of LBP and membrane-bound CD14 [371]. Lipoteichoic acid from *Staphylococcus aureus* appeared to be a ligand for TLR4 that could stimulate cells [382]. Interestingly, the heat-shock protein 60 (HSP60) that has been suggested to elicit a danger signal to the innate immune system and to induce the production of pro-inflammatory mediators by macrophages, would be the first known protein ligand for TLR4, and the first known endogenous TLR agonist [383]. Taxol, an anti-tumoral agent that displays a LPS-mimetic activity in murine cells but not in human cells, was described to mediate cell activation through the complex TLR4/MD-2 [384]. The description and the role of MD-2 will be further detailed.

TLR9 has been involved in the recognition of bacterial CpG DNA through the generation of TLR9-deficient mice [385]. In these mice, the cellular response to CpG including B cell proliferation, macrophage production of inflammatory cytokines and dendritic cell maturation was shown to be abolished.

Recent investigations showed that TLR5 was responsible for the recognition of flagellin, a monomeric constituent of bacterial flagella, a polymeric rod-like appendage extending from the outer membrane of bacteria. The interaction of flagellin and TLR5 leads to cell activation [386, 387].

4.1.3 Binding of LPS to TLR2 and TLR4

As mentioned before, TLR2 and TLR4 have been recently identified to play a major role in LPS signaling. Although, interactions of LPS with these receptors remain not very well understood, some data have already been published. In experiments using a soluble form of the TLR2 extracellular domain, ^{3}H-labeled LPS could bind to TLR2 and could be displaced from the receptor by cold LPS, but not by deacylated LPS. The Kd was estimated at 500-700 nM and was considered rather low, suggesting that the kinetics of association were slow [388]. To be functional, TLR4 associates with a non-transmembrane coreceptor, MD-2. Interactions between LPS and this complex were studied using a chemically modified, radioiodinated LPS containing a covalently linked UV light-activated cross-linking group (^{125}I-ASD-Re595 LPS). It was found that LPS cross-linked to TLR4 and MD-2 only when co-expressed with CD14. Soluble MD-2 was recently shown to bind highly purified rough LPS derived from *Salmonella minnesota* and *Escherichia coli* with an apparent Kd of 65 nM [389]. These data strongly suggest that LPS binds to or is in close proximity to the three proteins, assembled in a multichain receptor complex [390]. The involvement of CD14, TLR4 and MD-2 in LPS-mediated cellular activation has been previously detailed.

4.1.4 Role of TLRs in LPS signaling

CD14 has been recognized for many years as a major receptor mediating LPS effects on macrophages, monocytes, and neutrophils [139]. Numerous studies have also suggested that CD14 alone was not able to induce a signal and should act by associating with (a) distinct transmembrane signal transducing protein(s).

In 1998, two groups independently reported that TLR2 could function as a signaling receptor for LPS in the presence of CD14 [388, 391]. These investigators described that human embryonic kidney (HEK) 293 epithelial cells stably transfected with a human TLR2 expression plasmid could respond to LPS in the presence of CD14 and LBP, as judged by the activation of a reporter gene under the control of the NF-κB-dependent ELAM-1 promoter. It is important to notice that micromolar concentrations of LPS were necessary to induce a cell response in 293 cells, whereas mouse or human macrophages respond to nanomolar or

subnanomolar concentrations of endotoxin. However, deletion mutants of TLR2 lacking the most C-terminal 13 aa of the intracellular portion failed to mediate LPS responsiveness in this assay [392]. It was later demonstrated that in fact TLR2 was not a receptor for LPS itself, but for lipoproteins contaminating LPS preparations [393].

Rather than TLR2, TLR4 was subsequently identified as the LPS-signaling receptor. In 1998, Beutler and co-workers mapped the Lps locus of LPS resistant C3H/HeJ and identified TLR4 as a candidate gene [394]. The same year, they showed that a point mutation in the TLR4 gene was responsible for the long known LPS hypo-responsiveness of the C3H/HeJ mouse. They also showed that a TLR4 deletion mutation was responsible for the LPS non-responsiveness of C57Bl/10ScCr mouse. In C3H/HeJ mice, a single missense mutation within the intracellular TLR4 coding sequence changing a proline into a histidine residue was identified. C57Bl/10ScCr mice do not express TLR4 mRNA [183, 364, 395]. The creation of a TLR4 knock-out mouse by Takeushi et al. further demonstrated the critical role of TLR4. These mice were non-responsive to LPS. TLR2 knock-out mice were in contrast perfectly responsive to LPS, but deficient in their response to Gram-positive cell wall components [362]. Mutations in the extracelullar domain of human TLR4 were found to be associated with endotoxin hyporesponsiveness [396]. Work from Chow et al. indicated that transfection of human TLR4 into LPS non-responding human cells restored LPS response [397]. To support the idea that TLR4 played an important role in cell stimulation by LPS, a soluble form of mouse TLR4 was constructed that inhibited LPS-induced signaling [398]. The first demonstration of a physical contact between LPS and TLR4 was suggested by genetic complementation [399]. Pivotal studies by Miyake and colleagues showed that TLR4 alone was not fully functional, but required an additional protein to confer LPS responsiveness of cells identified as MD-2. This protein lacks a membrane anchoring system, but physically associates with the extracellular portion of TLR4 [400-402]. MD-2 is homologous to MD-1, a protein associated with RP105, a murine TLR [403]. Interestingly, a point mutation in a conserved region of MD-2 abolished endotoxin-induced signaling. This point mutation corresponded to the change of a cysteine (C) for a tyrosine (Y) aa at position 95. It was further hypothesized that MD-2 affected the conformation of the extracellular domain of TLR4, possibly inducing a change in its affinity for LPS or functioning as a ligand for TLR4 [404]. As shown by UV light-activated cross-linking experiments, LPS was in close proximity to each of these proteins of the putative LPS receptor complex. Important biochemical studies have shown that LPS was transferred from CD14 to TLR4 and MD-2 [390]. Interestingly,

Viriyakosol et al. reported that recombinant human MD-2 was able to bind to LPS, but in a LBP- and CD14-independent manner. LBP actually competed with MD-2 for LPS. Soluble MD-2 enhanced the biological activity of LPS in TLR4-transfected Chinese hamster ovary cells but inhibited LPS activation of U373 astrocytoma cells and of monocytes in human whole blood. Thus, MD-2 is a genuine LPS-binding protein and could play a role in regulation of cellular activation by LPS depending on its local availability [389]. Recently, another study revealed that MD-2 was secreted as a large polymeric soluble protein by normal and MD-2-transfected cells. The soluble form of MD-2 was found to efficiently confer LPS sensitivity to TLR4 by binding to it with high affinity. [405]. It was also demonstrated that TLR4-mediated activation of NF-κB by LPS required N-linked glycosylations of human MD-2 found at asparagine(26) and asparagine(114) [406]. The amino-terminal ectodomain of human TLR4 was also found to contain 9 N-linked glycosylation sites. Interestingly, TLR4 mutants carrying substitutions in Asparagine (526) or Asparagine (575) failed to be transported to the cell surface. Moreover, a reduction in the LPS-induced cell activation could be observed when TLR4 mutants lacked three or more N-linked glycosylation sites, but this reduction was partially reversed with the co-expression of CD14. This suggests that the functional integrity of the LPS receptor depends both on the surface expression of at least three proteins, CD14, MD-2, and TLR4, and on N-linked sites of both MD-2 and TLR4, essential in maintaining the functional integrity of this receptor [407].

Human MD-2 was also found to confer on mouse TLR4 species-specific LPS recognition as supported by experiments performed with lipid IVa or Salmonella lipid A [408, 409]. As reported before, CD14 helped the complex TLR4/MD-2 to sense and signaled the presence of LPS. Interestingly, membrane phospholipids such as phosphatidylserine and phosphatidylinositol recognized by CD14 as ligands, could act as LPS antagonists in the signaling *via* TLR4/MD-2 [401]. Using a MD-2 knock-out mouse, Nagai et al. study has comfirmed the essential role of MD-2 in LPS-recognition by TLR4, and also revealed that MD-2 regulates the intracellular distribution of TLR4 [410]. Very recently, it has been found that an 82-amino-acid region of human TLR4, hypervariable across species, would be involved in LPS recognition by discriminating different *P. aeruginosa* LPS structures [411].

TLR4 is not only involved in LPS-signaling in monocytes/macrophages, but also in endothelial and epithelial cells. As a matter of fact, Faure et al. indicated that LPS activated NF-κB through TLR4 in cultured human dermal ECs. It was proposed that TLR4 and TLR2 differentially expressed in ECs, might have important implications in the participation of

vascular ECs in innate immune defense mechanisms against various infectious pathogens. [412]. In other cells such as intestinal epithelial cell lines expressing TLRs, LPS was shown to activate distinct signaling pathways [413]. Finally, decreased expression of TLR4 and MD-2 correlated with intestinal epithelial cell protection against dysregulated pro-inflammatory gene expression in response to LPS [414].

MD-2 does not only regulate TLR4 function, but also seems to influence TLR2 activity. In one study, MD-2 transfection enabled TLR2-positive cells to respond to LPS and enhanced TLR2-mediated responses to Gram-positive and Gram-negative bacteria and their cell wall components [415]. As indicated above, although repurification of LPS eliminated signaling through both human and murine toll-like receptor 2 [393], TLR2 may play a role in some LPS-signaling. As published by Werts et al., leptospiral LPS which has a different structure compared with enterobacterial LPS, activated cells through a TLR2-dependent mechanism [416]. Moreover, it was shown that the upregulation of TLR2 gene expression in macrophages induced NF-κB activation in response to peptidoglycan and high concentration of LPS [417]. Another group found that *P. gingivalis* LPS exhibited potent TLR2, rather than TLR4, agonist activity to elicit gene expression and cytokine secretion in murine macrophages and transfectants. More importantly, TLR2 stimulation by this *P. gingivalis* LPS preparation resulted in differential expression of a panel of genes that were normally induced by *Escherichia coli* LPS [377].

4.1.5 LPS-induced signaling cascades from TLR4

Since TLR4 has been found to be involved in LPS signaling, the LPS-induced intracellular signaling cascade leading to NF-κB activation has been deeply studied and subsequently described in more detail.

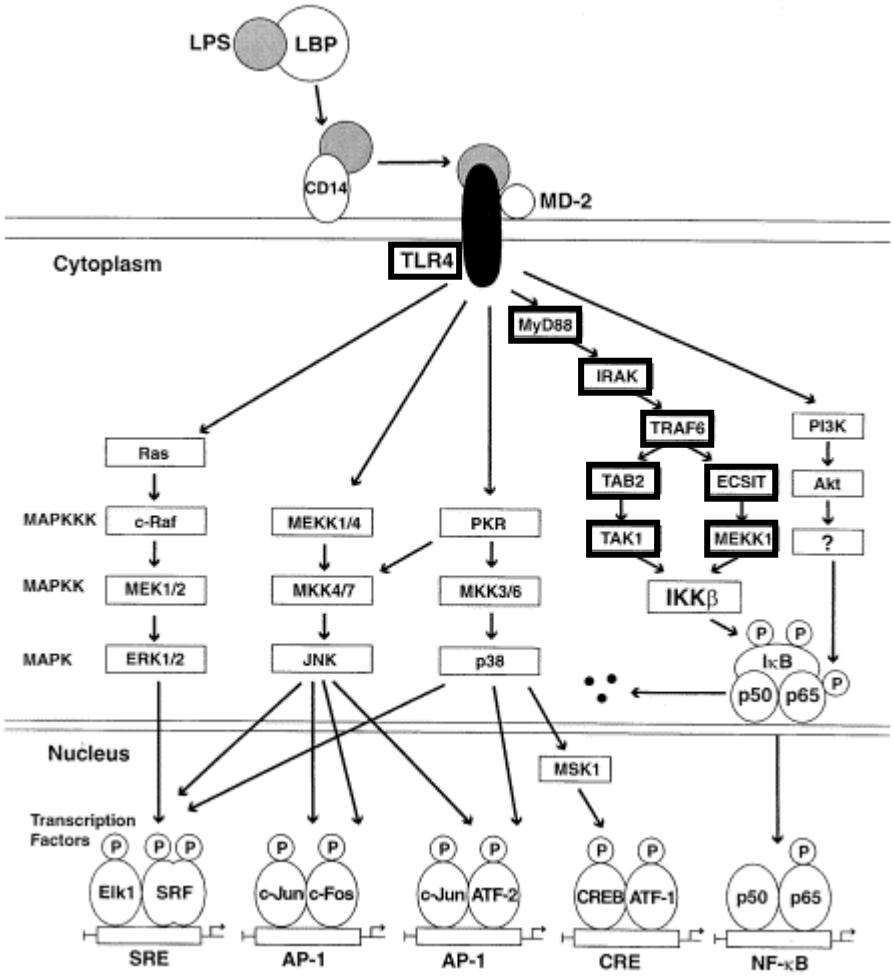

Figure 6: LPS activation of different signaling pathways and transcription factors in monocytes. TLR4/MD-2 interact with the LPS-LBP/CD14 complex and TLR4 transduces the LPS signal from the outside to the intracytoplasmic compartment (adapted from [2]).

In the presence of LPS, TLR4 recruits MyD88, an intracytoplasmic molecule [418]. MyD88 bound to the receptor interacts through its death domain with IL-1 receptor-associated kinase (IRAK) [419]. This serine/threonine kinase is phosphorylated and subsequently interacts with the TNF-α receptor associated-factor (TRAF) 6 [420]. Experiments performed with dominant-negative mutants of MyD88, IRAK and TRAF6 showed an inhibition of LPS-induced NF-κB activation in monocytic cells, suggesting an involvement of these intracellular molecules in the signaling pathway [421]. The NF-κB inducing kinase (NIK) is further activated by the transforming growth factor β-associated kinase 1 (TAK1), a kinase that is

itself activated by TRAF6 linked to TAK1 by an adapter protein called TAB2 [422, 423]. In parallel, MEKK1 is directly phosphorylated by TRAF6. These two kinases are coupled to each other by a protein called "evolutionarily conserved signaling intermediate in Toll pathways" (ESCIT) [424]. This adapter protein is specific for the Toll/IL-1 pathways and is a regulator of MEKK1 processing. IKKs are then activated and phosphorylate I-κB. Free in the cytoplasm, NF-κB translocates into the nucleus.

4.1.6 Problematic

Some of the most important tissue cell types in the recognition and elimination of bacteria and bacterial products are the monocyte/macrophage and the dendritic cell (DC). Another important common feature of these cells is that they participate in the activation of the adaptive immune system, by secreting specific cytokines and by presenting antigen to lymphocytes. The macrophage and the DC are derived from a common precursor, the blood monocyte. During haematopoiesis, the monocyte originates from the monoblast/promonocyte, itself differentiated from the myeloid stem cell. During differentiation steps, myeloid cells can acquire or loose specific and diverse functions according to the expression variation of intracellular molecules, cell surface receptors and also to their tissue localization. In the context of our topic, it has been observed that myeloid cells at various stages of differentiation respond differently to LPS. As an example, Martin et al. demonstrated that promonocytes acquired responsiveness to LPS during their maturation into mononuclear phagocytes. It was concluded that the maturation of the response to LPS was dependent in large part on the acquisition of CD14 on the cell surface [425]. In contrast, LPS response was shown to be lost when immature DCs were differentiated into mature DCs [426]. Several studies have also reported that LPS responses could vary in primary monocytes versus macrophages. Gessani et al. have demonstrated that the differentiation of human monocytes to macrophages led to an enhanced LPS response in terms of a progressive increase of IL-6/TNF-α production and the acquisition of an IFNβ-mediated antiviral state. These authors suggested that this enhanced response to LPS was largely CD14-independent [427]. Another study revealed that LPS-induced signals were greatly potentiated by an elevated intracellular calcium in undifferentiated monocytic cells, but not in the differentiated state of these cells [428]. With the recent discovery of TLR and MD-2 proteins involved in LPS-induced cell activation, we

were interested to investigate whether these variations of LPS-induced cell responses could be attributed to a differential expression of CD14 and TLRs in myeloid cells at various stages of differentiation. In the first part of this chapter, the expression of these receptors as well as the cell response to LPS has been analyzed in promonocytic cells differentiated with different agents. In order to compare, similar experiments were performed with human primary tissue macrophages. In the second part of this chapter, our study focused on similar experiments done during the differentiation of human circulating blood monocytes into immature DCs, further transformed into mature DCs.

4.2 Part I: «Myeloid cell responses to endotoxin during cell differentiation. Dependence on Toll-like receptor-2, -4, and MD-2 expression»

Myeloid cell responses to endotoxin during cell differentiation. Dependence on Toll-like receptor-2, -4, and MD-2 expression

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Running title: Toll-like receptors in myeloid cells

Key words: lipopolysaccharide, Toll-like receptors, CD14, monocytes/macrophages, endotoxin shock.

Abbreviations : TLR, Toll-like receptor ; AM, alveolar macrophages ; RA, retinoic acid ; D3, 1,25 $(OH)_2$ vitamin D_3.

Abstract

Myeloid cells develop a strong response to LPS during the process of cell differentiation. In a model of human THP-1 cell differentiation, we investigated the role of surface molecules implicated in LPS signaling as potential regulators of cell responses to LPS. Promonocytic THP-1 cells expressing high basal levels of CD14, TLR2 and TLR4 were subjected to a 72 hr-treatment with various differentiating agents: 1,25 $(OH)_2$ vitamin D_3 (D3), retinoic acid (RA) or γ-interferon (γ-IFN). Only γ-IFN increased TLR4 expression, whereas the levels of other surface molecules were not significantly modified by the differentiating agents. A correlation between expression of the TLRs and the magnitude of THP-1 cell response to LPS, as assessed by the secretion of IL-6 and IL-8, could not be established. Using specific mAbs we showed that undifferentiated THP-1 cells responded to LPS predominantly in a TLR2-dependent way, whereas cells differentiated with D3, RA or γ-IFN partially lost this TLR2-dependence. Blocking both TLR2 and TLR4 abrogated LPS responses in differentiated cells. MD-2 mRNA was upregulated in response to all differentiating agents. Differentiated primary human monocytes and alveolar macrophages, which express high levels of surface TLR2 and MD-2 mRNA, and low levels of TLR4, responded to LPS essentially in a TLR4-dependent manner. In all cases, the anti-CD14 mAb was the most efficient cytokine blocker in myeloid cells treated with LPS. We conclude that the surface molecules implicated in LPS signaling vary according to the level of cell differentiation. From TLR2-dependent progenitors, myeloid cells evolve progressively into cells that are TLR4/MD-2-dependent upon differentiation.

Introduction

Toll-like receptors (TLR) belong to a new and growing family of receptors of the innate immunity [1-5]. Two members, TLR2 and TLR4 are preferentially expressed by myeloid cells [6], and have been shown to mediate cell activation in response to various bacterial molecules. These include Gram-negative bacterial endotoxin [7-13], Gram-positive peptidoglycan, teichoid acids, mycobacterial lipoarabinomannan, and bacterial lipoproteins [14-18]. Interestingly, expression of MD-2, a TLR4-associated protein, is required for efficient LPS responses [19-21].

Little is known about the expression of TLRs and related proteins in human primary cells and cell lines, in particular during the process of myeloid cell differentiation. This latter process is complex and involves numerous genes [22, 23]. The dependence of TLRs and related protein(s) on the function of differentiated myeloid cells is unknown. We hypothesized that the pattern of TLR2, TLR4 and MD-2 expression was modulated during myeloid cell differentiation, and that those molecules accounted for differential responses to LPS. Using a classical model of promonocytic THP-1 cells differentiation [24-26], we show here that the LPS sensitivity acquired during cell differentiation correlates with the expression of MD-2 mRNA. A similar expression of TLR2 and TLR4 expression was found in undifferentiated and in differentiated THP-1 cells. However, undifferentiated cells responded to LPS predominantly in a TLR2-dependent manner, whereas differentiated THP-1 cells as well as primary human monocyte/macrophages responded in a TLR4-dependent way, possibly in correlation with MD-2 expression.

Materials and Methods

Cells

Human CD14 cDNA cloned in a pRc/RSV vector (Invitrogen, San Diego, CA) was used to transfect the human promonocytic, CD14-negative, THP-1 cell line (ATCC, Rockville, MD), as described elsewhere [26, 37]. Stable transfectants expressed high levels of GPI-anchored CD14 (CD14(+)THP-1 cells) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 U/mL penicillin, 10 µg/mL gentamicin, and 0.5 mg/mL G418 (Geneticin®), all from Life Technologies Ltd., Paisley, Scotland (= « culture medium »). Human alveolar macrophages (AMs) were obtained by bronchoalveolar lavage of lungs removed from patients with lung cancer as described [38], washed and resuspended in the culture medium. Whole blood obtained by venipuncture of healthy donors was heparinized (10 U/mL endotoxin-free heparin, Liquemine®, Roche Pharma, Basel, Switzerland), diluted 4-fold with RPMI 1640 medium and distributed in Eppendorf tubes for stimulation experiments.

Cell differentiation and stimulation

CD14(+)THP-1 cells were differentiated with 10^{-7} M 1,25 $(OH)_2$ vitamin D_3, (D3, Roche Pharma, Basel, Switzerland), 1 µM retinoic acid (RA, Sigma, S^t Louis, MO), or 500 U/mL γ-interferon (γ-IFN, Boehringer Ingelheim, Basel, Swizerland) for 72 hr. Cells were then collected by centrifugation and resuspended in RPMI 1640 medium containing 2 % FBS, 10 U/mL penicillin, 10 µg/mL gentamicin at the concentration of 10^6 cells/mL. Hundred µL of this cell suspension was distributed in each well of a 96 well-plate. Differentiated and

undifferentiated CD14(+)THP-1 cells, AMs and diluted whole blood were preincubated for 45 min with the following mouse monoclonal antibodies (mAb), all at a final concentration of 5 μg/mL: anti-human (h) CD14 mAb (28C5) [32], anti-hTLR2 mAb (2392, kindly provided by P.J. Godowski, Genentech Inc. South San Francisco) [12], anti-hTLR4 mAbs (HTA125 or HTA405) [21, 39], and an irrelevant isotype control mouse IgG1 mAb (Sigma). All mAbs were tested in a dose range of 5 to 20 μg/mL. The 5 μg/mL dose of anti-TLRs and anti-CD14 mAbs induced a maximal inhibition, consistent with saturation of the inhibitory effect. CD14(+)THP-1 cells were then stimulated at 37°C with 100 ng/mL 0111:B4 *E. coli* LPS (List Biological Laboratories Inc., Campbell, CA). AMs and diluted whole blood were stimulated with LPS concentrations ranging from 0.1 to 10 ng/mL. Controls included treatment with 10 ng/mL phorbol-12-myristate 13-acetate (PMA, Sigma) and treatment with culture medium only. The stimulation time was 14 hr for CD14(+)THP-1 cells, 6 hr for diluted whole blood and 7 hr for AMs. In some experiments, other anti-TLR4 mAbs (HTA414, HTA1216) and another anti-TLR2 mAb (TL2.1 mAb, a kind gift from T. Espevik, Norway) were tested for their capacity to inhibit LPS responses. Conditioned supernatants were collected by centrifugation, and interleukin (IL)-6 and IL-8 were assayed by ELISA, using commercially available antibody pairs, as described elsewhere [38]. Independent experiments in duplicates or in triplicates were repeated at least 3 times with the same conditions.

Flow cytometry analysis

To analyze the surface expression of Toll-like receptors and CD14, CD14(+)THP-1 cells were incubated with saturating concentrations (2 μg/mL) of the following murine mAbs: anti-hTLR2 (2392), anti-hTLR4 (HTA125), and anti-hCD14 (63D3, ATCC). The mAbs were incubated with cells in HBSS buffer (Sigma) containing 0.3% BSA (Sigma) and 0.05% NaN_3

(= “FACS medium”) for 45 min at 4°C. To assess the differentiation stage of THP-1 cells, cells were also stained with an anti-human intercellular adhesion molecule (ICAM)-1 mAb (R&D systems, Minneapolis, MN) [27, 28], and an anti-human HLA-DR mAb (Dako A/S, Denmark) [25]. Cells were washed once with FACS medium and incubated with 5 μg/mL secondary phycoerythrin-conjugated goat anti-mouse IgG (Anawa, Wangen, Switzerland) for 45 min at 4°C. After one wash, cells were resuspended in 0.5 mL FACS medium, and analyzed by flow cytometry using the FL2 channel (FACScan, Becton Dickinson). The specificity of the anti-TLR2 and anti-TLR4 mAbs was assessed by FACS using human embryonic kidney (HEK) 293 cells expressing either TLR2 or TLR4. Stable HEK 293 transfectants were produced using complementary DNA for Flag-tagged TLR2 and TLR4, kindly provided to us by C. Kirschning, Tularik Inc. South San Francisco, USA. Anti-TLR2 mAb recognized only TLR2-expressing cells and anti-TLR4 only TLR4-expressing cells. Levels of expression measured with these anti-TLRs mAbs were comparable to those measured using an anti-Flag (Sigma) mAb (data not shown).

RNA preparation and RT-PCR analysis of TLR2, TLR4, and MD-2

Differentiated and undifferentiated CD14(+)THP-1 cells, human primary AMs and whole blood-derived monocytes were lyzed in 1 mL of Trizol® (Life Technologies). Total RNA was extracted using the classical phenol/chloroform method and treated with RNase-free DNAse I (Roche Diagnostics GmbH Mannheim, Germany). Two μg of total RNA was reverse transcribed using an oligo(dT) primer (Roche) and M-MLV transcriptase (Life Technologies). PCR was performed using Taq polymerase (Roche) and specific primers designed for human TLR2 (5’ – GCC AAA GTC TTG ATT GAT TGG – 3’, sense; and 5’ – TTG AAG TTC TCC AGC TCC TG – 3’, antisense), human TLR4 (5’ – CAT GCC TGT

GCT GAG TTT GAA T – 3’ sense; and 5’ – TCC TGC CAA TTG CAT CCT GTA C – 3’ antisense), human MD-2 (5’ – TTC CAC CCT GTT TTC TTC CA – 3’ , sense; and 5’ – TAG GTT GGT GTA GGA TGA CA – 3’ , antisense) and GAPDH (5’ – GGT CAT CCA TGA CAA CTT TG – 3’ , sense ; and 5’ – CTA CAT GGC AAC TGT GAG GA – 3’ , antisense) for 32 cycles (for TLR2, TLR4, and MD-2) and 25 cycles (GAPDH) at 94°C for 1 min, 61°C (TLR2 and TLR4) or 55°C (MD-2 and GAPDH) for 1min, 72°C for 1 min 30. PCR products were electrophoresed on ethidium bromide-containing 1,5 % agarose gels, and imaged using a classical imaging system.

Results

Surface markers in THP-1 cells

In contrast to their parental CD14 negative THP-1 cells, CD14-transfected THP-1 cells (CD14(+)THP-1 cells) respond to LPS [26]. These CD14(+)THP-1 cells allowed us 1) to study the effect of differentiating agents on both the cell surface expression of various markers, 2) to compare LPS responses between differentiated and undifferentiated cells, and 3) to address the dependence of the surface receptors for the endotoxin-induced cell activation. The surface expression of TLR2 and TLR4 in CD14(+)THP-1 cells was determined by flow cytometry (FACS) using anti-TLRs mAbs. As shown in Fig. 1, we found that undifferentiated CD14(+)THP-1 cells expressed significant levels of both TLR2 and TLR4. The level of expression of these two receptors was equivalent to that measured in native (untransfected) THP-1 cells (not shown). Whereas 3 day-treatment of CD14(+)THP-1 cells with 1,25 $(OH)_2$ vitamin D_3 (D3) did not modify the expression of TLR2 and TLR4, the exposure of these cells to retinoic acid (RA) for 72 hr induced a small but consistent decrease in the surface expression of these molecules. Treatment of CD14(+)THP-1 cells with γ-IFN for 3 days induced an upregulation of TLR4. Surface expression of CD14, under the control of a constitutive viral RSV promoter, was unchanged after exposure to D3, and mildly decreased after RA and γ-IFN treatment. To control for the effect of the differentiating agents, we tested other surface molecules: intercellular adhesion molecule (ICAM)-1 expression increased with all differentiating agents (γ-IFN = RA > D3) [27, 28], and as expected, MHC class II molecules were specifically upregulated by γ-IFN [25], (Fig. 1). These surface marker controls indicate that the phenotype of differentiated CD14(+)THP-1 was that expected for a given differentiating agent.

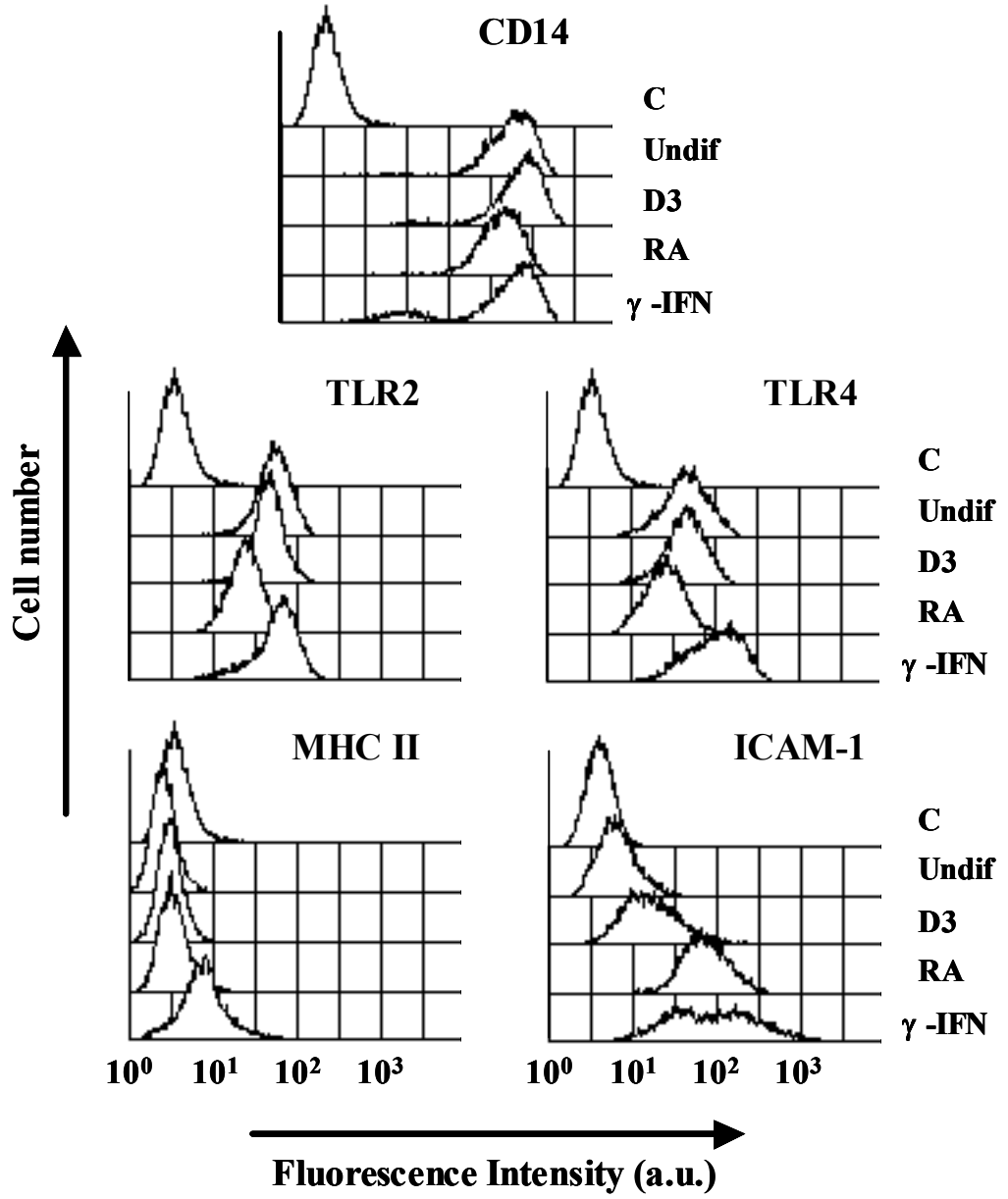

Figure 1. Flow cytometry analysis of the surface expression of TLR2, TLR4, CD14, MHC class II (MHC II), and ICAM-1 molecules in native CD14(+)THP-1 cells and in cells differentiated with 1,25 $(OH)_2$ vitamin D_3 (D3), retinoic acid (RA) and γ-interferon (γ-IFN). Cells were labeled with mAbs specific for each antigen or an irrelevant isotype control (C). A.u., arbitrary units.

The expression of TLR2, TLR4 and CD14 was also determined in human primary blood monocytes and in human alveolar macrophages (AMs). As shown in Fig. 2, panel A, monocytes expressed high surface levels of TLR2, and lower levels of TLR4. AMs expressed low levels of TLR2 and TLR4 by FACS, but FACS analyses of these cells was made difficult by the autofluorescence of alveolar macrophages recovered from patients who smoked. However, TLR2 and TLR4 mRNAs were easily detected by RT-PCR in AMs from 3 different donors (Fig. 2, panel B).

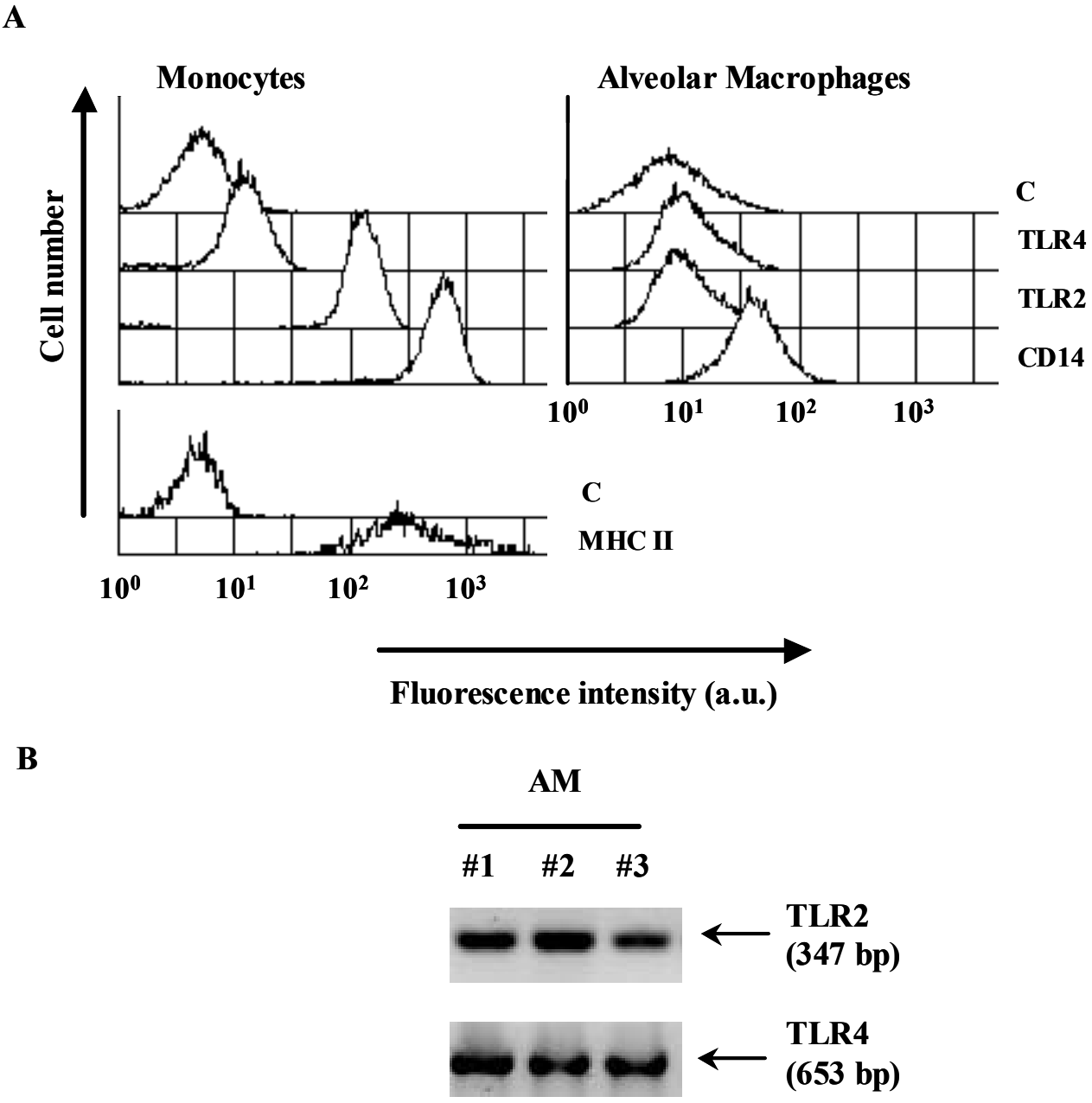

Figure 2. Panel A. Flow cytometry analysis of the surface expression of TLR2, TLR4, CD14, and MHC class II (MHC II) molecules in circulating human monocytes and in primary human alveolar macrophages. Cells were labeled with mAbs specific for each antigen or an irrelevant isotype control (C). A.u., arbitrary units.

Panel B. mRNA expression of TLR2 and TLR4 assessed by RT-PCR in untransfected HEK 293 cells (293), in human TLR2-transfected HEK 293 cells (293-TLR2), in TLR4-transfected HEK 293 cells (293-TLR4), and in human alveolar macrophages from 3 different donors.

LPS responses in differentiated THP-1 cells

Undifferentiated CD14(+)THP-1 cells and CD14(+)THP-1 cells treated 3 days with various differentiating agents did not produce IL-8 in the absence of a stimulus. They responded to a 14 hr treatment with 100 ng/mL LPS as shown in Table I. CD14 expression of THP-1 cells acquired by cell transfection induced LPS responsiveness. The treatment with γ-IFN and D3 markedly increased the production of IL-8 in response to LPS, whereas RA treatment only marginally enhanced LPS-induced IL-8 secretion. The blocking effect of the anti-CD14 mAb on LPS-induced IL-8 secretion was maximal (> 90%) in undifferentiated CD14(+)THP-1 cells (Fig. 3). This effect was less pronounced in differentiated cells (40-70% inhibition of IL-8 secretion). Similar results were obtained with anti-TLR2 mAbs (Fig. 3), coming from two different sources (results not shown with TL2.1 anti-TLR2 mAb). The LPS response of undifferentiated cells seemed to be highly dependent on TLR2 and CD14. However, once differentiated, the dependence on CD14, TLR2 and TLR4 was found to be roughly equivalent (Fig. 3). The greatest "TLR4-dependence" was observed in cells differentiated with retinoic acid. In all cases the addition of both anti-TLR2 and anti-TLR4 mAbs at the same time abrogated LPS-induced IL-8 secretion. The anti-TLRs and CD14 mAbs did not modify CD14(+)THP-1 cell responses when cells were stimulated with PMA instead of LPS (data not shown).

Table I. LPS- and PMA-induced IL-8 secretion (mean ± SEM) in CD14(+)THP-1 cells differentiated for 72 hours with various agents.

	LPS-induced IL-8 secretion (ng/mL)	**PMA-induced IL-8 secretion (ng/mL)**
CD14(-)THP-1 cells	0	
Undifferentiated CD14(+)THP-1 cells	7.8 ± 3	36 ± 7
1,25 $(OH)_2$ vitamin D_3 - CD14(+)THP-1 cells	63 ± 2	77 ± 14
Retinoic acid – CD14(+)THP-1 cells	9.5 ± 1	74 ± 3
γ-interferon – CD14(+)THP-1 cells	19.3 ± 7	47 ± 16

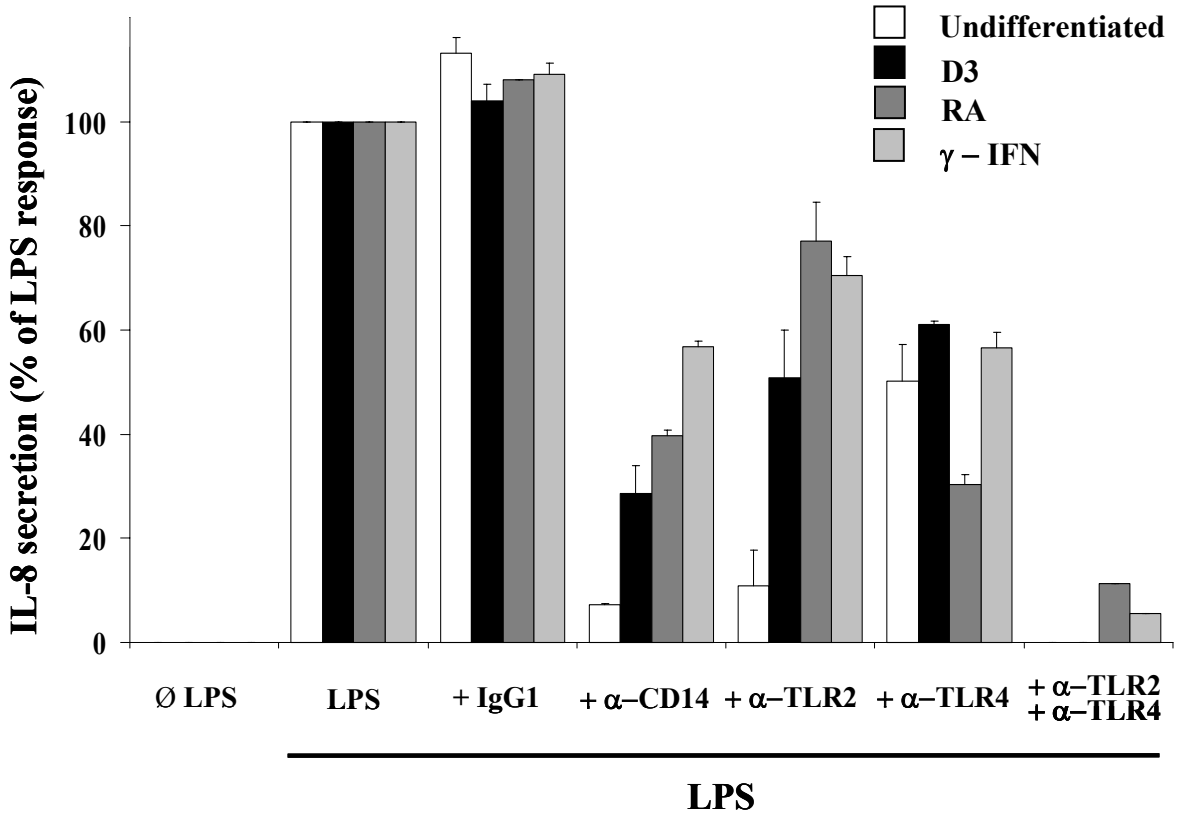

Figure 3. Effect of various monoclonal antibodies (α–hCD14, α–hTLR2, α–hTLR4 or irrelevant control (C) mAbs) on LPS-induced IL-8 response in undifferentiated CD14(+)THP-1 cells, and CD14(+)THP-1 cells differentiated with 1,25 $(OH)_2$ vitamin D_3 (D3), retinoic acid (RA) or γ-interferon (γ-IFN). Cells were treated for 14 hr with 100 ng/mL of 0111:B4 *E. coli* LPS. Results represent 3 independent experiments done in duplicates, and are expressed as % of LPS response (mean ± SEM), where 100 % corresponds to the condition with LPS but without antibodies.

LPS responses in human whole blood and primary alveolar macrophages

We next tested LPS responses in human whole blood and in primary human alveolar macrophages. In whole blood, the produced cytokines and chemokines come essentially from activated monocytes. As shown in Fig. 4, LPS-induced IL-6 and IL-8 secretion in whole blood and by AMs was primarily dependent on CD14, since anti-CD14 mAbs almost abrogated the response. Among the TLR mAbs tested on these models, only TLR4 mAbs had a marked inhibitory effect. Anti-TLR2 mAbs had either little or no effect on LPS-induced cell responses. The simultaneous addition of anti-TLR2 and TLR4 also had much less effect than that observed with CD14(+)THP-1 cells. The anti-TLRs and CD14 mAbs did not modify the whole blood and alveolar macrophage responses when cells were stimulated with PMA instead of LPS (data not shown).

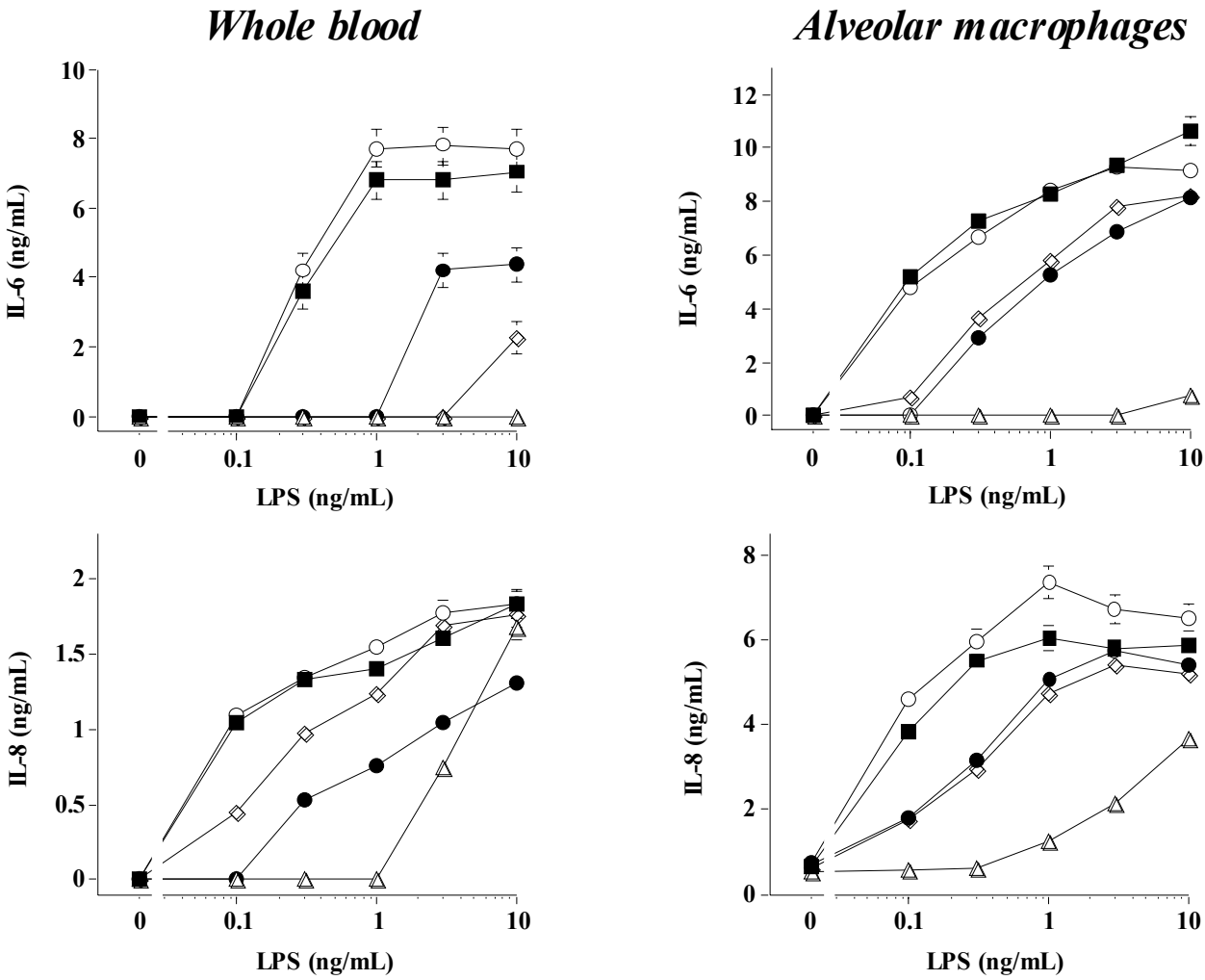

Figure 4. Effect of anti-hTLRs and anti-hCD14 mAbs on LPS-induced IL-6 and IL-8 secretion in whole human blood and in human primary alveolar macrophages. LPS alone (closed squares), α–hCD14 (open triangles), α–hTLR2 (open circles), α–hTLR4 (closed circles), αhTLR2 + αhTLR4 (open diamonds). Results are expressed as mean cytokine levels ± SEM. These results represent the mean values obtained in 2 donors in each condition. This is one representative experiment out of three similar experiments.

MD-2 expression during cell differentiation

MD-2 is required for efficient TLR4-dependent LPS activation of cells [19-21]. Here, we show by RT-PCR that MD-2 mRNA was differentially expressed in myeloid cells at various stages of differentiation. Undifferentiated CD14(+)THP-1 cells did not express detectable levels of MD-2 mRNA in the experiment shown in Fig. 5. In other experiments not shown here, a faint band corresponding to the amplified MD-2 PCR product could be detected. In all experiments, differentiation of CD14(+)THP-1 cells with 1,25 $(OH)_2$ vitamin D_3, retinoic acid, and γ-interferon consistently increased MD-2 mRNA expression compared with undifferentiated cells. Primary human blood monocytes and alveolar macrophages showed a strong MD-2 mRNA expression in all subjects tested.

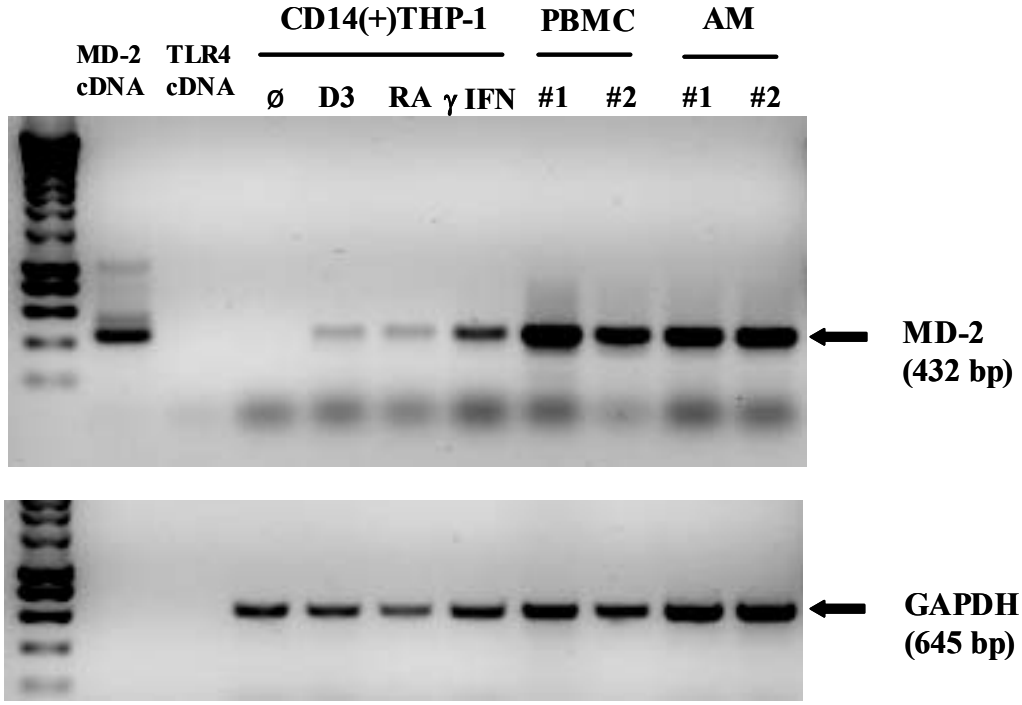

Figure 5. MD-2 mRNA expression (RT-PCR) in undifferentiated CD14(+)THP-1 cells, in cells differentiated with 1,25 $(OH)_2$ vitamin D_3 (D3), retinoic acid (RA), and γ−interferon (γ-INF), in peripheral blood monocytic cells from two healthy donors (#1 and #2) and in human primary alveolar macrophages (AM) from two different donors (#1 and #2). GAPDH mRNA housekeeping gene expression is shown in the lower panel. A vector containing MD-2 cDNA was used as a positive control for MD-2 expression and another vector containing TLR4 cDNA served as a negative control.

Discussion

We initially hypothesized that the differentiation process in myeloid cells modified the expression of surface molecules implicated in LPS responses, and that these changes were responsible for the acquisition of LPS responsiveness. We found that the surface levels of LPS putative receptor chains (CD14, TLR2, and TLR4) were not substantially modified by the differentiation process. Only γ-interferon significantly increased TLR4 expression. This is probably due to a functional interferon consensus responding element in its promoter region, as recently described by Rehli et al. [29]. 1,25 $(OH)_2$ vitamin D_3 did not modify TLR4 expression, whereas retinoic acid slightly but consistently decreased its surface expression. In contrast with studies on LPS tolerance [30], we were not able to correlate the magnitude of cellular LPS responses with TLR4 expression in this THP-1 model. Indeed, γ-interferon, which augmented TLR4 expression, was less potent than 1,25 $(OH)_2$ vitamin D_3 to enhance LPS responses in THP-1 cells. In addition, retinoic acid which induced a small reduction in TLR4 expression responded to endotoxin at least as well as parental CD14(+)THP-1 cells. CD14, whose expression is driven by a viral constitutive promoter in CD14(+)THP-1 cells, as well as TLR2 expression, were not significantly altered by the differentiation process, and could therefore not account for the differences observed in LPS responses between differentiated and undifferentiated cells.

Overall, we show that the involvement of TLR2 and TLR4 in the LPS response of myeloid cells depends on the degree of cell differentiation. Blocking experiments using monoclonal antibodies showed that the molecules critical for LPS responses were CD14 and TLR2 in undifferentiated CD14(+)THP-1 cells. Cells exposed to γ-interferon or 1,25 $(OH)_2$ vitamin D_3 became less TLR2-dependent. Cells differentiated with retinoic acid responded to

LPS prominently through TLR4. Importantly, in all situations, the addition of both anti-TLR2 and anti-TLR4 mAbs abrogated LPS responses. This and the observations described above tend to support the notion that TLR2 may function as an LPS receptor [12, 13], primarily in undifferentiated myeloid cells, but that cells become less TLR2-dependent as they mature. Although the level of expression of TLR2 in primary monocytes/macrophages was much higher than that of TLR4, LPS responses in these cells were essentially TLR4-dependent. The existence of TLR2-TLR4 heterocomplexes has been recently postulated [31], however the interplay and association between TLR2 and TLR4 in cells expressing both molecules remains to be determined.

We next asked whether the observed reduction of TLR2-dependence in LPS responses occurring during CD14(+)THP-1 cell maturation could be explained by a differential expression of the TLR4 co-factor MD-2 [19-21]. MD-2 was previously found to confer LPS responsiveness to TLR4-expressing cells, possibly by modifying the TLR4 tertiary structure [20]. We observed that MD-2 mRNA was upregulated after treatment with the three different differentiating agents. The increased MD-2 expression also correlated with the observation that differentiated cells became less TLR2-dependent. However, except for THP-1 cells differentiated with retinoic acid, MD-2 upregulation did not induce a significant increase in TLR4-dependence. One limitation of these observations is that MD-2 expression was only measured at the mRNA level due to the lack of specific anti-MD-2 mAbs. The reduction of the "CD14-dependence" of LPS-induced responses after THP-1 differentiation was somewhat surprising (Fig. 3). This could be due to an increased LPS sensitivity of differentiated cells, as previously shown by us and others [25, 32]. However, the cell response remained completely TLR-dependent as indicated by the double anti-TLR2 and anti-TLR4 mAb blockade experiment.

Our observation of a reduced TLR2-dependence and a skewing towards TLR4-dependence in LPS responses during the differentiation process could be explained by the possible involvement of an additional, as yet unknown component of the LPS multichain receptor. This component would "uncouple" LPS or LPS-CD14 complexes from TLR2 or alternatively augment the affinity of TLR4 for the signaling complex. It could also be argued that during myeloid cell differentiation, TLRs molecules (and/or CD14) localize in different plasma membrane subdomains or in different intracellular organelles that are important for transmembrane signaling [33, 34]. Recruitment of transmembrane receptors by GPI-anchored proteins has been observed in other systems, such as the molecules implicated in signaling by the GNDF family ligands [35], as well as in the contactin-associated protein (caspr)/F3 contactin system [36].

Because of the promonocytic origin and tumoral nature of THP-1 cells, this model has obvious limitation for generalization of the findings in primary cells. We felt important to further study the relevance of TLRs and MD-2 in LPS responses of fully differentiated primary human myeloid cells. Human monocytes and human alveolar macrophages express high levels of CD14 and TLR2. TLR4 was detected at much lower levels at the surface of primary human myeloid cells, consistent with the report of Beutler [7]. Despite this low expression level, LPS stimulation of primary myeloid cells was essentially dependent on TLR4, consistent with the increasing body of evidence that TLR4 is the key signaling chain of the LPS receptor [7]. The combination of anti-TLR2 and anti-TLR4 mAbs, most of the time did not increase inhibition of LPS responses compared with the anti-TLR4 mAb alone. Of note is the robust expression of MD-2 mRNA in all primary myeloid cells tested. This strong MD-2 expression could account for the definite TLR4 dependence of mature primary myeloid

cells stimulated with LPS. To reconcile data between THP-1 cells and primary myeloid cells, we postulate that differentiated THP-1 cells display a phenotype and functionality intermediate between undifferentiated promonocytes and fully differentiated primary myeloid cells. However, whether primary human promonocytes behave like THP-1 cells with regards to the differentiation process needs to be further determined.

It is also important here to stress that CD14 remains the primary acceptor molecule required for LPS signaling. In all cell lines and primary cells tested in this study, none of the anti-TLRs mAbs was as strong as the anti-CD14 mAb to block LPS effects. This may indicate a very proximal and pivotal role of CD14 in recognizing LPS before the putative transfer of LPS to a signaling molecule such as a member of TLRs occurs, or before a protein-protein interaction between CD14 and TLRs takes place [13]. Although anti-TLRs mAbs were used at saturating levels, these mAbs could obviously be of a lesser affinity as compared with the anti-CD14 mAb tested. This could also argue that in primary myeloid cells, CD14-dependent but TLR2 and TLR4-independent pathways co-exist.

In conclusion, we show that the relative involvement of membrane molecules in LPS recognition and signaling depends on the degree of myeloid cell differentiation. Whereas undifferentiated promonocytic cells respond to LPS in a TLR2-dependent manner, monocytes/macrophages predominantly signal through a TLR4/MD-2 pathway. Among the various cell surface molecules important for LPS signaling, CD14 remains the necessary and pivotal LPS acceptor.

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References

1 **Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. and Ezekowitz, R. A.**, Phylogenetic perspectives in innate immunity, *Science* 1999. **284**: 1313-1318.

2 **Janeway, C. A., Jr. and Medzhitov, R.**, Introduction: the role of innate immunity in the adaptive immune response, *Semin Immunol* 1998. **10**: 349-350.

3 **Modlin, R. L., Brightbill, H. D. and Godowski, P. J.**, The toll of innate immunity on microbial pathogens, *N Engl J Med* 1999. **340**: 1834-1835.

4 **O'Neill, L. A. and Dinarello, C. A.**, The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense, *Immunol Today* 2000. **21**: 206-209.

5 **Muzio, M. and Mantovani, A.**, Toll-like receptors, *Microbes Infect* 2000. **2**: 251-255.

6 **Muzio, M., Polentarutti, N., Bosisio, D., Prahladan, M. K. and Mantovani, A.**, Toll-like receptors: a growing family of immune receptors that are differentially expressed and regulated by different leukocytes, *J Leukoc Biol* 2000. **67**: 450-456.

7 **Beutler, B.**, Tlr4: central component of the sole mammalian LPS sensor, *Curr Opin Immunol* 2000. **12**: 20-26.

8 **Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B.**, Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, *Science* 1998. **282**: 2085-2088.

9 **Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J. and Gusovsky, F.**, Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction, *J Biol Chem* 1999. **274**: 10689-10692.

10 **Du, X., Poltorak, A., Silva, M. and Beutler, B.**, Analysis of Tlr4-mediated LPS signal transduction in macrophages by mutational modification of the receptor, *Blood Cells Mol Dis* 1999. **25**: 328-338.

11 **Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S.**, Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product, *J Immunol* 1999. **162**: 3749-3752.

12 **Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard, A., Wood, W. I., Gurney, A. L. and Godowski, P. J.**, Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling, *Nature* 1998. **395**: 284-288.

13 **Yang, R. B., Mark, M. R., Gurney, A. L. and Godowski, P. J.**, Signaling events induced by lipopolysaccharide-activated toll-like receptor 2, *J Immunol* 1999. **163**: 639-643.

14 **Lien, E., Sellati, T. J., Yoshimura, A., Flo, T. H., Rawadi, G., Finberg, R. W., Carroll, J. D., Espevik, T., Ingalls, R. R., Radolf, J. D. and Golenbock, D. T.**, Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products, *J Biol Chem* 1999. **274**: 33419-33425.

15 **Means, T. K., Wang, S., Lien, E., Yoshimura, A., Golenbock, D. T. and Fenton, M. J.**, Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis, *J Immunol* 1999. **163**: 3920-3927.

16 **Yoshimura, A., Lien, E., Ingalls, R. R., Tuomanen, E., Dziarski, R. and Golenbock, D.**, Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2, *J Immunol* 1999. **163**: 1-5.

17 **Aliprantis, A. O., Yang, R. B., Mark, M. R., Suggett, S., Devaux, B., Radolf, J. D., Klimpel, G. R., Godowski, P. and Zychlinsky, A.**, Cell activation and apoptosis by bacterial lipoproteins through toll- like receptor-2, *Science* 1999. **285**: 736-739.

18 **Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Bleharski, J. R., Maitland, M., Norgard, M. V., Plevy, S. E., Smale, S. T., Brennan, P. J., Bloom, B. R., Godowski, P. J. and Modlin, R. L.**, Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors, *Science* 1999. **285**: 732-736.

19 **Akashi, S., Shimazu, R., Ogata, H., Nagai, Y., Takeda, K., Kimoto, M. and Miyake, K.**, Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages, *J Immunol* 2000. **164**: 3471-3475.

20 **Kawasaki, K., Akashi, S., Shimazu, R., Yoshida, T., Miyake, K. and Nishijima, M.**, Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide- mimetic signal transduction by Taxol, *J Biol Chem* 2000. **275**: 2251-2254.

21 **Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. and Kimoto, M.**, MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll- like receptor 4, *J Exp Med* 1999. **189**: 1777-1782.

22 **Takahashi, T., Nakamura, K. and Iho, S.**, Differentiation of myeloid cells and 1,25-dihydroxyvitamin D3, *Leuk Lymphoma* 1997. **27**: 25-33.

23 **Glass, C. K., DiRenzo, J., Kurokawa, R. and Han, Z. H.**, Regulation of gene expression by retinoic acid receptors, *DNA Cell Biol* 1991. **10**: 623-638.

24 **Auwerx, J.**, The human leukemia cell line, THP-1: A multifacetted model for the study of monocyte-macrophage differentiation, *Experientia* 1991. **47**: 22-31.

25 **Vey, E., Zhang, J. H. and Dayer, J. M.**, IFN-gamma and 1,25(OH)2D3 induce on THP-1 cells distinct patterns of cell surface antigen expression, cytokine production, and responsiveness to contact with activated T cells, *J Immunol* 1992. **149**: 2040-2046.

26 **Pugin, J., Kravchenko, V. V., Lee, J. D., Kline, L., Ulevitch, R. J. and Tobias, P. S.**, Cell activation mediated by glycosylphosphatidylinositol-anchored or transmembrane forms of CD14, *Infect Immun* 1998. **66**: 1174-1180.

27 **Hsu, H. C., Tsai, W. H., Chen, P. G., Hsu, M. L., Ho, C. K. and Wang, S. Y.**, In vitro effect of granulocyte-colony stimulating factor and all-trans retinoic acid on the expression of inflammatory cytokines and adhesion molecules in acute promyelocytic leukemic cells, *Eur J Haematol* 1999. **63**: 11-18.

28 **Triozzi, P. L., Eicher, D. M., Smoot, J. and Rinehart, J. J.**, Modulation of leukemic cell sensitivity to lymphokine-activated killer cytolysis: role of intercellular adhesion molecule-1, *Exp Hematol* 1992. **20**: 1072-1076.

29 **Rehli, M., Poltorak, A., Schwarzfischer, L., Krause, S. W., Andreesen, R. and Beutler, B.**, PU.1 and interferon consensus sequence-binding protein regulate the myeloid expression of the human toll-like receptor 4 gene, *J Biol Chem* 2000. **275**: 9773-9781.

30 **Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K. and Akira, S.**, Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression, *J Immunol* 2000. **164**: 3476-3479.

31 **Lin, Y., Lee, H., Berg, A. H., Lisanti, M. P., Shapiro, L. and Scherer, P. E.**, LPS activated TLR-4 receptor induces synthesis of the closely related receptor TLR-2 in adipocytes., *J Biol Chem* 2000. **in press**:

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

33 **Poussin, C., Foti, M., Carpentier, J. L. and Pugin, J.**, CD14-dependent endotoxin internalization via a macropinocytic pathway, *J Biol Chem* 1998. **273**: 20285-20291.

34 **Underhill, D. M., Ozinsky, A., Hajjar, A. M., Stevens, A., Wilson, C. B., Bassetti, M. and Aderem, A.**, The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens, *Nature* 1999. **401**: 811-815.

35 **Tansey, M. G., Baloh, R. H., Milbrandt, J. and Johnson, E. M., Jr.**, GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival, *Neuron* 2000. **25**: 611-623.

36 **Faivre-Sarrailh, C., Gauthier, F., Denisenko-Nehrbass, N., Le Bivic, A., Rougon, G. and Girault, J. A.**, The glycosylphosphatidyl inositol-anchored adhesion molecule F3/contactin is required for surface transport of paranodin/contactin- associated protein (caspr), *J Cell Biol* 2000. **149**: 491-502.

37 **Schiff, D. E., Kline, L., Soldau, K., Lee, J. D., Pugin, J., Tobias, P. S. and Ulevitch, R. J.**, Phagocytosis of gram-negative bacteria by a unique CD14-dependent mechanism, *J Leukoc Biol* 1997. **62**: 786-794.

38 **Pugin, J., Dunn, I., Jolliet, P., Tassaux, D., Magnenat, J. L., Nicod, L. P. and Chevrolet, J. C.**, Activation of human macrophages by mechanical ventilation in vitro, *Am J Physiol* 1998. **275**: L1040-1050.

39 **Akashi, S., Ogata, H., Kirikae, F., Kirikae, T., Kawasaki, K., Nishijima, M.,**. **Shimazu, R., Nagai, Y., Fukudome, K., Kimoto, M. and Miyake, K.**, Regulatory roles for CD14 and phosphatidylinositol in the signaling via toll-like receptor 4-MD-2, *Biochem Biophys Res Commun* 2000. **268**: 172-177

4.3 Part II: «CD14 and Toll-like receptors condition LPS responsiveness during dendritic cell differentiation and maturation *in vitro*»

CD14 and Toll-like receptors condition LPS responsiveness during dendritic cell differentiation and maturation *in vitro*

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Abstract

Dendritic cells (DCs) represent a key cellular link between innate and adaptive immunity. During tissue infection, DCs sense the presence of microbial antigens, participate in the local inflammation, migrate to lymphoid organs, and turn into professional antigen-presenting cells. Using a model of human monocyte-derived DCs cultured *in vitro,* we show that LPS responsiveness of DCs varies greatly during the process of DC differentiation and maturation. Levels of LPS responsiveness correlated closely with the levels of CD14, TLR4 and MD-2, all key components of the LPS receptor. Monocyte-derived immature DCs retained their LPS sensitivity, and still expressed CD14 and TLR4. In contrast, DCs matured with endotoxin or pro-inflammatory cytokines lost their surface expression of CD14 and TLR4, as well as their LPS responsiveness. TLR3 expression was found restricted to differentiated and mature DCs. These findings show that the expression pattern and levels of microbial receptors in DCs govern their effector function, i.e. antigen capture and processing versus antigen presentation.

Introduction

Dendritic cells (DCs) are widely distributed in various organs throughout the body and play essential roles in antigen recognition and in the initiation of immune responses to non-self antigens (1). Immature DCs residing as a surveillance network in many tissues are very effective in capturing and processing antigens, particularly through an increased mannose receptor expression (2). Mature DCs are principally found in lymphoid organs and lose antigen capture capacity to acquire efficient antigen presenting function. These cells typically display high surface expression of major histocompatibility complex (MHC) molecules as well as T cell co-stimulatory proteins such as CD40, CD80, and CD86 (3). This maturation process is under the tight control of inflammatory cytokines and bacterial products (4). Whereas it has been recognized that the final maturation of DCs was associated with a loss of LPS responsiveness (5), the receptors implicated in the recognition of bacterial molecules and the pathway(s) of DC activation in response to these bacterial molecules remain poorly defined.

Toll-like receptors (TLRs) belong to a novel and growing family of receptors of the innate immunity, and are expressed by numerous cell types (6, 7). TLR2 and TLR4 are now recognized as receptors for molecules from the cell surface of Gram-positive and Gram-negative bacteria, mycobacteria and yeasts (6). TLR4, together with the acute phase lipopolysaccharide-binding protein and CD14 mediates cell activation in response to endotoxin (6). An “accessory” molecule, MD-2, binds to TLR4 and is required for efficient LPS signaling (8). Interestingly, Muzio et al. recently showed that TLR3 expression, a TLR of unknown function, was restricted to DCs (9).

We hypothesized that the surface expression of CD14, TLRs and MD-2 was regulated during the processes of human monocyte-derived DC differentiation and maturation, and accounted for the variations observed in LPS sensitivity. Herein, we show that the loss of endotoxin responsiveness in DCs matured with LPS or pro-inflammatory cytokines was due to the disappearance of TLR4 and CD14 from the cell surface.

Materials and Methods

Culture differentiation and maturation of human monocyte-derived dendritic cells.

Human monocytes were obtained from healthy blood donors. Peripheral blood mononuclear cells (PBMC) were isolated using a classical Ficoll-Paque® (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient and isolated by adherence (10). In order to obtain monocyte-derived dendritic cells, 10^6/mL monocytes were incubated in culture medium containing 10 ng/mL of both recombinant human GM-CSF (Immugenex Corp, CA, USA) and IL-4 (R&D Systems, MN, USA) for 6 days, according to the method published by Sallusto and Lanzavecchia (10). On day 6, immature dendritic cells (imDCs) were matured with 1 μg/mL 055:B5 *E.coli* LPS (Difco Laboratories, MI, USA), 50 ng/mL TNF-α (Biogen, Geneva, CH) or 50 ng/mL IL-1ß (a gift from Jean-Michel Dayer, University of Geneva, Switzerland) in culture medium containing GM-CSF and IL-4 for an additional period of 3 days.

Flow cytometry.

Monocytes from PBMC, monocytes which have adhered overnight, imDCs and mature DCs (mDCs) were analyzed by flow cytometry for the surface expression of TLRs, CD14, and specific markers of dendritic cells, CD86 and CD83. For flow cytometry analyses, cells were incubated with the following murine mAbs: anti-human (h)TLR2 (2392, from P.J. Godowski, Genentech Inc. South San Fransisco), anti-hTLR4 (HTA 125, provided by K. Miyake, Saga Medical School, Japan), anti-hCD86 (clone IT2.2, PharMingen, CA, USA), anti-hCD83 (clone HB15A, Immunotech), or an isotype control mouse IgG (clone 679.1Mc7,

Immunotech, Marseille, France), and followed by staining with a secondary phycoerythrin (PE)-conjugated goat anti-mouse IgG (Anawa, Wangen, Switzerland). CD14 was labeled with PE-conjugated anti-hCD14 mAb (clone TüK4, Dako, Denmark).

RNA preparation and RT-PCR analysis of TLRs and MD-2 mRNAs.

Buffy coats from healthy blood donors, adherent monocytes, imDCs and mDCs were lyzed in 1 mL of TRIzol® (Gibco). Total RNA was isolated with phenol/chloroform (11) and 1 μg of total RNA was reverse-transcribed with oligo(dT) primer (Pharmacia) and Expand Reverse Transcriptase (Roche,Basel, CH) for 60 min at 42°C to obtain cDNAs. PCR was performed on cDNAs using Taq polymerase (Roche) and specific primers designed for hTLR2 (5' - GCC AAA GTC TTG ATT GAT TGG - 3', sense and 5' - TTG AAG TTC TCC AGC TCC TG - 3', antisense), hTLR3 (5' - GAT TAA CGA GAC CCA TAC CA - 3', sense and 5' - AAC AGA GTG CAT GGT TCA GT - 3', antisense), hTLR4 (5' – GTC TGG CTG GTT TAG AAG TC - 3', sense and 5' - ACA CCA TTG AAG CTC AGA TC - 3', antisense), hMD-2 (5' - TTC CAC CCT GTT TTC TTC CA - 3', sense and 5' - TAG GTT GGT GTA GGA TGA CA - 3', antisense) or GAPDH (5' - GGT CAT CCA TGA CAA CTT TG - 3', sense and 5' - CTA CAT GGC AAC TGT GAG GA - 3', antisense) for 35 cycles at 94°C for 1 min, 55°C (MD-2 and GAPDH) or 58°C (TLR2, -3, -4) for 1 min, and 72°C for 90 sec (Perkin Elmer Thermocycler, Norwalk, CT). Amplified bands were imaged and quantitated using a computer-based imaging system (Kaiser/Vilber Lourmat system, France). Samples were screened for genomic DNA contamination by carrying samples through the PCR procedure with mRNA samples which did not undergo reverse transcription. Negative controls for each set of PCR reactions were performed with plasmids containing unrelated cDNAs. Positive controls for the PCR reaction included plasmids containing cDNAs of

TLR2, TLR4 (a gift from C. Kirschning) or MD-2 (kindly provided by K. Miyake). Positive controls for TLR3 consisted of cDNAs prepared from the human colonic HT29 cell line, known to express TLR3 (12).

Cell stimulation for cytokine production.

Cells at various stages of DC differentiation and maturation were stimulated at 37°C with 1 μg/mL 055:B5 *E.coli* LPS for 24 hr in a 24-well plate at the concentration of 10^6 cells/mL. Levels of TNF-α and IL-12 p70 proteins were measured in conditioned supernatants using ELISA kits from BioSource International (Camarillo, CA, USA) and R&D Systems, (Minneapolis, MN, USA), respectively. In addition, immature DCs were stimulated with LPS in the presence or absence of anti-CD14 (clone 28C5), and anti-TLRs mAbs.

Results

Surface markers on monocytes and DCs.

We first determined by flow cytometry the levels of various surface markers on monocytes as well as on immature and mature monocyte-derived DCs. As shown in Fig. 1, CD86, a classical DC marker, was not expressed at the surface of monocytes, but was acquired during DC differentiation and maturation. A similar expression profile was observed for the "co-stimulatory" molecules CD40 and CD83, which are specific markers of mature DC ((2) and data not shown). Since it is now thought that the LPS receptor is a multichain complex receptor associating CD14, a TLR and an "MD" molecule, we measured the expression of these molecules. CD14 was highly expressed on monocytes but was progressively lost after DC differentiation. TLR4 expression was easily detected in monocytes; its level of expression decreased in imDCs and became undetectable in mDCs. TLR2 expression also decreased during differentiation and maturation but remained expressed at low levels in mDCs. The changes in surface markers observed during maturation were similar in cells treated with LPS and TNF-α. IL-1ß was somewhat less potent in inducing DC maturation. Immature DCs not treated with maturation agents during the 3 d final maturation process did not modify surface levels of TLRs, CD14 and CD86.

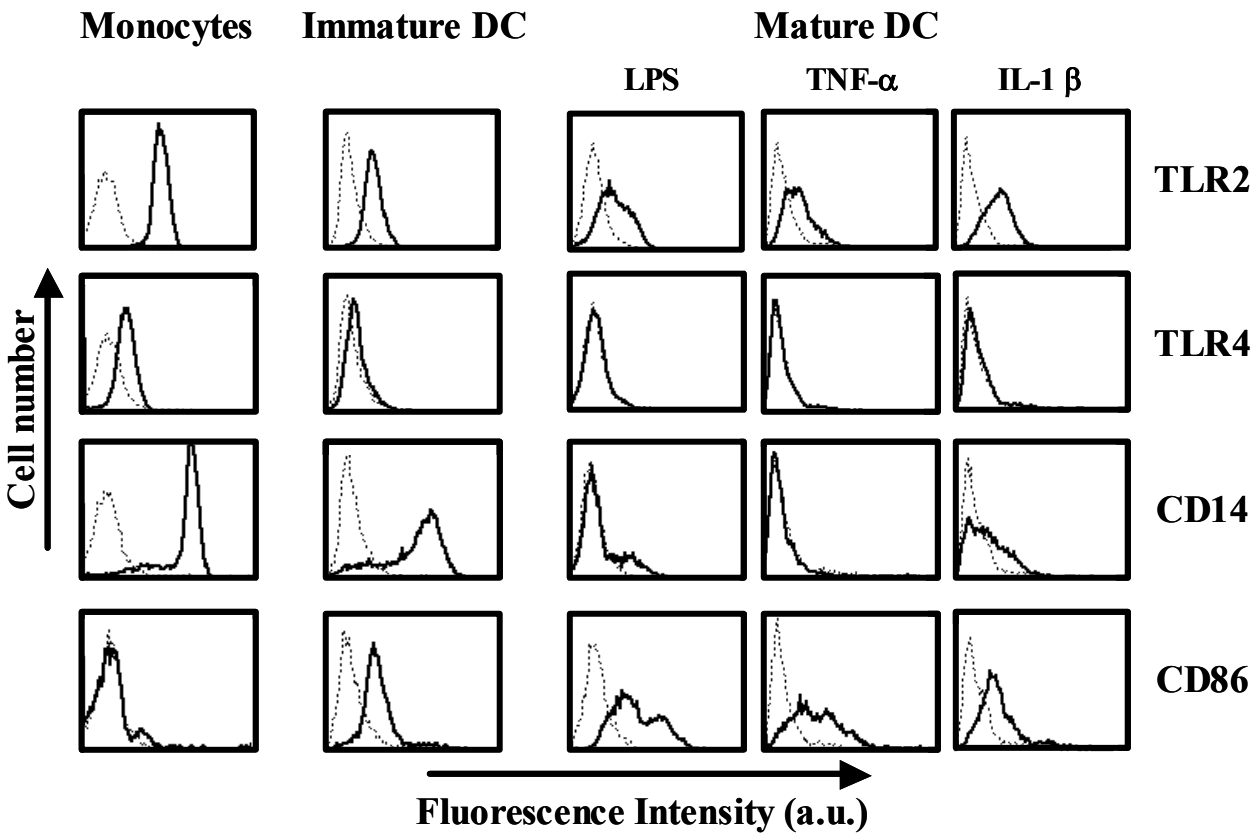

Figure 1. Flow cytometry analysis of the surface expression of TLR2, TLR4, CD14 and CD86 during differentiation and maturation of human monocyte-derived dendritic cells (DCs). Immature DCs were obtained after 6 d treatment of monocytes with GM-CSF and IL-4, and matured with an additional 3 d treatment with LPS, TNF-α or IL-1ß. At each step of cell differentiation and maturation, cells were collected and labeled with specific monoclonal antibodies (solid line), or an isotype control (dotted line). Cell fluorescence intensity (a.u. = arbitrary unit) was determined by flow cytometry.

Messenger RNA levels of TLRs and MD-2 during differentiation and maturation.

Using a semi-quantitative RT-PCR technique, we next investigated the mRNA expression levels of TLRs and MD-2 during the process of DC maturation (Fig. 2). TLR2 and MD-2 mRNA expression slightly decreased during DC differentiation and maturation, whereas TLR4 mRNA levels increased during differentiation and decreased during DC maturation (Fig. 2 for MD-2, data not shown for TLR2 and TLR4). TLR3 mRNA was not detected in monocytes, appeared in imDCs, and then decreased in mDCs (Fig. 2). The data presented in Fig. 2 correspond to one representative experiment out of 3 similar experiments. In one experiment, a faint TLR3 band could be amplified from monocytes isolated in two different donors.

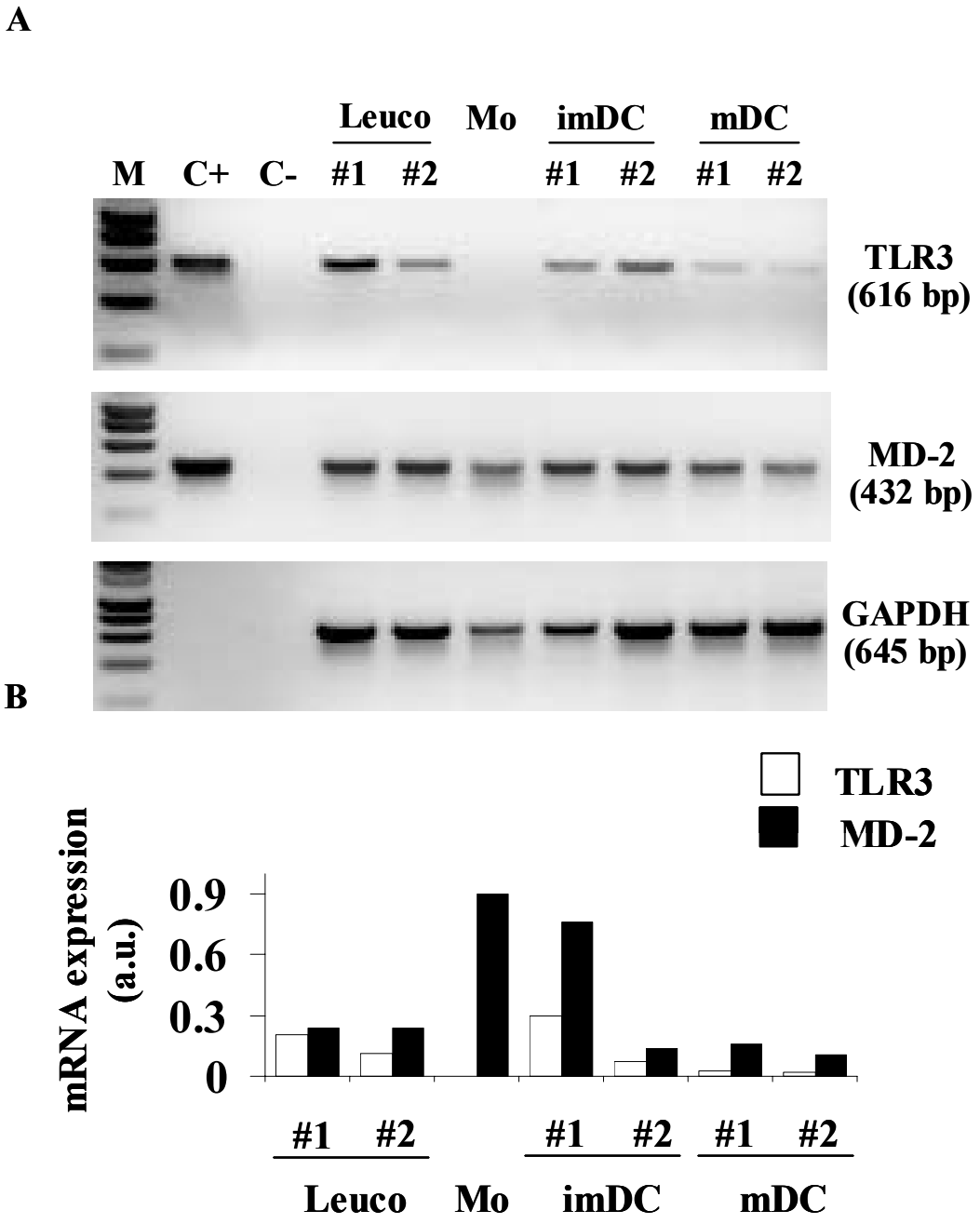

Figure 2. RT-PCR analysis of TLR3 and MD-2 mRNA expression during dendritic cell differentiation and maturation.

Panel A. Amplified TLR3, MD-2 and GAPDH cDNA bands in whole blood leucocytes (leuco), adherent monocytes (Mo), immature dendritic cells (imDCs) and mature dendritic cells (mDCs) from two healthy subjects (#1 and #2). cDNAs from HT29 cells and the plasmid containing MD-2 cDNA were used as positive controls (C+) for TLR3 and MD-2, respectively. A plasmid containing TLR4 cDNA was used as a negative control (C-).

Panel B. Semi-quantitative analysis of TLR3 and MD-2 mRNA expression. TLR3 (white bars) and MD-2 (black bars) band densities were determined by densitometry, reported to GAPDH band intensity in the same sample, and expressed as arbitrary units (a.u.).

Cell responses to LPS.

We next stimulated monocytes, imDCs and mDCs with LPS and measured cytokine production (Fig. 3, panel A). Monocytes responded to LPS by secreting TNF-α and IL-12. Immature DCs had increased cytokine responses to LPS as compared with monocytes. The LPS-induced TNF-α secretion by immature DCs was blocked by anti-CD14 mAb, decreased by 50% by anti-TLR4 mAb, and unchanged by anti-TLR2 mAb (Fig. 3, panel B). In all cases maturation with LPS or TNF-α was associated with a loss of LPS responsiveness (Fig. 4). Similarly to what we observed with surface markers, maturation with IL-1ß only partially decreased LPS responsiveness in mature DCs. No significant production of cytokines was observed in unstimulated cells.

A

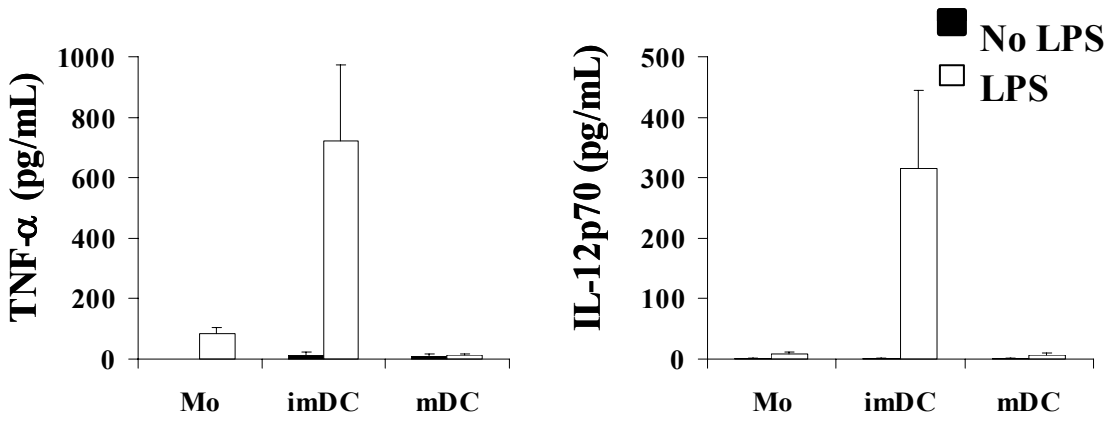

Figure 3. Panel A. TNF-α and IL-12p70 produced by monocytes, immature DCs and mature DCs after stimulation with LPS. The cytokines were measured in conditioned supernatants by ELISA. The results were expressed as the mean ± SEM of 6 independent experiments.

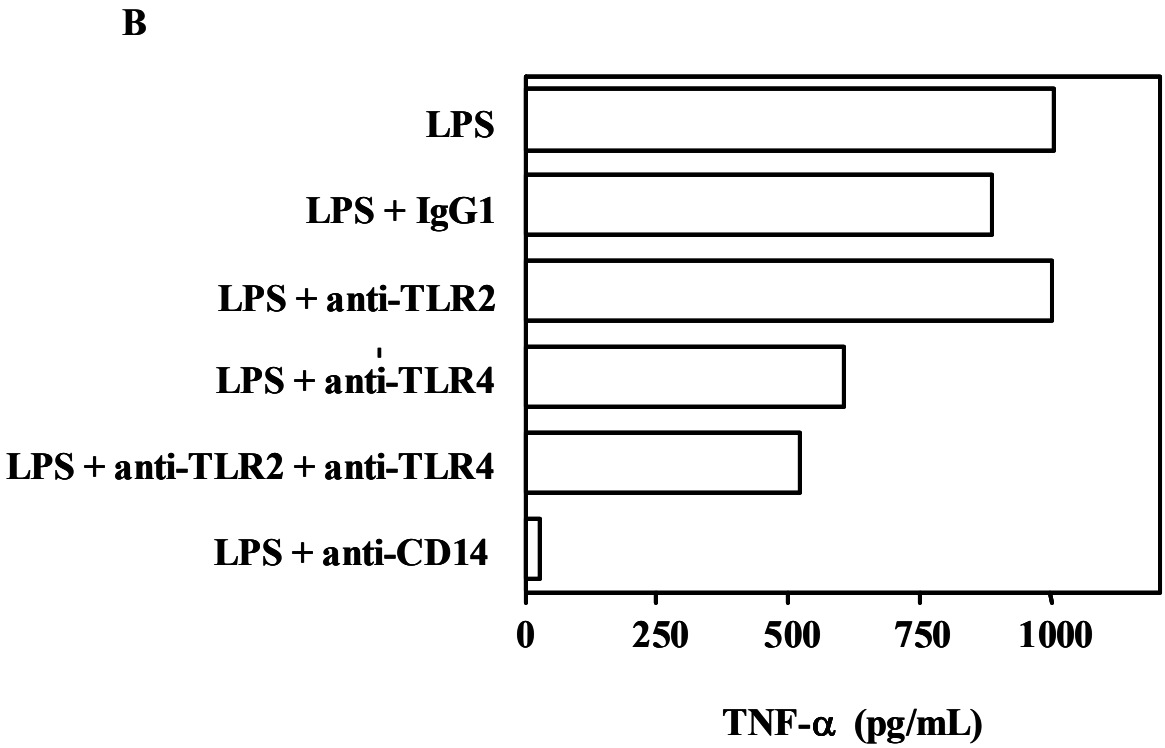

Figure 3. Panel B. TLRs and CD14 dependence in LPS-induced TNF-α secretion in immature DCs. Immature DCs were stimulated 18 hr. with 1 ng/mL LPS. One representative experiment out of three similar experiments.

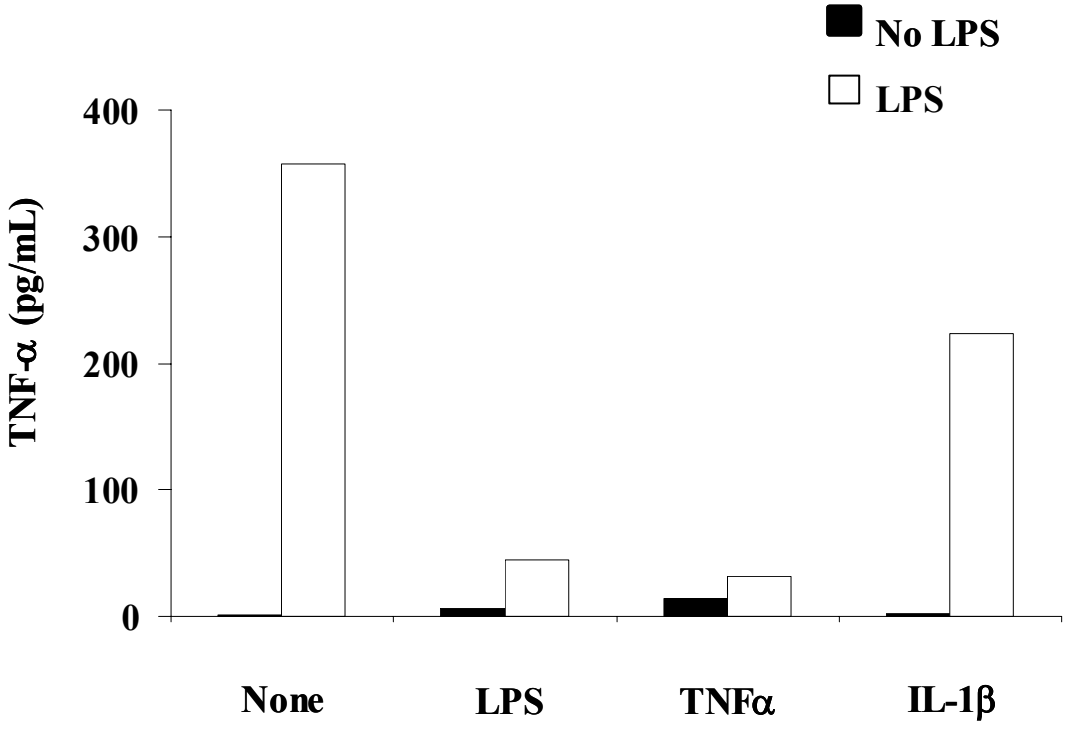

Figure 4. TNF-α production in dendritic cells matured with LPS, TNF-α or IL-1β for 3 days and stimulated with LPS for 24 hr. TNF-α was measured in conditioned supernatants by ELISA. This is one representative experiment out of three similar experiments.

Discussion

Using an *in vitro* model of monocyte-derived DC differentiation, we demonstrate here that DCs evolve from LPS-sensitive immature cells into LPS-unresponsive mature cells. This is correlated with the loss of expression of critical LPS recognition molecules such as CD14 and TLR4 during the process of DC maturation. This represents the molecular basis for the reprogramming of immature DCs, which become unresponsive to LPS, while acquiring the molecular equipment needed for full antigen-presenting capacity.

Immature DCs interface innate and adaptive immune responses (7, 13). Due to the surface expression of various pattern recognition receptors, such as the mannose, complement, scavenger and CD14 receptors, immature DCs specialize into cells recognizing, internalizing and processing non-self microbial antigens. They are typically concentrated in organs that are in close contact with non-self antigens, such as the skin, intestine, liver and lungs (14, 15). It has now been well demonstrated that the phenotype of *in vitro* differentiated DCs was very similar to that of tissue DCs, such as lung DCs (2) or skin Langerhans cells (16). These cells express a vast array of surface molecules important for the recognition and uptake of bacteria and bacterial products. Here, we show that, in addition to the mannose and scavenger receptors (2, 14), immature DCs also express significant amounts of CD14, TLR2, TLR4 and MD-2. These latter proteins associate on cell surface to form multichain receptor complexes and mediate cell activation to conserved microbial molecules such as LPS, peptidoglycan, and lipoarabinomannan (6). Importantly, we found that these cells were fully responsive to LPS and produce high amounts of TNF-α and IL-12. This response was dependent on CD14 and TLR4 but not TLR2. In our experiments, immature DCs responded somewhat better to LPS than adherent monocytes. This could be an effect of the 6 day-

treatment with GM-CSF, a growth factor which has previously been described to augment cell responsiveness to endotoxin (17).

The exposure of immature DCs to pro-inflammatory molecules such as LPS, TNF-α or CD40L leads to further maturation of the DCs. During maturation, DCs migrate to lymphoid organs where they acquire full antigen-presenting capacity, with high levels of surface expression of MHC and co-stimulatory molecules (4, 18). In the present study, we observed that DC maturation was accompanied with a loss in CD14 and TLR4 antigens expression, two molecules that are critical for LPS signaling in myeloid and endothelial cells (19-22) as well as for LPS internalization by macrophages (23, 24). MD-2, a molecule associated with TLR4 and required for efficient LPS signaling (8), was also decreased at the level of mRNA expression during DC maturation. This loss of CD14 and TLR4 expression paralleled the absence of LPS responsiveness in mature DCs. Interestingly, hyporesponsiveness of mature DCs to bacterial antigens had been previously reported by Kalinski et al. (5). Taken together, our results add to the notion that DC maturation is associated with a loss of surface expression molecules required for pathogen recognition and microbial uptake (2, 25). Mature cells, however acquire full antigen-presentation capacity and support a strong allogenic mixt lymphocyte reaction (2, 26, 27).

TLR-3 was found by Muzio et al. to be specifically expressed in human dendritic cells. (9). Herein, we confirm that mRNA for TLR3 was undetectable by RT-PCR in monocytes, appeared in immature DCs and decreased in mature DCs. To date, TLR3 remains of unknown function, but interestingly it is the only human TLR lacking a conserved proline residue important for signaling through the Toll/IL-1R domain (28, 29). We also found that TLR3 mRNA was expressed in buffy coats from blood donors. This could be due to the presence of

circulating DCs in blood leukocytes (30) or to the fact that non-monocytic leukocytes express TLR3 at levels below Northern blot detection (9). It is likely that the expression of TLR3 is tightly modulated by response elements sensitive to growth factors (such as GM-CSF) and/or IL-4. Further work is needed to unravel the potential role of TLR3 in DC-specific functions or in DC differentiation. The expression of additional TLRs by DCs also remains to be determined.

In conclusion, our findings provide additional support for the fact that immature DCs display a vast array of pathogen recognition receptors, including members of the important TLR family, and produce a strong pro-inflammatory response to LPS. This is important information given the phagocytic and inflammation-enhancing functions of tissue dendritic cells. Exposure of these cells to endotoxin and pro-inflammatory cytokines reprogram DCs into professional antigen-presenting cells which loose their response to microbial antigens, but are now capable of boosting T cell proliferation and activation. This unresponsiveness to endotoxin could be attributed to the downregulation of CD14, TLR4 and MD-2.

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References

1. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245*.

2. Cochand, L., P. Isler, F. Songeon, and L. P. Nicod. 1999. Human lung dendritic cells have an immature phenotype with efficient mannose receptors. *Am J Respir Cell Mol Biol 21:547*.

3. Cella, M., A. Engering, V. Pinet, J. Pieters, and A. Lanzavecchia. 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells [see comments]. *Nature 388:782*.

4. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med 182:389*.

5. Kalinski, P., J. H. Schuitemaker, C. M. Hilkens, E. A. Wierenga, and M. L. Kapsenberg. 1999. Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. *J Immunol 162:3231*.

6. Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature 406:782*.

7. Medzhitov, R., and C. Janeway, Jr. 2000. Innate immunity. *N Engl J Med 343:338*.

8. Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med 189:1777*.

9. Muzio, M., D. Bosisio, N. Polentarutti, G. D'Amico, A. Stoppacciaro, R. Mancinelli, C. van't Veer, G. Penton-Rol, L. P. Ruco, P. Allavena, and A. Mantovani. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol 164:5998*.

10. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med 179:1109*.

11. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem 162:156*.

12. Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. C. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol 164:966*.

13. Janeway, C. A., Jr., and R. Medzhitov. 1998. Introduction: the role of innate immunity in the adaptive immune response. *Semin Immunol 10:349*.

14. Clark, G. J., N. Angel, M. Kato, J. A. Lopez, K. MacDonald, S. Vuckovic, and D. N. Hart. 2000. The role of dendritic cells in the innate immune system. *Microbes Infect 2:257*.

15. Hart, D. N. 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood 90:3245*.

16. Geissmann, F., C. Prost, J. P. Monnet, M. Dy, N. Brousse, and O. Hermine. 1998. Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J Exp Med 187:961*.

17. Jones, T. C. 1996. The effect of granulocyte-macrophage colony stimulating factor (rGM-CSF) on macrophage function in microbial disease. *Med Oncol 13:141*.

18. Roake, J. A., A. S. Rao, P. J. Morris, C. P. Larsen, D. F. Hankins, and J. M. Austyn. 1995. Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J Exp Med 181:2237*.

19. Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science 249:1431*.

20. Pugin, J., C. C. Schurer-Maly, D. Leturcq, A. Moriarty, R. J. Ulevitch, and P. S. Tobias. 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc Natl Acad Sci U S A 90:2744*.

21. Akashi, S., H. Ogata, F. Kirikae, T. Kirikae, K. Kawasaki, M. Nishijima, R. Shimazu, Y. Nagai, K. Fukudome, M. Kimoto, and K. Miyake. 2000. Regulatory roles for CD14 and phosphatidylinositol in the signaling via toll-like receptor 4-MD-2. *Biochem Biophys Res Commun 268:172*.

22. Beutler, B. 2000. Tlr4: central component of the sole mammalian LPS sensor. *Curr Opin Immunol 12:20*.

23. Kitchens, R. L., P. Wang, and R. S. Munford. 1998. Bacterial lipopolysaccharide can enter monocytes via two CD14-dependent pathways. *J Immunol 161:5534*.

24. Poussin, C., M. Foti, J. L. Carpentier, and J. Pugin. 1998. CD14-dependent endotoxin internalization via a macropinocytic pathway. *J Biol Chem 273:20285*.

25. Granucci, F., E. Ferrero, M. Foti, D. Aggujaro, K. Vettoretto, and P. Ricciardi-Castagnoli. 1999. Early events in dendritic cell maturation induced by LPS. *Microbes Infect 1:1079*.

26. Kapsenberg, M. L., M. B. Teunissen, F. E. Stiekema, and H. G. Keizer. 1986. Antigen-presenting cell function of dendritic cells and macrophages in proliferative T cell responses to soluble and particulate antigens. *Eur J Immunol 16:345*.

27. Knight, S. C., S. Iqball, M. S. Roberts, S. Macatonia, and P. A. Bedford. 1998. Transfer of antigen between dendritic cells in the stimulation of primary T cell proliferation. *Eur J Immunol 28:1636*.

28. Rock, F. L., G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan. 1998. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A 95:588*.

29. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science 282:2085*.

30. Fearnley, D. B., L. F. Whyte, S. A. Carnoutsos, A. H. Cook, and D. N. Hart. 1999. Monitoring human blood dendritic cell numbers in normal individuals and in stem cell transplantation. *Blood 93:728*.

5 CHAPTER III: Cell response to other bacterial toxin : aerolysin from *Aeromonas hydrophila*

5.1 Introduction

As observed in previous studies, the GPI-anchor of CD14 does not seem to play a role in endotoxin-induced cell activation or internalization. Indeed, the replacement of the CD14 GPI-anchor by a transmembrane tail revealed that the GPI tail targets CD14 to lipid-rich domains, whereas it is not required for LPS-mediated myeloid cell activation and LPS internalization [429]. However, it was observed that anti-CD14 antibody-induced cross-linking caused a rapid calcium mobilization signal only in GPI-anchored CD14 THP-1 cells. For this observation, no physiological explanation could be provided [25]. However, it is possible that the GPI anchoring system may be important for other signaling functions.

Interestingly, aerolysin, one of the best characterized toxins secreted by the Gram-negative bacterium *Aeromonas (A.) hydrophila* as well as other *Aeromonas* species, has been reported to bind specifically to GPI-anchors. Aerolysin pathogenicity resides in its capacity to create pores in mammalian cell membranes and subsequently induces cell death by an osmotic process.

5.1.1 Pathologies induced by *Aeromonas hydrophila*

Aeromonas present in aqueous environments are at the origin of gastrointestinal diseases in humans. Moreover, in immuno-compromised patients, *Aeromonas* infection can lead to septicemia and meningitis [430, 431]. The implication of aerolysin also called alpha-haemolysin, in *A. hydrophila*- associated systemic infections was demonstrated by using mutant strains lacking the aerolysin gene product. These aerolysin mutant strains were neither hemolytic for blood erythrocytes nor cytotoxic for Chinese hamster ovary tissue culture cells compared to revertant strains [432]. In addition, specific neutralizing antibodies to aerolysin have been detected in animals surviving *Aeromonas* infection.

Few years before, some investigations had revealed that radiolabeled-aerolysin binds to rat erythrocytes, and that this binding could be inhibited by specific anti-aerolysin antibodies. It was defined that the aerolysin interaction with erythrocyte membranes was facilitated by a specific glycoprotein receptor. Moreover, aerolysin binding is followed by the formation of discrete holes or pores responsible for the hemolysis of erythrocytes [433].

5.1.2 Secretion of aerolysin from bacteria.

In order to get out of the bacteria, aerolysin utilizes the type II secretion system. This protein does not cross the inner and outer membranes simultaneously, but requires more than one step for an export outside [434]. First, the N-terminal signal sequence of the preproaerolysin targets the protein to sec machinery which allows translocation across the inner membrane and removal of the signal sequence [435]. Proaerolysin present in the periplasm, further traverses the outer membrane of bacteria in a ATP-dependent and an electromotive force, and is released in the extracellular medium [436]. Interestingly, though translocation across the inner membrane occurs in an unfolded state, translocation across the outer membrane occurs in an apparently fully folded state [437]. It is still unclear whether proaerolysin is secreted as a dimer or a monomer. Strong evidences indicated that the protein crosses the outer membrane as a fully folded dimer, suggesting that folding and dimerization occur in the periplasm [438]. As determined by mass spectrometry, no post-translational modification seems to occur in the aerolysin primary sequence.

5.1.3 Biochemical properties of aerolysin

Many biochemical investigations on aerolysin enabled to better characterize this protein. Purified aerolysin has a molecular weight of 52 kDa. Interestingly, its hemolytic action is inhibited by certain reducing agents including ferrous iron and cysteine [439]. The aerolysin sequence analysis indicated that although most of the sequence seems unrelated to that of *Staphylococcus aureus* alpha-toxin, both proteins are very hydrophilic, and they each contain a nearly identical string of 10 aa [440].

Proaerolysin is a highly soluble 470-aa protein of 52 kDa secreted as an inactive precursor of the toxin, aerolysin. Spontaneously, proaerolysin seems to be more stable as a dimer than as a monomer. In the dimer, adjacent monomers are orientated in an anti-parallel manner and the domain 1 is implicated in such a process as shown by truncation mutant experiments. However, as shown by Fivaz et al., the dimer gradually dissociates at concentrations below 0.1 mg/mL [441]. Such a low protein concentration prevents the

crystallization of monomers and subsequently, structural analysis of the monomer remains impossible. Meanwhile, crystallization of proaerolysin as a dimer is possible and forms tetragonal crystals that contain the dimer in the asymmetric unit [442]. Structural analysis revealed that the protoxin has a L-shaped form which can be divided into a small N-terminal globular domain and a large lobe corresponding to an elongated domain. This latter lobe contains 3 domains characterized by the presence of β-sheets, which characterize 42% of the whole structure, whereas helices mainly concentrated in domain 2, represent only 21% of the molecule. The presence of two disulfide bonds in domains 1 (Cysteine(C)19-C75) and 2 (C159-C164) significantly increases the overall stability of the protein [443]. Even if the exact function of each domain remains unknown, several important regions of the molecule were identified and hypothesis on the nature of their function could be raised using a combination of chemical modification and site-directed mutagenesis. Actually, domains 1 and 2 appear to be involved in receptor binding, domain 2 in triggering oligomerization, domains 3 and 4 in maintaining the oligomer assembled and domain 4 has been proposed to line the channel.

In contrast to other pore-forming toxins such as colicins, proaerolysin does not contain any hydrophobic stretches within its primary sequence that could form potential transmembrane regions.

5.1.4 Activation of proaerolysin into aerolysin

In order to protect themselves from killing effects of aerolysin, *Aeromonas* bacteria produce an inactive precusor, proaerolysin unable to oligomerize and to form a membrane-perforating channel. Activation of proaerolysin occurs through the cleavage of a C-terminal peptide present within a flexible loop located in domain 4. Several common proteases are able to perform this activation [444]. As determined by mass spectrometry, trypsin cleaves proaerolysin after the lysine at position 427, whereas chymotrypsin cuts after the arginine at 429 [445]. Members of the mammalian pro-protein convertase family including furin were also found to cut after the arginine 432 [446]. The above results also indicate that proaerolysin can be activated either in solution by soluble enzymes, or at the surface of the target cells by transmembrane proteases such as furin. As mentioned by van der Goot et al., a C-terminal peptide of 43 or 45 aa is removed after proteolytic cleavage and plays no further role in the process of channel formation [447]. Following activation, aerolysin circularly assembles into

heptamers that is extremely stable [443, 448, 449]. Actually, removal of the peptide from proaerolysin might trigger a conformational change that promotes aerolysin oligomerization. Indeed, a significant change in secondary structure, characterized by a decrease in random structure and an increase in beta-sheet content was observed by spectroscopy. This conformationnel change appeared to propagate along the molecule from the cleavage site [450]. This process could possibly reduce the energy barrier leading to heptamer. More precisely, it was shown that after proteolytic activation, movement of a loop in domain 3 of aerolysin is required to induce oligomerization [451]. In contrast, subsequent oligomerization of the mature toxin did not involve any change in overall secondary structure but involved a modification of the tertiary interactions [450]. Although oligomerization must normally occur on the surface of the target cell, it can also take place in solution if the protein concentration rises above about 100 μg/mL. The histidine (His-) 132 has been demonstrated to be a pivotal aa, since the mutation of this residue totally abolishes the channel formation in lipid bilayers [452, 453]. Later studies indicated that His-132 must in addition be protonated for oligomerization to proceed. This suggested that the environment of this residue might act as a nucleation site for the polymerisation process [454]. In contrast, mutations of tryptophan 371 and 373 were found to accelerate the heptamer formation process. This observation suggested that these mutations somewhat destabilize the protein thereby facilitating the rearrangements required for heptamer assembly [455]. Chemical experiments enabled to detect in domains 3 and 4, aa regions (180-307 and 401-427) presumably involved in monomer/monomer interactions in the heptameric protein and exclusively composed of beta structure [443]. In contrast, domains 1 and 2 do not seem to be crucial in such a function. However, it cannot be excluded that these two latter domains are important to initialise the formation of the complex as observed with the different aa mutations described above [452, 453, 455].

Structural analysis of the oligomer was limited because of extreme difficulties in the crytallization procedure. Nevertheless, the complex could be described to have a mushroom like shape similar to that of staphylococcal α-toxin using the low-resolution model of the heptamer defined from two-dimensional crystals [448]. In addition, combining this low-resolution model and the high-resolution structure of proaerolysin, a model of the channel has been proposed [456]. According to this model, domains 1 and 2 would lie on the membrane, domain 3 would form the mouth of the channel and domain 4 would cross the bilayer and line the pore. This model however remains to be confirmed experimentally.

5.1.5 Receptor binding of proaerolysin

For many years, it was wondered whether proaerolysin would bind to a receptor at the target cell surface and if so, whether this binding could induce the heptamerization after the proaerolysin activation. Interestingly, instead of recognizing one specific receptor, proaerolysin, and also aerolysin were found to interact with a specific post-translational modification, a GPI anchor [457-460]. The GPI-anchor is added in the endoplasmic reticulum, to the carboxy terminus of newly synthesized proteins that bear a GPI-anchoring signal [461, 462]. The GPI anchor then targets these proteins to the plasma membrane. The discovery of GPI-anchor proteins as receptors for proaerolysin came from different experiments. Indeed, the use of a phosphatidyl inositol-specific phospholipase C protected cells against aerolysin [458]. Moreover, cells deficient in GPI biosynthesis such as cells from patients suffering from the PNH syndrom, had a dramatically reduced sensitivity towards aerolysin [463]. Recent evidence suggested that the proaerolysin binds the GPI-anchor as a monomer and not as a dimer as previously thought [441]. Amongst the identified GPI-anchored proteins that bind proaerolysin, were found Thy-1, contactin, CD14, semaphorin K1 and the GPI-anchored isoform of N-cam (unpublished observations from L. Abrami et al.) [458, 460]. The apparent Kd for proaerolysin binding was estimated between 20 and 65 nM [459, 464]. Present as a stable dimer in solution, this dimeric protoxin is quite capable of receptor binding as recently shown [465, 466]. Regions of aerolysin involved in the binding were mapped by site-directed mutagenesis followed by plasmon resonance analysis. Interestingly, experiments revealed that many of the residues identified as being important for high-affinity binding corresponded to aromatic residues present in domain 1 and 2 [464]. It was suggested that aromatic residues could stack against the pyranose rings of sugars as already observed for other carbohydrate-binding proteins [467]. However, considering the large size of domains 1 and 2 of proaerolysin, it remains unclear how the toxin can interact with the glycan core of the GPI-anchor receptor. Conformational changes in the glycan core of the anchor and in the toxin are likely to be necessary. As shown by a recent study, aerolysin binding to the receptor also requires the polypeptide moiety of GPI-anchored proteins in addition to the glycan core [468]. Once bound to the cell surface, proaerolysin is activated by proteolytic cleavage as already described in a previous section. Oligomerization then occurs probably by lateral movement of the toxin within the plane of the membrane. This heptamerization is also possible in solution, but at a higher aerolysin concentration (1 μM) than that observed in experiments with living

cells (10 pM) [7, 445]. Actually, reducing the dimensionality from 3 to 2 (cell membrane surface), concentrates aerolysin and would facilitate interactions between aerolysin molecules for the oligomerization. One of the extensively characterized properties of the GPI-anchored protein family is that they have a complex and unusual mobility at the cell surface. They can navigate in the phosphoglyceride region of the plasma membrane with a higher mobility than transmembrane proteins [469]. They also have the ability to associate in a dynamic fashion with cholesterol and glycosphingolipid-rich microdomains also called "lipid rafts" and present in the plasma membrane [470, 471]. With such uncommon characteristics, it has been hypothesized for many years that lipid rafts could promote the cell surface oligomerization of aerolysin. Several studies led to controversial observations and interpretations. In 1999, Abrami et al. published that the capacity of proaerolysin to associate with microdomains was abrogated in cells treated with the cholesterol binding drug saponin. In the same time, the treatment dramatically affected the aerolysin oligomerization [7]. The oligomerization process was partially restored when higher concentrations of toxin were added to cells. Therefore, microdomains appeared to act as concentration platforms at the cell surface due to their capacity to recruit GPI-anchored proteins [472]. And aerolysin seemed to have use this property for its own purpose. On the opposite, recent investigations demonstrated that the chemical destruction of lipid-rich domains in T lymphocytes or in erythrocytes did not decrease the rate at which cells were lysed by the toxin. In a similar manner, the rate of aerolysin-induced channel assembly was not different in liposomes containing variable lipid compositions, which favoured or not lipid rafts formation [473].

Interestingly, several other bacterial pore-forming toxins have been found to share common properties: 1) the toxin oligomerization procedure for channel formation and 2) the use of raft components to achieve this process. In addition, it appeared that certain bacteria and viruses have chosen the site of lipid rafts to entry into cells [reviewed by 474].

After aerolysin heptamerization, insertion of the channel into the lipid bilayer remains a step very poorly understood. As shown by Van der Goot et al., the heptamer is an amphiphatic complex and is therefore prone to aggregation. From these results, it was also suggested that this amphiphatic property of the complex could constitute the driving force for membrane insertion [455]. It has been suggested that unfavourable energetic effects exist at the junctures between lipid rafts and the fluid-phase phosphoglyceride region of the plasma membrane [475]. These unstable boundaries might facilitate membrane insertion of the

aerolysin heptamer. Also, other characteristics of lipid rafts might promote membrane penetration. In a more recent study, Alonso et al. described that lipids composition would be an important factor for the heptamer insertion into lipid bilayers. Experiments were done using large unilamellar egg phosphatidylcholine vesicles containing varying amounts of other lipids. It appeared that the most favourable lipids were phosphatidylethanolamine, diacylglycerol, cholesterol, or hexadecane [476].

5.1.6 Consequences of channel formation

Aerolysin-induced channel formation leads to plasma membrane permeabilization to small ions such as calcium and potassium [459, 477]. As shown by voltage-clamp experiments, these channels are voltage-gated and slightly anion selective. Between -70 to +70 mV, channels present in artificial lipid membranes remain opened, whereas outside this range they are inactivated and subsequently closed. In addition to its inhibitory role in the oligomerization of monomeric aerolysin, zinc ions are able to close the channel by binding to a site within the channel lumen [478]. Using different technical analysis, the inner diameter of the channel was determined to range between 17 to 40 Å [433, 479]. In contrast to some other pore-forming toxins, no repair of plasma membrane lesions has been observed. This may be due to a high stability of the channel, its resistance to proteolysis and degradation. Alternatively, aerolysin might not be internalised by cells and therefore not be removed from the surface.

As measured with viability tests, cells can survive for several hours, depending on the aerolysin concentration. Few minutes after channel formation, cells start swelling and very often seem to die from an osmotic shock. Meanwhile, in some cell types, death seems to be induced by apoptosis, suggesting that underlying complex signals are involved in cell destruction. In T cells, degradation of genomic DNA, one of the property of apoptosis has been observed. This process could be overcome by overexpression of the anti-apoptotic protein Bcl-2. Aerolysin-induced apoptosis was suggested to be due to a massive entry of calcium.

As shown by Abrami et al., the interaction of aerolysin with a variety of polarized and non-polarized epithelial cells results in vacuolation of the ER, but does not affect other

intracellular compartments. This phenomenon in turn causes selective disorganization of early biosynthetic membrane dynamics, which led to an arrest in transport of newly synthesized proteins out of the ER. Although ER vacuolation can be observed for some forms of apoptosis, no degradation of genomic DNA was detected and moreover, vacuolation could not be prevented by Bcl-2 overexpression. But vacuolation was inhibited by ATP depletion of cells or depolymerization of the microtubule network, indicating that the process is dependent on the dynamic properties of ER membranes. It is not clear how this inhibition is achieved and several investigations remain to be perform to better understand how and why the ER vacuolation occurs. Interestingly, two other toxins (the *Serratia marcescens* hemolysin and the *Vibrio cholera* El tor hemolysin) have recently been described to also induce massive intracellular vacuole formation [480, 481].

In addition to these modifications, channel formation induces a number of cellular responses. Krause et al. have shown that aerolysin triggers release of calcium from the ER in human granulocytes [482]. Moreover, this process could be inhibited by pre-treating cells with pertussis toxin or by treating cells with phospholipase Cβ inhibitor. This observation indicated that aerolysin channel formation had triggered the activation of G-proteins and the production of inositol(1,4,5)-triphosphate. Activation of G-proteins still remains an unclear signaling cascade. One possibility is that the aerolysin channels affect the integrity of lipid rafts, which have been implicated in modulating and integrating signaling events at the plasma membrane.

5.1.7 Problematic

During millions of years, bacteria, higher fungi, cnidairians, snakes, insects and other arthropods have developed specific line of defense by producing very potent molecules : cytolytic peptides or proteins. Studies of some of such bacterial molecules revealed similarities in their sequences and also in their mode of action. This was the case of aerolysin compared to the α-toxin from *Clostridium speticum*, the *Leishmania amazonensis* cytolysin a pore-forming toxin called leishporin or the leukotoxin from *Pasteurella haemolytica* [483-485]. Interestingly, this latest pore-forming toxin was characterized to induce intracellular calcium elevation and also to activate the transcription factor NF-κB in ruminant leukocytes

and platelets [484]. As a result of this activation, pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8 were produced. It was further demonstrated that NF-κB activation was dependent on the calcium elevation induced by the leukotoxin. As previously mentioned, aerolysin provokes the increase of calcium in granulocytes [482]. In this study, we investigated whether aerolysin could also activate NF-κB and induce secretion of pro-inflammatory proteins that could be involved in inflammation observed in infection with *Aeromonas hydrophila*. Aerolysin enters in contact with cells *via* the GPI-anchor of proteins present at the cell surface. Using CD14, a GPI-anchored recepteur able to bind an array of microbial molecules, we were interested in studying a new mechanism of action of a bacterial molecule.

5.2 Aerolysin activates cells *via* a NF-κB pathway

Aerolysin activates cells *via* a NF-κB pathway

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Abstract

Bacterial exotoxins can affect host cell survival by their direct cytotoxic effects. Here, we show that aerolysin, a pore-forming toxin from Aeromonas hydrophila stimulates human cells to secrete pro-inflammatory mediators. Promonocytic THP-1 cells secreted significant amounts of the IL-8 neutrophil chemokine when exposed to aerolysin. Aerolysin treatment of epithelial HEK 293 cells was associated with a NF-κB-dependent cell activation, as assessed by a classical E-selectin luciferase reporter assay. Cell activation was markedly increased by the overexpression of surface GPI-anchored molecules such as CD14, CD55 and a GPI-engineered version of the green fluorescent protein, that act as receptors for the toxin. Experiments with hypoosmolar milieu as well as the small concentrations of aerolysin used in our assays rule out the possibility that the observed effect was due to cell swelling. These observations are important with regards to the possible pathogenic role of the toxin, being pro-inflammatory in addition to be cytotoxic.

Introduction

Aerolysin is a pore-forming toxin [1] secreted by Aeromonas hydrophila and plays an important pathogenic role in infections caused by these bacteria. [2-5]. The toxin is produced as an inactive precursor that requires proteolytic cleavage of a C-terminal peptide [6,7]. The GPI moiety of several GPI-anchored molecules has now been recognized as the necessary cell surface receptor for aerolysin binding [8-10]. The active toxin then associates into heptameric rings which are inserted into host cell membrane, to form a pore permeable to water and ions ultimately leading to cell death. In addition, channel formation triggers a number of unexpected cellular events such as apoptosis in T cells [11], vacuolation of the endoplasmic reticulum in epithelial cells [10,12] as well as calcium release from endoplasmic reticulum in granulocytes due to the activation of G-proteins and the production of inositol (1,4,5)-triphosphate [13]. The mechanisms by which aerolysin "activates" cells remain to be unraveled.

Here, we show that aerolysin triggers secretion of the chemokine IL-8 from human cells *via* a NF-κB-dependent pathway. This process requires binding of aerolysin to GPI-anchored proteins present at the surface of host cells, and is independent of the aerolysin-induced cell swelling.

Material and Methods

Cells

Human promonocytic, CD14-negative, THP-1 cells (ATCC, Rockville, MD) were stably transfected with a plasmid encoding for a green fluorescent protein (GFP, Clontech) under the control of the –546 bp interleukin-8 (IL-8) promoter (pIL-8/GFP-THP-1 cells) [14]. These pIL-8/GFP-THP-1 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 10 U/mL penicillin, 10 µg/mL gentamicin and 0.5 mg/mL G418 (= " culture medium "). To increase the surface expression of glycosylphosphatidyl inositol (GPI)-anchored CD14, pIL-8/GFP-THP-1 cells were differentiated with 10^{-7} M 1,25 $(OH)_2$ vitamin D_3 (VD3, Roche Pharma, Basel, Switzerland) in culture medium without G418 for 72 hr [15]. pIL-8/GFP-THP-1 cells differentiated or not with VD_3 did not express GFP unless stimulated with "pro-inflammatory" mediators such as LPS (unpublished data). VD3 differentiation (48 – 72 hrs) induces a marked upregulation of the GPI-anchored CD14 in THP-1 cells and confers LPS responsiveness [16]. Complementary DNAs encoding for GPI-anchored proteins (GPI-CD14, GPI-CD55, a GPI-engineered version of the green fluorescent protein, GPI-GFP) and for a transmembrane form of CD14 (tm-CD14) were used for experiments of transient transfection of HEK 293 cells. Briefly, these transfections were carried out using the Effecten™ transfection reagent. These cells were co-transfected with plasmids encoding firefly luciferase under the control of an E-selectin minimal promoter containing 3 NF-κB consensus sequences [17] and a plasmid encoding for a constitutively expressed renilla luciferase driven by the TK promoter (Promega). After 24 hr, the level of surface expression of these molecules in transient transfectants were determined by flow cytometry using the following antibodies: 63D3 (ATCC) for human CD14, MEM 118

(provided by V. Horejsi, Praha, Czechoslovakia) for human CD55 (decay accelerating factor, DAF), and a rabbit anti-GFP polyclonal antibody for GPI-GFP (provided by Van der Goot G), and appropriate PE-conjugated secondary antibodies. Controls included cells transfected with empty vectors. Cells were used 24 hrs after transfections.

Stimulation of pIL-8/GFP-THP-1 cells with aerolysin

Just before the assay, proaerolysin was cleaved into aerolysin with 2.5 μg/mL of trypsin (10 minutes at room temperature). Trypsin was inactivated with 12 μg/mL of trypsin inhibitor (Sigma). 3 x 10^5 cells/mL VD_3-differentiated pIL-8/GFP-THP-1 cells were stimulated at 37 °C with various concentrations of aerolysin in RPMI 1640 medium containing 2% FBS (= " stimulation medium "). At times 0, 1, 3, 6, and 24 hrs, 200 μL of conditioned supernatant were collected and kept frozen to further assay the secretion of IL-8 by ELISA (Endogen). Aerolysin-induced cell death was monitored using propidium iodide (PI, PharMingen) staining. GFP expression and PI cell uptake were analyzed by flow cytometry within the same sample using FL2 and FL3 channels, respectively. To control for inducibility of GFP under the control of the IL-8 promoter, cells were treated in parallel with 100 ng/mL LPS O111:B4 E. Coli LPS (List Biological Laboratories Inc., Campbell, CA). All cell activation experiments involving aerolysin were performed in the presence of 10 μg/mL polymyxin B in order to exclude a possible effect of contaminating LPS. In control experiments not shown here, polymyxin treatment of cells did not interfere with cell activation, and blocked LPS-dependent activation of these cells.

Stimulation of HEK 293 cells with aerolysin

HEK 293 cells expressing the GPI-anchored form of CD14, CD55, GFP, a chimeric transmembrane form of CD14, or transfected with their appropriate empty vectors were stimulated with aerolysin at various concentrations for 6 hr. Cells were then lysed and firefly and renilla luciferase activities were measured in 20 µL of cell lysates according to the Promega protocol [18]. Inducible luciferase activity in one sample was calculated using the ratio of: firefly luciferase/renilla luciferase activities. Fold induction was defined as the ratio of test sample/control sample inducible luciferase activities. Experiments were done in triplicates and results were expressed as mean of fold induction ± 1SD.

Hypo-osmotic shock

HEK 293 cells or pIL-8/GFP-THP-1 cells were submitted to a 6 hr hypo-osmotic shock by adding increasing concentrations of endotoxin-free dH_2O to the stimulation medium. Cell stimulation induced by osmotic shock was tested using the above-described double luciferase reporter gene assay (293 cells) and by the secretion by these cells of IL-8 (THP-1 cells) in the milieu. The cell viability was measured using a MTT assay [19] (Sigma). Briefly, MTT (yellow) is incorporated into mitochondria of live cells where it concentrates and crystallize (violet dye). Cell viability correlates with the intensity of the violet color after cell lysis, measured by spectrophotometry (570 nm).

Results

Aerolysin activates THP-1 cells

In order to study aerolysin induced cell activation, we have first used the well characterized cell line THP-1. As shown in Fig. 1A, this cell line is sensitive to the toxin as witnessed by a dose- and time-dependent induction of cell death. Interestingly, aerolysin treatment resulted in the secretion of the IL-8 chemokine in a dose- and time-dependent manner (Fig. 1B). This effect was most probably related to an augmented IL-8 gene transcription, since aerolysin induced the activation of the IL-8 promoter in our pIL-8/GFP reporter system (Fig. 1C). The maximum IL-8 secretion was observed with 2ng/mL (40 pM) aerolysin and corresponded to ~ 5 % of the secretion obtained with 100 ng/mL LPS, which is a strong agonist in this THP-system (data not shown). Aerolysin incuded IL-8 secretion was not due to LPS contamination of the toxin sample since polymixin B has no effect the chemokine production. These observations indicating that in addition, and most probably prior to, its cytotoxic action aerolysin could activate mammalian cells and promote the secretion of the pro-inflammatory IL-8 chemokine.

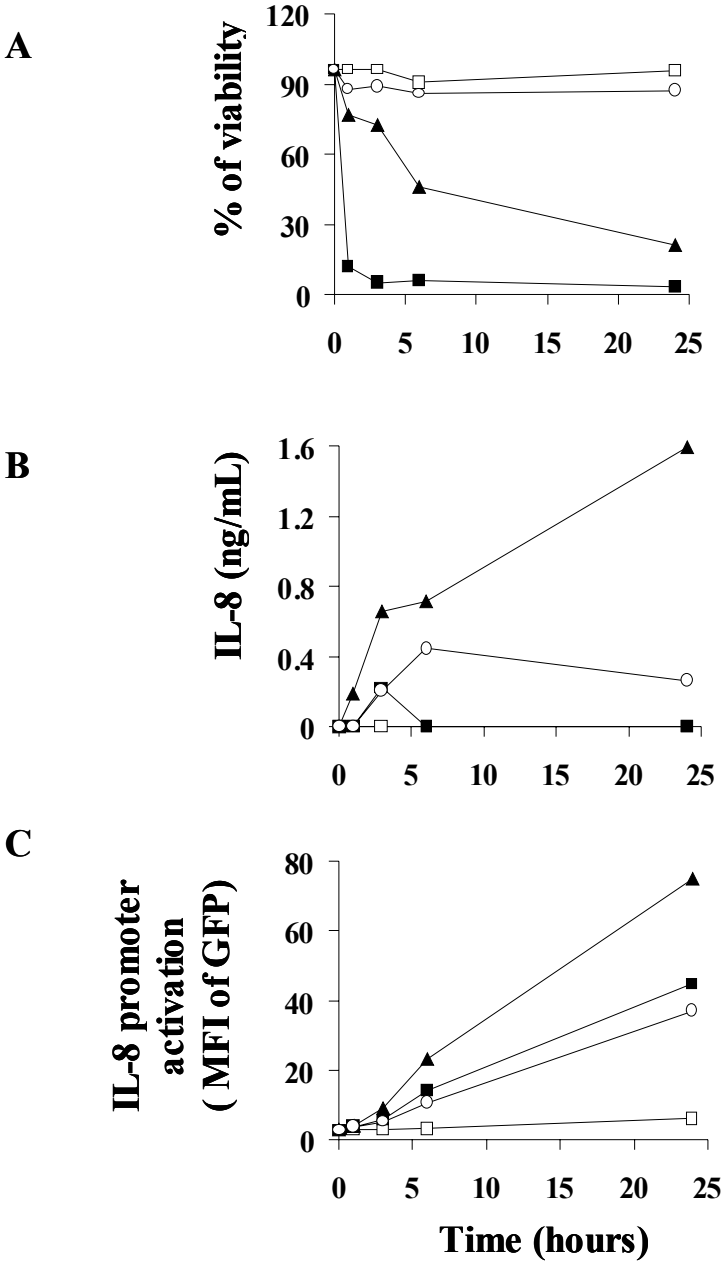

Figure 1. **Cell viability and IL-8 secretion by THP-1 cells.** Calcitriol-differentiated pIL-8/GFP-THP-1 cells were incubated for 24 hr with 0.5 (open circle), 2 (closed triangle), 10 ng/mL (closed square) aerolysin, or milieu only (open square). **(A)** Cell viability, as measured by propidium iodide staining, expressed as % viable cells; **(B)** IL-8 protein secretion in the supernatants of stimulated THP-1 cells; and **(C)** IL-8 promoter activation induced by aerolysin, measured by the capacity of aerolysin to induce the transcription of the GFP gene under the control of an IL-8 promoter construct, and expressed as mean fluorescence index, arbitrary units (see methods).

Cell surface molecules important for aerolysin activation

Channel formation by aerolysin in mammalian cells requires the presence of cell surface GPI-anchored proteins. In order to investigate whether the presently observed activation process was also GPI dependent, HEK 293 cells were transiently transfected with plasmids encoding for GPI-CD14 or, as a control, a transmembrane chimeric form of CD14 (tm-CD14). HEK293 cells were used because they do not express CD14-endogeously. Using a classical NF-κB reporter assay (minimal E-selectin-1 promoter driving the firefly luciferase), we found that even in the absence of transfection, HEK 293 cells were responsive to aerolysin. Aerolysin-dependent cell activation was markedly enhanced by the overexpression of GPI-CD14 but not by the expression of similar amounts of tm-CD14 (Fig. 2), in contrast to LPS sensitivity, which is promoted by GPI-CD14 as well as its transmembrane counterpart [20]. As a control, we have tested that for all transfected HEK 293 cells the response to TNF-α, a classical NF-κB agonist, was the same (data not shown).

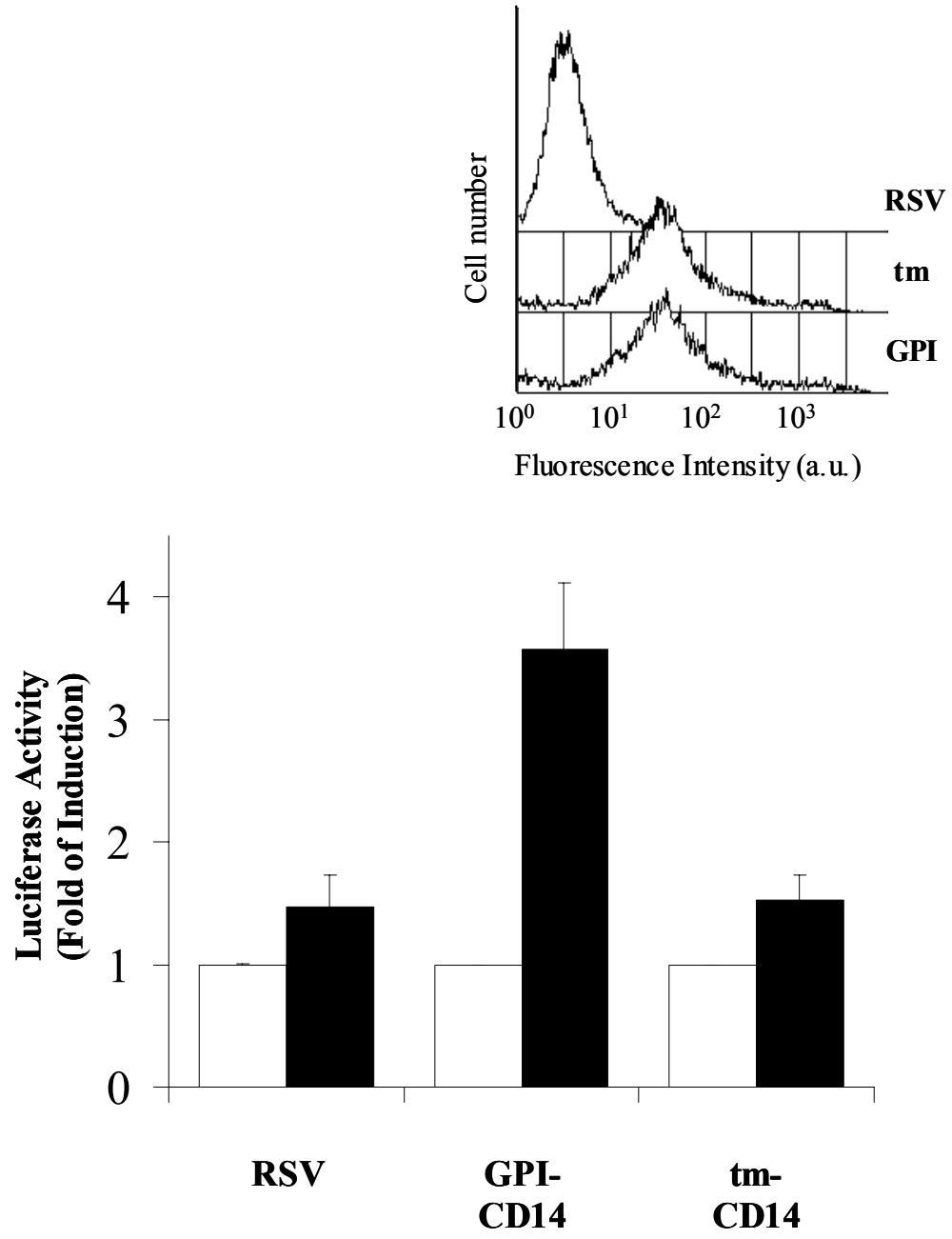

Figure 2. **Aerolysin-induced cell activation in cells overexpressing CD14.** HEK 293 cells were transiently transfected with plasmids encoding for a transmembrane (tm-) form of CD14, a GPI version of CD14 or an empty vector, together with a plasmid encoding for luciferase, driven by a minimal ELAM promoter. 24 hr post-transfection, cells were incubated for 6 hr with 2 ng/mL aerolysin (black bars) or medium only (white bars). **Insert.** Flow cytometry analysis of the surface expression of tm-CD14 and GPI-CD14 in transiently transfected HEK 293 cells after 24 hr. Cell fluorescence intensity was expressed as arbitrary unit (a.u.).

To rule out a role of CD14 itself in aerolysin induced activation process, the effects of over expression two other GPI-anchored proteins, CD55 (GPI-DAF) and a GPI-engineered version of the green fluorescent protein (GPI-GFP), were analyzed. Control HEK 293 cells expressed basal levels of endogenous GPI-DAF, but as expected neither CD14 nor GPI-GFP could be detected (Fig. 3A). Over-expression of both CD55 and GPI-GFP led to an increase sensitivity to aerolysin (Fig. 3B). The more pronounced effect of GPI-GFP is likely due to a higher degree of expression. These experiments further support the requirement for a GPI anchor-protein, independently of the polypeptide moiety, to observe aerolysin-dependent cell activation.

A

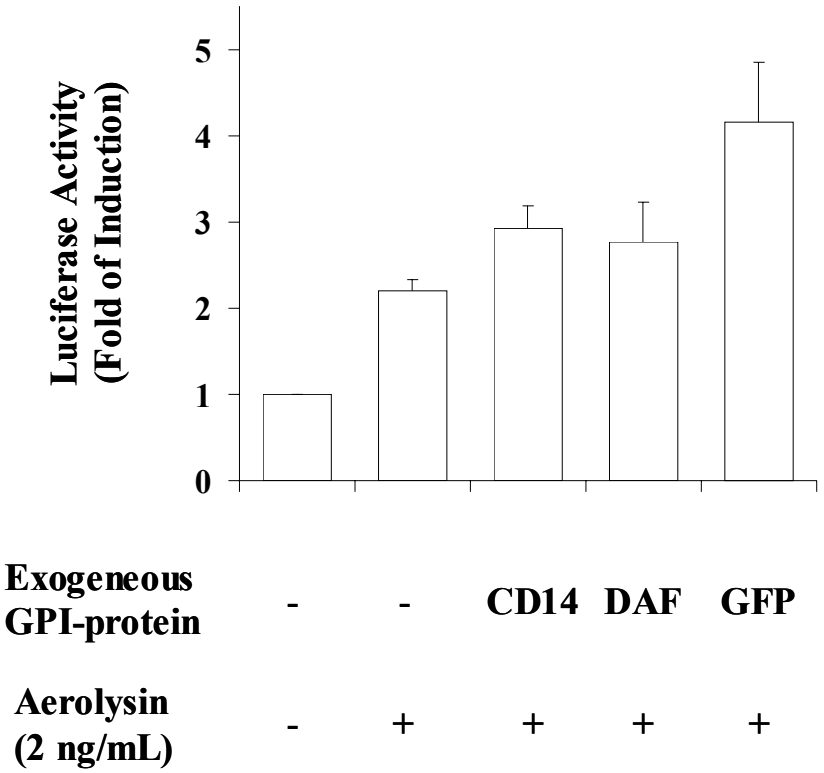

Figure 3. **Panel A. Overexpression of GPI-anchored proteins increases cell stimulation by aerolysin.** HEK 293 cells were transiently transfected with plasmids encoding for GPI-CD14, GPI-CD55 (DAF), GPI-GFP or an empty vector. 24 hr post-transfection, cells were incubated with aerolysin (2 ng/mL; black bars) or medium only (white bars) for 6 hr.

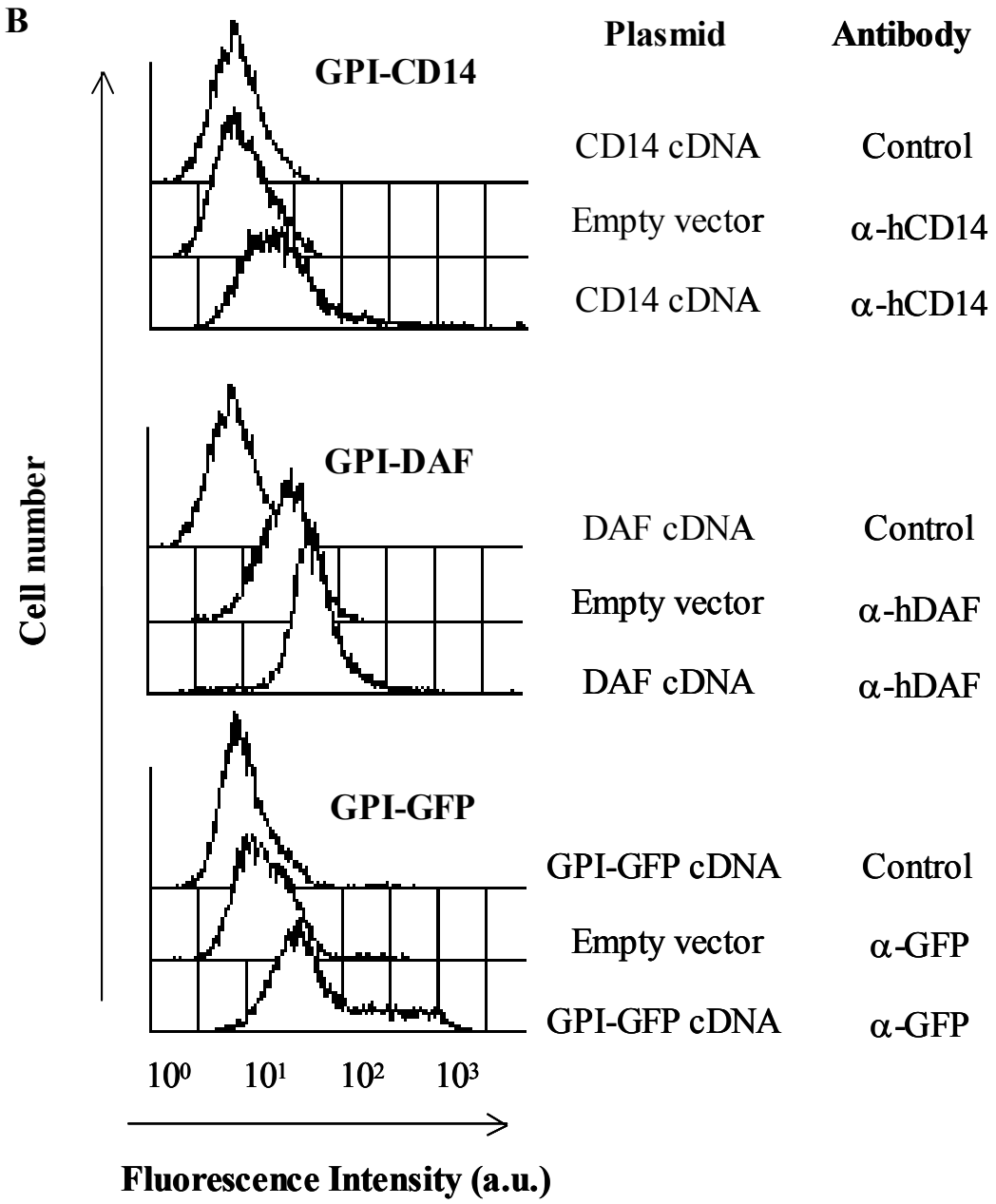

Figure 3. **Panel B. Flow cytometry analysis of the surface expression of GPI-CD14, GPI-CD55 (DAF), GPI-GFP in transiently transfected HEK 293 cells.** Cells were labeled with specific antibodies against the GPI proteins and a phycoerythrin-coupled secondary antibody. Cell fluorescence intensity was expressed as arbitrary unit (a.u.).

Hypo-osmotic shock does not reproduce aerolysin-dependent cell activation

Since we observed that aerolysin triggered cells swelling and since cell swelling has been associated in some cell systems with cell activation [21,22], we next investigated whether HEK 293 cells could be activated by a hypo-osmotic shock. Cells, expressing or not CD14, were submitted to culture media increasingly diluted with distilled water (dH_2O). Whereas cell swelling, in still viable cells, was clearly observed upon exposure to 60-80% dH_2O concentration, we were unable to detect NF-κB activation using our luciferase reporter assay (Fig. 4). In contrast, treatment with 2 ng/mL aerolysin induced NF-κB-dependent cell activation in a CD14 (or GPI) dependent manner (Fig. 4). Similar observations were made using VD3-differentiated THP-1 cells submitted to a hypo-osmotic shock: aerolysin treatment induced cell swelling and cell activation (IL-8 secretion) whereas the osmotic shock only lysed the cells without any IL-8 release (data not shown). These observation rule out the possibility that aerolysin activates cells due to osmotic swelling since this pathway does not appear to operate in HEK293 and THP-1 cells.

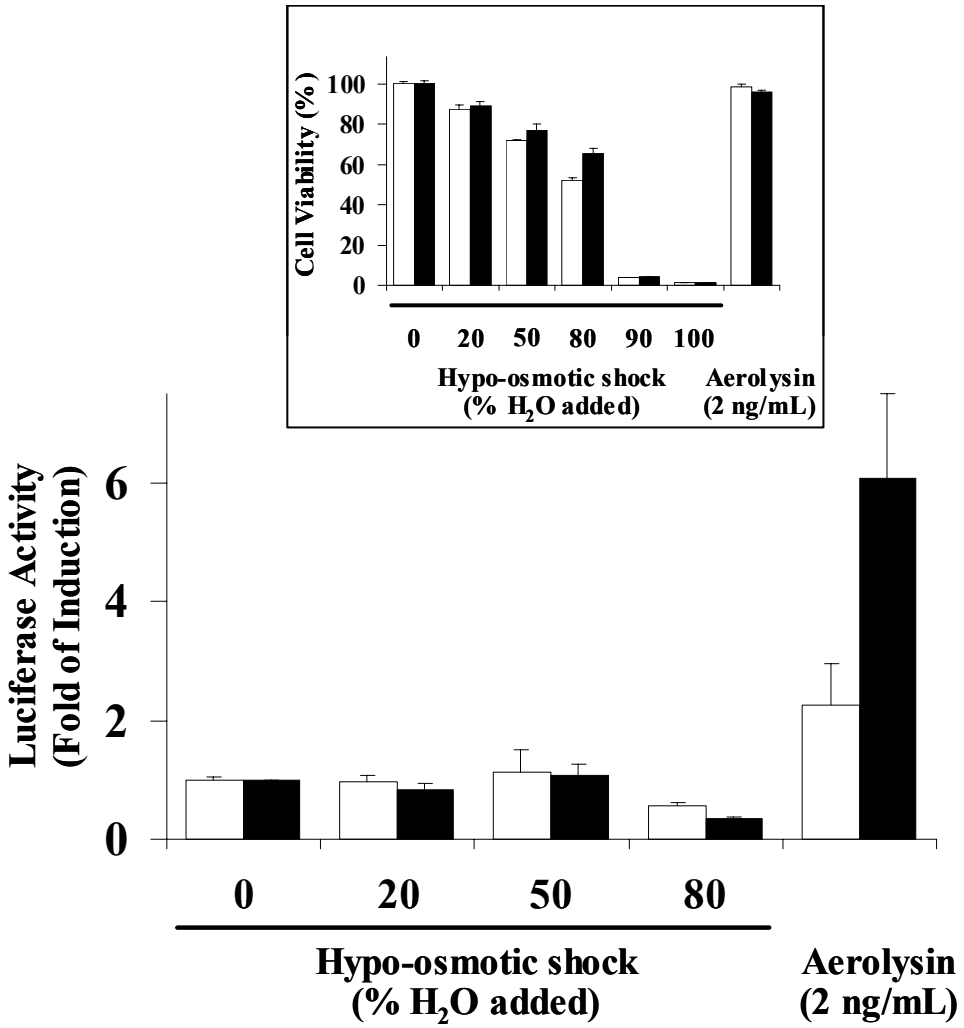

Figure 4. Aerolysin but not hypoosmotic shock induces cell activation *via* a NF-κB-dependent mechanism. HEK 293 cells were transiently transfected with plasmids encoding for GPI-CD14 (black bars) or with an empty vector (white bars). Cells were treated for 6 hrs with a hypoosmolar media obtained by increasing the percentage of water in the culture medium. Cells were also incubated with 2 ng/mL aerolysin in iso-osmolar medium; GPI-CD14-transfected cells (black bars) and HEK293 cells tranfected with an empty vector (white bars) for 6 hr. **Insert**. Effect of hypoosmolar milieu and of aerolysin on cell viability. In this experiment, cell viability was tested using a MTT assay (see methods).

Discussion

The pore-forming ability of aerolysin and other pore-forming toxins has been extensively analysed and is fairly well understood at the molecular level [23]. The biological consequences of pore-formation have however not been fully estimated. Some effects have been described for aerolysin such as G-protein activation [13], ER vacuolation [10] or induction of apoptosis [24]. Here we show that aerolysin triggers the secretion of IL-8, an important recruitment factor for polymorphonuclear neutrophils. Chemokine production was found to be paralleled by transcriptional activation *via* NF-κB.

The activating effects of aerolysin were observed at concentrations that were not cytotoxic. The mechanism underlying aerolysin induced NF-κB activation and chemokine production however remains unclear. The present transfection experiments clearly underline the importance of GPI-anchored proteins. These molecules could either be directly implicated in the signaling process [25] or exclusively act as aerolysin receptors. Several models for cell activation by aerolysin could be proposed: 1) oligomerized aerolysin bound to GPI molecules could induce crosslinking of these receptors, calcium mobilization and subsequent NF-κB activation; 2) the formation of a pore by aerolysin and the flux of ions may trigger cell activation; and 3) aerolysin bound to GPI molecules could encounter another signaling molecule and induce transmembrane signaling. Examples of the latter can be found in the interaction of other bacterial molecules, such as lipopolysaccharide (LPS) with its receptor; LPS receptor is a complex made of the GPI-anchored CD14 glycoprotein, and a transmembrane signaling complex, the Toll-like receptor (TLR)-4/MD-2 complex [26]. Although it is tempting to speculate that heptamer formation by aerolysin could promote clustering of GPI-anchored proteins and thereby cell activation, we favor the hypothesis that

cell activation is a consequence of channel formation. It has indeed previously been shown that aerolysin is able to activate G-proteins and intracellular calcium release *via* the IP3 pathway. This process absolutely required channel formation [13]. Similarly Nelson and co-workers found that aerolysin induced apoptosis of T-lymphocytes only occurred upon channel formation and that heptamer formation was not sufficient [11]. It has in addition been observed, during the course of the present study, that other pore-forming toxins such as Staphyloccocal α-toxin can activate cells *via* NF-κB and that membrane permeabilization was crucial [27]. Chemokine production was also observed with other pore-forming toxins [28-30]. Finally, since the NF-κB signaling pathway has been implicated in different types of stress [31], it is not unlikely to be triggered upon membrane damage. Nevertheless, it is important to highlight the fact that cell activation was independent on cell swelling caused by an hypo-osmotic shock following the aerolysin induced-membrane permeabilization.

The herein described the ability of aerolysin to trigger transcriptional activation and the production of pro-inflammatory mediators may represent a significant pathway in the pathogenesis of bacterial infections due to Aeromonas hydrophila.

Acknowledgments

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References

[1] Abrami, L., Fivaz, M. and van Der Goot, F.G. (2000) Trends Microbiol 8, 168-172.

[2] Donta, S.T. and Haddow, A.P. (1978) Infection and Immunity 21, 989-993.

[3] Daily, O.P. et al. (1981) J Clin Microbiol 13, 769-77.

[4] Janda, J.M., Clark, R.B. and Brenden, R. (1985) Current Microbiology 12, 163-168.

[5] Chakraborty, T., Huhle, B., Berghauer, H. and Goebel, W. (1987) Infection and Immunity 55, 2274-2280.

[6] Howard, S.P. and Buckley, J.T. (1985) Journal of Bacteriology 163, 336-340.

[7] van der Goot, F.G., Lakey, J.H., Pattus, F., Kay, C.M., Sorokine, O., Van Dorsselaer, A. and Buckley, T. (1992) Biochemistry 31, 8566-8570.

[8] Nelson, K.L., Raja, S.M. and Buckley, J.T. (1997) The Journal of Biological Chemistry 272, 12170-12174.

[9] Diep, D.B., Nelson, K.L., Raja, S.M., McMaster, R.W. and Buckley, J.T. (1998) The Journal of Biological Chemistry 273, 2355-2360.

[10] Abrami, L., Fivaz, M., Glauser, P.-E., Parton, R.G. and van der Goot, F.G. (1998) Journal of Cell Biology 140, 525-540.

[11] Nelson, K.L., Brodsky, R.A. and Buckley, J.T. (1999) Cellular Microbiology 1, 69-74.

[12] Abrami, L., Fivaz, M., Decroly, E., Seidah, N.G., François, J., Thomas, G., Leppla, S., Buckley, J.T. and van der Goot, F.G. (1998) J Biol Chem 273, 32656-32661.

[13] Krause, K.H., Fivaz, M., Monod, A. and van der Goot, F.G. (1998) J Biol Chem 273, 18122-9.

[14] Oliveira, I.C., Mukaida, N., Matsushima, K. and Vilcek, J. (1994) Mol Cell Biol 14, 5300-8.

[15] Vey, E., Zhang, J.H. and Dayer, J.M. (1992) J Immunol 149, 2040-6.

[16] Martin, T.R., Mongovin, S.M., Tobias, P.S., Mathison, J.C., Moriarty, A.M., Leturcq, D.J. and Ulevitch, R.J. (1994) J Leukoc Biol 56, 1-9.

[17] Schindler, U. and Baichwal, V.R. (1994) Mol Cell Biol 14, 5820-31.

[18] Sherf, B.A. (1996) Promega Notes 57, 2-9.

[19] Mosmann, T. (1983) J Immunol Methods 65, 55-63.

[20] Pugin, J., Kravchenko, V.V., Lee, J.D., Kline, L., Ulevitch, R.J. and Tobias, P.S. (1998) Infect Immun 66, 1174-80.

[21] Tilly, B.C., Gaestel, M., Engel, K., Edixhoven, M.J. and de Jonge, H.R. (1996) FEBS Lett 395, 133-6.

[22] Kim, R.D., Darling, C.E., Cerwenka, H. and Chari, R.S. (2000) J Surg Res 90, 58-66.

[23] van der Goot, F.G. (2001) in: Current Topics in Microbiology and Immunology, Vol. 257 Springer Verlag, Berlin Heidelberg.

[24] Nelson, K.L. and Buckley, J.T. (2000) J Biol Chem 275, 19839-43.

[25] Ilangumaran, S., He, H.T. and Hoessli, D.C. (2000) Immunol Today 21, 2-7.

[26] Aderem, A. and Ulevitch, R.J. (2000) Nature 406, 782-7.

[27] Dragneva, Y., Anuradha, C.D., Valeva, A., Hoffmann, A., Bhakdi, S. and Husmann, M. (2001) Infect Immun 69, 2630-5.

[28] Konig, B., Koller, M., Prevost, G., Piemont, Y., Alouf, J.E., Schreiner, A. and Konig, W. (1994) Infect Immun 62, 4831-7.

[29] Chopra, A.K., Xu, X., Ribardo, D., Gonzalez, M., Kuhl, K., Peterson, J.W. and Houston, C.W. (2000) Infect Immun 68, 2808-18.

[30] Cockeran, R., Theron, A.J., Steel, H.C., Matlola, N.M., Mitchell, T.J., Feldman, C. and Anderson, R. (2001) J Infect Dis 183, 604-11.

[31] Bours, V., Bonizzi, G., Bentires-Alj, M., Bureau, F., Piette, J., Lekeux, P. and Merville, M. (2000) Toxicology 153, 27-38.

6 GENERAL DISCUSSION

6.1 Internalization of LPS

6.1.1 Internalization pathways

The first aim of our studies was to investigate the LPS endocytic pathway, and the possible role of the receptor CD14 in this process. We found that LPS was internalised by a macropinocytotic pathway in a CD14-dependent manner. This pathway resembles that of phagocytosis observed by Kang et al for LPS uptake in monocytes and macrophages [250]. Interestingly, CD14 was also found to be implicated in phagocytosis of Gram-negative bacteria [24, 486]. The LPS endocytosis pathway as well as the involvement of CD14 in LPS internalization appeared to be two controversial points. In some studies, it could be demonstrated that LPS internalization was dependent on CD14, whereas other works indicated that it was a CD14-independent process. Vasselon et al. published that internalization of monomeric LPS occured after its transfer out of cell surface CD14 [294]. Following endocytosis, fluorescently-labeled LPS was found intracellularly near the cell surface in small vesicles that did not contain CD14. At later stages, LPS was observed in larger, punctate structures identified as the Golgi apparatus. Conversely, LPS aggregates were found to be internalised in association with CD14 [294]. Likewise in the study of Kitchens et al., CD14 was shown to internalise LPS in monocytes *via* at least two pathways, different from that we discovered in our study [487]. The bulk of LPS appeared to enter non-coated tubular invaginations of the plasma membrane, whereas endocytosis by clathrin-coated pits was found to be a minor pathway. Entry of LPS into these non-coated caveolae-like invaginations was related to the observation that LPS bound to mCD14 in low-density lipid enriched domains of the plasma membrane that have properties similar to those of caveolae [487]. These observations are consistent with our own work. Using sucrose density gradients isolating lipid “rafts”, CD14 was found in lipid-rich domains. The targeting of CD14 into these specific regions of the plama membrane was related to the GPI anchor. Furthermore, we found that CD14 was more concentrated on microvilli or ruffles at the cell surface, the typical morphological structures involved in phagocytosis and macropinocytosis. Similarly to our

results, electron microscopic images by Kang et al. revealed the presence of biotin-LPS on such cell surface protrusions and then in phagocytic vacuoles after internalization [250]. These vacuoles thightly resembles the large vesicular structures with which gold-labeled CD14 was observed to be associated during internalization [250]. Altogether, these results suggest that internalization of LPS occurs by several pathways. Furthermore, the physical state of LPS, monomeric versus aggregate LPS, is likely to greatly influence the route and the CD14-dependence of LPS endocytosis.

6.1.2 Influences on internalization

Since CD14 was discovered to be anchored by a GPI, it was wondered whether this specific anchor could play a role in cell activation. Until now, no difference could be observed in the LPS response of cells bearing the GPI-anchored CD14 and those bearing a chimeric transmembrane form of CD14 [25]. In our study, we addressed the possible role of the CD14 GPI-anchoring system for LPS internalization. Using CD14 anchored *via* a classical GPI anchor or an engineered transmembrane version, we concluded that the anchor of CD14 did not influence the pathway and also the rate of LPS endocytosis. Likewise, the rate of CD14 internalization, quantified by electron microscopy studies, was not modulated by the CD14 anchor. In contrast, different types of external stimuli can modulate LPS endocytosis. Interference in the CD14-mediated LPS internalization could be observed in several studies. Indeed, the addition of anti-LPS monoclonal antibodies modifies the route of LPS endocytosis by decreasing LPS uptake by CD14, while promoting complement-mediated opsonic uptake through membrane-associated complement receptor (CR) 1 or other cell receptors [488-490]. Similarly, it was demonstrated in a recent study that monoclonal antibodies raised against the deep-core of lipid A or the O-linked polysaccharide moieties of LPS accelerated internalization and detoxification of LPS without stimulating cytokine release, a process mediated by Fc-receptors [491]. Another phenomenon that influences LPS internalization was described by Ding et al. These authors suggested that secretory leukocyte protease inhibitor (SLPI) interfered with uptake of LPS by macrophages. This inhibitory effect of SLPI may have, in part, been due to the blockade by SLPI of LPS transfer to sCD14 and its interference with LPS uptake from LPS-sCD14 complexes in macrophages [492].

Despite influences of external stimuli on endotoxin endocytosis, LPS is able to regulate its own internalization, and also the ingestion of microbes and other particles. Two of the most characteristic properties of monocytes/macrophages are their capacity to take up large volumes by fluid-phase pinocytosis and to ingest microbes and other particles by phagocytosis. Effects of LPS on internalization processes have been reported in these cells. Peppelenbosch et al. showed that LPS regulated macrophage fluid phase pinocytosis *via* CD14-dependent and CD14-independent pathways. Indeed, LPS was shown to inhibit fluid phase pinocytosis at low concentrations, but at higher concentrations stimulated pinocytosis [493]. In addition to its role in the route of LPS endocytosis as mentioned in the previous section, the physical state in which LPS is presented to cells can influence its own initial rate of internalization. Indeed, the larger the LPS aggregates are, the faster they internalise into cells [494]. However, for each LPS physical state, LPS is internalised by a constitutive mechanism. These observations are consistent with our results showing that LPS is constitutively internalised in a CD14-dependent manner, and does not modulate its own endocytosis [429]. This conclusion is related to the fact that no difference was observed in the rate of CD14 internalization in the presence and in the absence of LPS.

6.1.3 Relationship between LPS signaling, LPS internalization and intracellular traffic

As mentioned above, a large majority of LPS molecules that bind to CD14 will eventually internalise. The reason for this process is to sequester LPS and limit its interactions with other cells. The relationship between LPS internalization and LPS signaling has been a matter of controversy. Some investigators proposed that internalization or endocytic movement of LPS may play a role in LPS-induced signaling. For example, Detmers et al. demonstrated that LPS internalization and early endosomal fusion may be required for signal transduction [179]. Moreover, it was reported that macrophages from C3H/HeJ mice genetically hyporesponsive to LPS exhibit defective endocytic uptake of LPS and ceramide, and point to a role of vesicular transport for responses to these mediators [495]. Subsequently, the same investigators published that LPS from *Rhodobacter sphaeroides* (RsLPS), a non-stimulatory LPS antagonist, exhibited defective traffic in normally LPS-responsive cells, and inhibited intracellular traffic of a stimulatory form of LPS [177]. In contrast, several studies supported the notion that LPS internalization and LPS signaling were independent processes.

It was for example shown that LPS partial structures inhibited responses to LPS in a human macrophage cell line without influencing LPS uptake by a CD14-mediated pathway [346]. Furthermore, Gegner et al. concluded that while LPS signal transduction and LPS clearance utilized both LBP and mCD14, the pathways bifurcated after LPS binding to mCD14 [114]. From these studies, it could be concluded that the bulk of LPS bound to CD14 was internalised, sequestrated and disposed, whereas only a small fraction of it was involved in signaling interactions at the cell surface. Consistent with these observations, our results showed that LPS-induced IL-8 production in THP-1 cells was not inhibited using cytochalasin D, a specific microfilament-disrupting agent, which prevented LPS and CD14 internalization. This observation strongly suggests that LPS-induced cell activation and LPS endocytosis are dissociated events. Another study reporting that LPS internalization occurs with identical kinetics in C3H/HeJ (non-LPS responding mouse strain) and C3H/HeN (LPS responding mouse strain) macrophages argues for this theory [494].

6.2 Role of CD14 and TLRs in LPS-induced activation and internalization in epithelial cells

Epithelial cells frequently represent the first line of defense against invading microorganisms due to their barrier function and to an array of antimicrobial molecules that they can produce. A better understanding of the involvement of epithelial cells in the host immune response would help us to prevent some infections leading to sepsis, and also some chronic diseases. Since our study, other investigators reported the constitutive expression of CD14 gene and protein in some intestinal and liver epithelial cells considered as CD14-negative cells [496]. In addition, sCD14 was described to be produced by some of these cells [131, 496-499]. CD14 is an important receptor that binds LPS and acts on epithelial cells *via* a soluble-dependent manner. Following the identification of some epithelial cells unresponsive to LPS, we found that these cells overexpressing CD14 after transfection remained resistant to LPS. We thus concluded that CD14 was not sufficient for LPS-induced cell activation and hypothesized that these cells were deficient in a molecule of the proximal LPS-dependent signaling pathway. The newly discovered TLR2 and TLR4 transmembrane proteins, demonstrated to be involved in LPS signaling, were proposed to be potential candidates. Several articles recently reported the presence of TLRs in intestinal and liver epithelial cells. Hepatocytes were shown to express TLR1 through TLR9 as well as MyD88 and MD-2

transcripts. Furthermore, these cells were shown to be activated by nanogram concentrations of LPS through a TLR4 response pathway [500]. Considering the LPS unresponsiveness of the HepG2 cell line used in our studies, our results appear to be in contradiction with such findings. To explain this discrepancy, we might point out that HepG2 cells are tumoral cells and could hypothesize that this cell line is mutated for either TLR4 or MD-2 protein. Mutation in the MyD88 gene seems to be less probable, since our HepG2 cells are responsive to IL-1. Indeed, the IL-1 receptor interacts with the MyD88 molecule by its intracytoplasmic tail (TIR domain) to activate the NF-κB signaling cascade [501]. To the opposite, MyD88 gene mutation could have occurred or a splice variant of MyD88 could be expressed in colonic T84 cells unresponsive to both LPS and IL-1, but not to TNF-α [501]. Given the function of hepatocytes which metabolise and clear molecules from the bloodstream, it is not surprising that these cells express a pattern of recognition receptors such as CD14 and TLRs. Furthermore, recent papers have shown that LPS treatment of hepatocytes induced the upregulation of CD14 mRNA and protein levels as well as TLR2 mRNA at the transcriptional level [131, 498, 502, 503].

Regarding intestinal epithelial cells, the constitutive expression pattern of CD14, TLRs (TLR2-5) receptors and MD-2, as well as LPS responsiveness differs from one cell type to another and also according to pathological disorders [413, 504, 505]. Surface expression of these receptors can be modulated by LPS and some cytokines [506]. Furthermore, TLRs appeared to activate specific intracellular signal transduction pathways in each intestinal epithelial cell type [413]. Responsiveness of these cells to LPS was recently shown to be correlated with TLR4, but not TLR2 or CD14 expression [499]. The IL-8 production by SW620 cells in response to LPS in a soluble CD14 dependent manner we observed is thus probably due to the presence of TLR4 and MD-2 expressed by these cells [507].

Many investigations have shown that, after interaction with the plasma membrane, LPS is endocytosed into myeloid cells to be cleared. In our study, we found that intestinal and liver epithelial cells could also internalise LPS. Hornef et al. confirmed this result and in addition, demonstrated that internalised LPS colocalized with TLR4 residing in the Golgi apparatus of intestinal epithelial cells [508]. In hepatocytes, LPS was shown to internalise *via* lipoprotein receptors and to traffic through the same endosomal pathway employed for the catabolism of triglyceride-rich lipoporoteins [509]. This result could provide an explanation to the so low CD14-dependence in LPS internalization we observed with HepG2 cells.

The lumenal surface of the colonic epithelium is continually exposed to Gram-negative commensal bacteria and LPS. Recognition of LPS by TLR4 results in pro-inflammatory gene expression in diverse cell types. Normally, however, commensal bacteria and their components do not elicit an inflammatory response from intestinal epithelial cells. Several hypothetic scenario could be put forward and would probably be worth exploring : i) little or no CD14 and TLRs receptors would be constitutively expressed by these cells. However, their expression could be upregulated by specific signals if some pathogenic bacteria manage to invade the epithelium. Abreu et al.'s study argues in this direction, since native colonic epithelial cell lines were shown to express a low level of TLR4 and MD-2 mRNA. Th1 cytokines such as γIFN or TNF-α were demonstrated to initiate intestinal inflammation by increasing TLR4 and MD-2 mRNA leading to LPS reactivity [506]. Another paper reported that TLR4 were barely detectable in human primary epithelial cells of normal mucosa, but was strongly upregulated in inflammatory situations [504]. In contrast, other investigations indicated that decreased expression of TLR4 and MD-2 correlated with intestinal epithelial cell protection against dysregulated pro-inflammatory gene expression in response to LPS [414]. ii) TLRs positioned at the apical pole, would monitor the sensitive balance of the lumenal microbial array and thus would control the cell response. As reported by Cario et al., TLR2 and TLR4 were shown to be constitutively expressed at the apical pole of differentiated T84 cells. After stimulation with LPS or peptidoglycan, TLRs were observed to selectively traffic to cytoplasmic compartments near the basolateral membrane [510]. This article also raised the idea that TLRs targeting in intestinal epithelial cells can be modulated. iii) It is tempting to imagine that CD14 and TLRs could rather be present at the basolateral surface of and inside epithelial cells constituting the lumen. Indeed, TLRs, localised at these strategic sites, could detect bacteria which use endocytosis or disrupt the paracellular epithelial barrier to infect the tissue. Interesting findings on *Shigella flexneri* way of invasion of the colonic epithelium would argue for this latter scenario [511].

6.3 Relationship between the differential expression of some innate immune receptors : CD14, MD-2 and TLRs, and the LPS-induced cell activation at different differenciation steps of monocytic cells

With the discovery of TLRs, receptors involved in cell response to LPS and other microbial molecules, we were interested in studying the expression profile of these receptors in parallel to the response to LPS in monocytic cells at different stages of differentiation. In the first part of chapter II, the study was performed with promonocytic THP-1 cells, treated or not with various differentiating agents. A comparative study was undergone with human primary tissue macrophages, fully differentiated monocytic cells and also blood leukocytes. In the second part of the chapter, the study concerned the differentiation of monocytes into immature DCs which further matured after treatment with IL-1, TNF-α or LPS. Here, we tried to summarize in this diagram the results of both studies.

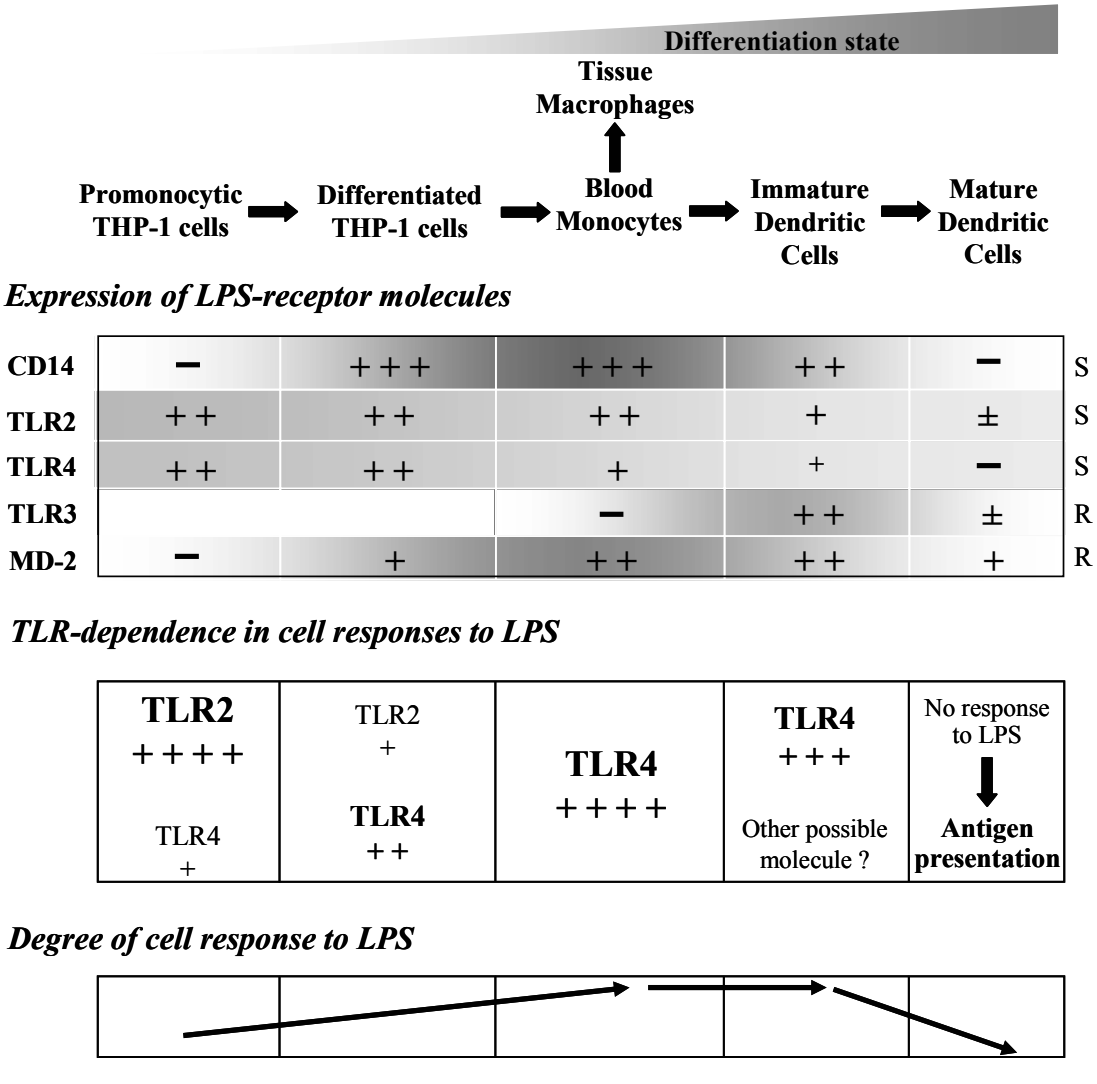

Figure 7: Expression of LPS-receptor molecules (S=cell membrane surface expression detected by FACS; R=mRNA expression measured by RT-PCR), TLR-dependence in and degree of cell response to LPS in myeloid cells at different stages of differentiation.

We found that the sensitivity of cells to LPS was acquired upon cell differentiation. This acquisition could be correlated with the appearance of MD-2 expression during the differenciation process, but not with the slight modulation of TLRs expression already present at the cell surface of undifferentiated myeloid cells. TLR receptors seem to be pivotal receptors in the innate immune defense. To support these results, Zarember et al. analyzed TLR expression in fractionated primary human leukocytes such as monocytes, granulocytes and indicated that professional phagocytes expressed the greatest variety of TLR mRNAs [512, 513].

In contrast, the loss of LPS-response in mature DCs could be correlated to the decrease of MD-2 mRNA expression, and the disappearance of TLR4 at the cell surface. The implication of TLR4 in the activation of immature DCs treated with LPS also resides in the partial blocking of cell activation with specific anti-TLR4 antibodies. In contrast, no blocking with anti-TLR2 antibodies was observed, suggesting that TLR2 was not implicated in LPS-induced activation of immature DCs. Using a mixture of anti-TLR2 and anti-TLR4 antibodies led to similar results to those obtained with anti-TLR4 antibodies only. This partial LPS inactivation led us to imagine that (an)other(s) possible component(s), as for instance TLR3 whose expression seems to be specific to immature DCs, as also found by Muzio et al., could be involved in LPS-induced DC activation. Recent papers have confirmed our results by describing identical modulation of TLRs expression during the differentiation process of monocytes into immature and then mature DCs [512, 514]. It was also unanimously accepted that TLR4 was responsible for LPS recognition as well as LPS-signal transduction in immature DCs, whereas TLR2 was involved in DC activation induced by Gram-positive bacterial products [514, 515]. DCs activation occurred through the activation of MAP kinase family members (ERK, p38), NF-κB and the secretion of various pro-inflammatory cytokines such as TNF-α, IL12p70 and IL-6 [516]. In several publications, immature DCs were described as cells with a large repertoire of TLRs, that enabled these cells to respond to a vast variety of stimuli. In addition to TLR2 and TLR4 involvement in DC activation, TLR9 turned out to be specialized in DC signal transducing for bacterial CpG DNA [517]. Although many TLRs led to comparable activation of NF-κB, striking differences in cytokine and chemokine gene transcription could be observed. Indeed, it was reported that some TLR agonists specifically promoted the production of cytokines which favored Th1-orientated immune

response, whereas other induced the release of cytokines producing conditions that were predicted to favor Th2 development [518]. Moreover, DC stimulation with different TLR agonists not only activated cells, but also orientated immature DCs towards maturation, and modulated the expression of many TLRs as shown in recent papers [512, 514, 516, 519].

Altogether these data suggest that myeloid cell precursors (i.e. promonocytes) evolve towards cells (i.e. monocytes, tissue macrophages or immature DCs) that have the capacity i) to sense their environment with the "pattern recognition" CD14 receptor and ii) to discriminate between microbial molecules such as LPS with their array of TLRs and MD-2, iii) to communicate with their environment by producing cytokines and other factors. After endocytosis and processing of "non-self" microbial molecules, these myeloid cells such as immature DCs evolve towards cells that loose the capacity of recognition with the important downregulation of CD14, some TLRs and MD-2 expression, and cell activation, but acquire the property of antigen-presentation to lymphocytes. This latter function as well as the kind of secreted cytokines create a link between innate and adaptive immunity by activating and orientating the secondary immune response. For a long time, the innate immunity was considered as a "non-specific" immune system and as a first line of defense compared to the adaptive immune system, which establishes long-lasting and very specific antimicrobial protection. Meanwhile, with the cloning of Toll-like receptors and other recent discoveries in the field, the innate immune system eventually appeared as a more complex and "refined" system.

6.4 Role of another bacterial product : Aerolysin as a pro-inflammatory signal inducer

Aerolysin from *Aeromonas hydrophila* has been shown to provoke serious and fatal diseases. This toxin first binds to a GPI-anchored protein and rapidly heptamerizes. This heptamerization leads to the creation of holes in the membrane of cells, which then swell and die by an osmotic shock, and, in some cases, by apotosis. Apart from cell death, we were interested in studying whether this bacterial product could induce pro-inflammatory signals, a critical step of innate immune defenses against foreign microorganisms. We found that aerolysin could induce a pro-inflammatory signal (IL-8 production and NF-κB activation) in a dose- and time-dependent manner only in cells expressing the GPI form of CD14 and not in

those expressing the transmembrane CD14. Meanwhile, we could demonstrate that this aerolysin-induced cell activation was not specific to CD14, since similar results were found with cells expressing other GPI-anchored proteins. The common feature between these surface receptors was the GPI-anchor. This observation strengthened the fact that the GPI-anchoring system seems to be an essential part of the receptor to induce cell activation. Supporting this, we observed that the degree of cell response to aerolysin paralleled the degree of GPI-receptors surface expression. Subsequently, all these results raised the question of the role of the GPI-anchoring system in the aerolysin-induced IL-8 synthesis and NF-κB activation measured in our study. Pugin at al. had shown that a calcium flux was induced when GPI-CD14 was cross-linked with anti-CD14 antibodies, an observation that did not have any physiological explanation in the context of LPS studies. Interestingly, as previously mentioned, Krause et al. have reported that when granulocytes were treated with aerolysin, a calcium flux from intracellular stores and chemotaxis could be observed [482]. From these results, it could be proposed that in heptamerizing, aerolysin bound to CD14, induces a cross-linking of the GPI-anchored receptor, that leads to cell activation through the increase of cytoplasmic calcium and NF-κB translocation into the nucleus. Despite its role in the targeting of GPI-anchored proteins into lipid-rich domains, the GPI-anchoring system could also have a role in the binding of bacterial toxins and in the induction of cell signaling different to that observed with LPS.

To support our results, other bacterial toxins such as act, a toxin secreted by *Aeromonas hydrophila* was shown to induce pro-inflammatory cytokine production and to activate arachidonic acid metabolism in macrophages [520]. It was also reported that leukotoxin stimulates cytokine gene expression in bovine alveolar macrophages which required calcium elevation and NF-κB activation. This paper hypothized that the calcium could be involved in I-κB inactivation by modulating the activity of kinases which could phosphorylate I-κB [484]. Jefferson et al. also found a role of intracellular calcium and NF-κB in the up-regulation and secretion of IL-8 induced by *Clostridium difficile* toxin A in human monocytic cells (PBMC and THP-1) [521]. In order to see whether the calcium is involved in NF-κB activation after aerolysin treatment, it would be judicious to block calcium by using specific chelators.

Treatment of cells with aerolysin led to the creation of holes in the plasma membrane and subsequently to the entry of water into cells. This event induced cell swelling as observed

during an hypoosmotic shock. Various authors have published that osmotic shock could stimulate cells by inducing MAPK and NF-κB activation [522, 523]. We thus wondered whether the cell activation observed after aerolysin treatment was caused by the toxin itself or by cell swelling. By performing an hypoosmotic stress with water, we could demonstrate that no cell activation was induced, suggesting that cells responded directly to aerolysin and probably not to the consequences of aerolysin treatment.

7 PERSPECTIVES

To summarize, the innate immunity is an ancestral immune system which constitutes a first line of defense when microorganisms invade the body. To neutralize these microbes, two major strategies have been developed: the soluble and the receptor pathways. The first pathway involves soluble proteins specialized in the detection of various carbohydrates and lipids present in microorganisms. This capacity of recognition renders these proteins efficient to attack and to kill pathogens. This may take place either directly, by inducing opsonization of these particles by phagocytic cells or indirectly, by presenting them to other cells. The second pathway which implicates receptors present on leukocytes, ECs and other cells, are also able to detect foreign molecules. After recognition, receptors internalise these molecules or microorganisms into the cell, and clear them from the body. In addition, some of these receptors such as CD14 and TLRs can mediate cell activation after binding of bacterial ligands. The Gram-negative endotoxin (or LPS) has been proposed to be one of the most potent cell stimulator through CD14 and TLR4/MD-2. Subsequently, activated cells produce biological mediators which initiate an inflammatory reaction. Such a reaction is essential to activate the defense system in order to destroy the invading microorganisms, to chemoattract effector cells, and to proceed into wound healing. Meanwhile, the production of pro-inflammatory mediators has to be tightly controled and has to be confined in a local area. Otherwise, “overproduction” of inflammatory mediators and release into the circulation may result in a systemic inflammatory reaction, a phenomenon typical of endotoxemia and septic shock. The starting point of this dysregulation remains unknown, but may involve genetical, biochemical, physiological and environmental predisposing factors. Each year, the syndrome of sepsis provokes more than 200 000 deaths in the United States alone. At the present time, there are only limited treatments available for septic shock, which are mainly confined to supportive measures. A better understanding of cellular and molecular mechanisms underlying the pathology of sepsis might lead to the discovery of new therapeutical targets. One of the critical and obligatory step in the pathogenesis of the sepsis syndrome is the recognition of bacteria and bacterial products by host cells. The recognition of LPS by immune cells, which was found to generate a syndrome similar to that of sepsis when LPS was injected to animals, has been extensively studied. More than ten years ago, CD14 has been identified as a receptor capable of mediating LPS effects on mammalian cells. LPS transfer to CD14 is catalysed by LBP, an acute phase protein present in the plasma, which

essential role is to monomerize LPS from LPS aggregates and to shuttle it to acceptors. After binding of LPS to CD14, cells are activated through TLR4 and MD-2, and LPS is internalised into cells to be cleared. The soluble form of CD14 present in plasma and body fluids can also bind LPS. The LPS-sCD14 complex activates some non CD14-bearing cells by binding to TLR4 [412].

CD14 is promiscuous in that it binds a large array of different bacterial ligands It recognizes various chemical structures from pathogens to apoptotic cells, to lipids, and has been proposed to be a "pattern recognition receptor" [23]. Now how CD14 can recognize all these various chemical structures remains unclear. It could be proposed that CD14 acts as a "concentrator" of pathogenic molecules to the surface of the cell that would allow both signaling and clearing processes through other receptors. In that sense, CD14 would be one chain of a multiple chain receptor complex for microbial molecules and other "non-self" ligands [524]. Supporting this, the discovery of TLRs revealed that each member of this family was specialized in the recognition of one or several ligands belonging to Gram-negative bacteria, Gram-positive bacteria, yeast and even viruses. Furthermore, it was shown that the heterodimerization of TLRs such as TLR2 with TLR6 or TLR1 increases the discrimination power between different ligands [380, 381]. It is therefore possible that other combinations between TLRs occur in a combinatorial manner. In addition to TLRs, other accessory proteins may be involved in this "receptor protein complex" such as "MD molecules", and may further explain different cell responses to a variety of ligands interacting with CD14. Indeed, depending on the nature of the ligands, CD14 may or not induce cell activation.

The association of CD14, TLR4, and MD-2 is a prototypical example of a "receptor protein complex" implicated in LPS signaling. This complex may also include other components as suggested in recent studies [525-527]. Meanwhile, interactions between each member of this complex and LPS remain to be explored more deeply. It was recently shown that both TLR4 and MD-2 directly bound LPS [389, 390, 405, 407]. It was questioned whether CD14 and TLR4 interacted through their respective leucine rich repeats when LPS is present. Understanding how MD-2, an anchorless protein, attaches to the transmembrane TLR4 is still an unresolved question. Membrane targeting studies of CD14 revealed that this protein is found in lipid-enriched domains [25, 429, 528]. With similar studies, it would be interesting to determine whether TLR4 or other TLRs are also constitutively present in such

lipidic rafts or whether these receptors are recruited into these membrane domains in a ligand-specific manner.

Previous studies have shown that LPS was internalised into cells in both CD14-dependent and CD14-independent manners and could follow different endocytosis pathways [250, 429, 487]. To date, it is still unknown whether TLRs are internalised and if so, which endocytosis pathways they used. In addition, their contribution in the endocytosis of LPS or other foreign molecules remained to be determined. Detection of specific "internalization motifs" in their transmembrane tail could bring new clues and may explain that a portion of LPS is endocytosed *via* coated pits as found by Kitchens et al. [487]. Interestingly, Underhill et al. found that membrane TLR2 was recruited to macrophage phagosomes during zymozan ingestion and able to discriminate the nature of pathogens [372]. This was rapidly followed by the surface recycling of TLR2. This observation again raises the question of a possible dependence between internalization and cell signaling.

Little is known on the modulation and on the implication of TLRs and MD-2 in the pathophysiology of sepsis. Measuring levels of surface expression of these receptors during the disease could identify new clinical markers. It has also to be demonstrated whether, like CD14, TLRs and MD-2 exist as soluble proteins and if they mediate cell activation in this form.

Until now, clinical trials using molecules or antibodies antagonizing the effects of individual pro-inflammatory mediators (eg. cytokines, PAF, prostaglandins, NO-synthase) have invariably failed. However, all these approaches had in common the inhibition of secondary inflammatory mediators occurring in sepsis. An alternative strategy would be to block interactions of noxious bacterial components with pivotal receptors that could prevent the initiation of an unappropriate inflammatory response. Encouraging results described by Schimke et al. showed that the use of anti-CD14 antibodies *in vivo* provided animal protection against endotoxin-induced septic shock [529]. However, a potential problem with this approach might be an undesired inhibition of the clearence of bacteria. Indeed, it has been shown that in the case of an infection with *Shigella*, the blockade of CD14 *in vivo* was associated with increased *Shigella*-mediated invasion and tissue destruction [530]. The key element to this problem could be the suppression or the modulation of cell response to bacterial products without affecting the internalization/clearance of these products or bacteria.

The discovery of accessory proteins in the "LPS receptor complex" may provide new therapeutic targets for the prevention of septic shock.

Apart from its role in CD14 targeting to lipid-rich domains, the GPI-anchor of CD14 has no clearly defined function in LPS-induced cell activation and LPS-endocytosis. After binding to a GPI-anchor, aerolysin, a pore-forming toxin from a Gram-negative bacteria *Aeromonas hydrophila* generates a pro-inflammatory signal in cells. In addition to deleterious cytolitic effects, this toxin seems to trigger a primary immune response which could synergise to that induced by endotoxin released from bacteria. Investigating this process could lead to a new mechanism of action of a bacterial toxin that could directly involve the GPI-anchor in cell activation.

In this manuscript are presented our results discussed in the context of the current state of knowledge concerning interactions of endotoxin with the innate system. Many topics have been covered ranging from LPS binding, internalization to signaling. The recent discovery of TLRs and MD-2 proteins provides exciting perspectives in the LPS field, and represents promising targets for the treatment of sepsis, but also rises key questions as to how LPS interacts with cells and how has innate immunity evolved.

8 REFERENCES

1. Lamaze C, Schmid SL. The emergence of clathrin-independent pinocytic pathways. Curr Opin Cell Biol 1995;7:573-80.

2. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001;13:85-94.

3. O'Neill LA, Greene C. Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. J Leukoc Biol 1998;63:650-7.

4. Rock FL, Hardiman G, Timans JC, Kastelein RA and Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A 1998;95:588-93.

5. Triantafilou M, Miyake K, Golenbock DT and Triantafilou K. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci 2002;115:2603-11

6. Agnese DM, Calvano JE, Hahm SJ, et al. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. J Infect Dis 2002;186:1522-5

7. Abrami L, van Der Goot FG. Plasma membrane microdomains act as concentration platforms to facilitate intoxication by aerolysin. J Cell Biol 1999;147:175-84.

8. Formanek H. A three dimensional model of the digestion of peptidoglycan by lysozyme. Biophys Struct Mech 1977;4:1-14.

9. Tabona P, Mellor A and Summerfield JA. Mannose binding protein is involved in first-line host defence: evidence from transgenic mice. Immunology 1995;85:153-9.

10. Gotschlich EC, Liu TY and Oliveira E. Binding of C-reactive protein to C-carbohydrate and PC-substituted protein. Ann N Y Acad Sci 1982;389:163-71

11. de Haas CJ. New insights into the role of serum amyloid P component, a novel lipopolysaccharide-binding protein. FEMS Immunol Med Microbiol 1999;26:197-202.

12. Mold C, Du Clos TW, Nakayama S, Edwards KM and Gewurz H. C-reactive protein reactivity with complement and effects on phagocytosis. Ann N Y Acad Sci 1982;389:251-62

13. Tapping RI, Gegner JA, Kravchenko VV and Tobias PS. Roles for LBP and soluble CD14 in cellular uptake of LPS. Prog Clin Biol Res 1998;397:73-8

14. Song WC, Sarrias MR and Lambris JD. Complement and innate immunity. Immunopharmacology 2000;49:187-98.

15. Suffredini AF, Fantuzzi G, Badolato R, Oppenheim JJ and O'Grady NP. New insights into the biology of the acute phase response. J Clin Immunol 1999;19:203-14.

16. Linehan SA, Martinez-Pomares L and Gordon S. Mannose receptor and scavenger receptor: two macrophage pattern recognition receptors with diverse functions in tissue homeostasis and host defense. Adv Exp Med Biol 2000;479:1-14

17. Gough PJ, Gordon S. The role of scavenger receptors in the innate immune system. Microbes Infect 2000;2:305-11.

18. Hampton RY, Golenbock DT, Penman M, Krieger M and Raetz CR. Recognition and plasma clearance of endotoxin by scavenger receptors. Nature 1991;352:342-4.

19. Dunne DW, Resnick D, Greenberg J, Krieger M and Joiner KA. The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. Proc Natl Acad Sci U S A 1994;91:1863-7.

20. Ezekowitz RA, Williams DJ, Koziel H, et al. Uptake of Pneumocystis carinii mediated by the macrophage mannose receptor. Nature 1991;351:155-8.

21. Taylor ME, Drickamer K. Structural requirements for high affinity binding of complex ligands by the macrophage mannose receptor. J Biol Chem 1993;268:399-404.

22. Sallusto F, Cella M, Danieli C and Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med 1995;182:389-400.

23. Pugin J, Heumann ID, Tomasz A, et al. CD14 is a pattern recognition receptor. Immunity 1994;1:509-16.

24. Schiff DE, Kline L, Soldau K, et al. Phagocytosis of gram-negative bacteria by a unique CD14-dependent mechanism. J Leukoc Biol 1997;62:786-94.

25. Pugin J, Kravchenko VV, Lee JD, Kline L, Ulevitch RJ and Tobias PS. Cell activation mediated by glycosylphosphatidylinositol-anchored or transmembrane forms of CD14. Infect Immun 1998;66:1174-80.

26. Liles WC, Van Voorhis WC. Review: nomenclature and biologic significance of cytokines involved in inflammation and the host immune response. J Infect Dis 1995;172:1573-80.

27. Kushner I. Semantics, inflammation, cytokines and common sense. Cytokine Growth Factor Rev 1998;9:191-6.

28. Geng JG. Directional migration of leukocytes: their pathological roles in inflammation and strategies for development of anti-inflammatory therapies. Cell Res 2001;11:85-8.

29. Atkins FM, Friedman MM, Subba Rao PV and Metcalfe DD. Interactions between mast cells, fibroblasts and connective tissue components. Int Arch Allergy Appl Immunol 1985;77:96-102

30. Teti A. Regulation of cellular functions by extracellular matrix. J Am Soc Nephrol 1992;2:S83-7.

31. Catchpole HR. Connective tissue, basement membrane, extracellular matrix. Pathobiol Annu 1982;12:1-33

32. Mandell GL. Phagocytic cells in host defense against infections. J Med 1980;11:263-6

33. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. Science 1996;272:50-3.

34. Smith GS, Lumsden JH. Review of neutrophil adherence, chemotaxis, phagocytosis and killing. Vet Immunol Immunopathol 1983;4:177-236.

35. Ranadive NS, Sajnani AN, Alimurka K and Movat HZ. Release of basic proteins and lysosomal enzymes from neutrophil leukocytes of the rabbit. Int Arch Allergy Appl Immunol 1973;45:880-98

36. Bainton DF. Neutrophil granules: A review. Am J Med Technol 1976;42:15-21.
37. Borregaard N, Lollike K, Kjeldsen L, et al. Human neutrophil granules and secretory vesicles. Eur J Haematol 1993;51:187-98.

38. Borregaard N, Kjeldsen L, Lollike K and Sengelov H. Granules and secretory vesicles of the human neutrophil. Clin Exp Immunol 1995;101 Suppl 1:6-9.

39. Zaoui P, Barro C and Morel F. Differential expression and secretion of gelatinases and tissue inhibitor of metalloproteinase-1 during neutrophil adhesion. Biochim Biophys Acta 1996;1290:101-12.

40. Hampton MB, Kettle AJ and Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 1998;92:3007-17.

41. Armstrong R. The physiological role and pharmacological potential of nitric oxide in neutrophil activation. Int Immunopharmacol 2001;1:1501-12.

42. Yoshida M, Gimbrone MA, Jr. Novel roles for E-selectin in endothelial-leukocyte adhesion. Ann N Y Acad Sci 1997;811:493-7.

43. Burns AR, Bowden RA, Abe Y, et al. P-selectin mediates neutrophil adhesion to endothelial cell borders. J Leukoc Biol 1999;65:299-306.

44. von Andrian UH, Chambers JD, Berg EL, et al. L-selectin mediates neutrophil rolling in inflamed venules through sialyl LewisX-dependent and -independent recognition pathways. Blood 1993;82:182-91.

45. Strieter RM, Kunkel SL, Showell HJ, et al. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1 beta. Science 1989;243:1467-9.

46. Smith CW, Kishimoto TK, Abbassi O, et al. Chemotactic factors regulate lectin adhesion molecule 1 (LECAM-1)-dependent neutrophil adhesion to cytokine-stimulated endothelial cells in vitro. J Clin Invest 1991;87:609-18.

47. Bokoch GM. Chemoattractant signaling and leukocyte activation. Blood 1995;86:1649-60.

48. Williams MA, Solomkin JS. Integrin-mediated signaling in human neutrophil functioning. J Leukoc Biol 1999;65:725-36.

49. Talstad I, Dalen H and Lehmann V. Degranulation and enzyme release during phagocytosis of inert particles and of bacteria by polymorphonuclear neutrophil granulocytes. Acta Pathol Microbiol Immunol Scand [C] 1983;91:403-11.

50. Smith JA. Neutrophils, host defense, and inflammation: a double-edged sword. J Leukoc Biol 1994;56:672-86.

51. Takahashi K, Naito M and Takeya M. Development and heterogeneity of macrophages and their related cells through their differentiation pathways. Pathol Int 1996;46:473-85.

52. Mantovani B, Rabinovitch M and Nussenzweig V. Phagocytosis of immune complexes by macrophages. Different roles of the macrophage receptor sites for complement (C3) and for immunoglobulin (IgG). J Exp Med 1972;135:780-92.

53. Griffin FM, Jr., Bianco C and Silverstein SC. Characterization of the macrophage receptro for complement and demonstration of its functional independence from the receptor for the Fc portion of immunoglobulin G. J Exp Med 1975;141:1269-77.

54. Tobias PS, Ulevitch RJ. Lipopolysaccharide binding protein and CD14 in LPS dependent macrophage activation. Immunobiology 1993;187:227-32.

55. MacMicking J, Xie QW and Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol 1997;15:323-50

56. Unanue ER. Perspective on antigen processing and presentation. Immunol Rev 2002;185:86-102.

57. Mantovani A, Sica A, Colotta F and Dejana E. The role of cytokines as communication signals between leukocytes and endothelial cells. Prog Clin Biol Res 1990;349:343-53

58. Luster AD. Chemokines--chemotactic cytokines that mediate inflammation. N Engl J Med 1998;338:436-45.

59. Movat HZ. Tumor necrosis factor and interleukin-1: role in acute inflammation and microvascular injury. J Lab Clin Med 1987;110:668-81.

60. Mannel DN, Echtenacher B. TNF in the inflammatory response. Chem Immunol 2000;74:141-61

61. Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett 1992;307:97-101.

62. Mukaida N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. Int J Hematol 2000;72:391-8.

63. Mantovani A, Sozzani S, Bottazzi B, et al. Monocyte chemotactic protein-1 (MCP-1): signal transduction and involvement in the regulation of macrophage traffic in normal and neoplastic tissues. Adv Exp Med Biol 1993;351:47-54

64. Lopez AF, Elliott MJ, Woodcock J and Vadas MA. GM-CSF, IL-3 and IL-5: cross-competition on human haemopoietic cells. Immunol Today 1992;13:495-500.

65. Lum H, Malik AB. Regulation of vascular endothelial barrier function. Am J Physiol 1994;267:L223-41

66. Majno G, Shea SM and Leventhal M. Endothelial contraction induced by histamine-type mediators: an electron microscopic study. J Cell Biol 1969;42:647-72.

67. Gabbiani G, Badonnel MC and Majno G. Intra-arterial injections of histamine, serotonin, or bradykinin: a topographic study of vascular leakage. Proc Soc Exp Biol Med 1970;135:447-52.

68. Bertrand C, Geppetti P, Baker J, Yamawaki I and Nadel JA. Role of neurogenic inflammation in antigen-induced vascular extravasation in guinea pig trachea. J Immunol 1993;150:1479-85

69. Piedimonte G, Bertrand C, Geppetti P, Snider RM, Desai MC and Nadel JA. A new NK1 receptor antagonist (CP-99,994) prevents the increase in tracheal vascular permeability produced by hypertonic saline. J Pharmacol Exp Ther 1993;266:270-3

70. Tharp MD. The interaction between mast cells and endothelial cells. J Invest Dermatol 1989;93:107S-112S.

71. Wright SD, Tobias PS, Ulevitch RJ and Ramos RA. Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. J Exp Med 1989;170:1231-41.

72. Schumann RR, Leong SR, Flaggs GW, et al. Structure and function of lipopolysaccharide binding protein. Science 1990;249:1429-31.

73. Lichtman SN, Wang J and Lemasters JJ. LPS receptor CD14 participates in release of TNF-alpha in RAW 264.7 and peritoneal cells but not in kupffer cells. Am J Physiol 1998;275:G39-46.

74. Amberger A, Maczek C, Jurgens G, et al. Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low- density lipoproteins. Cell Stress Chaperones 1997;2:94-103

75. Takahashi M, Ikeda U, Kasahara T, et al. Activation of human monocytes for enhanced production of interleukin 8 during transendothelial migration in vitro. J Clin Immunol 1997;17:53-62

76. Lingen MW. Role of leukocytes and endothelial cells in the development of angiogenesis in inflammation and wound healing. Arch Pathol Lab Med 2001;125:67-71.

77. Simeon A, Monier F, Emonard H, et al. Fibroblast-cytokine-extracellular matrix interactions in wound repair. Curr Top Pathol 1999;93:95-101

78. Glauser MP, Zanetti G, Baumgartner JD and Cohen J. Septic shock: pathogenesis. Lancet 1991;338:732-6.

79. Wenzel RP. Anti-endotoxin monoclonal antibodies--a second look. N Engl J Med 1992;326:1151-3.

80. Commitee ACoCPSoCCMC. Definitions for sepsis or organ failures and guidelinesfor the use of innovative therapies in sepsis. Chest 1992;101:1658-62

81. Brun-Buisson C, Doyon F, Carlet J, et al. Incidence, risk factors, and outcome of severe sepsis and septic shock in adults. A multicenter prospective study in intensive care units. French ICU Group for Severe Sepsis. Jama 1995;274:968-74.

82. Bates DW, Sands K, Miller E, et al. Predicting bacteremia in patients with sepsis syndrome. Academic Medical Center Consortium Sepsis Project Working Group. J Infect Dis 1997;176:1538-51.

83. Calandra T, Gerain J, Heumann D, Baumgartner JD and Glauser MP. High circulating levels of interleukin-6 in patients with septic shock: evolution during sepsis, prognostic value, and interplay with other cytokines. The Swiss-Dutch J5 Immunoglobulin Study Group. Am J Med 1991;91:23-9.

84. Suffredini AF, Reda D, Banks SM, Tropea M, Agosti JM and Miller R. Effects of recombinant dimeric TNF receptor on human inflammatory responses following intravenous endotoxin administration. J Immunol 1995;155:5038-45.

85. Cain BS, Meldrum DR, Harken AH and McIntyre RC, Jr. The physiologic basis for anticytokine clinical trials in the treatment of sepsis. J Am Coll Surg 1998;186:337-50.

86. Bone RC. Why sepsis trials fail. JAMA 1996;276:565-6

87. Denham W, Norman JG. Why sepsis trials fail. JAMA 1996;276:1723

88. Zeni F, Freeman B and Natanson C. Anti-inflammatory therapies to treat sepsis and septic shock: a reassessment. Crit Care Med 1997;26:1927-31

89. Greenman RL, Schein RM, Martin MA, et al. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of gram-negative sepsis. The XOMA Sepsis Study Group. Jama 1991;266:1097-102.

90. Ziegler EJ, Fisher CJ, Jr., Sprung CL, et al. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis Study Group. N Engl J Med 1991;324:429-36.

91. Fisher CJ, Jr., Dhainaut JF, Opal SM, et al. Recombinant human interleukin 1 receptor antagonist in the treatment of patients with sepsis syndrome. Results from a randomized, double-blind, placebo-controlled trial. Phase III rhIL-1ra Sepsis Syndrome Study Group. Jama 1994;271:1836-43.

92. Abraham E, Wunderink R, Silverman H, et al. Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. TNF-alpha MAb Sepsis Study Group. Jama 1995;273:934-41.

93. Abraham E, Glauser MP, Butler T, et al. p55 Tumor necrosis factor receptor fusion protein in the treatment of patients with severe sepsis and septic shock. A randomized controlled multicenter trial. Ro 45-2081 Study Group. Jama 1997;277:1531-8.

94. Bernard GR, Wheeler AP, Russell JA, et al. The effects of ibuprofen on the physiology and survival of patients with sepsis. The Ibuprofen in Sepsis Study Group. N Engl J Med 1997;336:912-8.

95. Abraham E, Anzueto A, Gutierrez G, et al. Double-blind randomised controlled trial of monoclonal antibody to human tumour necrosis factor in treatment of septic shock. NORASEPT II Study Group. Lancet 1998;351:929-33.

96. Munford RS, Pugin J. Normal responses to injury prevent systemic inflammation and can be immunosuppressive. Am J Respir Crit Care Med 2001;163:316-21.

97. Pugin J, Verghese G, Widmer MC and Matthay MA. The alveolar space is the site of intense inflammatory and profibrotic reactions in the early phase of acute respiratory distress syndrome. Crit Care Med 1999;27:304-12.

98. Goldie AS, Fearon KC, Ross JA, et al. Natural cytokine antagonists and endogenous antiendotoxin core antibodies in sepsis syndrome. The Sepsis Intervention Group. Jama 1995;274:172-7

99. Nikaido H, Nakae T. The outer membrane of Gram-negative bacteria. Adv Microb Physiol 1979;20:163-250

100. Lugtenberg B, Van Alphen L. Molecular architecture and functioning of the outer membrane of Escherichia coli and other gram-negative bacteria. Biochim Biophys Acta 1983;737:51-115.

101. Rietschel ET, Brade H. Bacterial endotoxins. Sci Am 1992;267:54-61.

102. Graber CD, Reinhold RB, Breman JG, Harley RA and Hennigar GR. Fatal heat stroke. Circulating endotoxin and gram-negative sepsis as complications. Jama 1971;216:1195-6.

103. Martich GD, Boujoukos AJ and Suffredini AF. Response of man to endotoxin. Immunobiology 1993;187:403-16.

104. Raetz CR, Ulevitch RJ, Wright SD, Sibley CH, Ding A and Nathan CF. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. Faseb J 1991;5:2652-60.

105. Tobias PS, Mathison J, Mintz D, et al. Participation of lipopolysaccharide-binding protein in lipopolysaccharide-dependent macrophage activation. Am J Respir Cell Mol Biol 1992;7:239-45.

106. Opal SM, Palardy JE, Marra MN, Fisher CJ, Jr., McKelligon BM and Scott RW. Relative concentrations of endotoxin-binding proteins in body fluids during infection. Lancet 1994;344:429-31.

107. Su GL, Freeswick PD, Geller DA, et al. Molecular cloning, characterization, and tissue distribution of rat lipopolysaccharide binding protein. Evidence for extrahepatic expression. J Immunol 1994;153:743-52.

108. Tobias PS, McAdam KP, Soldau K and Ulevitch RJ. Control of lipopolysaccharide-high-density lipoprotein interactions by an acute-phase reactant in human serum. Infect Immun 1985;50:73-6.

109. Tobias PS, Soldau K and Ulevitch RJ. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. J Biol Chem 1989;264:10867-71.

110. Mathison JC, Tobias PS, Wolfson E and Ulevitch RJ. Plasma lipopolysaccharide (LPS)-binding protein. A key component in macrophage recognition of gram-negative LPS. J Immunol 1992;149:200-6.

111. Tobias PS, Soldau K, Gegner JA, Mintz D and Ulevitch RJ. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. J Biol Chem 1995;270:10482-8.

112. Han J, Mathison JC, Ulevitch RJ and Tobias PS. Lipopolysaccharide (LPS) binding protein, truncated at Ile-197, binds LPS but does not transfer LPS to CD14. J Biol Chem 1994;269:8172-5.

113. Lamping N, Hoess A, Yu B, et al. Effects of site-directed mutagenesis of basic residues (Arg 94, Lys 95, Lys 99) of lipopolysaccharide (LPS)-binding protein on binding and transfer of LPS and subsequent immune cell activation. J Immunol 1996;157:4648-56.

114. Gegner JA, Ulevitch RJ and Tobias PS. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. J Biol Chem 1995;270:5320-5.

115. Gray PW, Flaggs G, Leong SR, et al. Cloning of the cDNA of a human neutrophil bactericidal protein. Structural and functional correlations. J Biol Chem 1989;264:9505-9.

116. Day JR, Albers JJ, Lofton-Day CE, et al. Complete cDNA encoding human phospholipid transfer protein from human endothelial cells. J Biol Chem 1994;269:9388-91.

117. Tobias PS, Soldau K, Iovine NM, Elsbach P and Weiss J. Lipopolysaccharide (LPS)-binding proteins BPI and LBP form different types of complexes with LPS. J Biol Chem 1997;272:18682-5.

118. Ulevitch RJ, Johnston AR and Weinstein DB. New function for high density lipoproteins. Their participation in intravascular reactions of bacterial lipopolysaccharides. J Clin Invest 1979;64:1516-24.

119. Van Lenten BJ, Fogelman AM, Haberland ME and Edwards PA. The role of lipoproteins and receptor-mediated endocytosis in the transport of bacterial lipopolysaccharide. Proc Natl Acad Sci U S A 1986;83:2704-8.

120. Harris HW, Grunfeld C, Feingold KR and Rapp JH. Human very low density lipoproteins and chylomicrons can protect against endotoxin-induced death in mice. J Clin Invest 1990;86:696-702.

121. Munford RS, Andersen JM and Dietschy JM. Sites of tissue binding and uptake in vivo of bacterial lipopolysaccharide-high density lipoprotein complexes: studies in the rat and squirrel monkey. J Clin Invest 1981;68:1503-13.

122. Read TE, Grunfeld C, Kumwenda ZL, et al. Triglyceride-rich lipoproteins prevent septic death in rats. J Exp Med 1995;182:267-72.

123. Flegel WA, Wolpl A, Mannel DN and Northoff H. Inhibition of endotoxin-induced activation of human monocytes by human lipoproteins. Infect Immun 1989;57:2237-45.

124. Pajkrt D, Doran JE, Koster F, et al. Antiinflammatory effects of reconstituted high-density lipoprotein during human endotoxemia. J Exp Med 1996;184:1601-8.

125. Netea MG, Demacker PN, Kullberg BJ, et al. Low-density lipoprotein receptor-deficient mice are protected against lethal endotoxemia and severe gram-negative infections. J Clin Invest 1996;97:1366-72.

126. Antal-Szalmas P, Strijp JA, Weersink AJ, Verhoef J and Van Kessel KP. Quantitation of surface CD14 on human monocytes and neutrophils. J Leukoc Biol 1997;61:721-8.

127. Morabito F, Prasthofer EF, Dunlap NE, Grossi CE and Tilden AB. Expression of myelomonocytic antigens on chronic lymphocytic leukemia B cells correlates with their ability to produce interleukin 1. Blood 1987;70:1750-7.

128. Labeta MO, Landmann R, Obrecht JP and Obrist R. Human B cells express membrane-bound and soluble forms of the CD14 myeloid antigen. Mol Immunol 1991;28:115-22.

129. Fearns C, Kravchenko VV, Ulevitch RJ and Loskutoff DJ. Murine CD14 gene expression in vivo: extramyeloid synthesis and regulation by lipopolysaccharide. J Exp Med 1995;181:857-66.

130. Diamond G, Russell JP and Bevins CL. Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. Proc Natl Acad Sci U S A 1996;93:5156-60.

131. Liu S, Khemlani LS, Shapiro RA, et al. Expression of CD14 by hepatocytes: upregulation by cytokines during endotoxemia. Infect Immun 1998;66:5089-98.

132. Goyert SM, Ferrero E, Rettig WJ, Yenamandra AK, Obata F and Le Beau MM. The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. Science 1988;239:497-500.

133. Setoguchi M, Nasu N, Yoshida S, Higuchi Y, Akizuki S and Yamamoto S. Mouse and human CD14 (myeloid cell-specific leucine-rich glycoprotein) primary structure deduced from cDNA clones. Biochim Biophys Acta 1989;1008:213-22.

134. Takai N, Kataoka M, Higuchi Y, Matsuura K and Yamamoto S. Primary structure of rat CD14 and characteristics of rat CD14, cytokine, and NO synthase mRNA expression in mononuclear phagocyte system cells in response to LPS. J Leukoc Biol 1997;61:736-44.

135. Ikeda A, Takata M, Taniguchi T and Sekikawa K. Molecular cloning of bovine CD14 gene. J Vet Med Sci 1997;59:715-9.

136. Haziot A, Lin XY, Zhang F and Goyert SM. The induction of acute phase proteins by lipopolysaccharide uses a novel pathway that is CD14-independent. J Immunol 1998;160:2570-2.

137. Stelter F, Pfister M, Bernheiden M, et al. The myeloid differentiation antigen CD14 is N- and O-glycosylated. Contribution of N-linked glycosylation to different soluble CD14 isoforms. Eur J Biochem 1996;236:457-64.

138. Ferrero E, Hsieh CL, Francke U and Goyert SM. CD14 is a member of the family of leucine-rich proteins and is encoded by a gene syntenic with multiple receptor genes. J Immunol 1990;145:331-6.

139. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ and Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990;249:1431-3.

140. Hailman E, Lichenstein HS, Wurfel MM, et al. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med 1994;179:269-77.

141. Grunwald U, Kruger C, Westermann J, Lukowsky A, Ehlers M and Schutt C. An enzyme-linked immunosorbent assay for the quantification of solubilized CD14 in biological fluids. J Immunol Methods 1992;155:225-32.

142. Schopf RE, Dobmeyer J, Dobmeyer T and Morsches B. Soluble CD14 monocyte antigen in suction blister fluid and serum of patients with psoriasis. Dermatology 1993;186:45-9

143. Pforte A, Schiessler A, Gais P, et al. Expression of CD14 correlates with lung function impairment in pulmonary sarcoidosis. Chest 1994;105:349-54.

144. Nockher WA, Wigand R, Schoeppe W and Scherberich JE. Elevated levels of soluble CD14 in serum of patients with systemic lupus erythematosus. Clin Exp Immunol 1994;96:15-9.

145. Lien E, Aukrust P, Sundan A, Muller F, Froland SS and Espevik T. Elevated levels of serum-soluble CD14 in human immunodeficiency virus type 1 (HIV-1) infection: correlation to disease progression and clinical events. Blood 1998;92:2084-92.

146. Kruger C, Schutt C, Obertacke U, et al. Serum CD14 levels in polytraumatized and severely burned patients. Clin Exp Immunol 1991;85:297-301.

147. Endo S, Inada K, Kasai T, et al. Soluble CD14 (sCD14) levels in patients with multiple organ failure (MOF). Res Commun Chem Pathol Pharmacol 1994;84:17-25.

148. Martin TR, Rubenfeld GD, Ruzinski JT, et al. Relationship between soluble CD14, lipopolysaccharide binding protein, and the alveolar inflammatory response in patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 1997;155:937-44.

149. Pugin J, Schurer-Maly CC, Leturcq D, Moriarty A, Ulevitch RJ and Tobias PS. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc Natl Acad Sci U S A 1993;90:2744-8.

150. Bazil V, Strominger JL. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J Immunol 1991;147:1567-74.

151. Bufler P, Stiegler G, Schuchmann M, et al. Soluble lipopolysaccharide receptor (CD14) is released via two different mechanisms from human monocytes and CD14 transfectants. Eur J Immunol 1995;25:604-10.

152. Durieux JJ, Vita N, Popescu O, et al. The two soluble forms of the lipopolysaccharide receptor, CD14: characterization and release by normal human monocytes. Eur J Immunol 1994;24:2006-12.

153. Stelter F, Bernheiden M, Menzel R, et al. Mutation of amino acids 39-44 of human CD14 abrogates binding of lipopolysaccharide and Escherichia coli. Eur J Biochem 1997;243:100-9.

154. Fan X, Stelter F, Menzel R, et al. Structures in Bacillus subtilis are recognized by CD14 in a lipopolysaccharide binding protein-dependent reaction. Infect Immun 1999;67:2964-8.

155. Kusunoki T, Hailman E, Juan TS, Lichenstein HS and Wright SD. Molecules from Staphylococcus aureus that bind CD14 and stimulate innate immune responses. J Exp Med 1995;182:1673-82.

156. Dziarski R, Tapping RI and Tobias PS. Binding of bacterial peptidoglycan to CD14. J Biol Chem 1998;273:8680-90.

157. Espevik T, Otterlei M, Skjak-Braek G, Ryan L, Wright SD and Sundan A. The involvement of CD14 in stimulation of cytokine production by uronic acid polymers. Eur J Immunol 1993;23:255-61.

158. Savedra R, Jr., Delude RL, Ingalls RR, Fenton MJ and Golenbock DT. Mycobacterial lipoarabinomannan recognition requires a receptor that shares components of the endotoxin signaling system. J Immunol 1996;157:2549-54.

159. Weidemann B, Brade H, Rietschel ET, et al. Soluble peptidoglycan-induced monokine production can be blocked by anti-CD14 monoclonal antibodies and by lipid A partial structures. Infect Immun 1994;62:4709-15.

160. Cleveland MG, Gorham JD, Murphy TL, Tuomanen E and Murphy KM. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect Immun 1996;64:1906-12.

161. Hattor Y, Kasai K, Akimoto K and Thiemermann C. Induction of NO synthesis by lipoteichoic acid from Staphylococcus aureus in J774 macrophages: involvement of a CD14-dependent pathway. Biochem Biophys Res Commun 1997;233:375-9.

162. Sellati TJ, Bouis DA, Kitchens RL, et al. Treponema pallidum and Borrelia burgdorferi lipoproteins and synthetic lipopeptides activate monocytic cells via a CD14-dependent pathway distinct from that used by lipopolysaccharide. J Immunol 1998;160:5455-64.

163. Yu B, Hailman E and Wright SD. Lipopolysaccharide binding protein and soluble CD14 catalyze exchange of phospholipids. J Clin Invest 1997;99:315-24.

164. Wang PY, Kitchens RL and Munford RS. Phosphatidylinositides bind to plasma membrane CD14 and can prevent monocyte activation by bacterial lipopolysaccharide. J Biol Chem 1998;273:24309-13.

165. Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL and Gregory CD. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature 1998;392:505-9.

166. Lee JD, Kato K, Tobias PS, Kirkland TN and Ulevitch RJ. Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. J Exp Med 1992;175:1697-705.

167. Golenbock DT, Bach RR, Lichenstein H, Juan TS, Tadavarthy A and Moldow CF. Soluble CD14 promotes LPS activation of CD14-deficient PNH monocytes and endothelial cells. J Lab Clin Med 1995;125:662-71.

168. Schutt C, Schilling T, Grunwald U, et al. Human monocytes lacking the membrane-bound form of the bacterial lipopolysaccharide (LPS) receptor CD14 can mount an LPS-induced oxidative burst response mediated by a soluble form of CD14. Res Immunol 1995;146:339-50.

169. Haziot A, Ferrero E, Kontgen F, et al. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. Immunity 1996;4:407-14.

170. Ferrero E, Jiao D, Tsuberi BZ, et al. Transgenic mice expressing human CD14 are hypersensitive to lipopolysaccharide. Proc Natl Acad Sci U S A 1993;90:2380-4.

171. Schutt C, Schilling T, Grunwald U, Schonfeld W and Kruger C. Endotoxin-neutralizing capacity of soluble CD14. Res Immunol 1992;143:71-8.

172. Haziot A, Rong GW, Bazil V, Silver J and Goyert SM. Recombinant soluble CD14 inhibits LPS-induced tumor necrosis factor-alpha production by cells in whole blood. J Immunol 1994;152:5868-76.

173. Troelstra A, Giepmans BN, Van Kessel KP, Lichenstein HS, Verhoef J and Van Strijp JA. Dual effects of soluble CD14 on LPS priming of neutrophils. J Leukoc Biol 1997;61:173-8.

174. Haziot A, Rong GW, Lin XY, Silver J and Goyert SM. Recombinant soluble CD14 prevents mortality in mice treated with endotoxin (lipopolysaccharide). J Immunol 1995;154:6529-32.

175. Hailman E, Vasselon T, Kelley M, et al. Stimulation of macrophages and neutrophils by complexes of lipopolysaccharide and soluble CD14. J Immunol 1996;156:4384-90.

176. Wurfel MM, Wright SD. Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers: preferential interaction with particular classes of lipid. J Immunol 1997;158:3925-34.

177. Thieblemont N, Thieringer R and Wright SD. Innate immune recognition of bacterial lipopolysaccharide: dependence on interactions with membrane lipids and endocytic movement. Immunity 1998;8:771-7.

178. Joseph CK, Wright SD, Bornmann WG, et al. Bacterial lipopolysaccharide has structural similarity to ceramide and stimulates ceramide-activated protein kinase in myeloid cells. J Biol Chem 1994;269:17606-10.

179. Detmers PA, Thieblemont N, Vasselon T, Pironkova R, Miller DS and Wright SD. Potential role of membrane internalization and vesicle fusion in adhesion of neutrophils in response to lipopolysaccharide and TNF. J Immunol 1996;157:5589-96.

180. Lee JD, Kravchenko V, Kirkland TN, et al. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. Proc Natl Acad Sci U S A 1993;90:9930-4.

181. Duchow J, Marchant A, Crusiaux A, et al. Impaired phagocyte responses to lipopolysaccharide in paroxysmal nocturnal hemoglobinuria. Infect Immun 1993;61:4280-5.

182. Sultzer BM, Castagna R, Bandekar J and Wong P. Lipopolysaccharide nonresponder cells: the C3H/HeJ defect. Immunobiology 1993;187:257-71.

183. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282:2085-8.

184. Ulevitch RJ, Tobias PS. Recognition of endotoxin by cells leading to transmembrane signaling. Curr Opin Immunol 1994;6:125-30.

185. Delude RL, Savedra R, Jr., Zhao H, et al. CD14 enhances cellular responses to endotoxin without imparting ligand-specific recognition. Proc Natl Acad Sci U S A 1995;92:9288-92.

186. Colucci M, Balconi G, Lorenzet R, et al. Cultured human endothelial cells generate tissue factor in response to endotoxin. J Clin Invest 1983;71:1893-6.

187. Schleimer RP, Rutledge BK. Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters. J Immunol 1986;136:649-54.

188. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS and Gimbrone MA, Jr. Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci U S A 1987;84:9238-42.

189. Dustin ML, Springer TA. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J Cell Biol 1988;107:321-31.

190. Carlos TM, Schwartz BR, Kovach NL, et al. Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. Blood 1990;76:965-70.

191. Miossec P, Cavender D and Ziff M. Production of interleukin 1 by human endothelial cells. J Immunol 1986;136:2486-91.

192. Jirik FR, Podor TJ, Hirano T, et al. Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. J Immunol 1989;142:144-7.

193. Meyrick BO, Ryan US and Brigham KL. Direct effects of E coli endotoxin on structure and permeability of pulmonary endothelial monolayers and the endothelial layer of intimal explants. Am J Pathol 1986;122:140-51.

194. Patrick D, Betts J, Frey EA, Prameya R, Dorovini-Zis K and Finlay BB. Haemophilus influenzae lipopolysaccharide disrupts confluent monolayers of bovine brain endothelial cells via a serum-dependent cytotoxic pathway. J Infect Dis 1992;165:865-72.

195. Haziot A, Rong GW, Silver J and Goyert SM. Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. J Immunol 1993;151:1500-7.

196. Arditi M, Zhou J, Dorio R, Rong GW, Goyert SM and Kim KS. Endotoxin-mediated endothelial cell injury and activation: role of soluble CD14. Infect Immun 1993;61:3149-56.

197. Goldblum SE, Brann TW, Ding X, Pugin J and Tobias PS. Lipopolysaccharide (LPS)-binding protein and soluble CD14 function as accessory molecules for LPS-induced changes in endothelial barrier function, in vitro. J Clin Invest 1994;93:692-702.

198. Striz I, Mio T, Adachi Y, Bazil V and Rennard S. The CD14 molecule participates in regulation of IL-8 and IL-6 release by bronchial epithelial cells. Immunol Lett 1998;62:177-81.

199. Loppnow H, Stelter F, Schonbeck U, et al. Endotoxin activates human vascular smooth muscle cells despite lack of expression of CD14 mRNA or endogenous membrane CD14. Infect Immun 1995;63:1020-6.

200. Hayashi J, Masaka T, Saito I and Ishikawa I. Soluble CD14 mediates lipopolysaccharide-induced intercellular adhesion molecule 1 expression in cultured human gingival fibroblasts. Infect Immun 1996;64:4946-51.

201. Verhasselt V, Buelens C, Willems F, De Groote D, Haeffner-Cavaillon N and Goldman M. Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway. J Immunol 1997;158:2919-25.

202. Yu B, Wright SD. Catalytic properties of lipopolysaccharide (LPS) binding protein. Transfer of LPS to soluble CD14. J Biol Chem 1996;271:4100-5.

203. Zhang JK, Morrison TK, Falk MC, Kang YH and Lee CH. Characterization of the binding of soluble CD14 to human endothelial cells and mechanism of CD14-dependent cell activation by LPS. J Endotoxin Res 1996;3:307-15

204. Vita N, Lefort S, Sozzani P, et al. Detection and biochemical characteristics of the receptor for complexes of soluble CD14 and bacterial lipopolysaccharide. J Immunol 1997;158:3457-62.

205. Tapping RI, Tobias PS. Cellular binding of soluble CD14 requires lipopolysaccharide (LPS) and LPS-binding protein. J Biol Chem 1997;272:23157-64.

206. Sweet MJ, Hume DA. Endotoxin signal transduction in macrophages. J Leukoc Biol 1996;60:8-26.

207. Gerondakis S, Grumont R, Rourke I and Grossmann M. The regulation and roles of Rel/NF-kappa B transcription factors during lymphocyte activation. Curr Opin Immunol 1998;10:353-9.

208. Foo SY, Nolan GP. NF-kappaB to the rescue: RELs, apoptosis and cellular transformation. Trends Genet 1999;15:229-35.

209. Muller JM, Ziegler-Heitbrock HW and Baeuerle PA. Nuclear factor kappa B, a mediator of lipopolysaccharide effects. Immunobiology 1993;187:233-56.

210. May MJ, Ghosh S. IkappaB kinases: kinsmen with different crafts. Science 1999;284:271-3.

211. Yamaoka S, Courtois G, Bessia C, et al. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. Cell 1998;93:1231-40.

212. O'Connell MA, Bennett BL, Mercurio F, Manning AM and Mackman N. Role of IKK1 and IKK2 in lipopolysaccharide signaling in human monocytic cells. J Biol Chem 1998;273:30410-4.

213. Lee FS, Hagler J, Chen ZJ and Maniatis T. Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. Cell 1997;88:213-22.

214. Ling L, Cao Z and Goeddel DV. NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. Proc Natl Acad Sci U S A 1998;95:3792-7.

215. Nakano H, Shindo M, Sakon S, et al. Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci U S A 1998;95:3537-42.

216. Herrera-Velit P, Knutson KL and Reiner NE. Phosphatidylinositol 3-kinase-dependent activation of protein kinase C-zeta in bacterial lipopolysaccharide-treated human monocytes. J Biol Chem 1997;272:16445-52.

217. Stefanova I, Corcoran ML, Horak EM, Wahl LM, Bolen JB and Horak ID. Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56lyn. J Biol Chem 1993;268:20725-8.

218. Henricson BE, Carboni JM, Burkhardt AL and Vogel SN. LPS and Taxol activate Lyn kinase autophosphorylation in Lps(n), but not in Lpsd), macrophages. Mol Med 1995;1:428-35.

219. Jakway JP, DeFranco AL. Pertussis toxin inhibition of B cell and macrophage responses to bacterial lipopolysaccharide. Science 1986;234:743-6.

220. Reimann KA, Waite BC, Lee-Parritz DE, et al. Use of human leukocyte-specific monoclonal antibodies for clinically immunophenotyping lymphocytes of rhesus monkeys. Cytometry 1994;17:102-8.

221. Geppert TD, Whitehurst CE, Thompson P and Beutler B. Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway. Mol Med 1994;1:93-103.

222. Scherle PA, Jones EA, Favata MF, et al. Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E2 production in lipopolysaccharide-stimulated monocytes. J Immunol 1998;161:5681-6.

223. Marais R, Wynne J and Treisman R. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell 1993;73:381-93.

224. Watson DK, Robinson L, Hodge DR, Kola I, Papas TS and Seth A. FLI1 and EWS-FLI1 function as ternary complex factors and ELK1 and SAP1a function as ternary and quaternary complex factors on the Egr1 promoter serum response elements. Oncogene 1997;14:213-21.

225. Rolli M, Kotlyarov A, Sakamoto KM, Gaestel M and Neininger A. Stress-induced stimulation of early growth response gene-1 by p38/stress-activated protein kinase 2 is mediated by a cAMP-responsive promoter element in a MAPKAP kinase 2-independent manner. J Biol Chem 1999;274:19559-64.

226. Hambleton J, Weinstein SL, Lem L and DeFranco AL. Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. Proc Natl Acad Sci U S A 1996;93:2774-8.

227. Bagrodia S, Derijard B, Davis RJ and Cerione RA. Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J Biol Chem 1995;270:27995-8.

228. Brown SB, Kluck RM and Ellem KA. Loss and shedding of surface markers from the leukemic myeloid monocytic line THP-1 induced to undergo apoptosis. J Cell Biochem 1996;60:246-59.

229. Brown JL, Stowers L, Baer M, Trejo J, Coughlin S and Chant J. Human Ste20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway. Curr Biol 1996;6:598-605.

230. Hirai S, Izawa M, Osada S, Spyrou G and Ohno S. Activation of the JNK pathway by distantly related protein kinases, MEKK and MUK. Oncogene 1996;12:641-50.

231. Hirai S, Katoh M, Terada M, et al. MST/MLK2, a member of the mixed lineage kinase family, directly phosphorylates and activates SEK1, an activator of c-Jun N-terminal kinase/stress-activated protein kinase. J Biol Chem 1997;272:15167-73.

232. Karin M. Mitogen-activated protein kinase cascades as regulators of stress responses. Ann N Y Acad Sci 1998;851:139-46.

233. Yang D, Tournier C, Wysk M, et al. Targeted disruption of the MKK4 gene causes embryonic death, inhibition of c-Jun NH2-terminal kinase activation, and defects in AP-1 transcriptional activity. Proc Natl Acad Sci U S A 1997;94:3004-9.

234. Tournier C, Whitmarsh AJ, Cavanagh J, Barrett T and Davis RJ. The MKK7 gene encodes a group of c-Jun NH2-terminal kinase kinases. Mol Cell Biol 1999;19:1569-81.

235. Pombo CM, Kehrl JH, Sanchez I, et al. Activation of the SAPK pathway by the human STE20 homologue germinal centre kinase. Nature 1995;377:750-4.

236. Aronheim A, Zandi E, Hennemann H, Elledge SJ and Karin M. Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. Mol Cell Biol 1997;17:3094-102.

237. Han J, Richter B, Li Z, Kravchenko V and Ulevitch RJ. Molecular cloning of human p38 MAP kinase. Biochim Biophys Acta 1995;1265:224-7.

238. Zhang S, Han J, Sells MA, et al. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J Biol Chem 1995;270:23934-6.

239. Tibbles LA, Ing YL, Kiefer F, et al. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. Embo J 1996;15:7026-35.

240. Derijard B, Raingeaud J, Barrett T, et al. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science 1995;267:682-5.

241. Han J, Lee JD, Jiang Y, Li Z, Feng L and Ulevitch RJ. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). J Biol Chem 1996;271:2886-91.

242. Goh KC, deVeer MJ and Williams BR. The protein kinase PKR is required for p38 MAPK activation and the innate immune response to bacterial endotoxin. Embo J 2000;19:4292-7.

243. Zhang D, Gaussin V, Taffet GE, et al. TAK1 is activated in the myocardium after pressure overload and is sufficient to provoke heart failure in transgenic mice. Nat Med 2000;6:556-63.

244. Wang XZ, Ron D. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. Science 1996;272:1347-9.

245. Janknecht R, Hunter T. Convergence of MAP kinase pathways on the ternary complex factor Sap-1a. Embo J 1997;16:1620-7.

246. Fukunaga R, Hunter T. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. Embo J 1997;16:1921-33.

247. Deak M, Clifton AD, Lucocq LM and Alessi DR. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. Embo J 1998;17:4426-41.

248. Lee JC, Kassis S, Kumar S, Badger A and Adams JL. p38 mitogen-activated protein kinase inhibitors--mechanisms and therapeutic potentials. Pharmacol Ther 1999;82:389-97.

249. Young PR, McLaughlin MM, Kumar S, et al. Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. J Biol Chem 1997;272:12116-21.

250. Kang YH, Dwivedi RS and Lee CH. Ultrastructural and immunocytochemical study of the uptake and distribution of bacterial lipopolysaccharide in human monocytes. J Leukoc Biol 1990;48:316-32.

251. Luchi M, Munford RS. Binding, internalization, and deacylation of bacterial lipopolysaccharide by human neutrophils. J Immunol 1993;151:959-69.

252. Zlydaszyk JC, Moon RJ. Fate of 51Cr-labeled lipopolysaccharide in tissue culture cells and livers of normal mice. Infect Immun 1976;14:100-5.

253. Ulevitch RJ. The preparation and characterization of a radioiodinated bacterial lipopolysaccharide. Immunochemistry 1978;15:157-64.

254. Fox ES, Thomas P and Broitman SA. Clearance of gut-derived endotoxins by the liver. Release and modification of 3H, 14C-lipopolysaccharide by isolated rat Kupffer cells. Gastroenterology 1989;96:456-61.

255. Kang YH, Carl M, Maheshwari RK, Watson LP, Yaffe L and Grimley PM. Incorporation of bacterial lipopolysaccharide by human Leu-11a+ natural killer cells. Ultrastructural and functional correlations. Lab Invest 1988;58:196-209.

256. Morrison DC. Nonspecific interactions of bacterial lipopolysaccharides with membranes and membrane components. In: Cellular Biology of Endotoxin, Berry, L. J. (ed.), Elsevier, New York 1985:25-55

257. Haeffner-Cavaillon N, Cavaillon JM, Etievant M, Lebbar S and Szabo L. Specific binding of endotoxin to human monocytes and mouse macrophages: serum requirement. Cell Immunol 1985;91:119-31.

258. Couturier C, Haeffner-Cavaillon N, Caroff M and Kazatchkine MD. Binding sites for endotoxins (lipopolysaccharides) on human monocytes. J Immunol 1991;147:1899-904.

259. Heumann D, Gallay P, Barras C, et al. Control of lipopolysaccharide (LPS) binding and LPS-induced tumor necrosis factor secretion in human peripheral blood monocytes. J Immunol 1992;148:3505-12.

260. Tobias PS, Soldau K, Kline L, et al. Cross-linking of lipopolysaccharide (LPS) to CD14 on THP-1 cells mediated by LPS-binding protein. J Immunol 1993;150:3011-21.

261. Gallay P, Jongeneel CV, Barras C, et al. Short time exposure to lipopolysaccharide is sufficient to activate human monocytes. J Immunol 1993;150:5086-93.

262. Kirkland TN, Finley F, Leturcq D, et al. Analysis of lipopolysaccharide binding by CD14. J Biol Chem 1993;268:24818-23.

263. Kitchens RL, Munford RS. Enzymatically deacylated lipopolysaccharide (LPS) can antagonize LPS at multiple sites in the LPS recognition pathway. J Biol Chem 1995;270:9904-10.

264. Troelstra A, Antal-Szalmas P, de Graaf-Miltenburg LA, et al. Saturable CD14-dependent binding of fluorescein-labeled lipopolysaccharide to human monocytes. Infect Immun 1997;65:2272-7.

265. Corrales I, Weersink AJ, Verhoef J and van Kessel KP. Serum-independent binding of lipopolysaccharide to human monocytes is trypsin sensitive and does not involve CD14. Immunology 1993;80:84-9.

266. Schromm AB, Brandenburg K, Rietschel ET, Flad HD, Carroll SF and Seydel U. Lipopolysaccharide-binding protein mediates CD14-independent intercalation of lipopolysaccharide into phospholipid membranes. FEBS Lett 1996;399:267-71.

267. Juan TS, Kelley MJ, Johnson DA, et al. Soluble CD14 truncated at amino acid 152 binds lipopolysaccharide (LPS) and enables cellular response to LPS. J Biol Chem 1995;270:1382-7.

268. Viriyakosol S, Kirkland TN. A region of human CD14 required for lipopolysaccharide binding. J Biol Chem 1995;270:361-8.

269. McGinley MD, Narhi LO, Kelley MJ, et al. CD14: physical properties and identification of an exposed site that is protected by lipopolysaccharide. J Biol Chem 1995;270:5213-8.

270. Rustici A, Velucchi M, Faggioni R, et al. Molecular mapping and detoxification of the lipid A binding site by synthetic peptides. Science 1993;259:361-5.

271. Hoess A, Schneider-Mergener J and Liddington RC. Identification of the LPS-binding domain of an endotoxin neutralising protein, Limulus anti-LPS factor. Prog Clin Biol Res 1995;392:327-37

272. Schumann RR, Lamping N and Hoess A. Interchangeable endotoxin-binding domains in proteins with opposite lipopolysaccharide-dependent activities. J Immunol 1997;159:5599-605.

273. Beamer LJ, Carroll SF and Eisenberg D. The BPI/LBP family of proteins: a structural analysis of conserved regions. Protein Sci 1998;7:906-14

274. Shapiro RA, Cunningham MD, Ratcliffe K, et al. Identification of CD14 residues involved in specific lipopolysaccharide recognition. Infect Immun 1997;65:293-7.

275. Cunningham MD, Shapiro RA, Seachord C, Ratcliffe K, Cassiano L and Darveau RP. CD14 employs hydrophilic regions to "capture" lipopolysaccharides. J Immunol 2000;164:3255-63.

276. Haeffner-Cavaillon N, Cavaillon J and Szabo M. Cellular receptors for endotoxin. In: Cellular Biology of Endotoxin, Berry, L. J. (ed.), Elsevier, North Holland, Amsterdam 1985:1-24

277. Schletter J, Brade H, Brade L, et al. Binding of lipopolysaccharide (LPS) to an 80-kilodalton membrane protein of human cells is mediated by soluble CD14 and LPS-binding protein. Infect Immun 1995;63:2576-80.

278. el-Samalouti VT, Schletter J, Chyla I, et al. Identification of the 80-kDa LPS-binding protein (LMP80) as decay-accelerating factor (DAF, CD55). FEMS Immunol Med Microbiol 1999;23:259-69.

279. Triantafilou K, Triantafilou M and Dedrick RL. Interactions of bacterial lipopolysaccharide and peptidoglycan with a 70 kDa and an 80 kDa protein on the cell surface of CD14+ and CD14- cells. Hum Immunol 2001;62:50-63.

280. Lei MG, Morrison DC. Specific endotoxic lipopolysaccharide-binding proteins on murine splenocytes. II. Membrane localization and binding characteristics. J Immunol 1988;141:1006-11.

281. Kirikae T, Inada K, Hirata M, Yoshida M, Kondo S and Hisatsune K. Identification of Re lipopolysaccharide-binding protein on murine erythrocyte membrane. Microbiol Immunol 1988;32:33-44

282. Hampton RY, Golenbock DT and Raetz CR. Lipid A binding sites in membranes of macrophage tumor cells. J Biol Chem 1988;263:14802-7.

283. Kirkland TN, Virca GD, Kuus-Reichel T, et al. Identification of lipopolysaccharide-binding proteins in 70Z/3 cells by photoaffinity cross-linking. J Biol Chem 1990;265:9520-5.

284. Wright SD, Jong MT. Adhesion-promoting receptors on human macrophages recognize Escherichia coli by binding to lipopolysaccharide. J Exp Med 1986;164:1876-88.

285. Wright SD, Detmers PA, Aida Y, et al. CD18-deficient cells respond to lipopolysaccharide in vitro. J Immunol 1990;144:2566-71.

286. Mathew EC, Shaw JM, Bonilla FA, Law SK and Wright DA. A novel point mutation in CD18 causing the expression of dysfunctional CD11/CD18 leucocyte integrins in a patient with leucocyte adhesion deficiency (LAD). Clin Exp Immunol 2000;121:133-8

287. Zarewych DM, Kindzelskii AL, Todd RF, 3rd and Petty HR. LPS induces CD14 association with complement receptor type 3, which is reversed by neutrophil adhesion. J Immunol 1996;156:430-3.

288. Ingalls RR, Arnaout MA and Golenbock DT. Outside-in signaling by lipopolysaccharide through a tailless integrin. J Immunol 1997;159:433-8.

289. Greaves DR, Gough PJ and Gordon S. Recent progress in defining the role of scavenger receptors in lipid transport, atherosclerosis and host defence. Curr Opin Lipidol 1998;9:425-32.

290. Peiser L, Gough PJ, Kodama T and Gordon S. Macrophage class A scavenger receptor-mediated phagocytosis of Escherichia coli: role of cell heterogeneity, microbial strain, and culture conditions in vitro. Infect Immun 2000;68:1953-63.

291. Krieger M. The other side of scavenger receptors: pattern recognition for host defense. Curr Opin Lipidol 1997;8:275-80.

292. Haworth R, Platt N, Keshav S, et al. The macrophage scavenger receptor type A is expressed by activated macrophages and protects the host against lethal endotoxic shock. J Exp Med 1997;186:1431-9.

293. Bara J, Lallier R, Trudel M, Brailovsky C and Nigam VN. Molecular models on the insertion of a Salmonella minnesota R-form glycolipid into the cell membrane of normal and transformed cells. Eur J Biochem 1973;35:495-8.

294. Vasselon T, Hailman E, Thieringer R and Detmers PA. Internalization of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14. J Exp Med 1999;190:509-21.

295. Morrison DC, Rudbach JA. Endotoxin-cell-membrane interactions leading to transmembrane signaling. Contemp Top Mol Immunol 1981;8:187-218

296. Kriegsmann J, Gay S and Brauer R. Endocytosis of lipopolysaccharide in mouse macrophages. Cell Mol Biol (Noisy-le-grand) 1993;39:791-800.

297. Maruyama N, Kakuta Y, Yamauchi K, et al. Quinine inhibits production of tumor necrosis factor-alpha from human alveolar macrophages. Am J Respir Cell Mol Biol 1994;10:514-20.

298. Hu Y, Fisette PL, Denlinger LC, et al. Purinergic receptor modulation of lipopolysaccharide signaling and inducible nitric-oxide synthase expression in RAW 264.7 macrophages. J Biol Chem 1998;273:27170-5.

299. de Duve C. Lysosomes. In: Ciba Foundation Symposium, edited by A. V. S. de Reuck and M. P. Cameron. London: Churchill 1963:411-2

300. Roth TF, Porter KR. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti L*. J Cell Biol 1964;20:313-32

301. Kanaseki T, Kadota K. The "vesicle in a basket". A morphological study of the coated vesicle isolated from the nerve endings of the guinea pig brain, with special reference to the mechanism of membrane movements. J Cell Biol 1969;42:202-20.

302. Willingham MC, Pastan IH, Sahagian GG, Jourdian GW and Neufeld EF. Morphologic study of the internalization of a lysosomal enzyme by the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. Proc Natl Acad Sci U S A 1981;78:6967-71.

303. Anderson RG, Brown MS and Goldstein JL. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. Cell 1977;10:351-64.

304. Anderson RG, Brown MS, Beisiegel U and Goldstein JL. Surface distribution and recycling of the low density lipoprotein receptor as visualized with antireceptor antibodies. J Cell Biol 1982;93:523-31.

305. Dunn WA, Hubbard AL. Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: ligand and receptor dynamics. J Cell Biol 1984;98:2148-59.

306. Miettinen HM, Matter K, Hunziker W, Rose JK and Mellman I. Fc receptor endocytosis is controlled by a cytoplasmic domain determinant that actively prevents coated pit localization. J Cell Biol 1992;116:875-88.

307. Schmid SL. The mechanism of receptor-mediated endocytosis: more questions than answers. Bioessays 1992;14:589-96.

308. Ohno H, Stewart J, Fournier MC, et al. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science 1995;269:1872-5.

309. Heuser J. Three-dimensional visualization of coated vesicle formation in fibroblasts. J Cell Biol 1980;84:560-83.

310. Schmid SL, Carter LL. ATP is required for receptor-mediated endocytosis in intact cells. J Cell Biol 1990;111:2307-18

311. Voorhees P, Deignan E, van Donselaar E, et al. An acidic sequence within the cytoplasmic domain of furin functions as a determinant of trans-Golgi network localization and internalization from the cell surface. Embo J 1995;14:4961-75.

312. Rabinovitch M. Professional and non-professional phagocytes: an introduction. Trends Cell Biol 1995;5:85-8

313. Greenberg S, Silverstein SC. Phagocytosis. In: Fundamental Immunology, edited by W. E. Paul. New York: Raven 1993:941-64

314. Griffin FM, Jr., Griffin JA, Leider JE and Silverstein SC. Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. J Exp Med 1975;142:1263-82.

315. Griffin FM, Jr., Griffin JA and Silverstein SC. Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow-derived lymphocytes. J Exp Med 1976;144:788-809.

316. Greenberg S. Signal transduction of phagocytosis. Trends Cell Biol 1995;5:93-9

317. Swanson JA, Baer SC. Phagocytosis by zippers and triggers. Trends Cell Biol 1995;5:89-93

318. Silverstein SC, Greenberg S, Di Virgilo F and Steinberg TH. Phagocytosis. In: Fundamental Immunology, edited by W. E. Paul. New York: Raven 1989:703-33

319. Goldman R. Lectin-mediated attachment and ingestion of yeast cells and erythrocytes by hamster fibroblasts. Exp Cell Res 1977;104:325-34.

320. Falkow S, Isberg RR and Portnoy DA. The interaction of bacteria with mammalian cells. Annu Rev Cell Biol 1992;8:333-63

321. Daukas G, Zigmond SH. Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes. J Cell Biol 1985;101:1673-9.

322. Sandvig K, Olsnes S, Petersen OW and Van Deurs B. Acidification of the cytosol inhibits endocytosis from coated pits. J Cell Biol 1987;105:679-89

323. Doxsey SJ, Brodsky FM, Blank GS and Helenius A. Inhibition of endocytosis by anti-clathrin antibodies. Cell 1987;50:453-63.

324. Novak JM, Ward DM, Buys SS and Kaplan J. Effect of hypo-osmotic incubation on membrane recycling. J Cell Physiol 1988;137:235-42.

325. Carpentier JL, Sawano F, Geiger D, Gorden P, Perrelet A and Orci L. Potassium depletion and hypertonic medium reduce "non-coated" and clathrin-coated pit formation, as well as endocytosis through these two gates. J Cell Physiol 1989;138:519-26.

326. Myers JN, Tabas I, Jones NL and Maxfield FR. Beta-very low density lipoprotein is sequestered in surface-connected tubules in mouse peritoneal macrophages. J Cell Biol 1993;123:1389-402.

327. Mukherjee S, Ghosh RN and Maxfield FR. Endocytosis. Physiol Rev 1997;77:759-803.

328. Van Deurs B, Holm PK, Sandvig K and Hansen SH. Are caveolae involved in clathrin-independent endocytosis? Trends Cell Biol 1993;3:249-51

329. Peters KR, Carley WW and Palade GE. Endothelial plasmalemmal vesicles have a characteristic striped bipolar surface structure. J Cell Biol 1985;101:2233-8.

330. Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR and Anderson RG. Caveolin, a protein component of caveolae membrane coats. Cell 1992;68:673-82.

331. Kurzchalia TV, Dupree P and Monier S. VIP21-Caveolin, a protein of the trans-Golgi network and caveolae. FEBS Lett 1994;346:88-91.

332. Stefanova I, Horejsi V, Ansotegui IJ, Knapp W and Stockinger H. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science 1991;254:1016-9.

333. Bamezai A, Goldmacher VS and Rock KL. Internalization of glycosyl-phosphatidylinositol (GPI)-anchored lymphocyte proteins. II. GPI-anchored and transmembrane molecules internalize through distinct pathways. Eur J Immunol 1992;22:15-21.

334. Keller GA, Siegel MW and Caras IW. Endocytosis of glycophospholipid-anchored and transmembrane forms of CD4 by different endocytic pathways. Embo J 1992;11:863-74.

335. Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 1992;68:533-44.

336. Sargiacomo M, Sudol M, Tang Z and Lisanti MP. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J Cell Biol 1993;122:789-807.

337. Anderson RG. Plasmalemmal caveolae and GPI-anchored membrane proteins. Curr Opin Cell Biol 1993;5:647-52.

338. Smart EJ, Ying YS, Mineo C and Anderson RG. A detergent-free method for purifying caveolae membrane from tissue culture cells. Proc Natl Acad Sci U S A 1995;92:10104-8.

339. Parton RG. Caveolae and caveolins. Curr Opin Cell Biol 1996;8:542-8.

340. Kiss AL, Geuze HJ. Caveolae can be alternative endocytotic structures in elicited macrophages. Eur J Cell Biol 1997;73:19-27.

341. Swanson JA, Watts C. Macropinocytosis. Trends Cell Biol 1995;5:424-8

342. Ruiter DJ, van der Meulen J, Brouwer A, et al. Uptake by liver cells of endotoxin following its intravenous injection. Lab Invest 1981;45:38-45.

343. Freudenberg MA, Freudenberg N and Galanos C. Time course cellular distribution of endotoxin in liver, lungs and kidney of rats. Br J exp Path 1982;63:56-65

344. Freudenberg MA, Kleine B and Galanos C. The fate of lipopolysaccharide in rats: evidence for chemical alteration in the molecule. Rev Infect Dis 1984;6:483-7.

345. Munford RS. Deacylation of bacterial endotoxins by neutrophils and macrophages: early observations and hypotheses. Methods Find Exp Clin Pharmacol 1986;8:63-5.

346. Kitchens RL, Ulevitch RJ and Munford RS. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. J Exp Med 1992;176:485-94.

347. Peterson AA, Munford RS. Dephosphorylation of the lipid A moiety of Escherichia coli lipopolysaccharide by mouse macrophages. Infect Immun 1987;55:974-8.

348. Kang YH, Carl M, Watson LP and Yaffe L. Immunoultrastructural studies of human NK cells: II. Effector-target cell binding and phagocytosis. Anat Rec 1987;217:290-304.

349. McGivney A, Bradley SG. Effects of bacterial endotoxin on lysosomal enzyme activities of human cells in continuous culture. J Reticuloendothel Soc 1978;23:223-30.

350. Jonas L, Schutt C, Neels P, Walzel H and Siegl E. Electron microscopic study of receptor mediated endocytosis of a monoclonal antibody (RoMo-1) against the surface marker CD 14 of human monocytes. Acta Histochem Suppl 1990;39:339-44

351. Hashimoto C, Hudson KL and Anderson KV. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 1988;52:269-79.

352. Eldon E, Kooyer S, D'Evelyn D, et al. The Drosophila 18 wheeler is required for morphogenesis and has striking similarities to Toll. Development 1994;120:885-99.

353. Tauszig S, Jouanguy E, Hoffmann JA and Imler JL. From the cover: toll-related receptors and the control of antimicrobial peptide expression in drosophila [In Process Citation]. Proc Natl Acad Sci U S A 2000;97:10520-5

354. Lemaitre B, Nicolas E, Michaut L, Reichhart JM and Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 1996;86:973-83.

355. Williams MJ, Rodriguez A, Kimbrell DA and Eldon ED. The 18-wheeler mutation reveals complex antibacterial gene regulation in Drosophila host defense. Embo J 1997;16:6120-30.

356. Gay NJ, Keith FJ. Drosophila Toll and IL-1 receptor. Nature 1991;351:355-6.

357. Wasserman K, Subklewe M, Pothoff G, Banik N and Schell-Frederick E. Expression of surface markers on alveolar macrophages from symptomatic patients with HIV infection as detected by flow cytometry. Chest 1994;105:1324-34.

358. Hultmark D. Insect immunology. Ancient relationships. Nature 1994;367:116-7.

359. Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C and Baker B. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. Cell 1994;78:1101-15.

360. Medzhitov R, Preston-Hurlburt P and Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388:394-7.

361. Chaudhary PM, Ferguson C, Nguyen V, et al. Cloning and characterization of two Toll/Interleukin-1 receptor-like genes TIL3 and TIL4: evidence for a multi-gene receptor family in humans. Blood 1998;91:4020-7

362. Takeuchi O, Kawai T, Sanjo H, et al. TLR6: A novel member of an expanding toll-like receptor family. Gene 1999;231:59-65.

363. Qureshi ST, Lariviere L, Leveque G, et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4) [see comments] [published erratum appears in J Exp Med 1999 May 3;189(9):following 1518]. J Exp Med 1999;189:615-25

364. Hoshino K, Takeuchi O, Kawai T, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 1999;162:3749-52.

365. Janeway CA. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today 1992;13:11-6

366. Medzhitov R, Janeway CA, Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997;91:295-8.

367. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R and Golenbock D. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 1999;163:1-5.

368. Underhill DM, Ozinsky A, Smith KD and Aderem A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc Natl Acad Sci U S A 1999;96:14459-63.

369. Lehner MD, Morath S, Michelsen KS, Schumann RR and Hartung T. Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different Toll-like receptors independent of paracrine mediators. J Immunol 2001;166:5161-7.

370. Means TK, Lien E, Yoshimura A, Wang S, Golenbock DT and Fenton MJ. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J Immunol 1999;163:6748-55.

371. Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT and Fenton MJ. Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. J Immunol 1999;163:3920-7.

372. Underhill DM, Ozinsky A, Hajjar AM, et al. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 1999;401:811-5.

373. Aliprantis AO, Yang RB, Mark MR, et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 1999;285:736-9.

374. Brightbill HD, Libraty DH, Krutzik SR, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 1999;285:732-6.

375. Lien E, Sellati TJ, Yoshimura A, et al. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem 1999;274:33419-25.

376. Aliprantis AO, Yang RB, Weiss DS, Godowski P and Zychlinsky A. The apoptotic signaling pathway activated by Toll-like receptor-2. Embo J 2000;19:3325-36.

377. Hirschfeld M, Weis JJ, Toshchakov V, et al. Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect Immun 2001;69:1477-82.

378. Thoma-Uszynski S, Stenger S, Takeuchi O, et al. Induction of direct antimicrobial activity through mammalian toll-like receptors. Science 2001;291:1544-7.

379. Takeuchi O, Kaufmann A, Grote K, et al. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. J Immunol 2000;164:554-7.

380. Ozinsky A, Underhill DM, Fontenot JD, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 2000;97:13766-71.

381. Hajjar AM, O'Mahony DS, Ozinsky A, et al. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J Immunol 2001;166:15-9.

382. Takeuchi O, Hoshino K, Kawai T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999;11:443-51.

383. Ohashi K, Burkart V, Flohe S and Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol 2000;164:558-61.

384. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K and Nishijima M. Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol. J Biol Chem 2000;275:2251-4.

385. Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740-5.

386. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001;410:1099-103.

387. Eaves-Pyles T, Murthy K, Liaudet L, et al. Flagellin, a novel mediator of Salmonella-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. J Immunol 2001;166:1248-60.

388. Yang RB, Mark MR, Gray A, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling [see comments]. Nature 1998;395:284-8

389. Viriyakosol S, Tobias PS, Kitchens RL and Kirkland TN. MD-2 binds to bacterial lipopolysaccharide. J Biol Chem 2001;276:38044-51.

390. da Silva Correia J, Soldau K, Christen U, Tobias PS and Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. J Biol Chem 2001;276:21129-35.

391. Kirschning CJ, Wesche H, Merrill Ayres T and Rothe M. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J Exp Med 1998;188:2091-7.

392. Yang RB, Mark MR, Gurney AL and Godowski PJ. Signaling events induced by lipopolysaccharide-activated toll-like receptor 2. J Immunol 1999;163:639-43.

393. Hirschfeld M, Ma Y, Weis JH, Vogel SN and Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol 2000;165:618-22.

394. Poltorak A, Smirnova I, He X, et al. Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region [published erratum appears in Blood Cells Mol Dis 1999 Feb;25(1):78]. Blood Cells Mol Dis 1998;24:340-55

395. Qureshi ST, Lariviere L, Leveque G, et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med 1999;189:615-25.

396. Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000;25:187-91.

397. Chow JC, Young DW, Golenbock DT, Christ WJ and Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J Biol Chem 1999;274:10689-92.

398. Iwami KI, Matsuguchi T, Masuda A, Kikuchi T, Musikacharoen T and Yoshikai Y. Cutting edge: naturally occurring soluble form of mouse Toll-like receptor 4 inhibits lipopolysaccharide signaling. J Immunol 2000;165:6682-6.

399. Poltorak A, Ricciardi-Castagnoli P, Citterio S and Beutler B. Physical contact between lipopolysaccharide and toll-like receptor 4 revealed by genetic complementation. Proc Natl Acad Sci U S A 2000;97:2163-7.

400. Shimazu R, Akashi S, Ogata H, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll- like receptor 4. J Exp Med 1999;189:1777-82.

401. Akashi S, Ogata H, Kirikae F, et al. Regulatory roles for CD14 and phosphatidylinositol in the signaling via toll-like receptor 4-MD-2. Biochem Biophys Res Commun 2000;268:172-7.

402. Yang H, Young DW, Gusovsky F and Chow JC. Cellular events mediated by lipopolysaccharide-stimulated toll-like receptor 4. MD-2 is required for activation of mitogen-activated protein kinases and Elk-1. J Biol Chem 2000;275:20861-6.

403. Miyake K, Shimazu R, Kondo J, et al. Mouse MD-1, a molecule that is physically associated with RP105 and positively regulates its expression. J Immunol 1998;161:1348-53.

404. Schromm AB, Lien E, Henneke P, et al. Molecular genetic analysis of an endotoxin nonresponder mutant cell line: a point mutation in a conserved region of MD-2 abolishes endotoxin-induced signaling. J Exp Med 2001;194:79-88.

405. Visintin A, Mazzoni A, Spitzer JA and Segal DM. Secreted MD-2 is a large polymeric protein that efficiently confers lipopolysaccharide sensitivity to Toll-like receptor 4. Proc Natl Acad Sci U S A 2001;98:12156-61.

406. Ohnishi T, Muroi M and Tanamoto K. N-linked glycosylations at Asn(26) and Asn(114) of human MD-2 are required for toll-like receptor 4-mediated activation of NF-kappaB by lipopolysaccharide. J Immunol 2001;167:3354-9.

407. da Silva Correia J, Ulevitch RJ. MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. J Biol Chem 2002;277:1845-54.

408. Akashi S, Nagai Y, Ogata H, et al. Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition. Int Immunol 2001;13:1595-1599.

409. Muroi M, Ohnishi T and Tanamoto K. MD-2, a novel accessory molecule, is involved in species-specific actions of Salmonella lipid A. Infect Immun 2002;70:3546-50.

410. Nagai Y, Akashi S, Nagafuku M, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat Immunol 2002;3:667-72.

411. Hajjar AM, Ernst RK, Tsai JH, Wilson CB and Miller SI. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat Immunol 2002;3:354-9.

412. Faure E, Equils O, Sieling PA, et al. Bacterial lipopolysaccharide activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. J Biol Chem 2000;275:11058-63.

413. Cario E, Rosenberg IM, Brandwein SL, Beck PL, Reinecker HC and Podolsky DK. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. J Immunol 2000;164:966-72.

414. Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET and Arditi M. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J Immunol 2001;167:1609-16.

415. Dziarski R, Wang Q, Miyake K, Kirschning CJ and Gupta D. MD-2 enables Toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to Gram-positive and Gram-negative bacteria and their cell wall components. J Immunol 2001;166:1938-44.

416. Werts C, Tapping RI, Mathison JC, et al. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nat Immunol 2001;2:346-52.

417. Liu Y, Wang Y, Yamakuchi M, et al. Upregulation of toll-like receptor 2 gene expression in macrophage response to peptidoglycan and high concentration of lipopolysaccharide is involved in NF-kappa b activation. Infect Immun 2001;69:2788-96.

418. Kawai T, Adachi O, Ogawa T, Takeda K and Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 1999;11:115-22.

419. Wesche H, Henzel WJ, Shillinglaw W, Li S and Cao Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity 1997;7:837-47.

420. Cao Z, Xiong J, Takeuchi M, Kurama T and Goeddel DV. TRAF6 is a signal transducer for interleukin-1. Nature 1996;383:443-6.

421. Zhang FX, Kirschning CJ, Mancinelli R, et al. Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. J Biol Chem 1999;274:7611-4.

422. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z and Matsumoto K. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 1999;398:252-6.

423. Takaesu G, Kishida S, Hiyama A, et al. TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol Cell 2000;5:649-58.

424. Kopp EB, Medzhitov R. The Toll-receptor family and control of innate immunity. Curr Opin Immunol 1999;11:13-8

425. Martin TR, Mongovin SM, Tobias PS, et al. The CD14 differentiation antigen mediates the development of endotoxin responsiveness during differentiation of mononuclear phagocytes. J Leukoc Biol 1994;56:1-9.

426. Kalinski P, Schuitemaker JH, Hilkens CM, Wierenga EA and Kapsenberg ML. Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. J Immunol 1999;162:3231-6.

427. Gessani S, Testa U, Varano B, et al. Enhanced production of LPS-induced cytokines during differentiation of human monocytes to macrophages. Role of LPS receptors. J Immunol 1993;151:3758-66.

428. Hurme M, Viherluoto J and Nordstrom T. The effect of calcium mobilization on LPS-induced IL-1 beta production depends on the differentiation stage of the monocytes/macrophages. Scand J Immunol 1992;36:507-11.

429. Poussin C, Foti M, Carpentier JL and Pugin J. CD14-dependent endotoxin internalization via a macropinocytic pathway. J Biol Chem 1998;273:20285-91.

430. Austin B, Altweg M, Gosling PJ and Joseph SW. The genus *Aeromonas*. Whiley, Chichester 1996

431. Chang CY, Thompson H, Rodman N, Bylander J and Thomas J. Pathogenic analysis of Aeromonas hydrophila septicemia. Ann Clin Lab Sci 1997;27:254-9.

432. Chakraborty T, Huhle B, Hof H, Bergbauer H and Goebel W. Marker exchange mutagenesis of the aerolysin determinant in Aeromonas hydrophila demonstrates the role of aerolysin in A. hydrophila- associated systemic infections. Infect Immun 1987;55:2274-80.

433. Howard SP, Buckley JT. Membrane glycoprotein receptor and hole-forming properties of a cytolytic protein toxin. Biochemistry 1982;21:1662-7.

434. Howard SP, Buckley JT. Protein export by a gram-negative bacterium: production of aerolysin by Aeromonas hydrophila. J Bacteriol 1985;161:1118-24.

435. Jahagirdar R, Howard SP. Isolation and characterization of a second exe operon required for extracellular protein secretion in Aeromonas hydrophila. J Bacteriol 1994;176:6819-26.

436. Letellier L, Howard SP and Buckley JT. Studies on the energetics of proaerolysin secretion across the outer membrane of Aeromonas species. Evidence for a requirement for both the protonmotive force and ATP. J Biol Chem 1997;272:11109-13.

437. Dalbey RE, Robinson C. Protein translocation into and across the bacterial plasma membrane and the plant thylakoid membrane. Trends Biochem Sci 1999;24:17-22.

438. Hardie KR, Schulze A, Parker MW and Buckley JT. Vibrio spp. secrete proaerolysin as a folded dimer without the need for disulphide bond formation. Mol Microbiol 1995;17:1035-44

439. Buckley JT, Halasa LN, Lund KD and MacIntyre S. Purification and some properties of the hemolytic toxin aerolysin. Can J Biochem 1981;59:430-5.

440. Howard SP, Garland WJ, Green MJ and Buckley JT. Nucleotide sequence of the gene for the hole-forming toxin aerolysin of Aeromonas hydrophila. J Bacteriol 1987;169:2869-71.

441. Fivaz M, Velluz MC and van der Goot FG. Dimer dissociation of the pore-forming toxin aerolysin precedes receptor binding. J Biol Chem 1999;274:37705-8.

442. Tucker AD, Parker MW, Tsernoglou D and Buckley JT. Crystallization of a proform of aerolysin, a hole-forming toxin from Aeromonas hydrophila. J Mol Biol 1990;212:561-2.

443. Lesieur C, Frutiger S, Hughes G, Kellner R, Pattus F and van der Goot FG. Increased stability upon heptamerization of the pore-forming toxin aerolysin. J Biol Chem 1999;274:36722-8.

444. Garland WJ, Buckley JT. The cytolytic toxin aerolysin must aggregate to disrupt erythrocytes, and aggregation is stimulated by human glycophorin. Infect Immun 1988;56:1249-53.

445. van der Goot FG, Lakey J, Pattus F, et al. Spectroscopic study of the activation and oligomerization of the channel-forming toxin aerolysin: identification of the site of proteolytic activation. Biochemistry 1992;31:8566-70.

446. Abrami L, Fivaz M, Decroly E, et al. The pore-forming toxin proaerolysin is activated by furin. J Biol Chem 1998;273:32656-61.

447. van der Goot FG, Hardie KR, Parker MW and Buckley JT. The C-terminal peptide produced upon proteolytic activation of the cytolytic toxin aerolysin is not involved in channel formation. J Biol Chem 1994;269:30496-501.

448. Wilmsen HU, Leonard KR, Tichelaar W, Buckley JT and Pattus F. The aerolysin membrane channel is formed by heptamerization of the monomer. Embo J 1992;11:2457-63.

449. Moniatte M, van der Goot FG, Buckley JT, Pattus F and van Dorsselaer A. Characterisation of the heptameric pore-forming complex of the Aeromonas toxin aerolysin using MALDI-TOF mass spectrometry. FEBS Lett 1996;384:269-72.

450. Cabiaux V, Buckley JT, Wattiez R, Ruysschaert JM, Parker MW and van der Goot FG. Conformational changes in aerolysin during the transition from the water-soluble protoxin to the membrane channel. Biochemistry 1997;36:15224-32.

451. Rossjohn J, Raja SM, Nelson KL, et al. Movement of a loop in domain 3 of aerolysin is required for channel formation. Biochemistry 1998;37:741-6.

452. Green MJ, Buckley JT. Site-directed mutagenesis of the hole-forming toxin aerolysin: studies on the roles of histidines in receptor binding and oligomerization of the monomer. Biochemistry 1990;29:2177-80.

453. Wilmsen HU, Buckley JT and Pattus F. Site-directed mutagenesis at histidines of aerolysin from Aeromonas hydrophila: a lipid planar bilayer study. Mol Microbiol 1991;5:2745-51.

454. Buckley JT, Wilmsen HU, Lesieur C, et al. Protonation of histidine-132 promotes oligomerization of the channel- forming toxin aerolysin. Biochemistry 1995;34:16450-5.

455. van der Goot FG, Pattus F, Wong KR and Buckley JT. Oligomerization of the channel-forming toxin aerolysin precedes insertion into lipid bilayers. Biochemistry 1993;32:2636-42.

456. Parker MW, Buckley JT, Postma JP, et al. Structure of the Aeromonas toxin proaerolysin in its water-soluble and membrane-channel states. Nature 1994;367:292-5.

457. Cowell S, Aschauer W, Gruber HJ, Nelson KL and Buckley JT. The erythrocyte receptor for the channel-forming toxin aerolysin is a novel glycosylphosphatidylinositol-anchored protein. Mol Microbiol 1997;25:343-50.

458. Nelson KL, Raja SM and Buckley JT. The glycosylphosphatidylinositol-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin aerolysin. J Biol Chem 1997;272:12170-4.

459. Abrami L, Fivaz M, Glauser PE, Parton RG and van der Goot FG. A pore-forming toxin interacts with a GPI-anchored protein and causes vacuolation of the endoplasmic reticulum. J Cell Biol 1998;140:525-40.

460. Diep DB, Nelson KL, Raja SM, Pleshak EN and Buckley JT. Glycosylphosphatidylinositol anchors of membrane glycoproteins are binding determinants for the channel-forming toxin aerolysin. J Biol Chem 1998;273:2355-60.

461. Kinoshita T, Ohishi K and Takeda J. GPI-anchor synthesis in mammalian cells: genes, their products, and a deficiency. J Biochem (Tokyo) 1997;122:251-7.

462. Ferguson MA. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. J Cell Sci 1999;112:2799-809.

463. Brodsky RA, Mukhina GL, Nelson KL, Lawrence TS, Jones RJ and Buckley JT. Resistance of paroxysmal nocturnal hemoglobinuria cells to the glycosylphosphatidylinositol-binding toxin aerolysin. Blood 1999;93:1749-56.

464. MacKenzie CR, Hirama T and Buckley JT. Analysis of receptor binding by the channel-forming toxin aerolysin using surface plasmon resonance. J Biol Chem 1999;274:22604-9.

465. van der Goot FG, Ausio J, Wong KR, Pattus F and Buckley JT. Dimerization stabilizes the pore-forming toxin aerolysin in solution. J Biol Chem 1993;268:18272-9.

466. Barry R, Moore S, Alonso A, Ausio J and Buckley JT. The channel-forming protein proaerolysin remains a dimer at low concentrations in solution. J Biol Chem 2001;276:551-4.

467. Quiocho FA. Carbohydrate-binding proteins: tertiary structures and protein-sugar interactions. Annu Rev Biochem 1986;55:287-315

468. Abrami L, Velluz MC, Hong Y, et al. The glycan core of GPI-anchored proteins modulates aerolysin binding but is not sufficient: the polypeptide moiety is required for the toxin- receptor interaction. FEBS Lett 2002;512:249-54.

469. Zhang F, Crise B, Su B, et al. Lateral diffusion of membrane-spanning and glycosylphosphatidylinositol- linked proteins: toward establishing rules governing the lateral mobility of membrane proteins. J Cell Biol 1991;115:75-84.

470. Harder T, Simons K. Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. Curr Opin Cell Biol 1997;9:534-42.

471. Brown DA, London E. Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol 1998;14:111-36

472. Abrami L, Fivaz M and van der Goot FG. Surface dynamics of aerolysin on the plasma membrane of living cells. Int J Med Microbiol 2000;290:363-7.

473. Nelson KL, Buckley JT. Channel formation by the glycosylphosphatidylinositol-anchored protein binding toxin aerolysin is not promoted by lipid rafts. J Biol Chem 2000;275:19839-43.

474. Fivaz M, Abrami L and van der Goot FG. Landing on lipid rafts. Trends Cell Biol 1999;9:212-3.

475. Brown RE. Sphingolipid organization in biomembranes: what physical studies of model membranes reveal. J Cell Sci 1998;111:1-9.

476. Alonso A, Goni FM and Buckley JT. Lipids favoring inverted phase enhance the ability of aerolysin to permeabilize liposome bilayers. Biochemistry 2000;39:14019-24.

477. Chakraborty T, Schmid A, Notermans S and Benz R. Aerolysin of Aeromonas sobria: evidence for formation of ion-permeable channels and comparison with alpha-toxin of Staphylococcus aureus. Infect Immun 1990;58:2127-32.

478. Wilmsen HU, Pattus F and Buckley JT. Aerolysin, a hemolysin from Aeromonas hydrophila, forms voltage-gated channels in planar lipid bilayers. J Membr Biol 1990;115:71-81.

479. Tschodrich-Rotter M, Kubitscheck U, Ugochukwu G, Buckley JT and Peters R. Optical single-channel analysis of the aerolysin pore in erythrocyte membranes. Biophys J 1996;70:723-32.

480. Hertle R, Hilger M, Weingardt-Kocher S and Walev I. Cytotoxic action of Serratia marcescens hemolysin on human epithelial cells. Infect Immun 1999;67:817-25.

481. Coelho A, Andrade JR, Vicente AC and Dirita VJ. Cytotoxic cell vacuolating activity from Vibrio cholerae hemolysin. Infect Immun 2000;68:1700-5.

482. Krause KH, Fivaz M, Monod A and van der Goot FG. Aerolysin induces G-protein activation and Ca2+ release from intracellular stores in human granulocytes. J Biol Chem 1998;273:18122-9.

483. Ballard J, Crabtree J, Roe BA and Tweten RK. The primary structure of Clostridium septicum alpha-toxin exhibits similarity with that of Aeromonas hydrophila aerolysin. Infect Immun 1995;63:340-4.

484. Hsuan SL, Kannan MS, Jeyaseelan S, et al. Pasteurella haemolytica leukotoxin and endotoxin induced cytokine gene expression in bovine alveolar macrophages requires NF-kappaB activation and calcium elevation. Microb Pathog 1999;26:263-73.

485. Noronha FS, Cruz JS, Beirao PS and Horta MF. Macrophage damage by Leishmania amazonensis cytolysin: evidence of pore formation on cell membrane. Infect Immun 2000;68:4578-84.

486. Grunwald U, Fan X, Jack RS, et al. Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism. J Immunol 1996;157:4119-25.

487. Kitchens RL, Wang P and Munford RS. Bacterial lipopolysaccharide can enter monocytes via two CD14-dependent pathways. J Immunol 1998;161:5534-45.

488. Pollack M, Espinoza AM, Guelde G, Koles NL, Wahl LM and Ohl CA. Lipopolysaccharide (LPS)-specific monoclonal antibodies regulate LPS uptake and LPS-induced tumor necrosis factor-alpha responses by human monocytes. J Infect Dis 1995;172:794-804.

489. Pollack M, Ohl CA, Golenbock DT, et al. Dual effects of LPS antibodies on cellular uptake of LPS and LPS-induced proinflammatory functions. J Immunol 1997;159:3519-30.

490. Ohl CA, Pollack M. Lipopolysaccharide (LPS) antibodies regulate cellular uptake of LPS and LPS induced proinflammatory responses. Prog Clin Biol Res 1998;397:227-34

491. Lazaron V, Leslie DB, Wasiluk KR and Dunn DL. Accelerated internalization and detoxification of endotoxin by anti-lipopolysaccharide antibody is an Fc receptor--mediated process. Surgery 2001;130:192-7.

492. Ding A, Thieblemont N, Zhu J, Jin F, Zhang J and Wright S. Secretory leukocyte protease inhibitor interferes with uptake of lipopolysaccharide by macrophages. Infect Immun 1999;67:4485-9.

493. Peppelenbosch MP, DeSmedt M, ten Hove T, van Deventer SJ and Grooten J. Lipopolysaccharide regulates macrophage fluid phase pinocytosis via CD14-dependent and CD14-independent pathways. Blood 1999;93:4011-8.

494. Kitchens RL, Munford RS. CD14-dependent internalization of bacterial lipopolysaccharide (LPS) is strongly influenced by LPS aggregation but not by cellular responses to LPS. J Immunol 1998;160:1920-8.

495. Thieblemont N, Wright SD. Mice genetically hyporesponsive to lipopolysaccharide (LPS) exhibit a defect in endocytic uptake of LPS and ceramide. J Exp Med 1997;185:2095-100

496. Funda DP, Tuckova L, Farre MA, Iwase T, Moro I and Tlaskalova-Hogenova H. CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells in vitro: lipopolysaccharide activation of epithelial cells revisited. Infect Immun 2001;69:3772-81.

497. Su GL, Dorko K, Strom SC, Nussler AK and Wang SC. CD14 expression and production by human hepatocytes. J Hepatol 1999;31:435-42.

498. Li SW, Gong JP, Wu CX, Shi YJ and Liu CA. Lipopolysaccharide induced synthesis of CD14 proteins and its gene expression in hepatocytes during endotoxemia. World J Gastroenterol 2002;8:124-7.

499. Bocker U, Yezerskyy O, Feick P, et al. Responsiveness of intestinal epithelial cell lines to lipopolysaccharide is correlated with Toll-like receptor 4 but not Toll- like receptor 2 or CD14 expression. Int J Colorectal Dis 2003;18:25-32.

500. Liu S, Gallo DJ, Green AM, et al. Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide. Infect Immun 2002;70:3433-42.

501. Janssens S, Burns K, Tschopp J and Beyaert R. Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. Curr Biol 2002;12:467-71.

502. Liu S, Salyapongse AN, Geller DA, Vodovotz Y and Billiar TR. Hepatocyte toll-like receptor 2 expression in vivo and in vitro: role of cytokines in induction of rat TLR2 gene expression by lipopolysaccharide. Shock 2000;14:361-5.

503. Vodovotz Y, Liu S, McCloskey C, Shapiro R, Green A and Billiar TR. The hepatocyte as a microbial product-responsive cell. J Endotoxin Res 2001;7:365-73

504. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 2000;68:7010-7.

505. Naik S, Kelly EJ, Meijer L, Pettersson S and Sanderson IR. Absence of Toll-like receptor 4 explains endotoxin hyporesponsiveness in human intestinal epithelium. J Pediatr Gastroenterol Nutr 2001;32:449-53.

506. Abreu MT, Arnold ET, Thomas LS, et al. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. J Biol Chem 2002;277:20431-7.

507. Uehara A, Sugawara S, Tamai R and Takada H. Contrasting responses of human gingival and colonic epithelial cells to lipopolysaccharides, lipoteichoic acids and peptidoglycans in the presence of soluble CD14. Med Microbiol Immunol (Berl) 2001;189:185-92.

508. Hornef MW, Frisan T, Vandewalle A, Normark S and Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. J Exp Med 2002;195:559-70.

509. Harris HW, Brady SE and Rapp JH. Hepatic endosomal trafficking of lipoprotein-bound endotoxin in rats. J Surg Res 2002;106:188-95.

510. Cario E, Brown D, McKee M, Lynch-Devaney K, Gerken G and Podolsky DK. Commensal-associated molecular patterns induce selective toll-like receptor-trafficking from apical membrane to cytoplasmic compartments in polarized intestinal epithelium. Am J Pathol 2002;160:165-73.

511. Kohler H, Rodrigues SP and McCormick BA. Shigella flexneri Interactions with the Basolateral Membrane Domain of Polarized Model Intestinal Epithelium: Role of Lipopolysaccharide in Cell Invasion and in Activation of the Mitogen-Activated Protein Kinase ERK. Infect Immun 2002;70:1150-8.

512. Muzio M, Bosisio D, Polentarutti N, et al. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol 2000;164:5998-6004.

513. Zarember KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J Immunol 2002;168:554-61.

514. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK and Segal DM. Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol 2001;166:249-55.

515. Thoma-Uszynski S, Kiertscher SM, Ochoa MT, et al. Activation of toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. J Immunol 2000;165:3804-10.

516. An H, Yu Y, Zhang M, et al. Involvement of ERK, p38 and NF-kappaB signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. Immunology 2002;106:38-45.

517. Hemmi H, Kaisho T, Takeda K and Akira S. The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. J Immunol 2003;170:3059-64.

518. Re F, Strominger JL. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J Biol Chem 2001;276:37692-9.

519. Michelsen KS, Aicher A, Mohaupt M, et al. The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCS). Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. J Biol Chem 2001;276:25680-6.

520. Chopra AK, Xu X, Ribardo D, et al. The cytotoxic enterotoxin of Aeromonas hydrophila induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. Infect Immun 2000;68:2808-18.

521. Jefferson KK, Smith MF, Jr. and Bobak DA. Roles of intracellular calcium and NF-kappa B in the Clostridium difficile toxin A-induced up-regulation and secretion of IL-8 from human monocytes. J Immunol 1999;163:5183-91.

522. Michalke M, Cariers A, Schliess F and D Ha. Hypoosmolarity influences the activity of transcription factor NF- kappaB in rat H4IIE hepatoma cells. FEBS Lett 2000;465:64-8.

523. Kim RD, Darling CE, Cerwenka H and Chari RS. Hypoosmotic stress activates p38, ERK 1 and 2, and SAPK/JNK in rat hepatocytes. J Surg Res 2000;90:58-66.

524. Ulevitch RJ. Recognition of bacterial endotoxins by receptor-dependent mechanisms. Adv Immunol 1993;53:267-89

525. Vidal VF, Casteran N, Riendeau CJ, et al. Macrophage stimulation with Murabutide, an HIV-suppressive muramyl peptide derivative, selectively activates extracellular signal-regulated kinases 1 and 2, C/EBPbeta and STAT1: role of CD14 and Toll- like receptors 2 and 4. Eur J Immunol 2001;31:1962-71.

526. Vogel S, Hirschfeld MJ and Perera PY. Signal integration in lipopolysaccharide (LPS)-stimulated murine macrophages. J Endotoxin Res 2001;7:237-41

527. Medvedev AE, Henneke P, Schromm A, et al. Induction of tolerance to lipopolysaccharide and mycobacterial components in Chinese hamster ovary/CD14 cells is not affected by overexpression of Toll-like receptors 2 or 4. J Immunol 2001;167:2257-67.

528. Wang PY, Kitchens RL and Munford RS. Bacterial lipopolysaccharide binds to CD14 in low-density domains of the monocyte-macrophage plasma membrane. J Inflamm 1995;47:126-37

529. Schimke J, Mathison J, Morgiewicz J and Ulevitch RJ. Anti-CD14 mAb treatment provides therapeutic benefit after in vivo exposure to endotoxin. Proc Natl Acad Sci U S A 1998;95:13875-80.

530. Wenneras C, Ave P, Huerre M, et al. Blockade of CD14 increases Shigella-mediated invasion and tissue destruction. J Immunol 2000;164:3214-21.