IL-1β and sIL-1Ra Production in acute and chronic inflammation : regulation and signaling in human monocytes

Brandt, Karim

UNIVERSITÉ DE GENÈVE

FACULTÉ DES SCIENCES
Professeur M.Gonzalez-Gaitan

Section de chimie et biochimie
Département de biochimie

FACULTÉ DE MÉDECINE
Professeur Joerg Seebach

Département de médecine interne
Service d'immunologie et d'allergologie
Docteur Danielle Burger

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Karim Brandt

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« *La sagesse ne repose sur aucune certitude scientifique et la certitude scientifique ne conduit à aucune sagesse* »

Jean-François Revel

RESUME EN FRANÇAIS

INTRODUCTION

L'inflammation correspond à l'ensemble des mécanismes biologiques complexes générés par l'organisme en réponse à un pathogène ou à une agression physique ou chimique, qui requiert la mise en œuvre de nombreux effecteurs cellulaires et biochimiques tels que des cellules du système immunitaire ainsi que des médiateurs solubles. Celsus, en 40 avant JC, a définit l'inflammation par quatre termes: rubor, dolor, calor, tumor. Ces dénominations font référence à différentes manifestations physiques de l'inflammation qui correspondent à la rougeur, douleur, chaleur et gonflement. Ces manifestations sont le résultat de l'activation séquentielle de médiateurs solubles, incluant les cytokines et les chimiokines, participant au recrutement des leucocytes aux sites inflammatoires. Ces leucocytes, ou cellules blanches immunitaires sanguines, comprennent entre autres, les lymphocytes et les monocytes. L'inflammation est généralement un processus protecteur, toutefois, elle peut être pathologique quand elle est dérégulée. De plus, l'inflammation peut se manifester sous deux formes : la forme aiguë/physiologique et chronique/pathologique. L'inflammation suit généralement le schéma suivant : induction, progression puis résolution. Lorsque ces trois phases se déroulent dans un temps restreint, on parle d'inflammation aiguë/physiologique. L'inflammation aiguë présente histologiquement l'infiltration de neutrophiles et de monocytes. Inversement, l'inflammation chronique/pathologique est généralement de longue durée et caractérisée par l'infiltration de lymphocytes et de monocytes. De plus, dans l'inflammation chronique, les processus de destruction et de réparation sont actifs simultanément. Finalement, bien qu'elle puisse succéder à une inflammation aiguë, l'inflammation chronique commence fréquemment de façon sournoise, par une réponse de moindre degré, discrète et souvent asymptomatique comme dans le cas de deux maladies : la sclérose en plaque (SEP) et l'arthrite rhumatoïde (PR). Ainsi, c'est souvent l'activation

persistante des leucocytes dans l'inflammation chronique qui permet d'établir une nette distinction avec la forme aigue.

Le processus inflammatoire est déclenché en réponse à différents facteurs dont les cytokines pro-inflammatoires telles que l'interleukine-1 β (IL-1β) ou le facteur nécrosant des tumeurs (TNF). En revanche, la sécrétion de cytokines anti-inflammatoires, telles que la forme sécrétée de l'antagoniste du récepteur de l'interleukine 1 (sIL-1Ra) ou l'IL-10, participe à sa résolution. Les cytokines sont des protéines de faible poids moléculaire qui participent à la communication intercellulaire par cascades. Elles sont produites par un grand nombre de cellules immunitaires et non immunitaires de façon transitoire. Leurs rayons d'action sont souvent limités, et de cette manière, elles agissent sur les cellules voisines (action paracrine) ou sur la cellule sécrétrice elle-même (action autocrine).

La présence de récepteurs spécifiques à la surface des cellules permet la reconnaissance de nombreux ligands. Ces ligands, *e.g.* les lipopolysaccharides (LPS) ou l'interféron beta (IFNβ), induisent par l'intermédiaire de leur récepteur respectif des signaux intracellulaires. En effet, sous l'action d'un ligand, le récepteur spécifique recrute un ensemble d'adaptateurs permettant d'interagir spécifiquement avec des effecteurs en aval appartenant à des voies de signalisations précises. Ainsi, l'activation de ces voies de signalisation permet de répondre spécifiquement à un signal à travers de nombreux processus moléculaires incluant la régulation de la synthèse, de la stabilité ainsi que la sécrétion de protéines tel que des cytokines. Les voies des phosphatidylinositide-3-kinases (PI3K) et des mitogen-activated protein kinases (MAPK) font partie de ces cascades de signalisation impliquées dans l'induction de cytokines. Ainsi, certains effecteurs appartenant à ces voies jouent un rôle clé dans la régulation de la production des cytokines pro- et/ou anti-inflammatoires. Par conséquent, ces kinases, par leurs implications dans la régulation de l'inflammation,

représentent des cibles thérapeutiques potentielles qui permettraient des modulations plus fines des processus inflammatoires impliqués dans des pathologies telles que la SEP ou la PR.

La violation de l'intégrité corporelle par un agent pathogène ou un processus mécanique induit une inflammation aiguë par l'intermédiaire de différents facteurs lesquels comprennent entre autres les cytokines pro-inflammatoires. Ces cytokines sont principalement produites par des cellules d'origine myéloïde : les monocytes/macrophages. Il est de plus en plus évident que les monocytes/macrophages participent activement à diverses pathologies liées à l'inflammation. En effet, en plus de leurs rôles prépondérant dans l'inflammation aiguë, ils possèdent également une fonction cruciale dans l'inflammation chronique/pathologique. De fait, bien que les causes déclenchant des maladies chroniques comme la SEP ou la PR ne soient pas connues, on suspecte fortement que les monocytes/macrophages ainsi que les lymphocytes T soient impliqués dans l'induction ainsi que dans la chronicité de ces maladies.

La SEP ainsi que la PR sont des pathologies qui touchent des systèmes très différents, la SEP affectant le système nerveux central alors que la PR est une maladie des articulations (cartilages et os). En apparence, ces maladies semblent avoir des physiopathologies distinctes, toutefois, elles sont très proches. En effet, la SEP ainsi que la PR sont des maladies auto-immunes caractérisées par un état inflammatoire chronique associé à une infiltration importante de lymphocytes T et de monocytes/macrophages. L'hypothèse prépondérante quant à la pathogenèse de l'inflammation chronique suggère que les lymphocytes T y jouent un rôle pathogénique car, dans les modèles animaux, ils sont les premiers présents dans les lésions. Cependant, la présence de monocytes/macrophages dès le début de l'inflammation suggère également un rôle prépondérant de ces cellules. En effet, de récentes études ont mis en évidence que l'injection de monocytes/macrophages stimulés dans des souris ne possédant pas de lymphocytes T induit une encéphalomyélite démyélinisante (une pathologie proche de l'encéphalite autoimmune experimentale, un modèle animal de la SEP). Ainsi, il semblerait

que la pathogenèse de cette maladie nécessite la présence de monocytes/macrophages, lesquels s'avéreraient y participer en favorisant la différentiation des lymphocytes pathogéniques Th17 dans les tissus cible via leur habilité à sécréter de l'IL-1β. De plus, nos travaux de recherche, ainsi que ceux d'autres groupes, tendent à suggérer qu'un mécanisme impliquant une interaction cellulaire directe entre les lymphocytes T et les monocytes/macrophages soit important dans la chronicité de ces maladies. En effet, *in vitro*, l'activation de monocytes via un contact direct avec des lymphocytes T stimulés est capable d'induire, *per se*, la production d'IL-1β ainsi que d'autres cytokines pro-inflammatoires par les monocytes/macrophages. Ce mécanisme pourrait ainsi jouer un rôle important dans la persistance de maladies inflammatoires telles que la SEP et la PR, mais également dans leur résolution au travers la sécrétion de médiateurs anti-inflammatoires, tel que la forme sécrétée de l'IL-1Ra ainsi que d'autres inhibiteurs de cytokines.

BUT DE L'ETUDE

Le but général de cette thèse est l'étude et la caractérisation des acteurs intracellulaires régissant la production de cytokines pro- et anti-inflammatoires, en particulier celle de l'IL-1β et de son inhibiteur naturel qu'est la forme sécrétée de l'IL-1Ra (sIL-1Ra), par les monocytes/macrophages humains. Pour aborder cette problématique, nous avons étudié trois aspects primordiaux de ces mécanismes, dont la caractérisation a mené à l'écriture de trois publications intégrées dans cette thèse. Trois manuscrits supplémentaires, en relation directe avec les travaux de recherche publiés, font actuellement l'objet de soumission dans des journaux internationaux et figurent également parmi les résultats présentés dans cette thèse.

RESULTATS

Régulation différentielle de la production de cytokines par la PI3Kδ chez les monocytes humains dans des conditions inflammatoires chroniques et aiguës (Molnarfi et al, 2008)

Les précédentes études de notre laboratoire ont permis de caractériser les PI3K comme des effecteurs clé de la production des cytokines pro- et anti-inflammatoires dans les monocytes humains activés, en réponse soit au LPS soit aux membranes solubilisée de lymphocytes T activés (CE_{sHUT}), utilisées afin d'imiter le contact cellulaire. Le LPS ainsi que les CE_{sHUT} permettant, respectivement, de reproduire des modèles d'inflammation aiguë et chronique. Le but de notre recherche a été d'identifier les isoformes des PI3K impliquées dans la sécrétion de cytokines telles que l'IL-1β, le TNF, IL-6 et le sIL-1Ra dans les monocytes humains stimulés par ces deux modèles.

Des expériences recourant à différents inhibiteurs généraux et spécifiques nous ont permis de mettre en évidence la fonction différentielle de l'isoforme δ des PI3K de classe I dans nos modèles d'inflammation aiguë et chronique. En effet, nous avons observé que seule l'inhibition de la PI3Kδ, mais pas celle des autres isoformes, permet la modulation de la production des cytokines susmentionnées. Au niveau mécanistique, nos études ont montré par Western blot une réduction importante de la phosphorylation/activation de l'Akt, un effecteur important en aval des PI3K, lors de l'utilisation du Ly294002 (un inhibiteur non spécifiques des différentes isoformes des PI3K) et de l' l'IC87114 (un inhibiteur spécifique de la PI3Kδ), mais pas lors de l'usage d'un inhibiteur spécifique de la PI3Kγ, dans les monocytes humains activés en réponse au LPS ou aux CE_{sHUT}. De façon intéressante, nos données indiquent que l'inhibition de la PI3Kδ diminue la production des cytokines pro- et anti-inflammatoires dans les monocytes activés en réponse aux CE_{sHUT}, alors qu'en réponse au LPS, la production des cytokines pro-inflammatoire est augmentée et celle de sIL-1Ra légèrement diminuée. En accord avec les niveaux protéiques de ces cytokines, nos résultats ont démontrés que les transcrits des différentes cytokines étudiées subissent les mêmes variations en réponse aux

inhibiteurs. Finalement, une série d'expériences supplémentaires ont permis de démontrer l'implication de la GSK3, un substrat direct de l'Akt, en aval de l'activité de la PI3Kδ dans la régulation des cytokines pro- et anti-inflammatoires. Ainsi, nos observations indiquent que l'inhibition de la GSK3 conduit à des effets opposés à ceux obtenus lors de l'inhibition de la PI3Kδ, un phénomène en accord avec la démonstration que la GSK3 est constitutivement active et que l'Akt réprime son activité par phosphorylation. Par conséquent, l'activité de la PI3Kδ, au travers l'axe Akt/GSK3, est utilisée dans les monocytes humains afin de contrôler la production des cytokines pro- et anti-inflammatoires dans des modèles d'inflammation aiguë et chronique.

Cette étude démontre également que la PI3Kδ contrôle de manière différentielle la production de cytokines dans les monocytes humains dans des conditions d'inflammation chronique/stérile et aiguë/infectieuse. Ainsi, la PI3Kδ représente une cible thérapeutique spécifique intéressante dans des maladies inflammatoires chroniques comme la PR ou la SEP, car son inhibition devrait permettre la diminution de la production des cytokines pro-inflammatoires induite dans ces pathologies, mais pas celle, bénéfique, induite lors de la défense contre un agent infectieux. Une telle approche permettrait de pouvoir traiter l'inflammation chronique d'une pathologie sans rendre les patients sensibles à des infections opportunistes.

Une nouvelle voie MEK2/PI3Kδ contrôle l'expression du sIL-1Ra dans les monocytes humains activés avec de l'interféron beta (Brandt et al, submitted)

Nous avons entrepris d'étudier le rôle de la PI3Kδ ainsi que de la kinase MEK2, dans la production de cette dernière cytokine dans les monocytes humains en réponse à l'IFNβ suite à nos observations indiquant que la PI3Kδ contrôle la production de multiple cytokines dans des modèles d'inflammation aiguë et chronique, et que l'IFNβ peut induire directement la forme sécrétée de l'IL-1Ra par un mécanisme dépendent des PI3K mais pas de MEK1. Les fonctions de MEK2 sont très largement décrites comme redondantes avec celles de MEK1, en

grande partie à cause de la grande homologie de séquence (qui dépasse les 80%) entre ces deux kinases. Toutefois, il a été observé que ces deux kinases peuvent aussi présenter des activités différentes.

Dans cette étude, nous avons utilisé une approche utilisant des inhibiteurs pharmacologiques ainsi que la technologie du siRNA. Nos résultats ont montré que l'utilisation du U0126, un inhibiteur de MEK1 et MEK2, diminue la production ainsi que les niveaux d'ARN messager de sIL-1Ra dans les monocytes activés avec de l'IFNβ, alors que l'utilisation du PD98059, un inhibiteur spécifique de MEK1, n'a pas d'effet sur ces mêmes niveaux. Ainsi, après confirmation par «silencing» de MEK1 et MEK2, MEK2 uniquement est requis pour l'induction de sIL-1Ra. Les effets des deux inhibiteurs, U0126 et PD98059, ont été testés sur la phosphorylation de ERK1/2, le substrat direct de MEK1 et MEK2. De manière surprenante, les deux inhibiteurs diminuent de manière similaire la phosphorylation de ERK1 et ERK2, alors qu'ils ont des effets différentiels sur la production de sIL-1Ra. Ces résultats ont été confirmés par siRNA. Ainsi, la diminution de l'expression de ERK1 et ERK2 n'a pas d'influence sur la production du sIL-1Ra suggérant que ERK1 et ERK2 ne sont pas impliqués dans le contrôle de sa production.

D'autre part, l'IC84117 (un inhibiteur spécifique de la PI3Kδ) ainsi que le siRNA de la PI3Kδ diminuent significativement les niveaux de production de sIL-1Ra des monocytes activés par l'IFNβ alors que l'inhibition ou «silencing» des isoformes α, β, γ n'a pas d'effets. L'addition simultanée de l'inhibiteur de MEK2 ainsi que celui de la PI3Kδ ne montre pas d'action synergique sur la production de sIL-1Ra, suggérant que MEK2 et PI3Kδ font partie de la même voie. Cette hypothèse a été vérifiée par le fait que l'inhibiteur de la PI3Kδ diminue significativement la phosphorylation de l'Akt alors que l'inhibiteur de MEK1/2 supprime la phosphorylation de ERK1/2 ainsi que celle de Akt. De plus, U0126 abroge la translocation de la PI3Kδ à la membrane, donc son activation. Ensemble, ces résultats

confirment que MEK2 ainsi que l'Akt font partie de la même voie mais aussi que MEK2 agit en amont de la PI3Kδ.

En conclusion, MEK2 régule la production de sIL-1Ra de manière ERK1/2 indépendante. De plus, bien que les interactions entre les PI3K et MAPK soient nombreuses, cette étude est la première à montrer que MEK2 peut contrôle l'activité de la PI3Kδ.

L'acétate de glatiramer augmente la production de sIL-1Ra mais diminue la production d'IL-1β induit par les membranes activées de lymphocytes T et dans la sclérose en plaque (Burger et al, 2009)

L'IFNβ est le traitement de la forme rechute-rémission (RR) de la sclérose en plaques (RRSEP). Toutefois, un autre traitement est aussi disponible : l'acétate de glatiramer (GA) lequel présente des effets cliniques similaires à l'IFNβ. Le GA est composé d'un mélange de peptides synthétiques de 50 à 90 acides aminés constitués d'acide glutamique, de lysine, d'alanine et de tyrosine. Développé à l'origine pour induire une encephalite expérimental auto-immune (EAE), un modèle murin de la SEP, le GA a montré de manière surprenante qu'au lieu d'induire une EAE, elle en inhibait l'induction et la progression. Le GA est maintenant un médicament approuvé pour le traitement de la SEP depuis 1995. Toutefois, les mécanismes d'action du GA restent en grande partie à élucider.

Notre étude, montre que le GA est capable de réduire le score clinique de la EAE comme montré préalablement. Toutefois, nous montrons qu'au pic de la maladie, les sérums de souris EAE traitées avec du GA ont un niveau de sIL-1Ra plus élevé que les sérums de souris EAE non-traitées. De plus, les patients avec RRSEP traité avec du GA, au même titre que les patients traités à l'IFNβ, présentent également des niveaux sériques supérieurs de sIL-1Ra comparé aux patients non-traités. Ainsi, il semblerait que le GA ait la propriété d'augmenter les niveaux circulant de sIL-1Ra, présentant ainsi des caractéristiques anti-inflammatoires.

En effet, nous avons observé que le GA est capable d'induire lui-même une production de sIL-1Ra dans les monocytes humains sans y induire la production d'IL-1β. Dans un modèle

d'inflammation chronique représenté par l'utilisation de membranes solubilisées de lymphocytes T activés, le GA montre une capacité d'augmenter la production de sIL-1Ra et de diminuer la production d'IL-1β des monocytes humains. D'autre part, dans un modèle d'inflammation aiguë représenté par l'utilisation de LPS, le GA augmente les niveaux d'IL-1β et de sIL-1Ra dans les monocytes activés.

Cette étude a mis en évidence un mécanisme par lequel le GA peut avoir ses effets bénéfiques sur des patients avec une RRSEP. En effet, l'augmentation des niveaux de sIL-1Ra est probablement la conséquence d'une activation directe des monocytes par le GA. De plus, la diminution de l'activité de l'IL-1β, par le GA, est corrélée avec la sévérité de la maladie. Le GA possède des effets immunomodulateurs périphériques et au site inflammatoire à travers l'inhibition de l'IL-1β, ainsi que par la production de sIL-1Ra qui passe la barrière hématoencéphalique.

L'acetate de glatiramer active les voie PI3Kδ/Akt et MEK/ERK afin d'induire la production de sIL-1Ra dans les monocytes humains (Carpintero et al, submitted)

Comme décrit précédemment, le GA est un composé utilisé dans le traitement de la SEP dont les effets bénéfiques présentent de fortes similitudes avec ceux observés avec l'IFNβ. En effet, il est supposé qu'une partie des résultats positifs du traitement avec le GA provient, à l'instar de l'IFNβ, de sa capacité à induire un facteur anti-inflammatoire : sIL-1Ra.

Notre étude avait pour but de déterminer les voies de signalisations intracellulaires induites par le GA dans les monocytes humains. Etant donné que nous avons déjà décrit les voies des PI3K et des MAPK comme des voies de signalisations nécessaires à la production de sIL-1Ra par l'IFNβ, nous avons décidé d'investiguer le rôle que pourrait jouer les isoformes de class I des PI3K ainsi que la voie des ERK1/2 dans la production de sIL-1Ra dans les monocytes humains activés avec du GA.

Nos résultats montrent que le GA active la voie des PI3K et des MAPK. En effet, le GA induit la phosphorylation des ERK1/2 ainsi que l'effecteur classique en aval des PI3K: l'Akt à 2h. Le « silencing » des différentes isoformes de class I des PI3K montrent que la PI3Kδ est l'unique isoforme requise pour la production de sIL-1Ra dans monocytes humains activés par le GA. Ces résultats sont confirmés par la translocation à la membrane de la PI3Kδ mais pas des autres isoformes des PI3K de classe I. Finalement, on montre que le « silencing » de l'Akt diminue significativement la production de sIL-1Ra dévoilant celle-ci comme impliqué dans ce mécanisme. D'autre part, l'utilisation du U0126, un inhibiteur de MEK1 et MEK2, diminue la production ainsi que les niveaux d'ARN messagers de sIL-1Ra dans les monocytes activés avec du GA. De plus, l'utilisation du PD98059, un inhibiteur spécifique de MEK1, contrairement à ce qui a été observé avec l'IFNβ, diminue également la production de sIL-1Ra. Ainsi, après confirmation par «silencing», MEK1 et MEK2 sont requis pour l'induction de sIL-1Ra par le GA. Les effets des deux inhibiteurs, U0126 et PD98059, ont été testés sur la phosphorylation de ERK1/2, le substrat direct de MEK1 et MEK2. Les deux inhibiteurs diminuent de manière similaire la phosphorylation de ERK1 et ERK2, ce qui correspond aux résultats obtenus sur les niveaux de sIL-1Ra. Ces résultats ont été confirmés par siRNA. Ainsi, la diminution de l'expression de ERK1 et ERK2 diminue significativement la production du sIL-1Ra suggérant que ERK1 et ERK2 sont également impliqués dans le contrôle de sa production.

Contrairement aux résultats obtenus avec l'IFNβ, nous montrons que la voie PI3Kδ/Akt ainsi que MEK/ERK contrôle de manière parallèle la production de sIL-1Ra. Néanmoins, nous montrons qu'elles convergent sur la GSK3α/β. En effet, nos observations indiquent que l'inhibition de la GSK3 conduit à des effets opposés à ceux obtenus lors de l'inhibition de la PI3Kδ ou de MEK1/2, un phénomène en accord avec la démonstration que la GSK3 est constitutivement active et que l'Akt ou les ERK1/2 réprime son activité par phosphorylation.

Donc, l'inhibition ainsi que le « silencing » de la GSK3 augmente la production de sIL-1Ra. De plus, les inhibiteurs de MEK1/2 ainsi que celui de la PI3Kδ diminuent la phosphorylation de la GSK3, et par conséquent, augmente son activité, ce qui indique que la GSK3α/β est le point de convergence de la PI3Kδ/Akt et MEK/ERK dans l'induction de sIL-1Ra dans les monocytes humains.

Cette étude montre pour la première fois que le GA est capable d'induire, *per se*, un signal intracellulaire, se qui laisse entrevoir l'existence d'un(de) récepteur(s) spécifique(s). De plus, une partie des effets bénéfiques du GA dans la SEP proviennent probablement de sa capacité à induire la production de sIL-1Ra à travers l'activation des voies PI3Kδ/Akt et MEK/ERK dans les monocytes. Cette étude appuie également l'hypothèse que la PI3Kδ est une kinase clé dans la voie d'induction de sIL-1Ra dans les monocytes humains.

Les lymphocytes T stimulés génèrent des microparticules qui imitent l'activation des monocytes humains par contact cellulaire : régulation différentielle de la production des cytokines pro- et anti-inflammatoires par les lipoprotéines de haute-densité (Scanu et al, 2008)

Les microparticules (MP) sont des fragments sphériques de membrane plasmique avec un diamètre d'environ 0.1 à 0.8 μm générées par des cellules stimulées ou apoptotiques. Longtemps considérées comme des débris inertes, les MP sont maintenant connues pour avoir des fonctions effectrices impliquées dans la communication intercellulaire. Les MP sont présentes dans la circulation de donneur sains mais leur concentration augmente dans des conditions pathologiques comme la SEP ou la PR. En effet, bien que la présence de MP dans le liquide cérébrospinal n'ait pas été montrée, elles sont en concentration significative dans le plasma de patients atteints de SEP. De manière similaire, les patients PR présente une concentration élevée de MP au niveau du liquide synovial.

Le but de cette étude était de déterminer l'implication des MP isolées de lymphocytes T stimulés dans l'induction de la production de cytokines dans les monocytes humains. En outre, nos travaux ont aussi porté sur la possibilité de moduler ce mécanisme en présence de

lipoprotéines de haute-densité (HDL), connues pour bloquer de façon spécifique la production de cytokines pro-inflammatoires lors de l'activation des monocytes par les de membranes de lymphocyes T stimulés.

Nos travaux montrent que les lymphocytes T isolés de sang périphérique et les lignées lymphocytaires sont capable, lorsqu'elles sont activées, de produire des MP qui ont la propriété d'induire la production de cytokines pro et anti-inflammatoires chez les monocytes humains. En effet, les MP induisent la production d'IL-1β; de TNF et de sIL-1Ra, mais uniquement les cytokines pro-inflammatoires que sont l'IL-1β et le TNF sont inhibés par la présence des HDL. Les mêmes résultats sont obtenus lorsqu'on mesure les niveaux d'ARN messager des différentes cytokines. Ces résultats sont consistants avec l'hypothèse que les activateurs de surfaces présents dans les membranes solubilisées de lymphocytes T activés (CE_{sHUT}) sont également présents à la surface des MP. En effet, le contact cellulaire (CE_{sHUT}) est inhibé de la même manière que les MP par les HDL.

En conclusion, les MP peuvent avoir une activité pro- et anti-inflammatoire par l'induction d'IL-1β, de TNF et de sIL-1Ra. Cette production est régulée par les HDL. Ainsi, les MP générées par les lymphocytes T pourraient représenter des vecteurs capables d'induire une activation par contact sur des cellules qui ne sont pas en contact avec les lymphocytes T.

Les HDL interfèrent avec la liaison des microparticules et les monocytes humains afin d'inhiber la production de cytokines pro-inflammatoires (Carpintero et al, submitted)

Nous avons entrepris d'étudier l'effet des HDL sur la liaison des MP sur les monocytes après avoir démontré que les MP, générées à partir lymphocytes T stimulés, induisent la production de cytokines pro- et anti-inflammatoires de manière similaire aux membranes ou aux membranes solubilisées de lymphocytes T activés et que cette induction est contrôlée par les HDL.

Les MP ont été générées à partir de lymphocytes T activé (MP_T), de cellules endothéliales (MP_{EC}) ou de plaquettes. Seul les MP générées à partir de lymphocytes T activés ont la propriété d'activer les monocytes humains. De plus, les MP_T se lient aux monocytes mais pas aux cellules $CD3^+$ (lymphocytes T), alors que les MP_{EC} ou les MP_T non-stimulés n'interagissent ni avec les monocytes ni avec les cellules $CD3^+$.

Comme la production d'IL-1β est inhibée par les HDL, nous avons regardé les effets des HDL sur l'interaction entre les MP_T et les monocytes par cytométrie en flux et avons constaté que les HDL inhibent la liaison entre ces derniers. La question résultante de ces constatations a été de comprendre si les HDL se lient aux monocytes ou aux MP_T. Nos résultats montrent que les HDL se lient aux facteurs d'activation de surface des MP_T empêchant la liaison avec les monocytes. Toutefois, les HDL inhibent la production d'IL-1β mais pas la production de sIL-1Ra. Ceci suggère qu'il existe au-moins deux facteurs d'activation de surface contrôlant l'un l'IL-1Ra et l'autre l'IL-1β.

Les MP_T induisent la production de l'IL-1β et de sIL-1Ra mais aussi d'autres cytokines ou chimiokines telles que l'IL-6, le TNF, l'IL-8, le CCL2, le CCL3 et le CCL4. De la même manière que pour l'IL-1β, les HDL inhibent la production des cytokines et chimiokines pro-inflammatoires à l'exception du CCL2 dont la production, de manière analogue au sIL-1Ra, n'est pas affectée. Ces observations confirment la présence de deux molécules de surface différentes impliquées dans l'activation des monocytes humains, l'une des deux étant inhibée par les HDL.

Cette étude révèle que les MP_T interagissent spécifiquement avec les monocytes afin d'induire la production de cytokines et chimiokines. La liaison des MP_T et des monocytes est inhibée par les HDL qui s'attachent au(x) facteur(s) activateur(s) de surface des MP_T, inhibant à son tour la production de cytokines et chimiokines dans les monocytes humains.

CONCLUSION

Dans ce travail de thèse, nous avons tout d'abord déterminé et caractérisé l'implication de la PI3Kδ dans la production des facteurs pro- et anti-inflammatoires que sont l'IL-6, l'IL-1β, le TNF et le sIL-1Ra, dans des monocyte/macrophages stimulés par le contact avec des membranes de lymphocytes T activés, un mécanisme qui semble avoir un rôle dans le déclanchement et la persistance des maladies inflammatoires chroniques telle que la SEP ou la PR. Par ailleurs, au vue de nos précédents travaux indiquant que l'IFNβ, un traitement pour la SEP et potentiellement pour la PR, induit la production du sIL-1Ra à travers une voie PI3K-dépendante, mais STAT1- et MEK1-indépendante, nous avons dès lors investigué le rôle de la PI3Kδ ainsi que de celle de MEK2 dans la production du sIL-1Ra par les monocytes humains. Nous avons pu démontrer l'implication de la PI3Kδ et de MEK2 dans la même voie d'activation du sIL-1Ra par l'IFNβ, MEK2 agissant en amont de la PI3Kδ. Nos recherches ont donc mis à jour une interaction encore jamais décrite entre la voie des MAPK et celle des PI3Ks. Nous avons par ailleurs renforcé l'idée que l'activité des différentes isoformes des PI3Ks et des MEKs ne sont pas forcément redondantes et peuvent être importantes dans la régulation de certains facteurs inflammatoires. D'autre part, nous nous sommes aussi intéressés aux effets du GA, un traitement de la SEP dont les mécanismes d'action sont mal connus, sur la production de médiateurs inflammatoires par les monocytes humains. Nous avons pu montrer que le GA *per se* induit la production du sIL-1Ra *in vitro* ainsi que chez les patients atteint d'une SEP. De plus, le GA diminue la production d'IL-1β dans les monocytes stimulés par le contact cellulaire alors qu'il augmente la sécrétion du sIL-1Ra dans les mêmes conditions. Ces processus pouvant être à l'origine d'une partie des effets bénéfiques de ce traitement. En dernier lieu de ce travail de thèse, nous avons déterminé que des MP_T produites par des lymphocytes T activés sont capables d'activer des cellules cibles, et en particulier des monocytes humains, par contact direct. Ce contact avec les MP_T présente les mêmes caractéristiques que le contact avec des membranes solubilisées de lymphocytes T stimulés

(CE_{sHUT}). En effet, l'interaction des HDL avec les MP_T inhibe la production d'IL-1β sans perturber celle de sIL-1Ra, de façon similaire à l'action des HDL sur les CE_{sHUT}. Cette interaction diminue significativement la liaison des MP_T sur les monocytes. Ainsi, bien que l'identité du(des) facteur(s) activateur(s) reste à être déterminée, son interaction avec les HDL donne un indice ainsi qu'une possibilité de l'(les) identifier.

L'étude des voies de signalisation apporte une nouvelle vision des moyens permettant de contrôler l'inflammation. Ce contrôle pouvant être effectif via la répression de la production des facteurs pro-inflammatoires où, à l'inverse, en stimulant la sécrétion de facteurs anti-inflammatoires. Les maladies inflammatoires chroniques fonctionnent souvent par phases d'inflammation aiguë suivie d'une phase de rémissions. Ce schéma pourrait être associé à un cycle d'activation et/ou de répression de voies de signalisation spécifiques relatives aux différents facteurs de l'inflammation. Les signaux de transduction étant des régulateurs intrinsèques de ces différents facteurs de l'inflammation, ils représentent des cibles potentiels dans une perspective de contrôle plus spécifique des productions endogènes de facteurs pro et anti-inflammatoires. Par conséquent, une meilleure compréhension de ces processus permettrait de développer des thérapies plus adaptées et plus ciblées aux différentes pathologies inflammatoires.

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1 INTRODUCTION

1.1 Innate Immunity

The innate immune system provides a firth line of defense against many pathogens without requirement of prior exposure to foreign antigen. Granulocytes, macrophages, neutrophils and dendritic cells as well as nonprofessional cells such as epithelial cells, endothelial cells and fibroblasts contribute to innate immunity. However, the innate immune system is not able to always eliminate the infectious organism. Thus, the adaptive immune system takes over from innate immune system to provide a more versatile response and keep the pathogen in an immunological memory in order to increase protection against a new infection. Moreover, the innate immune system plays an important role in initiation and subsequent direction of adaptive immunity. The innate immune system requires invariant receptors recognizing conserved features of pathogens called NLD-like receptors (NOD), Toll-like receptors (TLR), Retinoic acid-inducible gene (RIG)-I-like receptors and C-type lectin receptors (CLR) . TLR are very important to trigger an early inflammatory response and their characteristic and functions will be developed in chapter 1.3 while NLR will be developed in chapter 2.3, Finally, RIG-I-like receptors will not be described in this thesis.

1.2 Inflammation

Unlike the adaptive immune response which is specific, long-term to take hold and has an immunological memory, the inflammation is non-specific, rapid to take hold and has no immunological memory. Inflammation takes place in response to mechanical injury, chemical toxins, invasion by pathogens, and hypersentivity reactions. It is a natural response which is expected to occur. Thus, the inflammation is defined as acute or chronic. Indeed, the acute inflammation is required to engage the resolution of the injury or bacteria invasion while the chronic inflammation derives from the uncontrolled acute inflammation. Several diseases

defined as T-cell mediated diseases are characterized by a chronic inflammatory state. Multiple sclerosis (MS) is a disease of central nervous system (CNS) long time considered only as a T-cell mediated diseases but, currently, considered as chronic inflammatory disease. In addition, rheumatoid arthritis (RA) is a chronic inflammatory disorder that could affect many tissues and organs, but principally attacks synovial joints. Thus, inflammation is not necessarily the primary cause of diseases but it is fundamental contributor. Consequently, the chronic inflammation is pathologic. Therefore, the differences between acute and chronic inflammation are mainly in duration and cells types recruited within the inflammation site [31]. Despite the difficulty to define specifically, chronic inflammatory state is characterized by prolonged time of active inflammation, *i.e.* up to months or years. Another characteristic is the infiltration of monocytes/macrophages and T-cells. On other hand, the acute inflammatory state is characterized by an immediate onset with duration of few days and by a dominance of neutrophils, macrophages and dendritics cells infiltration [31-33].

Celsus, about 40 BC, described the inflammation as redness, swelling, heat and pain giving a picture of numbers of process involved. Thus, inflammation appears as complex biological processes involving cells and soluble mediators. The processes corresponding to Celsus's images reflect increased vascular dilatation of blood vessel and permeability of vascular endothelium allowing leukocyte recruitment into the tissues. Moreover, those mediators, involved into cell-to-cell communication, comprise cytokines which play an important role in inflammation induction and resolving. Indeed, a plethora of cytokines are involved in inflammation and an overview is given in [33]. Thus, inflammation progresses through the action of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), IL-6 or interferon γ (IFNγ) and resolved through anti-inflammatory cytokines including secreted interleukin-1 receptor antagonist (sIL-1Ra), IL-10 or IFNβ [4, 31, 33, 34]

Today, inflammation provides a wide variety of research domains including signal transduction and cells interactions which interest the pharmaceutical industry because, often, the origin of diseases is not known, *e.g.* MS and RA. Consequently, the control of inflammation is often the best alternative. Moreover, some diseases have been characterized as "inflammatory" or undetermined origins but appear derived from infectious or viral causes, *e.g* gastric ulcers and uterus cervix cancer. Finally, the better understanding of inflammation is important because it could be more damageable than the pathogen itself [35]

1.3 The innate immune sensors

The innate immune system was considered as less complex and less flexible as compared to the adaptive immune system until the discovery of TLR [36]. Indeed, they have been related to pattern-recognition receptors (PRR) and, thus, their crucial roles in regulation of immune responses. PRR recognize conserved structure of microorganisms called pathogen-associated molecular patterns (PAMP) or endogenous molecules released from damaged cells called damage-associated molecular patterns (DAMP). Several classes of PRR family have been indentifying: TLR, RIG-I, NLR and CLR. These PRR are expressed, by both immune and non-immune cells, at the plasma membrane or into the cytoplasm [4, 36]. Here, I described one of the best characterized PRR families: TLR.

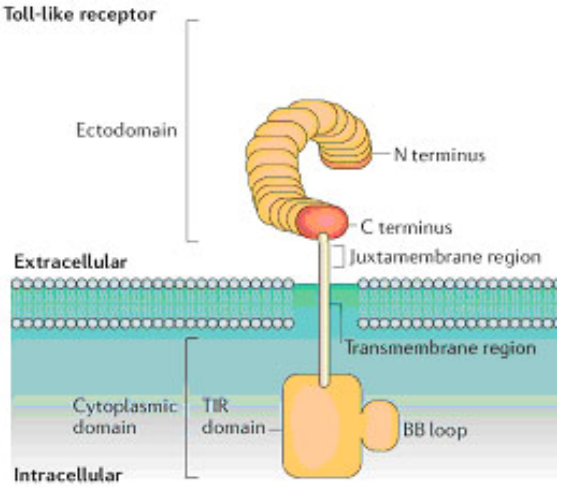

Figure 1: Schematic diagram of Toll-like receptors, showing the extracellular, transmembrane and cytoplasmic domains of the receptors. (Adapted from [7])

Table 1. PRRs and Their Ligands			
PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown

Table 1 : PRR and their Ligands (from [4])

TLR are type I transmembrane proteins and share a common conserved structure [37]. They are composed by three major domains: N-terminal leucine-rich repeats (LRR) which mediates the recognition of their respective PAMP, a transmembrane domain and a Toll/IL-1R (TIR) cytoplasmic domain that is required to trigger signaling pathways (Figure 1) [4, 38].

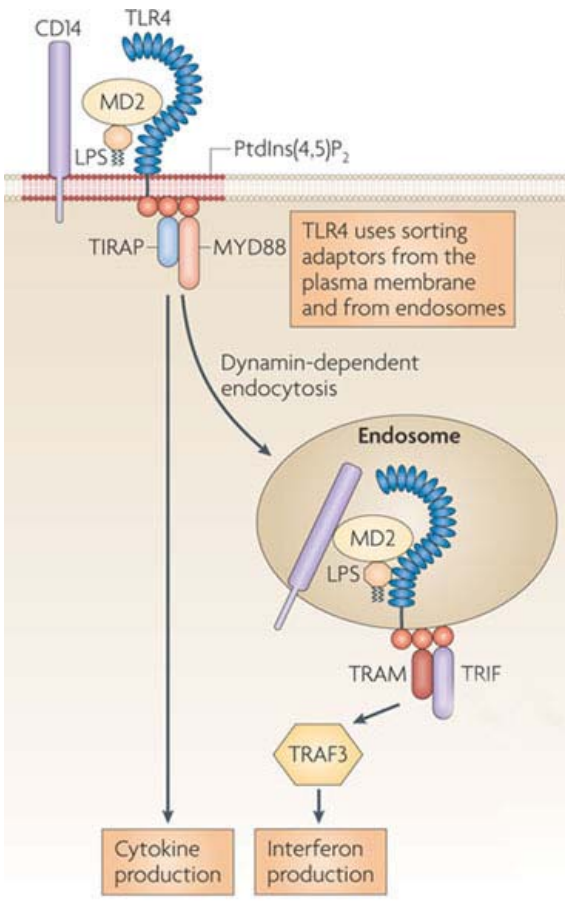

Figure 2: The Toll/IL-1 receptor (TIR) domain-containing adaptors have one of two roles. MYD88 and TRIF function as signaling adaptors link Toll-like receptors (TLRs) that are found in several organelles to the downstream kinases that define a given signalling pathway. The recruitment of MYD88 to plasma membrane TLRs and of TRIF to endosomal TLRs depends on the sorting adaptors (TIRAP; TRAM). (Adapted from [19]

There is ten human TLR recognizing different PAMP (Table 1). TLR could be divided into two subpopulations related to their cellular localization. Thus, TLR1; 2; 4; 5; 6 and 11 are localized on the cell surface whereas TLR3; 7; 8 and 9 are localized in intracellular vesicles (endosomes) and recognize mainly microbial nucleic acid [39, 40]. In addition to regulation of TLR through the compartmentalization, TLR ligands recognition requires oligomerization to trigger signal transduction [41, 42].

TLR4 is the best characterized cell surface TLR. It recognizes lipopolysacharide (LPS), a major component of the outer membrane of Gram negative bacteria, in association

with myeloid differentiation factor2 (MD-2), cluster of differentiation 14 (CD14) and LPS-binding protein (LBP). LBP is a soluble protein binding LPS. LBP-LPS complex is recognized by CD14 which delivers this complex to the TLR4-MD-2 heterodimer [4, 39, 43]. TLR4 is known to activate two different intracellular signaling pathways that they will be described further in *chapter 5*. However, these different pathways are triggered through a process localization-dependent. Indeed, TLR4 which is localized to plasma membrane triggers a pathway MyD88-dependent, while when TLR4 is endocytosed through a dynamin-dependent process, the signaling is TRIF-dependent (Figure 2) [19, 44].

The knowledge about TLR grows every year, gains from biochemical and biophysical studies. However, the structure and the signaling of TLR remain to be completely solved. [45].

2 CYTOKINES

As stated above, the immunological responses are controlled in part by the generation and maintenance of a network of small, nonstructural regulatory proteins that mediate a multiplicity of immunological and non-immunological functions. These proteins are called cytokines. They are pleiotropic, often display redundant activities and may act as autocrine or paracrine factors [46-48]. Cytokines are secreted in response to various stimuli including bacterial products and themselves. Thus, the generation of cytokines plays crucial roles in inflammation since it is involved in the regulation of the magnitude, nature and duration of the inflammatory responses and determines the clinical course. Initially, cytokine production determines whether an immune response develops and subsequently whether that response is cytotoxic, humoral, cell-mediated, or allergic [49]. Moreover, cytokines are involved in a broad range of biological processes such as bone marrow differentiation, cellular recruitment, activation and antigen presentation [49, 50]. In addition, cytokines may have other involvement including depression or coronary artery diseases [51, 52].

Cytokines are small and soluble proteins. The active form can be monomeric, *e.g.*IL-1α/β, homodimeric, *e.g.* IFNγ or homotrimeric, *e.g.*TNF. Some cytokines are active as heteromeres (IL-12) [53-56]. Cytokine actions can be regulated outside and/or inside the cells. Intracellular regulation is performed via the control of cytokine production and secretion. Extracellular regulation is performed via the control of cytokine receptor expression, by other cytokines activity and soluble cytokine-binding factors (*e.g.* soluble receptors) and receptor-binding factors/ inhibitors [50]. Furthermore, cytokine expression can be regulated at transcription, translation and processing levels. The modulations of mRNA half-lives involve adenylate/uridylate (AU)-rich elements (AREs), often consisting of one to several copies of the AUUUA sequence located in the 3' untranslated region of the transcript [57-59]. Some cytokine processing requires complex mechanisms to be matured and secreted, as exemplified

by IL-1β [60]. Outside the cells, cytokine receptors or soluble forms of receptors are generally expressed at low level by resting cells but their expression and release can be up-regulated upon all activation [61]. Thus, cytokine effects are regulated by the duration of cytokine availability in the microenvironment and by the amount of cytokine produced [61, 62]. Finally, the excessive production of cytokines can be harmful and more dangerous than original stimulus.

A particular interest is given to TNF, IL-6, IL-1β and sIL-1Ra in this thesis. Indeed, these cytokines are used as readout of the different studies, includes in this work, which investigate different features of chronic inflammatory processes involved in the severity of T-cell mediated diseases and their treatment such as RA or MS. Thus, despite the wishes to descript exhaustively TNF, IL-6 and IL-1 family, I have to limit the descriptions only to several members, which allow understanding the results.

2.1 The Interleukin-1 family

The term Interleukin-1 (IL-1) is used for 6 members (IL-1F) (Table 2) and for a restricted family of ligands composed of 5 other members, constituting a family of 11 members. The restricted family includes IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18 and IL-33. IL-1, IL-18 and IL-33 are related by three-dimensional structure, gene location, receptor structure and binding, and signal transduction pathways usage. This restricted family includes the receptors IL-1RI; IL-RII and IL-1RAcP and their soluble forms which bind IL-1α; IL-1β and IL-1Ra; for IL-18, the IL-18 receptor and IL-18 accessory proteins (IL-18RAcP); and for IL-33, the IL-1-related protein T1/ST2 and IL-1RAcP [13, 63]. After induction, IL-1β, IL-18 and IL-33 are synthesized as inactive zymogen inside the cell and, after secretion, induce local and systemic inflammation. However, there is a controversial discussion about the relevant relation between IL-1β, IL-18 and IL-33. Their activity may and has to be tightly regulated at

many levels. In consequences, many proteins, *e.g*. IL-1Ra, sIL-RII or IL-18 Binding Protein, are secreted or released in order to regulate IL-1 and IL-18 activity. [12, 64].

The IL-1 family (agonist and antagonist)

Cytokine	Other names	Systematic name	Immunological function
IL-1α	IL-1 and leucocyte activating factor (LAF) (both collectively with IL-1β)	IL-1F1	IL-1R1 agonist with proinflammatory action but mainly acts as an intracellular transcriptional regulator
IL-1β	IL-1 and leucocyte activating factor (LAF) (both collectively with IL-1α)	IL-1F2	Acts synergistically with TNF-α, activates proinflammatory responses in a wide range of cells, increases expression of adhesion molecules in endothelial cells and promotes diapedesis and the acute phase response
IL-1Ra		IL-1F3	IL-1RI antagonist, prevents IL-1-dependent signalling
IL-18	IFN-γ inducing factor (IGIF), IL-1γ	IL-1F4	Induces IFN-γ production from T lymphocytes and NK cells and acts synergistically with IL-12 to promote the Th1 response
IL-1F5	IL-1Hy1, FIL1δ, IL-1L1, IL-1δ, IL-1H3, IL-1RP3	IL-1F5	Possible IL-1Rrp2 receptor antagonist
IL-1F6	FIL1ε	IL-1F6	Agonist via the IL-1Rrp2 receptor. Increases IL-6, IL-8 production in epithelial cells
IL-1F7	FIL1ζ, IL-1H4, IL-1RP1, IL-1H1	IL-1F7	Interacts with IL-18 binding protein to reduce IL-18 activity
IL-1F8	FIL-1η, IL-1H2	IL-1F8	Agonist via the IL-1Rrp2 receptor. Increases IL-6, IL-8 production in epithelial cells. Also up-regulates IL-6 and IL-8 production in chondrocytes and synovial fibroblasts
IL-1F9	IL-1H1, IL-1RP2, IL-1ε	IL-1F9	Agonist via the IL-1Rrp2 receptor. Increases IL-6, IL-8 production in epithelial cells
IL-1F10	IL-1Hy2, FKSG75	IL-1F10	Binds soluble IL-1RI, function unknown
IL-33	NF-HEV	IL-1F11	ST2 receptor agonist. Induces Th2 cytokine expression. Intracellular transcriptional regulator in endothelial cells

IFN: interferon; NK: natural killer; Th: T helper; TNF: tumour necrosis factor.

Table 2: [12]

2.1.1 The IL-1 agonists

As stated above, the IL-1 family includes 7 agonists (IL-1α; IL-1β; IL-18; IL-1F6; IL-1F8; IL-1F9; IL-33). This section will be devoted to IL-1α and β because my thesis work was focused on the IL-1/IL-1Ra system.

IL-1 is one of the first cytokine described in the early 1980s. IL-1 was studied for its ability to cause fever, headache, stimulate hepatic acute-phase proteins, leukocytosis, sleepiness and anorexia [30]. At that time, it was unclear whether IL-1 was a single substance or several different molecules. The confusion originated from molecular heterogeneity, since four isoelectric points and both large and small molecular weight forms were found [30, 65, 66]. Two cDNA were reported, one coding for acidic IL-1 and the other for neutral IL-1, now referred to as IL-1α and IL-1β, respectively. Both cDNA encode large precursor molecules that lack a classical leader sequence of secreted proteins [30, 65]. The proIL-1α and proIL-1β

have a molecular mass of 31 kDa and are cleaved by calpain, a membrane-associated calcium-dependent cystein protease, and by interleukin-1 converting enzyme (ICE)/caspase-1, respectively [63, 67-70]. Whereas the recombinant forms of mature IL-1α and IL-1β are active agonists, only the precursor form of IL-1α is also active.

2.1.2 IL-1α and IL-1β genes structure

IL-1α and IL-1β have 26% amino acid sequence homology while their two proIL-1 genes display 45% homology [71]. Human IL-1α and IL-1β genes are both located on the long arm of chromosome 2, distributed over 430 kbp and show a high conservation of exon/intron structure. IL-1α promoter does not contain TATA box motifs, whereas this motif is presented on IL-1β gene. Inducible gene expression of IL-1α involves both a 4.2 kbp upstream induction sequence (UIS) and a proximal promoter region of 200bp [72, 73]. Furthermore, in human, both promoters contain binding sites for minimal induction region: NF-κB regulatory elements, activator protein-1 (AP-1), NF-IL6; and cyclic adenosine 3', 5'–cyclic monophosphate (cAMP) response element binding protein (CREB) [74 75, 76].

Since a deregulated production of IL-1α and IL-1β can be harmful to the host through an uncontrolled inflammation, it is not surprising that both are controlled at several levels including (i) gene transcription [77]; (ii) mRNA turnover (AU–rich region present on 3'UTR) [75, 78]; (iii) protein translation and secretion [2, 30, 79, 80]; and activity [81]. Moreover, both activity is post-secretory regulated by different other processes as described below.

2.1.3 IL-1α

IL-1α is synthesized in zymogen form of 31kDa [82]. ProIL-1α is constitutively present in primary cells including keratinocytes or epithelial cells and the mature IL-1α is not commonly found in the circulation or in body fluids [70]. Even under conditions of cell stimulation, phagocytic and non-phagocytic cells do not process or readily secrete mature IL-1α, unless from dying cells. Thus, IL-1α is synthesized and accumulates as an intracellular precursor molecule [13, 82]. ProIL-1$\alpha_{(1-271)}$ is synthesized in association with microtubules and possesses a N-terminal KVLKKRR motif, which is a nuclear localization signal (NLS) (Figure 3 A). Mature IL-1$\alpha_{(113-271)}$ is cytoplasmic [70, 82, 83]. proIL-1α is considered as an proinflammatory activator of transcription that regulates normal cellular differentiation specifically in endothelial and epithelial cells [84]. Moreover, proIL-1α can be myristoylated on specific lysine residues, a process which facilitates transport to the cell membrane where it is referred to as membrane IL-1α [13, 70, 83, 85]. Membrane IL-1α present at the surface of monocytes/macrophages is biologically active and is temporarily dissociated from secreted IL-1α (Figure 3 B) [86]. The nuclear form of proIL-1α seems to modulate gene expression through the interaction of proIL-1α/IL-1RI complexes with DNA structures into the nucleus [87, 88]. The processing of proIL-1$\alpha_{(1-271)}$ in mature IL-1$\alpha_{(113-271)}$ generates an IL-1$\alpha_{(1-112)}$ propiece. IL-1α propiece contains the nuclear KVLKKRR motif present in N-terminus of proIL-1$\alpha_{(1-271)}$ and, consequently, is localized in the nucleus (Figure 3 C). The nuclear localization is facilitated by myristoylation on lysine 82 and 83 into its NLS. Within the

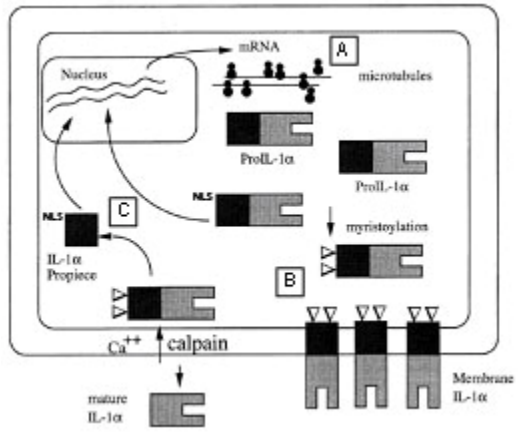

Figure 3 : IL-1α mRNA coding for proIL-1α is translated in association with microtubules (A). ProIL-1α remains in the cytosol where it could be myristoylated or translocated to nucleus through its NLS sequence. ProIL-1α is translocated to the cell membrane when it is myristoylated and it can be anchored (B). After cleavage by calpain, 17 kDa IL-1α is released into the extracellular compartment. ProIL-1α can also be secreted in rare cases. Intracellularly, either pro IL-1α or the 16 kDa IL-1α propiece can bind to nuclear DNA (C). (Adapted from [11])

nucleus, the IL-1α propiece localized to interchromatin granule clusters (IGCs) that enclose proteins of the RNA processing pathway [89]. In addition, IL-1α propiece is associated with HAX-1 that binds several intracellular proteins such as polycystin-2, cortactin and Epstein-Barr virus nuclear antigen leader protein [70, 85]. In conclusion, although IL-1α seems to have several different functions, the exact biology of proIL-1α and IL-1α propiece remain to be completely solved.

2.1.4 IL-1β

IL-1β lacks classical signal peptide and is not released by the canonical mechanisms from endoplasmic reticulum/Golgi apparatus [30, 63, 90]. IL-1β has a molecular weight of 17 kDa. It exerts its activity in the extracellular compartment. IL-1β is secreted by multiples cells including monocytes, macrophages, neutrophiles or hepatocytes in response to cytokines or engagement of TLRs [2, 63] but can also remains cytosolic in nonphagocytic cells [91]. The mechanisms by which IL-1β is processed and secreted begin to be understood. Indeed, IL-1β mRNA is synthesized in large amounts without significant translation into protein in monocytes stimulated by lipopolysaccharides (LPS) [13, 92]. Even more, less than 10% of IL-1β is released into the extracellular space after activation in vitro while the remaining 90% is accumulated intracellularly [93]. Thus, it is hypothesized that two signals are required for IL-1β secretion. The first one drives gene transcription and proIL-1β (31 kDa) accumulation, while the second one controls processing and release [63, 90, 93-95]. When bacterial products or whole bacteria are the stimuli, TLRs appear to be the first signal. LPS, which is recognized by TLR4, is sufficient to induce the maturation and secretion of a fraction of cellular IL-1β. (Figure 4 1; 2 and 3) [63, 69]. However, the second signal enhances the externalization by the secretory stimulus ATP acting via the P_2X_7 receptor (Figure 4, 4) [69, 96]. P_2X_7 receptor is a potassium-selective channel, which belongs to plasma membrane receptors for extracellular nucleotide family referred to as P2 receptors [93]. Extracellular ATP binds and activates P_2X_7.

This activation induces the formation of a reversible plasma membrane pore permeable to hydrophilic solutes of molecular mass up to 900 Da, [90] resulting in a massive efflux of intracellular K^+ by recruiting a larger pore: the hemichannel pannexin-1 (Figure 4, 5b)[97-99]. The diminution of intracellular K^+ concentration is critical for secretion of high levels of IL-1β by activation of caspase-1 through a molecular complex named the inflammasome [95, 100, 101]. However, a number of other proteases can also process proIL-1β into the mature, bioactive cytokine, but the focus will be here on the main protease involved, *i.e* caspase-1 [102], and its activation by the NALP3 inflammasome.

The inflammasomes are large macromolecular complexes, which include nucleotide-

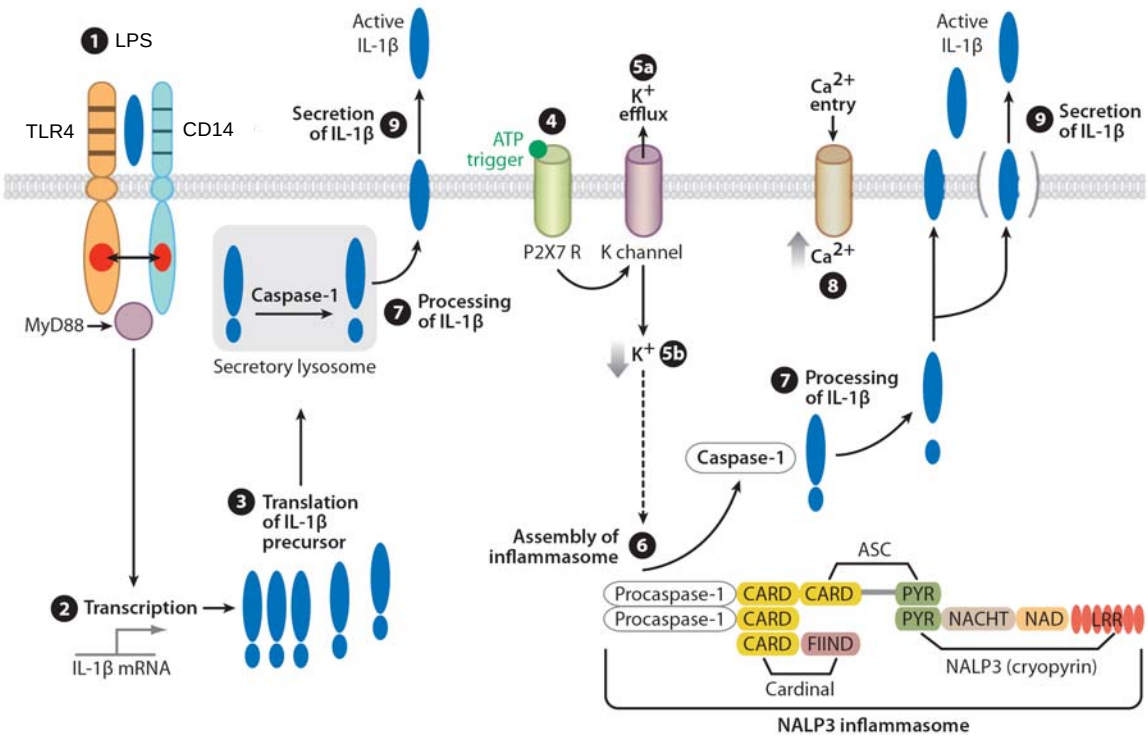

Figure 4 : Steps in the synthesis and secretion of IL-1β induced by LPS. Primary blood monocytes are activated by IL-1β (step 1). The formation of the IL-1 receptor complex heterodimer results in approximation of the Toll IL-1 receptor (TIR) domains (*red*) and the recruitment of MyD88. The transcription (step 2) and translation into the IL-1β precursor (step 3) are separate events. Translation takes place in the cytosol, not in the endoplasmic reticulum. The activated monocyte releases ATP into the extracellular space. Upon activation of the P2X7 receptor by ATP (step 4), there is a rapid efflux of potassium from the cell (step 5a) resulting in a fall in intracellular levels of potassium (step 5b). The fall in intracellular potassium levels triggers the assembly of the components of the NALP3 inflammasome (step 6). Given that NALP3 does not contain a caspase-activating recruiting domain (CARD), the adaptor protein termed ASC is required together for oligomerization. The assembled components of the inflammasome initiate the processing of procaspase-1, resulting in the formation of the active caspase-1. Active caspase-1 processes the IL-1β precursor (step 7) in the cytosol or in the secretory lysosome, resulting in the generation of the mature IL-1β. An influx of calcium into the cell (step 8) with an increase in intracellular calcium levels provides a mechanism by which mature IL-1β is released from the cell (step 9). The rise in intracellular calcium activates phosphatidylcholine-specific phospholipase C and calcium-dependent phospholipase A, which facilitate the secretion of IL-1β (step 9).[13] and [30]

binding oligomerization domain (NOD) like receptor (NLR) family members and the adaptor called Apoptosis-associated Speck-like protein containing a CARD domain (ASC), are assembled through CARD-CARD, *i.e*. N-terminal caspase recruitment domain (CARD) [103] and pyrin domain-pyrin protein-protein interactions to form a scaffold for the recruitment and activation of procaspase-1 (see Figure 4 n°6)[104]. NLR family is a group of cytoplasmic PRR NODs, NALPs, IPAF (ICE-protease activating factor), NAIPs (neuronal apoptosis inhibitor factors) and CIITA [105]. Furthermore, NLRs are defined by the architecture of their C-terminal variable domain: a nucleotide-binding domain (NBD), also known as NACHT domain, which binds nucleotides, possesses an ATPase activity and regulates oligomerization. A leucine-rich repeat (LRR) domain, which varies in the number and composition of repeats, is considered to be the ligand-sensing motif [106] and is required for protein-protein interaction [103, 105]. There are four possible N-terminal domains: (i) a CARD domain, (ii) a pyrin domain (PYR), (iii) a baculovirus inhibitor of apoptosis protein repeat (BIR) domain, and (iv) an N-terminal PYR domain [103, 106]. ASC contains an PYR domain, which acts as an oligomerization domain [107], and a C-terminal CARD domain, which acts as an effector domain in the caspase-1 activation pathway [108, 109]. There are three prototypes of inflammasomes: the NALP1 inflammasome (NLRP1), the NALP3 inflammasome (NLRP3; cryopyrin) and the IPAF inflammasome (NLRC4) [103]. ASC is the common adaptor to these inflammasomes [104]. The NALP1 inflammasome was the first caspase-1-activating platform to be indentified [110]. It can be activated by muramyl dipeptides (MDP) and anthrax lethal toxin. NALP1 gene mutations have been involved in Vitiligo [60]. The NALP3 inflammasome is probably the best-understood inflammasome because it is involved in IL-1β processing. It is activated by several microbial stimuli, including multiple TLR agonists [111], and by non-microbial components such as monosodium urate (MSU) and silica [112, 113]. Mutations within the NBD of NALP3 lead to several diseases characterized by spontaneous systemic inflammation such as Muckle-Well

syndrome, familial cold urticaria and chronic infantile neurological cutaneous and articular (CINCA) syndrome [111] [60]. The NALP3 inflammasome also contains a protein called CARDINAL whose functional role is still unclear [67].

IPAF inflammasome includes Ipaf itself (ICE-protease activating factor) and is required for caspase-1 activation in response to *S.typhimurium, S.flexneri, P.aeruginosa* and *L.pneumophila* flagellin [103, 104, 114]. IPAF belongs to the CED-4/Apaf-1 family and is composed of an N-terminal CARD followed by a NBD and a C-terminal LRR domain [114]. Five different mechanisms of IL-1β secretion have been proposed. The firth mechanism involves the transport of multivesicular bodies (MVB) towards the cell periphery and their docking and fusion with the plasma membrane and subsequent release of IL-1β-containing exosomes. This pathway is Ca^{+}-influx independent (Figure 5 Pathway A)[115]. The second mechanism suggests that IL-1β and caspase-1 co-localize in endolysosomes after an active transport [28]. Then, IL-1β maturation occurs inside these endolysosomes during trafficking to the plasma membrane. Finally, ATP stimulation induces exocytotic fusion and release of mature IL-1β. This process seems to be regulated through an influx of Ca^{+}, an efflux of K^{+} and a phospholipase-dependent mechanism (Figure 5 Pathway B)[116-118]. The third mechanism suggests that ATP stimulation induces a local accumulation of caspase-1 and pro-IL-1β within a microdomain below the plasma membrane. Subsequently, the evaginations of plasma membrane blebs detach themselves from the cell surface. This blebbing process is completely dissociated from IL-1β processing. This process seems to be Rho and p38 MAPKs dependent (Figure 5 Pathway C) [28, 119, 120]. The fourth mechanism suggests that mature IL-1β directly crosses the plasma membrane via molecular transporters, which are still undefined (Figure 5 Pathway D) [69, 120]. The fifth mechanism proposed is the lysis of IL-1β-containing cells and subsequent passive release of cell contents. Despite, a variety of recent studies that have clearly demonstrated that IL-1β release occurs independently of cell lysis, one cannot

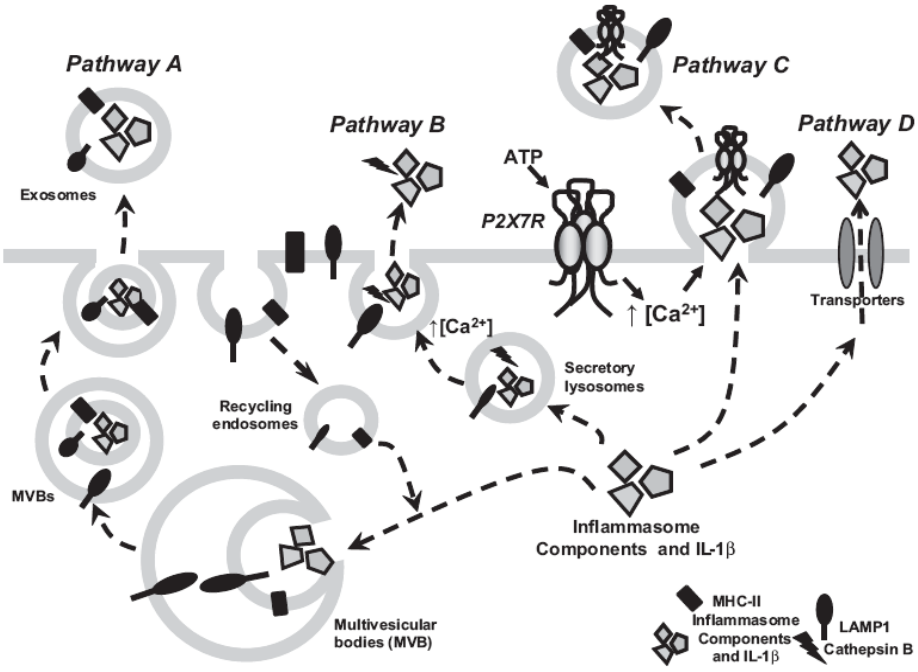

Figure 5: Four models of nonclassical IL-1β secretion. Pathway A describes the MVB/exosome model. This may involve the P_2X_7R-induced concentration/assembly of caspase-1 inflammasomes and pro-IL-1β within the cytosolic vicinity of recycling endosomes, formation of MVB that contains exosomes with entrapped IL-1β, and fusion of these MVB with the plasma membrane to release exosomes containing IL-1β, other inflammasome components. Pathway B describes the Ca^{2+}-dependent secretory lysosome exocytosis model, in which pro-IL-1β and its processing enzyme caspase-1 are targeted to a subset of secretory lysosomes. Pathway C describes the microvesicular shedding model, in which cytosolic pro-IL-1β and caspase-1 inflammasome complexes are entrapped within Ca^{2+}-dependent evaginating blebs of plasma membrane that are then released as microvesicles containing IL-1β, caspase-1. Pathway D posits the direct efflux of cytosolic IL-1β through as-yet-uncharacterized transporters localized in the plasma membrane.(From [28])

completely rule out that cell lysis-induced IL-1β release contributes to the amount of IL-1β produced under pathological conditions [115].

2.2 The IL-1 receptor family

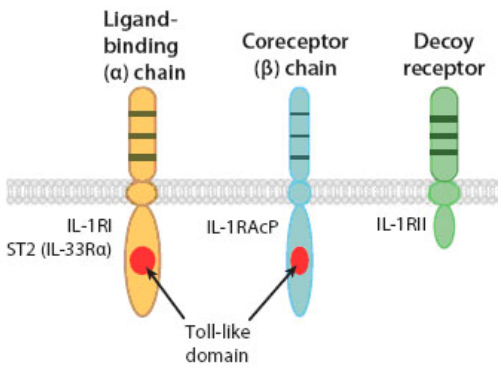

Figure 6: The various members of the interleukin-1 (IL-1) receptors are shown. IL-1α et β bind the -IL-1RI, type I IL-1receptor, -IL-1RII, type II IL-1 receptor, -IL-1Ra, IL-1-receptor antagonist and IL-1RAcP, IL-1-receptor accessory protein. IL-33 binds ST2 while IL-18 binds IL-18Rα and IL-18β. (Adapted from [13])

The IL-1 receptor family comprises ten members, some of which remain orphan receptors. Of particular importance, the three members IL-1RI, IL-1RII and ST2 are well characterized (Figure 6). In human, these receptors derive from a common receptor gene localized on the long arm of chromosome 2. Their extracellular parts are homologous and contain three IgG-like domains [13, 30]. IL-1RI and IL-1RII bind IL-1α, IL-1β and sIL-1Ra while

IL-33 is the only ligand known for ST2. IL-1RI is an 80 kDa glycoprotein ubiquitously present at cell surfaces, but its expression is higher in keratinocytes, dentritic cells, hepatocytes, fibroblasts, and T lymphocytes [121]. IL-1RI contains a single transmembrane domain and a 212 amino acids cytosolic domain that transduces signals. The N-glycosylation of 7 Asn residues is required for IL-1 binding and signaling [122]. The strong biological effects of IL-1 can be explained by the increased binding affinity of IL-1 to the complex formed with the IL-1R-Accessory protein (IL-1R-AcP) [11, 30]. IL-1R-AcP is a 66 kDa glycoprotein that shares homology with IL-RI. Moreover, IL-1R-AcP is required for IL-1 signaling. Indeed, IL-1 binds IL-1RI leading to conformational changes in the latter that allow the recruitment of IL-1R-AcP and, in turn, signal transduction. However, IL-1R-AcP alone does not specifically bind IL-1 [122]. Despite the presence of IL-1R-AcP which increase the receptor affinity for IL-1 agonists, IL-1RI shows a stronger affinity for sIL-1Ra as compared to IL-1β or IL-1α [123, 124].

IL-1RII is a 68 kDa glycoprotein expressed by monocytes, macrophages, neutrophiles, and B cells. It shares 28% amino acid sequence homology with the extracellular domain of IL-1RI. Having a short cytoplasmic domain of 29 amino acid as compared to that of IL-1RI [125], IL-1RII is unable to transduce signal and display decoy receptor functions [122]. Therefore, it is not surprising that IL-1RII and displays a stronger affinity for IL-1α/β than for sIL-1Ra [123, 124, 126].

The activity of IL-1 is regulated by soluble form of IL-1R-AcP, IL-1RI and IL-1RII, and by secreted IL-1Ra. While the extracellular domains of IL-1RI and IL-1RII can be cleaved by a metalloprotease, probably TACE (TNF converting enzyme) in order to produce soluble forms of IL-1RI and IL-RII (sIL-1RI; II), IL-1R-AcP is generated from an alternatively spliced mRNA [1]. sIL-1RI binds sIL-1Ra more avidly that IL-1α or IL-1β whereas IL-1β or IL-1α binding to sIL-1RII is nearly irreversible [11, 13, 126-128]. The role of sIL-1R-AcP is actually not fully understood. Some studies have shown that low levels of sIL-1RI are present in both

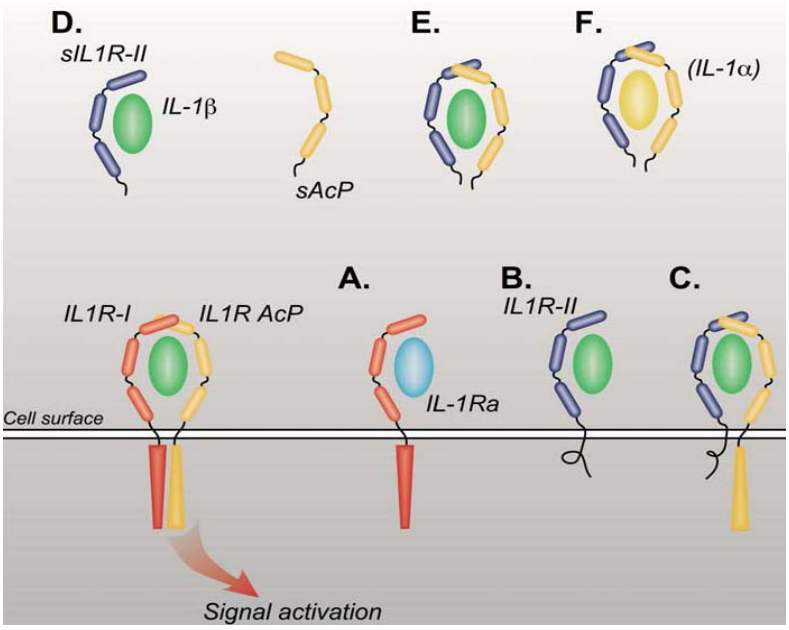

Figure 7: Regulation of mature IL-1 activity. This figure depicts the various regulatory complexes formed by IL-1R and IL-1 ligands, compared to the active IL-1 signaling complex shown in the lower left. IL-1Ra binding to the IL-1RI (A) blocks receptor activation by IL-1α and IL-1β. sIL-1RII binds IL-1β with high affinity (B) and decoys its activity. This complex can also compete IL-1R-AcP away from productive signaling complexes (C). sIL-RII maintains high neutralizing affinity for IL-1β in solution (D), but obtains even higher affinity due to cooperation with sIL-1R-AcP (sAcP) (E). Finally, the combination of sIL-RII and sIL-1R-AcP is also able to bind IL-1α with high affinity (F). [1, 2]

inflammatory and non-inflammatory synovial fluids whereas sIL-1RII is found at high levels in the circulation of patients with RA [11, 121]. Thus, the activity of IL-1 is regulated by a combination of an antagonist (sIL-1Ra, see below), soluble receptors (sIL-1R-AcP and sIL-1RII) and cell surface expression of a decoy receptor (IL-1RII) (Figure 7) [1, 11, 129].

2.3 Interleukin-1 Receptor Antagonist

IL-1F3, the third member of the IL-1 family, also named IL-1Ra, functions as a competitive receptor antagonist. sIL-1Ra is an acute phase protein, which is an IL-1 inhibitor of 22-25 kDa found in the supernatants of human monocytes activated by immune complexes [30, 130]. IL-1Ra displays 19% and 26% amino acid sequence homology with IL-1α and IL-1β, respectively [6]. The IL-1Ra gene, *IL-1RN*, mapped to band q14-21 in the long arm of chromosome 2 [6, 131]. It generates two types of products encoded from alternative spicing of two different first exons [132]. One type is termed intracellular IL-1Ra (icIL-1Ra1; icIL-1Ra2; icIL-1Ra3) [133] because it lacks a full secretory peptide and remains intracellular [134] as opposed to the second type pattern which is termed secreted IL-1Ra because it is released into extracellular space. sIL-1Ra is released as a variably glycosylated protein. sIL-1Ra mRNA

sequence, *i.e.* 1740 bp, has a single open reading frame (ORF) coding for 177 amino acids, which is composed of a 31 nucleotides 5'untranslated region (UTR) followed by a long 3'UTR of 1'133 nucleotides without poly-A tail [135, 136]. sIL-1Ra is an inducible gene in various cells (monocytes/macrophages; neutrophils; neurons; fibroblasts; epithelial tissues; hepatocytes; glial cells; etc.) [137] [6].

The three isoforms of icIL-1Ra (icIL-1Ra1; icIL-1Ra2; icIL-1Ra3) derive from alternative splicing of a single transcript that gives rise to molecules with similar C-terminal parts but different N-terminal parts (Figure 6) [138, 139]. However, all forms of IL-1Ra conserved the ability to inhibits the IL-1 activity when they are applied extracellularly *in vitro* [140]. The icIL-1Ra1 is expressed constitutively in epithelial cells and could be released in rare cases [138, 139] while icIL-1Ra2 mRNA has been detected in keratinocytes, neutrophils and monocytes, the corresponding protein was not found. IcIL-1Ra1; icIL-1Ra2 and icIL-1Ra3 are inducible in monocytes/macrophages but only icIL-Ra2 is inducible in neutrophils [133, 141]. In spite of the fact that the function of icIL-1Ra has been unknown until recently and still remains unclear. Watson and al. [142] demonstrated that the icIL-1Ra inhibited the IL-1β signaling. This inhibition was not due to competition for binding IL-1RI, but rather by blocking intracellular

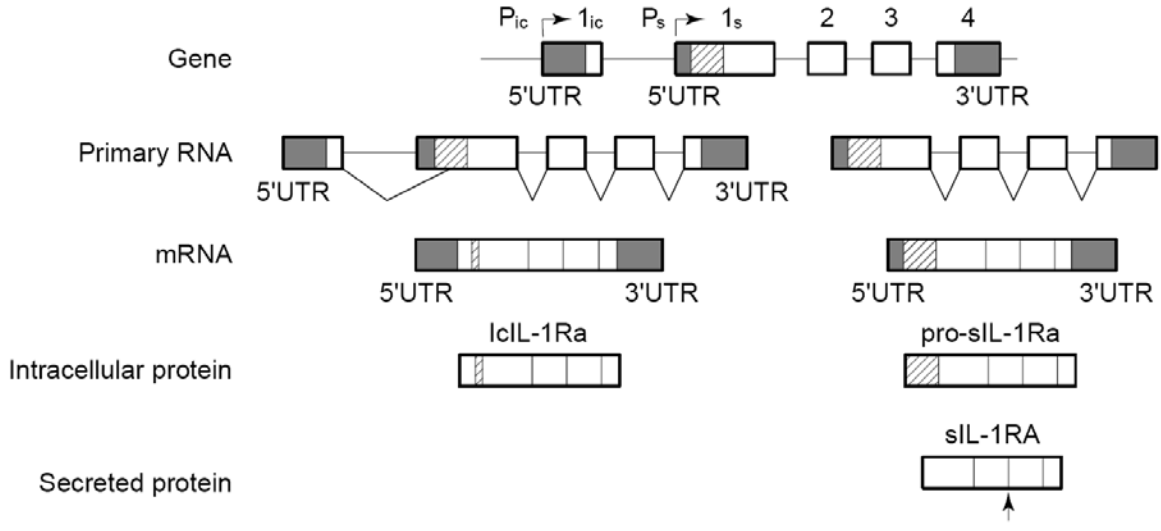

Figure 8: IL-1Ra isoforms are generated from the same gene. icIL-1Ra is produced by splicing of exon 1ic into exon 1s excluding a large proportion of the leader peptide sequence (dashed zone). The production of sIL-1Ra is controlled by promoter Ps. sIL-1Ra pro-peptide is processed and glycosylated prior to secretion. [6]

action of IL-1β. Accordingly, the functions of icIL-1Ra are not to block necessarily the immediate signaling above IL-1 receptors but to attenuate and destabilize some IL-1β-inducible immediate early gene mRNA, *e.g.* GRO and IL-8 [2, 142]. On the other hand, it was also shown that icIL-1Ra1 dampens the IL-6 and IL-8 production in Caco-2 cells through p38 MAP and NF-κB pathways inhibition [138]. Thus, icIL-1Ra may act inside the cells via mRNA destabilization or by inhibition of IL-1-mediated signaling in living cells. Nevertheless, it is also conceivable that icIL-1Ra isoforms have extracellular effects by secretion via vesicles or cell death [139] or may function as intracellular store of IL-1Ra, being released by necrotic or apoptotic cells to block IL-1 binding to IL-1RI [143].

2.3.1 sIL-1Ra gene structure

The *IL-1RN* gene comprises five exons. The first exon I_{ic} (exon present only in $_{ic}$IL-1Ra) 9.4kb 5'-upstream of the exon I_s (first exon present in both types of IL-1Ra), is alternatively spliced and the four other exons are common to all IL-1Ra isoforms (Figure 8) [144]. The transcription of IL-1Ra is controlled by two different promoters, *i.e.* $P_{ic;}$ P_s. The promoter P_{ic} control the exon I_{ic}, in order to produce icIL-Ra and the promoter P_s control the exon I_s to drive sIL-1Ra expression [137, 144]. Unlike P_{ic} that contains a TATA box at -26 and -697 from ATG start site [135], P_s has no TATA-like sequence [144]. Moreover, several potential *cis*-acting binding sites are indentified such as NF-κB, NFIL-1β, AP-1, CRE and CT rich region in -84 and -93, -37 and -26, -39/ -510/ -1197, -399 and -134, and -946 and -882, respectively [135]. PU-1 has been described as the most critical transcription factor for the sIL-1Ra gene expression in response to LPS but not in the regulation of basal activity in human monocytes and macrophages [145, 146]. Indeed, two PU-1 binding sites are located between positions -80 and -90 on the minus stand and both PU-1 and GA-binding protein (GABP) bind those sites. As referred above, an overlap of several transcription factors binding sites is composed by NF-κB/PU-1/GABP binding sites. However, 50% of the response to LPS is NF-κB and PU-1-

dependent. More recently it was shown that STAT3 is required for an optimal binding of NF-κB p65 and p50 to the sIL-1Ra promoter [147]. GABP seems to play a role in the regulation of basal promoter activity, while NF-κB and PU-1 are likely to display redundant functions [146, 148]. Nevertheless, it has been suggested that a major role for PU.1 is to recruit other factors into the transcription complex that are necessary to regulate sIL-1Ra gene downstream LPS-activation [145]. Finally, STAT6 and STAT3 are described as additional *cis*-acting elements required for a full activation of sIL-1Ra gene in IL-4- or LPS-activated macrophages, respectively [149] [150].

Some studies have been performed to characterize the icIL-1Ra promoter. The icIL-1Ra promoter contains three transcription initiation sites at -4, -11, -70 relative to start codons [137, 151]. The region between -4525 to -1438,-288 to -156 and -156 to -49 are necessary for icIL-1Ra promoter activity in epithelial cells [151] [130]. Furthermore, the -156 to -49 region is rather devoted to regulation than to activation in epithelial cells and macrophages. Finally, full control of sIL-1Ra transcription is under positive and/or negative *cis*-acting elements such as NF-κB, NF-IL6 (C/ EBPβ), Oct-1, SP-1, and 3 YY1 [130, 144, 151].

The association of genetic polymorphism to diseases may be of relevance in terms of susceptibility and disease severity [152]. An allelic polymorphism is present in intron 2 of the sIL-1Ra gene. This polymorphism consists in two to six tandem repeats of 86 bp [153] [154]. The allele A1 (IL1RN*1) is found in 73.6% of normal population while the allele A2 (IL1RN*2) is present in 21.4% [155]. These variations may influence the rate of gene transcription, mRNA stability, or the quantity and activity of the resulting protein [154]. Thus, individuals homozygous for IL1RN*2 have higher circulating sIL-1Ra levels than individuals with other genotypes.

Increased frequency of IL1RN*2 has been associated with a variety of human diseases but the association is more related to disease severity than to disease predisposition [156].

IL1RN*2 was shown associated to psoriasis, systemic lupus erythematosus, ulcerative colisis, arthritis, RA, and MS [157, 158] and many other pathologies such as cancer, osteoporosis, and coronary artery disease [156], most of these associations being dependent on ethnic group [159]. Furthermore, IL1RN*2 is in linkage disequilibrium with a diallelic polymorphism of IL-1β that is associated with increased IL-1β production [155, 160]. Thus, genetic associations might be important in explaining possible pathogenic mechanisms that are relevant for the clinical expression of a disease [152]. Consequently, the lowest sIL-1Ra/IL-1β ratio is associated with high, prolonged pro-inflammatory immune responses such as MS [156, 161].

2.4 The IL-1/sIL-1Ra balance in pathology

Cytokines perform important functions in normal physiology, host response to infection and exogenous damages to the organism. The cytokine network is self-regulated through the action of opposite cytokines, the production of antagonists of cytokine binding to receptor and the release of soluble receptors. Since few years, it has been suggested that the net biologic response in a diseased organ or tissue reflected an imbalance between level of pro- and anti-inflammatory cytokines and played an important role in patients' susceptibility to and severity of many inflammatory diseases [130, 155]. Therefore, the excessive production of pro-inflammatory cytokines or the inadequate production of anti-inflammatory cytokines may lead to a chronic disease [162].

The relative IL-1/sIL-1Ra ratio may play an important role in normal physiology of various organs and tissues [155].This ratio determines whether the inflammatory response hands toward resolution or persistence [137, 163-165]. Indeed, the concentration of circulating sIL-1Ra is higher than that of IL-1 in diseases. However, sIL-1Ra production is delayed and prolonged in comparison to IL-1 production. Thus, the relative concentration of IL-1 and sIL-1Ra, rather than the absolute concentration of IL-1 alone, reflects the real activity of IL-1 [166]. The IL-1/sIL-1Ra ratio was studied in variety of experimental animal models of diseases such as

kidney diseases, RA, MS, granulomatous colitis or inflammatory bowel disease (IBD) [155]. Indeed, in IBD, an increase of IL-1α and IL-1β/sIL-1Ra ratio has been described in freshly isolated intestinal mucosal cells and in colonic mucosal biopsies from IBD patients [167, 168]. Besides, a decrease of sIL-1Ra level increases the severity of intestinal inflammation in rabbit immune colitis [169]. Finally, the implication of the IL-1 system in neurodegeneration is supported by the dramatic enhancement of IL-1 expression after a brain injury and by the improvement of the outcome and facilitation of the recovery when IL-1 signaling is blocked by sIL-1Ra [170]. Consequently, a tight modulation of IL-1β may have a therapeutic interest in order to resolve the chronic inflammatory state present in IBD, MS and RA [171].

2.5 The IL-1/sIL-1Ra balance in MS

MS is primarily an inflammatory disorder of the CNS in which focal lymphocytic and monocytic infiltration leads to myelin and axon damages [172]. It is increasingly recognized that the innate immune system plays a central role in the immunopathogenesis of MS [173] characterized by an important release of pro- and anti-inflammatory cytokines [174].

Extensive evidence indicates that both IL-1 and sIL-1Ra are produced within the CNS by several cell types including glial cells, microglial cells, neurons, vascular endothelial cells and monocytes/macrophages. A pathological development in the CNS could be influenced by the ratio between these both cytokines [161, 173, 175]. Although the presence of IL-1β has been rarely demonstrated in the normal CNS, its increased levels have been shown in the MS brain lesion. Moreover, the IL-1β and sIL-1Ra production may differ depending on the stage of disease [129]. Actually, the circulating sIL-1Ra levels have been shown to correlate with MS activity [176]. Furthermore, rats or mice with actively induced EAE, *i.e,* an animal model of MS, and treated with Anakinra (recombinant sIL-1Ra) develop disease with milder severity [129, 177]. Finally, it has been demonstrated that IL-1 contributes to enhance blood-brain-barrier (BBB)

permeability [178] thus allowing circulating sIL-1Ra to infiltrate the CNS. Accordingly, IFNβ, a treatment of MS, is a strong inducer of sIL-1Ra that is long lasting. Therefore, it may contribute to the anti-inflammatory effect of IFNβ [179, 180]. Thus, it is more the relative level between sIL-1Ra and IL-1β rather absolute level of each cytokine alone that is an important therapeutic strategy for MS treatment [163, 168, 169, 181].

2.6 The IL-1/IL-1Ra balance in RA

RA can be considered as a "cytokine deregulation disease" leading to cartilage and bone destruction in synovial joints. Joint damage is mediated in part by the macrophage-derived cytokines, IL-1β and TNF [182], that induce the production of proteases, *e.g.,* matrix metalloproteinases (MMPs), but also $PGE_{2,}$ by synovial fibroblasts. Indeed, the rheumatoid synovium contains high levels of IL-1β and TNF without any deficiency of induction of natural inhibitors of these potent cytokines. The mechanisms inducing such high levels of pro-inflammatory cytokines have not been fully characterized but involved macrophages-stimulated by T-cells [183-185]. Moreover, the injection of IL-1 into rat knee joints results into the development of features related to chronic arthritis [186, 187] while the injection of neutralizing antibodies against IL-1 reverses synovial inflammation. On the other hand, antibodies against sIL-1Ra exacerbate LPS-induced arthritis in rabbit [155] and sIL-1Ra-deficient mice exhibit a more severe form of collagen-induced arthritis (CIA) [188]. Those studies suggest that an inadequate ratio between sIL-1Ra and IL-1β may be one factor predisposing to RA [167, 189]. Indeed, it was shown a high concentration of IL-1β and sIL-1Ra into the synovium of patients with RA or in mice with CIA [190]. Nevertheless, a large molar excess of sIL-1Ra is required in order to efficiently inhibit the IL-1β effects. Therefore, the production of sIL-1Ra into the synovium is not sufficient to abrogate the cartilage degradation [191, 192].

A restoration of the balance between IL-1β and sIL-1Ra in RA or more generally in inflammatory diseases can be achieved through several strategies such as soluble receptors administration, inhibition of IL-1β production or gene therapy [193, 194]. Thus, the administration of soluble IL-1RII, results in a marked inhibition of joint swelling and joint damage [193, 195]. Moreover, some experiments have shown that the rabbit joints are protected from inflammation and cartilage degradation by infections of adenoviral vector containing sIL-1Ra gene. [196]. Besides, clinical trials of recombinant human sIL-1Ra (Anakinra®; Kineret®) in RA have also decreased radiologic damages, pain and mononuclear cell infiltration [155, 191, 193, 197]. However, the treatment of RA with soluble IL-RII or sIL-1Ra was actually abandoned in favor to administration of monoclonal antibodies against TNF which led to obvious improvement of clinical signs of inflammation and joints destruction [198].

2.7 The IL-1/IL-1Ra balance in infection

In a context of viral or bacterial infection, it is hypothesized that the physiological consequences of infection-induced inflammation and shock might be improved by modulation of IL-1 system. Indeed, it is shown that the mortality of mice infected by *E.coli* is reduced by the injection of sIL-1Ra [199]. Therefore, the ratio of IL-1β/sIL-1Ra in local tissues may influence the course of different infectious diseases. In fact, live *B. burgdorferi* preferentially induce synthesis of IL-1β, *i.e* six fold more than a strong inducer such as LPS, compared to sIL-1Ra [30, 200]. Inversely, bacteria and viruses evolve towards the inhibition of the synthesis and/or release of pro-inflammatory cytokines such as IL-1β. Thus, respiratory syncytial virus disrupts IL-1β synthesis through the release of the anti-inflammatory cytokine IL-10 [2, 201]. The physiological inhibition of IL-1 by sIL-lRa appears to protect the organism against responses to infection. Nevertheless, there is a risk of impairing the host's ability to eliminate infection [202]. Mutations present into the *IL1RN* gene prevent the production of sIL-1Ra leading to hypersecretion of pro-inflammatory cytokines and chemokines in response to IL-1β. The pro-

inflammatory effects of non-inhibition or overexpression of IL-1β (through *NALP3* gene mutations gain-of-function) induce several diseases including skin diseases, bones lesion and wide variety of other diseases which could also lead to death. Thus, the control of IL-1 system in several levels is crucial [165].

2.8 Tumor necrosis factor (TNF)

TNF is a pro-inflammatory cytokine with a wide range of biological functions formulate through a complex mechanism of action. TNF is present in two forms: the soluble form and the transmembrane form. The majority of TNF cellular actions described in the literature are related to the soluble form. Indeed, the 17kDa mature form of TNF is generated through a cleavage, by a protease of metalloprotease/cysteine–rich family called TNF converting enzyme (TACE), resulting in release of extracellular portion of the 26kDa type II transmembrane TNF [203, 204]. The active form of mature TNF has been determined to be a compact homotrimer [53]. TNF is released by several cells including macrophages, monocytes, neutrophils, NK-cells, T-cells, astrocytes and microglia [205].

The human TNF gene consists in a single copy gene located on the short arm of human chromosome 6, closely linked within the cluster of major histocompatibility complex genes (MHC) [205, 206]. The TNF gene is constituted of approximately 3.6 kbp and consists of four exons and three introns. The mature TNF sequence is about 80% encoded, in the fourth exon, while exons I and II encode only the leader peptide sequence [205]. The TNF mRNA transcript is a short-lived mRNA that contains an ARE in its 3'untranslated region (3'UTR). Thus, its stability is controlled by trans-acting factors, which bind the ARE, such as tristetraprolin (TTP) or HuR [207].

TNF homotrimer (Figure 9) exerts its effects through the binding and induction of high-affinity clustering of TNF homotrimer-receptors. The receptors, present in great number on cell membranes, are metabolically labile, with a half-life of 30 to 120min. The receptor-TNF

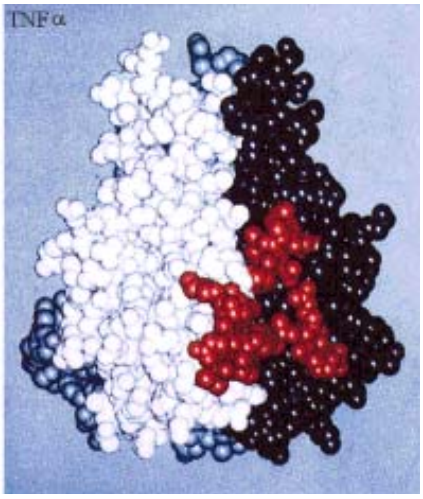

Figure 9: Structure of human TNF trimer (from [5])

complex is internalized via clathrin-coated pits and finally degraded in the lysosomes [5, 204]. There are two distinct TNF receptors, the 55kDa (TNF-R1) [208] and the 75kDa (TNF-R2) [209]. The extracellular portions of the two receptors display similar sequences but the intracellular portions are different, suggesting that the two receptors activate different intracellular signaling pathways. Furthermore, TNF has a different affinity for TNF-R1 and TNF-R2 [210]. In addition, the TNF activity is neutralized or modulated by soluble form of TNF-R1 and TNF-R2. Indeed, TNF-R1 and TNF-R2 can be proteolytically cleaved. Thus, soluble TNF-R1 or soluble TNF-R2 are able to block TNF activity (Figure 10) [203]. The presence of TNF-R1 is essential for TNF receptor function. TNF-R1 alone is sufficient for high affinity binding and full biological activity. However, unlike TNF-R1, the activation of TNF-R2 is not sufficient to initiate a variety of responses to TNF [206, 211, 212].

TNF and its receptors play several physiologic, pathologic and/or benefic roles such as cells growth, cells differentiation, cells survival, immunostimulation, resistance to tumors, sleeping regulation and embryonic development [203, 206, 213]. Parasitic, bacterial and viral infections become more pathogenic or fatal due to the presence of TNF in the circulation [204]. However, TNF seems to have a major role in the mediation of protection against

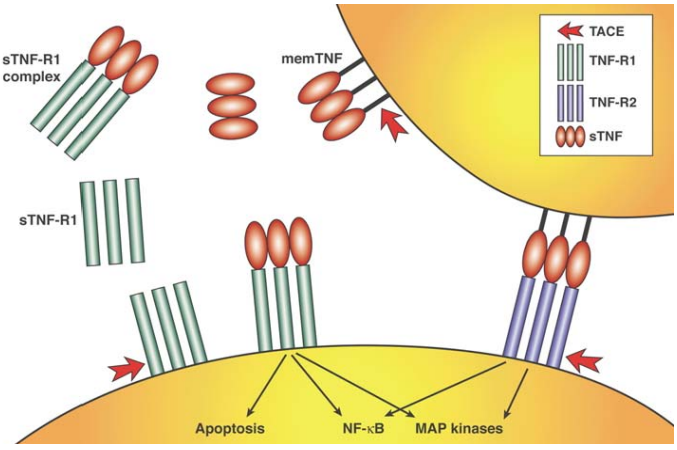

Figure 10: Interaction of soluble TNF and transmembrane form TNF with TNF receptors (TNF-R1 and TNF-R2) and down-stream signalling pathways. Arrow (in red) shows cleavage sites for TACE. (Adapted from [16]

parasitic, bacterial and viral infections [5, 214]. Indeed, the administration of TNF prolongs survival in lupus prone NZB mice and appears to have an important anti-inflammatory role in MOG-induce Experimental autoimmune encephalomyelitis (EAE) [215]. TNF was evaluated in several arthritis models such as collagen-induced arthritis (CIA), and in patient with RA. Thus, the inhibition of TNF with neutralizing monoclonal, polyclonal antibodies or soluble TNF receptors ameliorates but did not completely bock CIA in mice[216, 217]. In human, TNF inhibitors have demonstrated effectiveness in decreasing the severity of RA and in slowing the progression of bone erosion. However, anti-TNF therapy could be associated with inflammatory demyelinating procedures and aggravate MS [218]. Some studies have shown evidences for the effectiveness of TNF inhibitors in Crohn's disease, psoratic arthritis, psorasis, juvenile inflammatory arthritis and andylosing spondylitis [16]. Those examples highlight the dual functions of TNF which may have some benefic or pathologic effects depending disorder context.

2.9 Interleukin-6

Interleukin-6 (IL-6) is a four-helix bundle cytokine of 21 to 28 kDa depending on post-translational processing such as glycosylation belongs to the IL-6-superfamily. This superfamilly, whose members are structurally and functionally (*e.g.*gp130) related, includes IL-6, IL-11, oncostratin M (OSM), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-like cytokines (CLC), cardiotropin-1 (CT-1) and neuropoietin (NPN) and has recently been supplemented by two newly cytokines, IL-27 and IL-31 [219, 220]. IL-6 is a prototypical pleiotropic cytokine, originally indentified as a factor in the induction of immunoglobulin production in B lymphocytes [221]. Various types of lymphoid and non-lymphoid cells produce IL-6, such as T cell, B cells, monocytes, fibroblasts, keratinocytes, osteoblasts, endothelial cells, and several tumor cells [222]. Once inflammatory mechanisms are activated, IL-6 plays a pivotal role in acute amplification. Besides, the acute phase proteins

production, whose plasma concentrations increase or decrease in response to inflammation, is stimulated by IL-6. Thus, IL-6 participates to the transition into the chronic phase of inflammation by mononuclear cell accumulation at the site of injury [223]. In addition to its immunologic/inflammatory role, IL-6 has some functions in a wide variety of other systems such as spermatogenesis, skin cell proliferation, and neural cell differentiation and proliferation [221].

The human IL-6 gene is located on chromosomes 7p21 [221]. Its size is 5kbp and consists of five exons and four introns [222, 224]. There are three transcriptional initiation sites which correspond to three TATA-like sequences between -92 and -85, -115 and -109; -207 and -201[225]. The control of IL-6 promoter activity in the 5' flanking region is very complex. Indeed, the 5' flanking region of 300bp length upstream the start codon and consists in several *cis*-acting response elements including activator protein-1 (AP-1), nuclear factor IL-6 (NF-IL6), NF-κB, c-fos serum responsive element (SRE) and the glucocorticoid receptor binding (GRE) [221, 222, 224, 226]. Finally, alike TNF, the 3'-UTR contains an AU-rich region, which is important for the regulation of IL-6 mRNA stability [224].

IL-6 exerts its activity on target cells through the functional membrane IL-6 receptor, mIL-6R (also called gp80 or IL-6Ra) and gp130 [220, 227]. The mIL-6R has a molecular weight of 80 kDa and is located on human chromosome 1q21. mIL-6R is expressed by several cell types including monocytes, hepatocytes, neutrophils, T- and B-lymphocytes [221]. A naturally occurring soluble form of IL-6 receptor (sIL-6R) is generated by two different and independent mechanisms: (i) by proteolytic cleavage from the mIL-6R and (ii) by alternative splicing of mRNA [228-230]. The glycoprotein gp130 (130 kDa) is ubiquitously expressed. gp130 has no IL-6-binding capacity but plays an important role in transduction of the IL-6 signal [219]. Indeed, into "classic signaling", gp130 play the role of co-receptors of mIL-6R. Therefore, gp130 is required to transduce the IL-6-signal through mIL-6R although there is no direct IL-

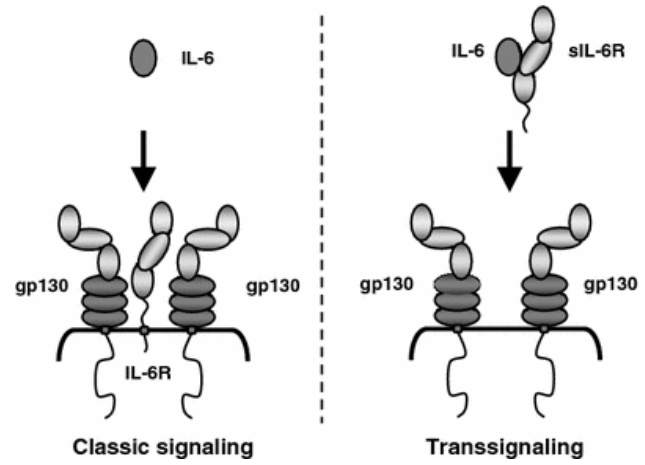

Figure 11: Classic and trans-signaling of IL-6. Trans-signaling of receptors for the IL-6 family of cytokines. The sIL-6R generated by proteolytic cleavage or alternative splicing binds its ligand with comparable affinity as the membrane expressed IL-6R. The IL-6/sIL-6R complex associates with gp130 expressed on a cell with no IL-6R expression, induces dimerization and initiates signaling. Cells that only express gp130 but no IL-6R are unable to respond to IL-6. Activation of such cells in the presence of sIL-6R is called trans-signaling since the sIL-6R generated by one cell type enables a second cell type to respond to the cytokine (Adapted from [22])

6-binding to gp130 (Figure 11, Classic signaling) [219-221, 227, 231]. On the other hand, into "transsignaling", a complex which is composed of one IL-6 and one sIL-6R binds to two gp130 proteins present in cell surface and induces a signal [220, 232]. Thus, in contrast to most soluble cytokines, the specific membrane receptor is dispensable to initiate a signal. By this mechanism, the cells which generate the sIL-6R can make other cells, which do not express mIL-6R, responsive to IL-6 (Figure 11, Transsignalling) [22, 220, 232-234]. No single cytokine of the IL-6 family can stimulate gp130 without additional receptor proteins. Thus, the additional receptor determines whether or not a cell is able to respond to IL-6 family member [61, 220].

IL-6 activities are critical for resolving innate immune response and promoting acquired immunity. The switch between innate and acquired immunity is a central event in the resolution of any inflammatory conditions and disruption of this transition could potentially affect the onset of autoimmune or chronic inflammatory disorders. Indeed, IL-6 is an acute phase protein that induces leukocytosis, fever, and angiogenesis. IL-6 participates to the chronic phase of inflammation by cells recruitment, B-cell differentiation, and T-cells activation [223, 232]. There is substantial evidence that IL-6 plays an important role in rheumatoid inflammation. The concentration of IL-6 in blood stream of RA patients, which is six-fold higher than in healthy persons, has been directly correlated with disease activity [235, 236]. Moreover, IL-6 deficient mice are resistant to the induction of antigen-induced arthritis

(AIA). Whereas the articular cartilage in IL-$6^{-/-}$ mice are preserved, it was completely destroyed in IL-$6^{+/+}$ mice [237]. Furthermore, the blocking anti-IL-6 receptor antibody leads to significant clinical response and amelioration of joint damage in RA patients [227, 238].

Astrocytes are also a major source of IL-6 expression in the CNS and the serum level of sIL-6R is found increased in MS patients. Furthermore, the IL-$6^{-/-}$ mice is not sensitive to EAE induction, suggesting that the IL-6 system play a part in EAE. However, further studies have shown that IL-6 blockade by treatment with an monoclonal antibody against IL-6 receptor inhibited the development of EAE and inhibited the induction of myelin oligodendrocyte glycoprotein (MOG) peptide-specific CD4-positive, CD8-positive, and Th17 T cells [239]. Finally, IL-6 family has been shown to be involved in several other chronic inflammatory diseases such as Crohn's disease, asthma, peritonitis, colitis or colon cancer [22, 238].

3 INTERFERONS

IFN were discovered in 1957 by Issacs and Lindenman in response to viral exposure [240]. Interferons are a family of structurally related cytokines that exhibit antiviral, anti-proliferative, anti-tumoral functions and have immunomodulatory properties [27]. They are present near the beginning of the innate immune response and have also a role in regulating the adaptive immune system [241]. IFN are commonly grouped into two types designated type I and type II IFN [242]:

3.1 Type I IFN

Type I IFN are a main players of antiviral defense against viruses. Produce by several cell types including dendritic cells, NK cells, monocytes, T-cells and macrophages, type I IFN genes are triggered in virus infected cells, that induces antiviral mechanisms limiting the viral growth and spreading [243]. Biological activities against viruses comprise MHC class I and II expression, activation and repression of pro- and anti-apoptotic genes, respectively, and modulation of angiogenic activity as well as the differentiation [244-246].

Type I IFN comprises at least thirteen subspecies of IFNα and IFNβ, IFNω, IFNτ, IFNκ, IFNε, IFNλ (IL-28A,B; IL-29), IFNδ and IFNζ (limitin) [247]. IFNα, IFNβ and IFNω are glycosylated secreted proteins with a molecular weight about 16 to 24 kDa which form dimers. IFNα and IFNβ are the most extensively studies type I IFN with more than 29% amino acid homology [248, 249]. Type I IFN are composed by five

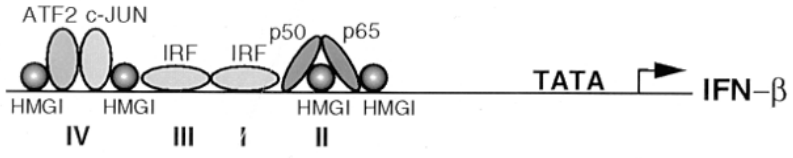

Figure 12: Human IFNβ gene promoter. The enhancer region contains four positive regulatory domains designated PRDI, II, III, and IV. Transcription factors that bind to each of the elements are shown: the ATF2/c-JUN heterodimer binds to PRDIV, an IRF family member protein binds to both PRDI and PRDIII, the p50/p65 heterodimer of NF-kB binds to PRDII, and HMG I(Y) binds to the three AT-rich sequences within the enhancer and to one site between PRDII and the TATA box. (Adapted from [17])

α-helices linked by one AB loop, which is crucial for high-binding affinity with receptors, and three conserved segments through type I IFN [250, 251].

IFN-α, IFNβ, IFNε, IFNκ, IFNω and IFNλ are express in humans whereas IFNτ and IFNδ are not. Indeed, the human genes loci are 9p22-p21-p13 [241, 249]. The genes encoding IFN are not expressed in resting cells but can be activated rapidly by viruses, cytokines, bacterial products or other diverse groups of natural and synthetic agents. IFNα and IFNβ transcripts possess 45% homology in nucleotide sequence [248]. The regulatory domains of IFNβ gene are present within a 110 bp region upstream of the transcription initiation site [252, 253]. Four distinct regions have been revealed as DNA binding proteins called positive regulatory domains I to IV (PRD). The fourth are required for an optimal induction of IFNβ promoter. PRDI and PRDIII are required to the interaction with the IFN regulatory factors IRF-1; IRF-2 and PRD-BF-1 (repressor) [254], whereas PRDII domain binds RelA (NF-κB transcription factor). PRDIV interacts with ATF-2/c-Jun heterodimer and octomer binding proteins. Finally, high-mobility group (HMG) I(Y) binds to three AT-rich sequences into the enhancer as well as a other site between PRDII and TATA box (see Figure 12) [17, 252, 255, 256].

Type I IFN receptors (IFNR) is a dimer composed of IFNAR1 and IFNAR2. IFNAR1 has a molecular mass of 110 kDa and there are three forms of IFNAR2: two membrane-associated forms, IFNAR2c and IFNAR2b, and one soluble form, sIFNAR2a. IFNAR2c is the long form with a weight of 90 to 110kDa, and IFNAR2b is the short form with a weight of 51 kDa [257].sIFNAR2a has a molecular weight of 58 kDa. *IFNAR1* and *IFNAR2* genes, located on human chromosome 21q22.1-22.3, encode IFNAR1 and the three variants of IFNAR2 [258]. Both IFNAR1 and IFNAR2c are required to optimal signaling whereas the physiological role of IFNAR2b remains elusive. IFNAR1 and IFNAR2 belong to the class II helical cytokine receptor family. IFNAR1 extracellular domain consists of four fibronectin type III domains (FNIII) while IFNAR2 extracellular domain comprise two FNIII domains. IFNAR2 is the

major ligand-binding component of type I IFN receptor with a higher affinity for IFN than IFNAR1. Thus, receptor complex is triggered by a sequential process with IFNAR2-IFN binding followed by recruitment of IFNAR1 [258, 259]. In vitro, the sIFNAR2a receptors could also be generated by cleavage of transmembrane IFNAR2c by intramembrane proteases in response to IFN and other stimuli [260]. Therefore, IFN signaling is inhibited by soluble IFNAR2s receptors. However, other studies have shown that IFNAR2a could have a carrier function by stabilizing IFNβ interaction with the receptor complex, and in turn, enhancing IFNβ activities. Finally, type I IFN receptors lack intrinsic kinase activity and require associated proteins kinases to phosphorylate receptors and signal transduction molecules as described below chapter n°5[258, 261].

An important part of the responses through the type I what is due to positive feedback. It is known since a long time that type I IFN can promote their own expression at the cellular level. Thus, secreted IFN act on cells in both autocrine and paracrine manner, stimulating the receptors of the infected cells and neighboring cells, thereby strengthening responses against viruses [262].

3.2 Type II IFN

There is only a single type: IFN-γ. It is produced from a single gene, containing 4 exons and 3 introns, located on human chromosome 12q24.1. Its expression is regulated at both transcriptional and post-transcriptional levels. IFNγ has a molecular weight about 34 kDa and forms a homodimer. The production of IFNγ is restricted to a small group of cells. Therefore, type II IFN is produce mainly by activate T-cells and NK cells, in response to TNF, type I IFN, IL-2, IL-12 and IL-18. However, virus-infected cells are not able to produce IFNγ [27]. The cytokine network supporting the triggering of the IFNγ production varies significantly depending on the type of infection [241, 262].

IFNGR1, 90 kDa, has an extracellular ligand-binding domain comprising two FNIII and possesses the IFNγ binding site. IFNGR2, which has a mass of 62 kDa, possesses the same general structure that IFNGR1 and plays only a minimal part in the interaction with the ligand. IFN-γ modulates different immunes functions including $CD4^+$ Th1 and $CD8^+$ response development, phagocytosis, MHC antigen presentation, and apoptosis [27, 263, 264].

3.3 IFNβ in Multipe Sclerosis Therapy

MS is an autoimmune disease presenting an inflammation of the CNS that leads to neurological disability due to demyelization [265]. IFNβ was the first immunomodulatory therapy approved for the treatment of MS in 1993 [266]. The IFNβ usage arises from the belief that viruses could play a role in the pathogenesis of MS. In spite of the inability to isolate a viral or microbial agent from the tissue of MS patients, IFNβ was maintained as a treatment in regard to the benefices during the disease course [265]. Nevertheless, the exact mechanism of action of IFNβ in MS remains unclear. Several way have been investigated, which demonstrated that IFNβ affects many cell functions. Indeed, IFNβ reduces antigen presentation by DC [267], inhibits Th1 proliferation and diapedesis, up-regulates sIL-1Ra and IL-10 production [268-270], reduces TNF, IL-1β, and IL-17 production [271], increases the amount of $CD14^{high}$ monocytes (anti-inflammatory monocytes) [272], and decreases the permeability of blood brain barrier. In addition, the disease

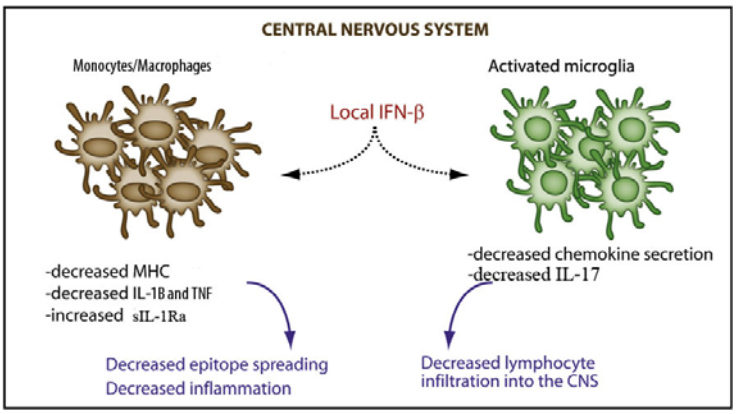

Figure 13: Role of Type 1 Interferons in Brain Inflammation. IFNβ in the CNS suppresses chemokine secretion, decreases antigen presentation, and decreases IL-17 production from microglia, infiltrating monocyte/macrophages. These effects attenuate inflammation by altering the effector function and trafficking of infiltrating T cells and other immune cells. (Adapted from [10])

activity is essentially determined by the shift of cytokine production from pro- to anti-inflammatory factors in MS patients (Figure 13) [273, 274].

IFNβ can reduce T cell migration into the MS patients' CNS through at least two processes: the inhibition of the T-cell MMP activity involved in the degradation of the basal lamina and by modulation of the expression of adhesion molecules. [10, 275, 276]. In MS patient, the treatment with IFNβ decreases the production of IL-6, IL-12, IFNγ and IL-17 and increases the production of IL-10, IL-2 and TGF-β. Moreover, IFNβ inhibits the production of IL-1β and TNF and increases strongly the production of sIL-1Ra in monocyte/macrophages activated by contact with stimulated-T-cells [277]. Thus, the beneficial effects of IFNβ seem to arise by the induction of anti-inflammatory shifts consisting in down-regulation of pro-inflammatory and up-regulation of anti-inflammatory factors. The anti-inflammatory shift of influenced by IFNβ plays a significant role in the migration and the differentiation of T-cell population. Indeed, through the changes of cytokines pattern, IFNβ restricts the inflammatory potential of $CD8^+$ and $CD4^+$Th1/Th17 T-cell through the boost of the Th2 T-cells [278, 279].

There are some opposite reports showing that IFNβ could have pro-inflammatory effects. Indeed, it was reported that TNF is increased in early stage of IFNβ treatment [280]. However, in contrast with the pathological potential of TNF, they could be necessary for CNS repair [281];Arnett, 2001 2660 /id}. In conclusion, despite a better understanding of IFNβ action, it remains that IFNβ has complex effects on the immune system in MS [282].

An alternative treatment to IFNβ, called Glatiramer acetate (GA), is used in MS patient. Indeed, GA shows similar therapeutic effects that IFNβ. GA is composed of a mixture of synthetic peptides of 50 to 90aa randomly composed of amino acid sequence EKAY. GA reduced relapse rate and progression of relapsing-remitting MS. However, mechanisms of action as well as cellular targets are unsolved although the GA has anti-inflammatory

properties illustrated by increasing sIL-1Ra and decreasing IL-1β level in chronic inflammatory conditions [283].

4 T-LYMPHOCYTES AND MONOCYTE/MACROPHAGES IN CHRONIC INFLAMMATORY DISEASE

4.1 Monocytes/ macrophage in chronic/ sterile inflammation

Monocytes/macrophages are derived from myeloid progenitor in bone marrow. Monocytes constitute until 10% of peripheral blood leukocytes. They go into the blood stream where they circulate up to 1 to 3 days. They are established as circulating precursors for alveolar macrophages in lung, macrophages or DC in connective tissue, microglia into CNS, Langerhans cells in epidermis and mature osteoclasts (see Figure 14) [29]. Altogether, these observations are consistent with the concept that monocytes repopulate macrophage or DC populations during inflammation. Thus, monocytes play critical role in innate and adaptive immunity [284, 285]. Furthermore, there is strong evidence that the deregulation of monocytes/macrophages leads to pathologic issue such as MS or RA [286, 287].

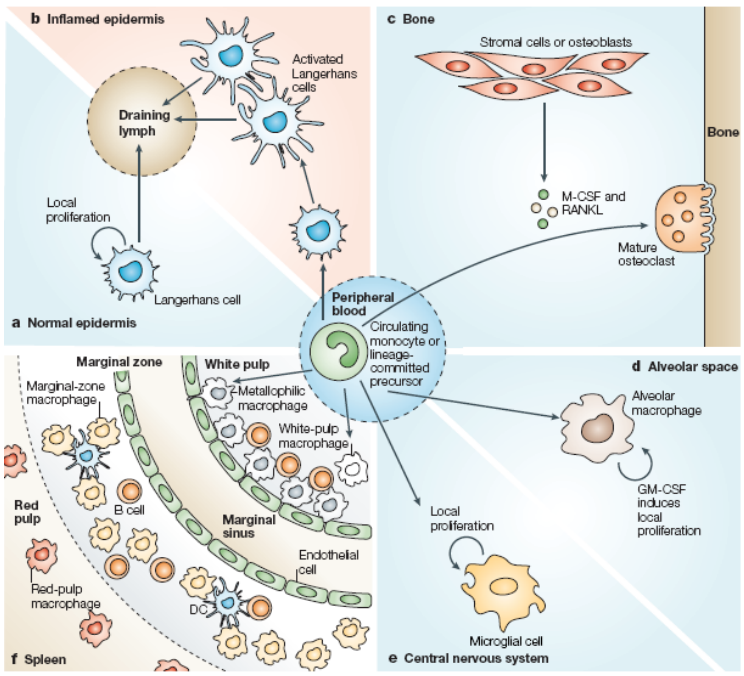

Figure 14 : The importance of circulating precursors in the repopulation of tissue-resident macrophage and dendritic cell (DC) populations is not fully understood. In the case of Langerhans cells, local proliferation in the normal epidermis (a) is adequate to maintain Langerhans-cell numbers without the requirement for replenishment by circulating precursors; however, after inflammation (b), the CC-chemokine receptor 2 (CCR2)-dependent recruitment of precursors to the epidermis allows repopulation of the tissue-resident populations. In bone (c), it is thought that circulating precursors are recruited to the bone surface, where they differentiate into mature osteoclasts, which are multinucleate and resorb bone. In the lungs (d), alveolar macrophages can be sustained for long periods by local proliferation; however, bone-marrow-transplantation experiments show that blood-borne precursors can repopulate and divide in the lungs. In the central nervous system (e), microglia are similarly maintained by local proliferation after an initial embryonic phase that populates the central nervous system, but peripheral-blood monocytes also contribute to this pool. In the spleen (f), there is marked heterogeneity of macrophages, with red-pulp, white-pulp, marginal-zone and metallophilic macrophages occupying specific anatomical niches. A simplified schema is shown, which also indicates B cells, DCs and endothelial cells. (from [29])

The classical immunophenotypic marker for monocytes is CD14, the co-receptor of TLR4 (see chapter 1.3). However, monocyte population is heterogeneous and different subsets

display different phenotypes and functions. Indeed, although the pattern of surface antigens presents different minor subset (referred in [29]), the peripheral blood population is divided into two major subsets characterized by the expression or not of CD16 (Fc-gamma receptors III). The monocytes of the "classical" subset are $CD14^{high}CD16^{-}$, representing about 90% of monocyte population. The "non-classical"subset, comprising the remaining fraction of monocyte, is either $CD14^{dim}CD16^{+}$or $CD14^{high}CD16^{+}$ [29, 284, 285, 288]. The $CD14^{dim}CD16^{+}$ population is able to express higher levels of MHCII, to produce pro-inflammatory cytokines including TNF, IL-1β and IL-6, but does not produce IL-10, following TLR-stimulation [272, 289]. In addition, $CD14^{high}CD16^{+}$ produce more IL-10 than $CD14^{high}CD16^{-}$ [290]. Consequently, $CD14^{dim}CD16^{+}$ are considered as pro-inflammatory monocytes, which is consistent with the fact that an expansion of this subset is found in septic patients [291]. Finally, it is considered that the $CD14^{dim}CD16^{+}$ monocytes are more mature than the $CD14^{high}CD16^{-}$ monocytes [292].

Monocyte migration into inflamed tissues has been shown to be mediated by chemokines as well as by other tissue-specific homing factors [293]. Therefore, $CD14^{high}CD16^{-}$ monocytes migration is mainly mediated by CCL2 (also called MCP-1 for monocyte chemotactic protein-1) while $CD14^{dim}CD16^{+}$, which do not express CCR2 (MCP-1 receptor), migrate in response to CX_3C-chemokine ligand 1 and CCL3 [292-294]. After inflammatory recruitment, monocytes undergo their differentiation in macrophages to participate in maintenance of tissue homeostasis and in the innate immune response by producing of large amounts of inflammatory effectors. The pattern of effectors is produced and regulated through the homing tissue microenvironment [295]. The migration of monocytes from the blood stream to tissues, which can occurs in absence of stimuli, remains unclear although the migration in response to inflammatory signal has been described by Imhof and Aurrand-Lions [293].

As stated above, although they are classified into two main subsets, monocyte/macrophage cell lineages are very heterogeneous and are involved in many

activities that may appear divergent such as pro- and anti-inflammatory, protective/restorative, and destructive activities. The implication of monocyte activities could affect homeostasis, tolerance, and immunity [296]. Although several manners to activate monocyte/macrophage have been described by Gordon, S 2003 [297], the process that regulates activation of monocytes in chronic/sterile inflammation remains broadly unsolved. However, it is likely that it is elicited by direct cellular contact with activated T-cells [298, 299].

4.2 Relevance of monocyte/macrophage in MS and RA

Monocytes/macrophages play a central role in RA. They are importantly involved into diverse aspects of the disease either through cell-cell interactions or through the production of inflammatory mediator [300]. Indeed, there is a good correlation between the number of synovial macrophages and joint destruction in RA [301]. Thus, the scores for local disease activity and the number of macrophage as well as the macrophage-associated cytokines have been correlated, suggesting that macrophages are directly responsible for the local inflammation and can be used to predict possible efficacy of anti-rheumatoid treatment [302]. Furthermore, only pro-inflammatory $CD14^{dim}CD16^{+}$ monocytes are associated with degree of joint destruction [303]. $CD14^{dim}CD16^{+}$ monocytes, which produce TGF-β, TNF, IL-1β, IL-12, IL-6, and other inflammatory molecules, infiltrate the synovial and may contribute to chronic inflammation and joint destruction [304]. Cytokine production by monocyte/macrophages could participate to shift from Th2 to Th1 and Th17. Since Th1 cells have shown to be more an initiator than a consequence of RA, IL-12-induced Th1 cells may increase synovial inflammation and destruction [305, 306]. IL-1, IL-6 and TGF-β production by T-cell contact-activated monocyte/macrophages might promote Th17 cells, which play a critical role in RA pathology [307]

Monocyte/macrophages and microglia cells represent related cell types involved in the development of MS and EAE. Indeed, the rapid recruitment of monocytes and the activation

of resident microglia cells are consistent with changes observed in MS. Indeed, a large numbers of activated T-cells and monocytes/macrophages are present in early MS lesion [308, 309]. Moreover, a proliferative activity of microglia is observed in the early active sites of demyelization [310]. Monocytes/macrophages and microglia cells appear to play a decisive role in the induction and resistance to MS because they are associated with a strong disease-promoting production of cytokines and other inflammatory mediators such as NO, chemokines, and growth factors. In addition, they are competent presenters of antigen and may activate or reactivate effectors T-cells [311]. Similarly to RA, the cytokines patterns produce by infiltrated monocytes or resident microglia cells support a T-cells bias towards Th1 and Th17. It is consistent for their importance in the pathogenesis of MS. Finally, monocyte/macrophages seem to be more potent to induce and perpetuate the disease. Indeed, in EAE, monocytes depletion reduces more significantly disease incidence and severity than $CD4^+$ and $CD8^+$ T-cells depletion [286]. In conclusion, monocytes/macrophages contribute to induction and perpetuation of the disease. However, the monocyte/macrophages produce also anti-inflammatory cytokines such as IL-10, sIL-1Ra and IL-4 leading to resolution of the disease. It is an illustration of the dual role of monocyte/macrophage in MS pathology.

4.3 T-cell contact-mediated activation of monocte/macrophage

As described above, cytokines contributing to the pathology of chronic inflammatory diseases, including MS and RA, are mainly produced by infiltrating as well as the resident cells of the monocyte/macrophage lineage. Unlike in acute/infectious inflammation where cytokine production is dependent on bacterial products as described for LPS, in chronic/sterile inflammation cytokines production occurs in the absence of infectious agents through mechanisms that are still elusive. Based on histology, function, animal models and clinical studies of MS and RA, it was suggested that T-cells are likely to play a pivotal role in the pathogenesis because the infiltration of T-cells within the target tissue precedes tissue damage

[298, 312]. Moreover, although T-cells are able to produce pro-inflammatory cytokines, the amounts generate are lower than expected for a chronic T-cells-mediated disease [312].Thus, it is suggested that the interaction between T-cells and monocyte/macrophage is a potent mechanism that induces massive upregulation of pro-inflammatory cytokines IL-1β and TNF by monocytes/macrophages [299, 313-316]. Both MS and RA illustrate the relevance of T-cells-activated monocyte/macrophage. Indeed, in RA, activated perivascular T-cells can be found in the synovium. Monocyte/macrophages migrate through scattered T-cells where they can be activated through direct cell contact (Figure 15). Thus, this activation induces the production of pro-inflammatory cytokines and MMP [298, 317, 318]. In early MS lesion, the rapid recruitment of monocytes and the activation of resident microglia through contact-dependent T-cell are consistent with the demyelization and axonal damages since it is found that the majority of T-cells that infiltrate MS lesions are activated. Thus, a large numbers of activated T-cells and monocytes/macrophages are present, in a close contact manner, suggesting that the direct T-cell contact with monocyte/macrophage is relevant in the inflammatory and destructive phase of RA and MS [276, 319-321]. Finally, TNF, IL-1β, IL-6 and sIL-1Ra are strongly induced in monocytes by direct contact with activated T-cells, however, TNF and IL-1β production is inhibited by high density lipoprotein (HDL) while there is not effects on sIL-1Ra production conferring an anti-inflammatory function to HDL. This mechanism may be critical in the pathogenesis of RA and MS [307, 322, 323].

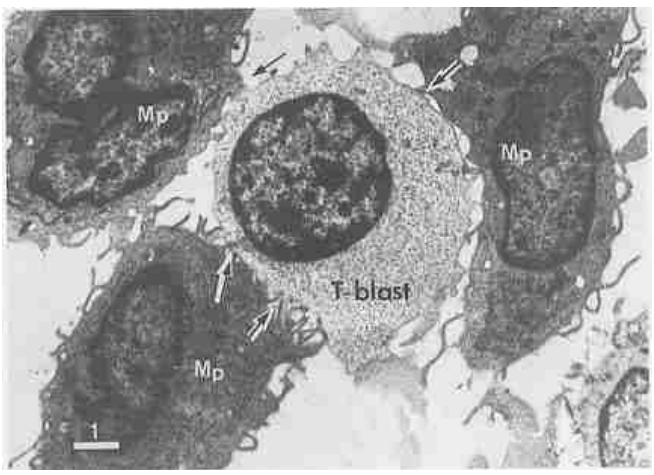

Figure 15 : Electron photomicrograph of cells in RA. T-cells (T-blast) activate monocytes/macrophages (Mp) by direct cellular contact in RA inflammatory site (from [3])

Although RA and MS seem to be initiated by antigen specific T-cells, chronic inflammation appears to be responsible for tissue lesion through antigen unspecific mechanisms mediated by T-cell contact with monocyte/macrophage, which is, in turn, regulated by the "negative" acute-phase protein Apo A-I.

4.4 Signaling pathways involved in T-cell contact-activated-human monocyte

The characterization of intracellular signaling pathway involved in cytokines production and regulation is a great challenge in order to control the inflammation in T-cell contact mediated diseases. Indeed, the intracellular pathways involved in cytokines production in human monocytes appear to be some potential targets to fine-tune the treatment of RA and MS. Although the identity of surface molecules involved in this process remains elusive and discussed in the next chapter, CD40L and CD40 are though to be implicated in TNF and IL-10 induction [324]. However, CD40L involvement remains controversial because it seems to be cells types-dependent [325]. Indeed, IL-10 and TNF are produced in response to CD40L through PI3K- and MAPK-dependent pathway in macrophages [324] whereas CD40L is not able to trigger cytokines production in monocytes. T-cell contact induces several pathways to trigger pro- and anti-inflammatory cytokines in macrophages. It was shown that, upon T-cell contact activation, IL-10, *i.e.* anti-inflammatory cytokines, is PI3K-dependent whereas TNF, *i.e.* pro-inflammatory cytokines in NF-κB-dependent in macrophages [326, 327]. Therefore, it seems that pro-inflammatory cytokines could be regulated by the PI3K pathway while NF-κB is more for pro-inflammatory cytokine [328]. It was also demonstrated that PI3K pathway represents a check-point signaling molecule favoring sIL-1Ra synthesis over that of IL-1β [329, 330]. Furthermore, it appears from recent studies performed in our laboratory that p110δ is involved in TNF, IL-1β and IL-6 regulation. This is a part of the subjects of my thesis, which results

are presented and debated in *Results*. Finally, the ratio between IL-1β and sIL-1Ra production is regulated by Ser/Thr phosphatases in T-cell contact-activated monocytes [316]. In conclusion, monocytes activation by contact with T-cell triggers the IL-1β, TNF, IL-6, sIL-1Ra and IL-10 production through intracellular pathways activation including PIK3 and MAPK pathways. The differential modulations of the pathway modify the pattern of cytokines produce by monocyte/macrophages, offering opportunities to improve disease treatment by modifications of the outcome of inflammatory processes.

4.5 Cell surface factors implicated in T-cell contact mediated monocyte/macrophage activation

One of the crucial questions is the identity of the molecules on the T-cell surface that are involved in contact-mediated signaling of monocyte/macrophage activation as well as their respective ligands. T-cell surface factor expression and activity is dependent of previous activation of T-cells, suggesting the involvement of expression of anchored-ligand that is T-cell activation dependent. T-cells membrane associated TNF was considered until $TNF^{-/-}$ T-cells line was still able to activate monocytes. Thus, TNF membrane associated could participate only a part of the induction of cytokine in monocytes/macrophages [331, 332]. By using cytokines inhibitor as well as monoclonal antibody,

Blocking antibody or inhibitor	Production of IL-1β (%)
Anti-CD11a	64
Anti-CD11b	47
Anti-CD11c	50
Anti-CD18	95
Anti-CD54	100
Anti-CD29	99
Anti-CD14	100
Anti-CD40L	**92**
Anti-CD2	85
Anti-CD69	55
Anti-GMCSF	100
Anti-IFNγ	100
Anti-IL-2	100
Anti-FasL	97
CTLA4-Ig fusion prot.	100
TNFsRI	99
TNFsRII	100
sIL-1Ra	99

Table 3 : Involvement of cell surface molecules in T cell-contact mediated activation of human monocytes

a role of several cell associated cytokines was ruled out (Table 3) ([325, 333, 334]} and Burger.D. unpublished data). In addition to cell associate cytokines, other surface molecules have been considered as to their ability to activate monocyte/macrophage. Thus, several proteins have been identified that have a partial activity in T-cells contact: CD11a, CD11b, CD11c and CD69 (Table 3) [333] and other surface molecules which are not involved: CD18, CD54, CD29, CD14, CD40L, CD2, FasL and CTLA4. In addition, the secondary structure of proteins, *i.e.* N-linked oligosaccharide, does not have any implication in T-cell contact (Burger.D unpublished data). These results suggest that the activating factor on T-cells responsible for the activation of monocytes/macrophages remains to be determined. However, transduction pathways involved in cytokines production are currently investigated and characterized providing new clues to elucidate the T-cell contact activation.

4.6 Microparticles

Upon chronic inflammation, after extravasation into the target tissue, most T-cells remain in the perivascular region and other infiltrating cells such as monocytes/macrophages have to cross the perivascular layer of T-cells and in turn to make contact with the latter cells before penetrating further into the target tissue. Consequently, direct cell-cell contact with T-cells is less frequent outside perivascular regions. However, cells can disseminate cell surface molecules by generating microparticles (MP) and thus ensure "distant" cellular contact. MP are fragments (0.1-0.8 μm diameter) shed from the plasma membrane of stimulated or apoptotic cells. Having long been considered inert debris reflecting cellular activation or damage, MP are now acknowledged as cellular effectors involved in cell-cell crosstalk [335]. Indeed, MP display membrane proteins as well as bioactive lipids implicated in a variety of fundamental processes and thus constitute a disseminated pool of bioactive effectors [336]. MP are present in the circulation of healthy subjects, and their numbers increase upon various pathological conditions [337]. Elevated MP have also been reported in chronic inflammatory

diseases [338-340] including RA [341-344] and MS [340, 345-348]. Although present in patients' plasma, MS cerebrospinal fluid has, to our knowledge, not been investigated for the presence of MP. In RA synovial fluid, MP are abundant and modulate fibroblast-like synoviocyte activity in vitro [343, 344, 349, 350]. We recently demonstrated that MP generated by stimulated T-cells (MPT) can activate monocytes to produce cytokines similarly to membranes or solubilized membranes of stimulated T-cells [351]. Furthermore, T-cell contact-induced production of IL-1β and TNF in monocytes is specifically inhibited by high-density lipoproteins (HDL)-associated apolipoprotein A-I (apo A-I) [322] as we have seen above. In contrast to the production of pro-inflammatory cytokines, HDL do not inhibit that of sIL-1Ra [351] suggesting the presence of different factors of the surface of T-cells which differentially induce IL-1β and sIL-1Ra.

4.7 T lymphocytes

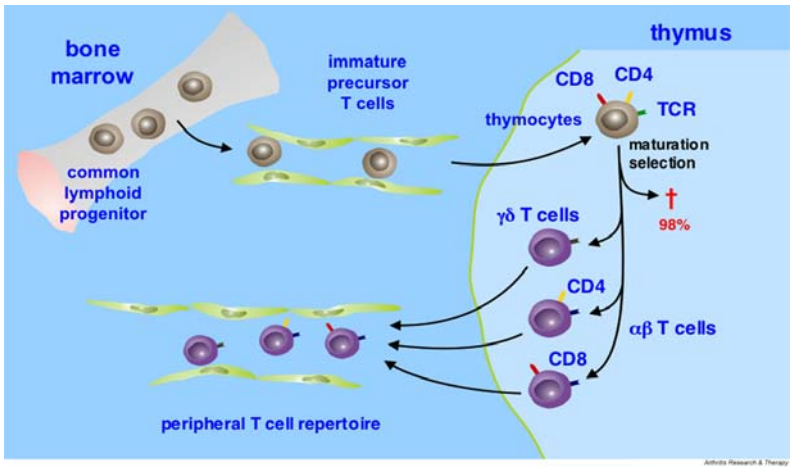

Figure 16: Schematic representation of T cell development. T cells originate from the common lymphoid progenitor cells in the bone marrow. They migrate as immature precursor T cells via the bloodstream into the thymus, which they populate as thymocytes. The thymocytes go through a series of maturation steps including distinct changes in the expression of cell surface receptors, such as the CD3 signaling complex (not shown) and the coreceptors CD4 and CD8, and the rearrangement of their antigen receptor (T cell receptor, TCR) genes. More than 98% of the thymocytes die during maturation by apoptosis (†), as they undergo positive selection for their TCR's compatibility with self-major histocompatibility molecules, and negative selection against those T cells that express TCRs reactive to autoantigenic peptides. In humans, the vast majority of peripheral blood T cells expresses TCRs consisting of α and β chains (αβ T cells). A small group of peripheral T cells bears an alternative TCR composed of γ and δ chains (γ/δ T cells). αβ and γδ T cells diverge early in T cell development. Whereas αβ T cells are responsible for the classical helper or cytotoxic T cell responses, the function of the γδ T cells within the immune system is largely unknown. αβ T cells that survive thymic selection lose expression of either CD4 or CD8, increase the level of expression of the TCR, and leave the thymus to form the peripheral T cell repertoire. (from [20])

Immunocompetent T lymphocytes or T cells are produced from lymphoid progenitors (see Figure 16) and play a central role in cell-mediated immunity. While several selection processes during T cell development ensure the removal of T cells capable of generating autoimmune diseases in the host, self-reactive T-cells are however constantly observed in peripheral blood of healthy individuals.

Several different subsets of T cells have been discovered, each with a distinct function. The majority of mature T lymphocyte possesses an αβ T cell receptor (TCR) which mediates the helper T cell ($CD4^+$) and cytotoxic T cell ($CD8^+$) responses through the recognition of peptide-MHCII and –MHCI complex, respectively. Besides αβ T cells, γδ T lymphocytes represent a small subset of T cells (2% of total T cells) that possess a distinct TCR on their surface, and which functions in immunity are not fully understood.

$CD8^+$ T cells, also known as cytotoxic T cells, provide protection against viral infections and tumors by inflicting cytotoxic damage to target cells that express MHCI with the relevant antigen. $CD8^+$ T cells have a strong potential to cause tissue damage as most cells of the body express MHCI molecules and that upon activation, $CD8^+$ T cells produce large among of TNF and IFNγ [20].

$CD4^+$ T cells, or T helper cell (Th cells), assist various leukocytes in immunologic processes, including, among other functions, differentiation of B cells into plasma cells and activation of macrophages and cytotoxic T cells. During TCR activation in a particular cytokine environment, naive $CD4^+$ T cells may differentiate into one of several T effector cell subsets: Th1, Th2, Th17 and natural regulatory T cells (called $CD4^+CD25^+FoxP3^+$ T_{reg}), as defined by their pattern of cytokine production and function. [24, 352]

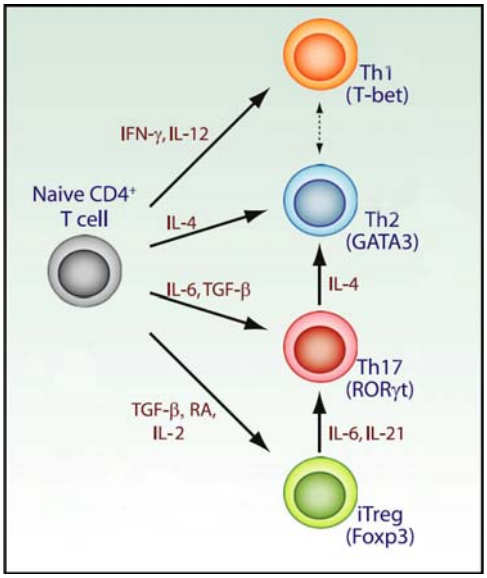

Figure 17: Upon encountering foreign antigens presented by antigen-presenting cells, naive CD4+ T cells can differentiate into Th1, Th2, Th17 and Treg. These differentiation programs are controlled by cytokines produced by innate immune cells, such as IL-12 and IFN-γ, which are important for Th1 cell differentiation, and IL-4, which is crucial for Th2 cell differentiation. TGF-β together with IL-6 induces Th17 cell differentiation, whereas Treg differentiation is induced by TGF-β, retinoic acid (RA), and IL-2. Although Th1 and Th2 cells display more stable phenotypes, Treg cells and Th17 cells can readily switch to other T helper cell programs under certain cytokine conditions. Th17 cells may also convert into IFN-γ-producing Th1 cells or IL-4-producing Th2 cells when stimulated by IL-12 or IL-4, respectively. (Adapted from [24]

Both $CD4^+$ and $CD8^+$ differentiation are controlled by cytokines present in the microenvironment secreted by professional

antigen-presenting cells (APC), such dendritic cells (DCs) and macrophages, after exposure to pathogen products (see Figure 18). The differentiation of naïve human $CD4^+$ T cells to Th1 is controlled by IFNγ and IL-12, Th2 by IL-4; Th17 by IL-6, TGF-β1, and IL-1, natural T_{reg} by TGF-β1, Retinoic acid and IL-2. (Figure 17) [353] Th1 cells are characterized by their production of IFNγ, IL-2, IL-3 and IL-27. Th2 cells, required for promoting a humoral immune response to extracellular pathogens, express IL-4, IL-5, IL-6, IL-10, IL-13 and IL-25. Th17 cells secrete IL-9, IL-17, IL-21 and IL-22 with strong pro-inflammatory effects [24, 352-354].

4.8 T lymphocytes in inflammation

Imbalance of T-helper cells differentiation and subsequent cytokine dysregulation is involved in inflammatory and autoimmune diseases. Th1-dominant immune responses are associated with inflammation and tissue injury as several cytokines produced by Th1 cells recruit and activate inflammatory leukocytes at the site of inflammation, such as Th17 cells in the CNS during EAE [355]. Moreover, Th1 cytokines promote the differentiation of $CD8^+$ T cells. Dysregulation of Th1 responses is believed to drive cell-mediated organ-specific autoimmune disorders such as RA and MS. Conversely, Th2 cells have been considered to display anti-inflammatory activities in these pathologies. Indeed, the foremost Th1 cytokines IFNγ and TNF, potent macrophages activators, are counteracted by the Th2 cytokines IL-4 and IL-13, know to suppress macrophages responses. Thus, it is generally accepted that Th2 activation results in the inhibition of acute and chronic inflammation [352, 356]. Th2 activity is however also involved

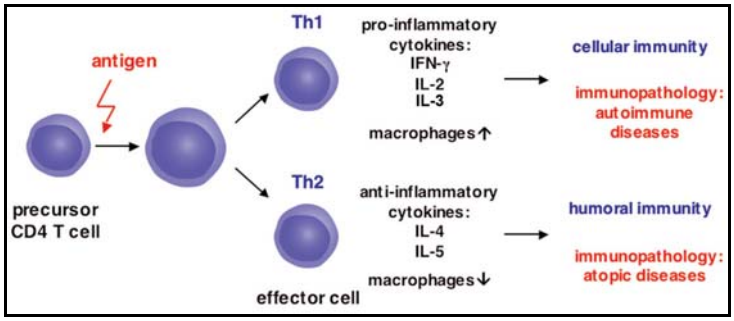

Figure 18 : Upon activation with antigen, CD4 T cells proliferate and differentiate into either the Th1 or the Th2 subset. Th1 cells promote cellular immunity and are involved in the development of autoimmune diseases; Th2 cells mediate humoral immunity and are involved in allergic immune responses. (Adapted from [20]

in allergic reaction, atopic diseases and asthma (Figure 18). While the Th1/Th2 balance has been associated to chronic inflammatory, autoimmune and allergic disorders, it is now clear that Th17 cells may be also critical for many of the inflammatory autoimmune responses once attributed to Th1 cells [353, 357-359]. One can summarize that dysregulated Th1 response drives cell-mediated autoimmune disorders, and enhanced Th2 activity is involved in atopy, whereas Th17 cells are probably responsible for chronic tissue inflammation. There are also other type of T cells that can influence the expression and activation of Th17 cells: the regulatory T cells (Tregs). Recent studies have demonstrated that Tregs, either natural or induced, suppress a variety of physiological and pathological immune responses mediated by Th1 and Th17 cells. In animal models, the opposite relation between Th17 and Tregs cell differentiation is related to autoimmunity. Several mechanisms highlight the reciprocal control of Tregs and Th17 cells, including the role of pro-inflammatory cytokines IL-1β. Th17/Treg balance modulation in favour of Tregs leads to restore immune tolerance [360].

4.9 Relevance of T cells in multiple sclerosis and rheumatoid arthritis

A central role is attributed to T cells in the pathogenesis of MS. Both $CD4^+$ and $CD8^+$ T cells have been observed in MS lesions, with $CD4^+$ T cells predominating in acute lesions and $CD8^+$ T cells being more frequent in chronic lesions [361]. MS has long been considered a prototypic $CD4^+$ Th1-mediated autoimmune disease [362] according to the cellular composition of the brain infiltrates of both MS patients and EAE mouse models. Furthermore, the observation that MHC II alleles are strongly associated with the susceptibility to the development of the disease is probably related to their function of antigen-presenting molecules to $CD4^+$ T-cells [363]. As mentioned earlier, it is however now recognized, that, in addition to Th1 cells Th17 cells may be as important in inducing central nervous system CNS

autoimmunity. The role of Th1 cells in CNS demyelinating disease has been challenged by the demonstration that IFNγ or IFNγ Receptor-deficient mice, as well as mice lacking other molecules involved in Th1 differentiation, were not protected but tended to have enhanced susceptibility to EAE. Moreover, the administration of anti-IFN-γ antibodies was found to aggravate disease in MS patients. In contrast, the development of EAE was suppressed in IL-17$^{-/-}$ mice [364, 365], suggesting that Th17 cells could be critically responsible for the induction and for the maintaining of the chronic inflammatory course [366]. In addition, the role of CD8^{+} T cells in MS pathogenesis is controversial. Seminal studies in the rodent MS model, EAE, and latter in MS patients, have suggested that CD8^{+} T cells contribute to the disease initiation and progression, yet recent mouse and human data indicate that they may also play a protective or suppressive role during disease resolution [367].

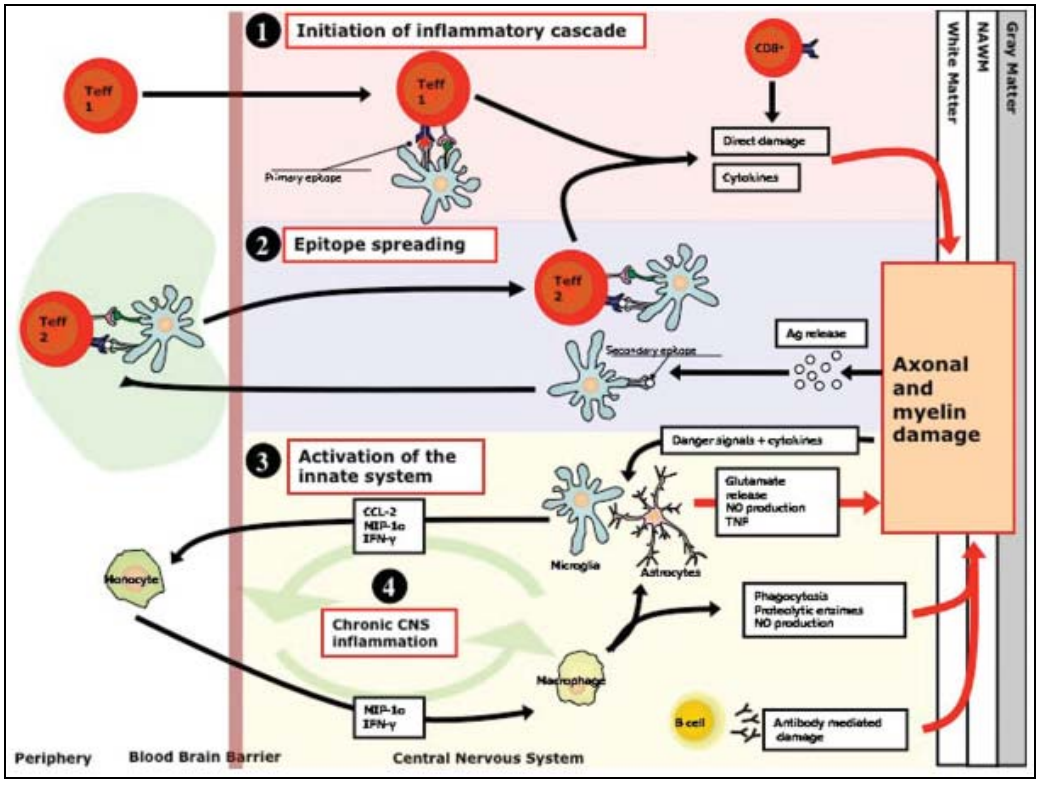

Figure 19 : Innate immunity in the progression of multiple sclerosis (MS). Inflammatory T cells that enter the CNS initiate a complex immunological cascade consisting of epitope spreading, which triggers new attacks, and activation of the innate immune system (microglia, dendritic cells, astrocytes, B cells), which leads to chronic CNS inflammation. (from {Weiner, 2009 4005 /id)

Despite the fundamental role of adaptive immune system (and in particular T cells) in the pathogenesis of MS, it becomes recognized that the contribution of the innate immune system is critical. While the adaptive immune system drives acute inflammatory events by producing TNF, IFNγ and IL-17, among others, the innate immunity drives progressive aspects of MS by producing a large amount of pro- and anti-inflammatory cytokines (Figure 19) [173]. The innate immune system has a dual immuno-pathogenic role through the release of inflammatory mediators necessary for the differentiation and maintenance of Th1 and Th17 effector cells, which ultimately favor and amplify the inflammatory response. Of importance, TNF and IFNγ production by innate cells damage the BBB integrity increasing the CNS leukocyte infiltration and activation [368, 369]. Consequently, the later progressive phase of MS is characterized by chronic inflammatory states associated with activation of monocytes and DCs leading, in turn, to activation of microglia and astrocytes into the CNS [370]. Activation of CNS resident and infiltrating cells leads to several pro-inflammatory processes such as phagocytosis and glutamate homeostasis perturbations (see Figure 19). Currently, there are no specific therapies to target innate immune cells in MS [368] although it supposed that a part of beneficial effects of IFNβ and GA in MS treatment comes from its potential: a) to induce anti-inflammatory cytokines such as sIL-1Ra, b) to decrease pro-inflammatory mediators such as IL-1β and TNF [179, 277, 283, 371]

Similarly to MS, RA has long been considered as a pathogenic inflammatory $CD4^+$ Th1-mediated disease, as the majority of synovial biopsies showed a high level of IFNγ and almost no IL-4. Consequently, Th1/Th2 ratio in the synovial fluid was consistent with diseases activity [305, 372]. However, several studies from animal models of RA, have recently shown that IL-17, mostly produced by mast cells and T lymphocytes, is over-expressed in joints, inducing joint inflammation, bone erosion and cartilage proteoglycan loss [373]. Data further indicate that IL-$17^{-/-}$ mice are resistant to CIA induction [374]. Although the role of TNF in RA

was clearly demonstrated, under abundance of IL-17, the pathogenic process loses its IL-1 and TNF dependency [375, 376]. Together, it appears that RA is a heterogeneous disease with a critical role of $CD4^+$ T cells and innate inflammatory mediators.

5 SIGNAL TRANSDUCTION

A basic property of living systems is the ability to dynamically respond to the environmental changes. The communication with the environment is achieved through a number of pathways that receive and process signals, not only from the external environment but also from different regions within the cell. These signaling pathways control most of vital processes in all cells [377-379]. The signal transduction pathways are often spatially restricted but not necessarily linear. Indeed, the signaling pathways are highly complex and interconnected, in order to respond to and to integrate multiple signals. This complexity arises from pathway switching, meaning that signals can be switched into alternative pathways, while achieving identical output [379-381].

A Key characteristic of signal transduction is the need to activate components for a brief period of time followed by deactivation. Activation is performed by: posttranslational modifications (phosphorylation, acetylation, methylation) and by binding of chemical messengers or ions. Deactivation involves the degradation or sequestration of chemical messengers and the reversion of posttranslational modifications [378]. Without signal ending, cells lose their responsiveness to new stimuli. Moreover, a constant activation leads to uncontrolled cell growth and can be responsible for cancer.

Despite the large number of cell signaling components and pathways, my studies were focused on two major pathways and their interconnections: the mitogen-activated proteins kinases (MAPK) and the phosphatidylinositol 3-kinases (PI3K); both pathways being involved in many immune processes including inflammation, virus infection and cancer. In this part, I will describe more in depth the current knowledge on these 2 pathways.

5.1 Mitogen-Activated Protein Kinanes (MAPK)

The MAPK are one of most highly conserved, primordial and intensely studied signaling pathway families. MAPK are involved in a wide variety of processes such as proliferation, survival, stress, apoptosis, differentiation and immune responses [382]. They regulate cellular activities through target protein substrates including other protein kinases, phospholipases, transcription factors, and cytoskeletal proteins. MAPK are part of a phosphorelay system composed of three sequentially activated kinases. The first are the activators, which have distinct motifs in their sequences that selectively confer their activation in response to different stimuli. The second are MAP kinase kinase kinase (MKKK), which phosphorylate and activate specific MAPK kinases (MKK). Finally, MKK phosphorylate specifically MAPK with a high degree of selectivity (Figure 20) [377, 383-385].

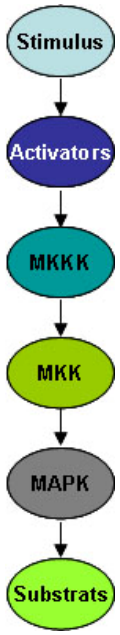

Figure 20 : MAPK phosphorelay systems.

MAPK comprise three subfamilies on the basis of sequence similarity, mechanisms of upstream regulation and sensitivity to activation by different MKK [386]. Thus, the following section will be focus on ERK1/2 family because during my thesis, I investigated further this pathway involvement in cytokines production in monocytes activated by IFNβ as well as by stimuli related to acute and chronic inflammation.

5.1.1 MAPK families

5.1.1.1 Extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway

The ERK1/2 pathway is activated in numerous cell types by diverse extracellular stimuli and is among the most thoroughly studied signaling pathways that connect different membrane receptors to the nucleus (Figure 21) [387]. Signals are commonly initiated through binding of a ligand to its cognate receptor which displays intrinsic or associated tyrosine

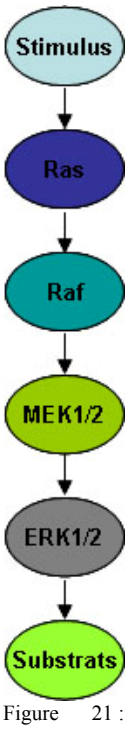

Figure 21 : ERK1/2 pathway.

kinase activity [388, 389]. Phosphorylation of critical tyrosine residues in the cytoplasmic domain of the receptor provides docking sites for adaptor molecules which facilitate recruitment of Ras. Ras belongs to the superfamily of GTPases and act as a molecular switch. Signal-induced conversion of the inactive to active state is mediated by guanine nucleotide exchange factors (GEF) that stimulate the exchange of GDP for GTP [390]. Ras activation allows the interaction with various effectors but particularly with members of the Raf kinase family. The latter family comprises three members designated A-, B- and C-Raf also named Raf1 that share common structure. For instance, their N-terminal contains two conserved region (CR), CR1 and CR2 embedded in the regulatory domain [388]. MEK1/2 are the major substrates of Rafs. The activation of MEK1/2 is facilitated by some scaffold molecules such as kinase suppressor of Ras (KSR) [391, 392].

MEK1/2 have a molecular weight of 47 kDa and display 80% of sequence homology. MEK1 and MEK2 are ubiquitously expressed in human cells and their genes are located on human chromosomes 15q22 and 19q13, respectively [392, 393]. To my knowledge, MEK1/2 are the only described MAPKKs in the ERK cascade. Thus, they are considered as the specificity determinants of the cascade [391, 392]. MEK1/2 are involved in a wide variety of diseases and processes including tissue hypoplasia [393], epidermal neoplasia [394], development [395], cancer [396] and cytokine secretion [330, 397]. MEK1 and MEK2 similarly phosphorylate ERK1/2. Despite their homologies, the functions of MEK1 and MEK2 are not always redundant [394]. Indeed, MEK1 knockout mice display recessive lethality, with homozygous mutant embryos dying by day 10.5 of gestation [398]. In contrast, MEK2 knockout mice are viable, fertile and show no phenotypic abnormalities [399] although its expression take place earlier in the mouse development than MEK1 [395].

Regulation of MEK1 and MEK2 occurs through a proline-rich loop region, inserted between protein kinase subdomains IX and X, that is not present in any of the other known MEK family members. This proline-rich loop is a loop-regulatory subdomain phosphorylated by Raf family on Ser218 and 222, and on Ser222 and 226, in MEK1 and MEK2, respectively [391, 395, 400, 401]. Furthermore, this insert region contains potential sites for interaction with SH3 domains suggesting that it might be phosphorylated by several protein kinases because proline-rich domain is not present in other MEK family member. In addition, MEK1 proline-rich region contains a site of feedback phosphorylation for ERK (Thr292) that is absent in MEK2 [384, 402].

The spatial organization of MAPK determines which signals could be transmitted and received at various possible sites of action. The complement of cellular signaling proteins and cell conditions together determine the spatial distribution of MAP which is a highly regulated process [384]. Thus, in resting cells, MEK1 and MEK2 are localized in the cytoplasm and possess cytoplasmic anchors such as KSR, MP1 and Sef1. Furthermore, MEK1/2 are rapidly translocated into cytoplasm after activation because they contain nuclear export signal (NES) [391].

The third part of the cascade is composed of the ERK1 and ERK2 proteins that are well conserved throughout the evolution. ERK1 and ERK2, also referred to as 44 and 42 kDa kinase, respectively, are activated by dual phosphorylation on their Tyr and Thr residues located within a loop containing a Thr-Glu-Tyr motif [387, 403] increasing of 1000 fold the specific activities. This activation is fulfilled by MEK1 and MEK2. Moreover, phosphorylation of the activation loop induces major conformation changes of ERK1/2 which are crucial for the translocation of the two proteins into the nucleus. Inactive ERK and MEK tend to interact with each other either directly or with the help of scaffold proteins [384, 404]. However, ERK1/2 remain into the nucleus for periods of time that may vary from few

minutes to several hours although the normal localization is cytoplasmic in resting cells [405]. Unlike MEK1/2, ERK1/2 have less redundancies functions. Indeed, evidences from ERK1$^{-/-}$ mice have provided support for a differential role for ERK1 and ERK2 in cell signaling. Indeed, ERK2 seems to have a positive role in controlling normal and Ras-dependent signaling whereas ERK1 seems affects the overall signaling output of the cell by antagonizing ERK2 activity [406].

ERK1/2 recognize a consensus phosphorylation sequence Pro-Xaa-Ser/ Thr-Pro in their substrates. Substrate phosphorylation occurs when ERK1/2 are phosphorylated on their regulatory Thr and Tyr residues in the activation loop [405]. The molecular mechanisms that allow the full activation of ERK1/2 involve the dual phosphorylation on Thr and Tyr but also two docking sites called CD-domain and DEF that interact with the N-terminus D-domain of MEK1/2 [391, 403]. The activation of ERK1/2 is down regulated by phosphatases. Thus, phospho-threonine, -tyrosine and -serine phosphatases participate in MAPK cascade functions both positively and negatively. Moreover, other post-translational modifications such as acetylation and interaction with scaffold proteins may modulate the MAPK activity [397, 404, 407].

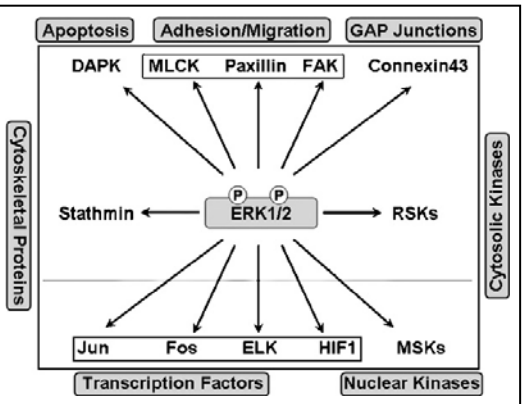

Figure 22 : The diversity of ERK1/2 substrates. ERK has more than 100 known substrates. The diversity of these substrates have divergent functions. [9]

ERK1/2 are able to phosphorylate a large number of substrates. Indeed, there are more than 100 possible targets for ERK1/2 with many divergent functions (Figure 22). For instance, ERK1/2 phosphorylate and activate a series of transcription factors such as c-Jun, c-Fos, Smad2/3/4, Fra1, but also other MAPK family members [403, 405]. The large number of substrates raise the question of ERK1/2 specificity, i.e., how can they regulate different signaling pathways by choosing/phosphorylating the right substrate? Several mechanisms

have been proposed for this specificity determination: (i) presence of similar isoforms; (ii) duration and strength of the signals; (iii) interaction with diverse scaffold proteins (Figure 23) [404, 407]; (iv) subcellular localization [9, 408]; and (v) wide-ranging of cross-talk and interplay between ERK1/2 and other intracellular signaling pathways [382, 403]. The ERK cascade is a central pathway that transmits signals from many extracellular ligands to regulate cellular processes. Thus, it is not surprising that with about 180 substrates described ERK1/2 pathway is highly regulated in time and space.

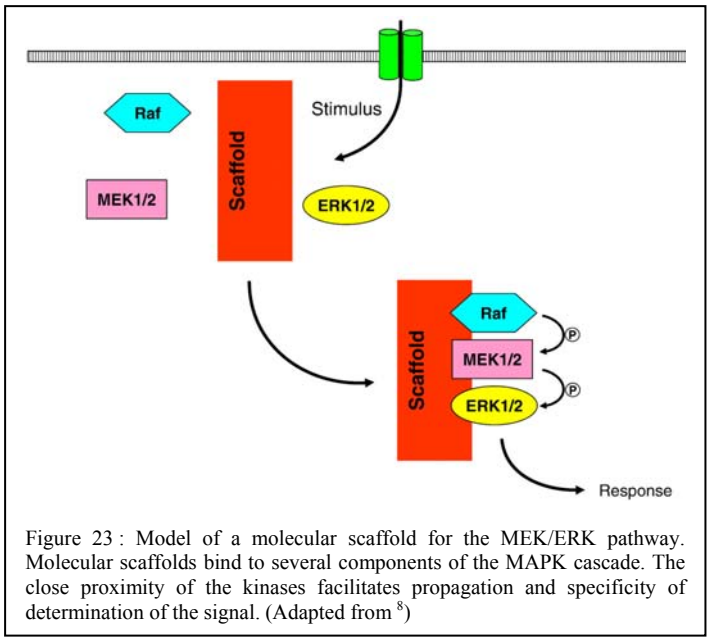

Figure 23 : Model of a molecular scaffold for the MEK/ERK pathway. Molecular scaffolds bind to several components of the MAPK cascade. The close proximity of the kinases facilitates propagation and specificity of determination of the signal. (Adapted from [8])

5.1.1.2 P38 and JNK pathways

p38 MAPK was discovered in 1994 and was originally called cytokine-suppressive anti-inflammatory drug binding protein (CSBP) or indentified as the molecular target of the pyridinyl imidazole.. The structure of the unphosphorylated form of p38, with a molecular weight of 38 kDa, is characterized by 2 domains: a N-terminal domain contains ATP-binding pocket and C-terminal domain containing the catalytic residues requires for phosphorylation [409-411]. There are four different isoforms with 60-70% homology: p38α, p38β, p38γ and p38δ. p38 MAPK pathway is activated in response to a large variety of stimuli such as interleukins, TNF, TLR, growth factors, heat shock, radiation or UV [409] and mediates induction of important cellular responses including transcriptional regulation, apoptosis, cell cycle progression or cytokine production [412].

Some evidences support the importance of the p38 MAPK in inflammatory diseases and cancer. Indeed, p38 has a key role in the production of many cytokines including IL-6, IL-2, IL-7, IL-8 and TNF [409, 413]. In addition, p38 pathway plays also a role in the regulation of cell proliferation and differentiation in the immune system. The involvement of p38 pathway inflammatory disease such as RA is characterized by the regulation of MMP, MMP1, MMP2, MMP3 and MMP13 [414, 415]. Furthermore, p38 MAPK pathway is involved in brain inflammation, inflammatory bowel disease, asthma and other pulmonary diseases [411, 416, 417]. Although large amount of data has been accumulated on p38 pathway and inflammation, it is clear that p38 MAPK pathway is also associated with cancer in view of new evidences. Besides, p38 have function of tumor suppressor. [413, 418-420].

JNK, initially described as stress-activated protein kinases (SAPK), phosphorylate c-Jun at the N-terminal Ser63 and 73 residues in response to environmental stresses, cytokines and TLRs activation [421-423]. However, JNKs can activate a variety of other substrates as it will be described further. There are JNK1, JNK2 and JNK3, with 80% homologies, which are expressed as a short form of 46 kDa and long form of 54 kDa. The multiple isoforms are produced from an alternative splicing of JNK1/2/3 genes [422]. JNK1/2/3 alternatives forms appear to differ in their ability to activate different substrate proteins [424]. Both JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 expression is restricted to CNS, testis and cardiac muscle [423, 425].

The JNK pathway is implicated several of physiological processes. Thus, under physio-pathological conditions, the abnormally activated JNK pathway leads to several neurological disorders such as Parkinson's or Alzheimer's diseases as well as cancer, diabetes or inflammatory diseases [383, 422, 424, 426-428].

5.1.2 MAPK pathways in inflammation

As stated above, TLR play a major role in inflammatory response to pathogens. The cytoplasmic tails of TLRs, except TLR3, allow recruiting an adaptor protein called MyD88. MyD88 assembles a signalosome leading to MAPK activation, which triggers the production of pro- and anti-inflammatory factors including cytokines and chemokines [429, 430]. Thus, MAPK pathways take up a central function in cellular responses and are necessary to immune system homeostasis [431]. The signal transduction mechanisms involved in inflammation provides abundant opportunities for the discovery of novel interfering compounds which may be useful in therapy of inflammatory disorders. Indeed, as mentioned above, MAPK are involved in a wide variety of inflammatory disorders. Therefore, MAPK signaling pathways are obviously attractive therapeutic targets because they are able to modulate both the transcription and secretion of pro-inflammatory cytokines and the signaling induced by the latter [431-433]. For instance, TNF, IL-1β and IL-6 expression are decreased after treatment with a MEK1/2 inhibitor diminishing the inflammatory state cerebral ischemia patient [434]. Recent studies have reveled several possible biological therapies, targeting MAPK pathways in colorectal cancer or in viral infection [435, 436].

The drugs targeting MAPK signaling arise to be a good alternative to other treatment, e.g. anti-TNF or IFNβ in ischemia or collagen induced-arthritis, because they are small molecules and can be administered orally. Moreover, a growing number of studies reveal their potential therapeutic benefits to controlling MAPK signaling in models of acute or chronic inflammation, highlighting the fabulous potential of intracellular drugs in general [315, 417, 437].

5.2 Phosphoinositide-3 Kinases (PI3K)

The term PI3K is applied to a lipid kinase family that phosphorylates the inositol ring OH groups in inositol phospholipids (PtdIns) [438, 439]. Inositol phospholipids comprise a diacylglycerol along with a phosphatidic acid to which an inositol ring is attached via its 1'-OH group. All free-OH groups of the inositol ring can be phosphorylated except those in 2' and 6' positions due to the sterical hindrance by the phosphate group of the phosphodiester link (Figure 24) [21]. These phosphorylations generate lipid second messengers that provide membrane docking sites for a diversity of effector proteins including protein kinases, regulators of small GTPases, and scaffold proteins [18, 440].

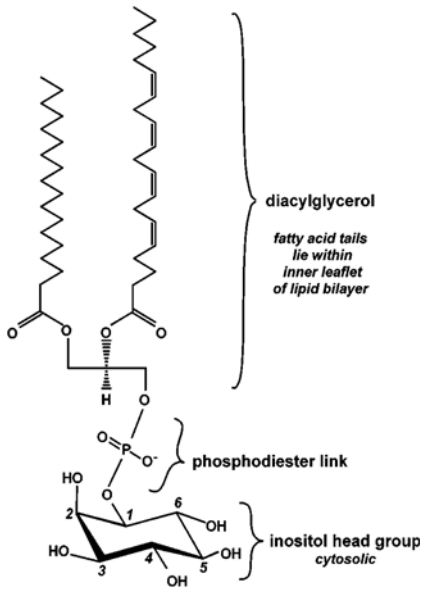

Figure 24 : Chemical structure of PtdIns. [14]

The activity of PI3K was first identified associated with different oncoproteins and growth factor receptors. Moreover, some evidence mounted up that PI3K can provide a crucial signal for growth factors, cell proliferation, cell survival and migration, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling and chemotaxis. The nature of PI3K multiple functional properties is reflected in their activation by various receptors, the existence of three classes and diverse isoforms of PI3K and multiple effectors that can cooperate with PI3K lipidic products by distinct structural motifs [438, 441].

Based on their selective substrates along with their structure specificity, PI3K are classified into three subfamilies: Class I (A and B); Class II and Class III (Figure 25) [21, 438]

5.2.1 Class I PI3K

Class I PI3K are heterodimers composed of a catalytic subunit of 110 kDa and a 50-85 kDa adaptor subunit [440]. They are the most extensively investigated subfamily of PI3K. Their major products are phosphatidylinositol 3, 4-bisphosphate and phosphatidylinositol 3, 4, 5-trisphosphate [18, 21, 438, 442]. The class I group of PI3K consists of two subgroups: the class IA and IB. The prototypical class IA is composed of one of the three catalytic isoforms: p110α; p110β and p110δ also called PI3Kα, PI3Kβ, PI3Kδ; and one of the five regulatory isoforms: p85α; p85β, p55γ, p55α, p50α. The isoforms p85α; p85β, and p55γ are encoded by specific genes while the isoforms p55α and p50α are produced by alternative splicing of the p85α gene [18, 21, 438]. The p110 catalytic subunits comprise an N-terminal p85-binding domain, a Ras-binding domain, a C2 domain and a kinase domain [443]. The p110α and p110β genes are located on chromosomes 3q26 and 3q23, respectively. They are ubiquitously expressed in mammalian cells and their knockout is lethal. In contrast, p110δ, which gene is located on chromosome 1q36, is mainly expressed in immune cells and its knockout is viable [444-446].The expression of p110δ in leukocytes suggests

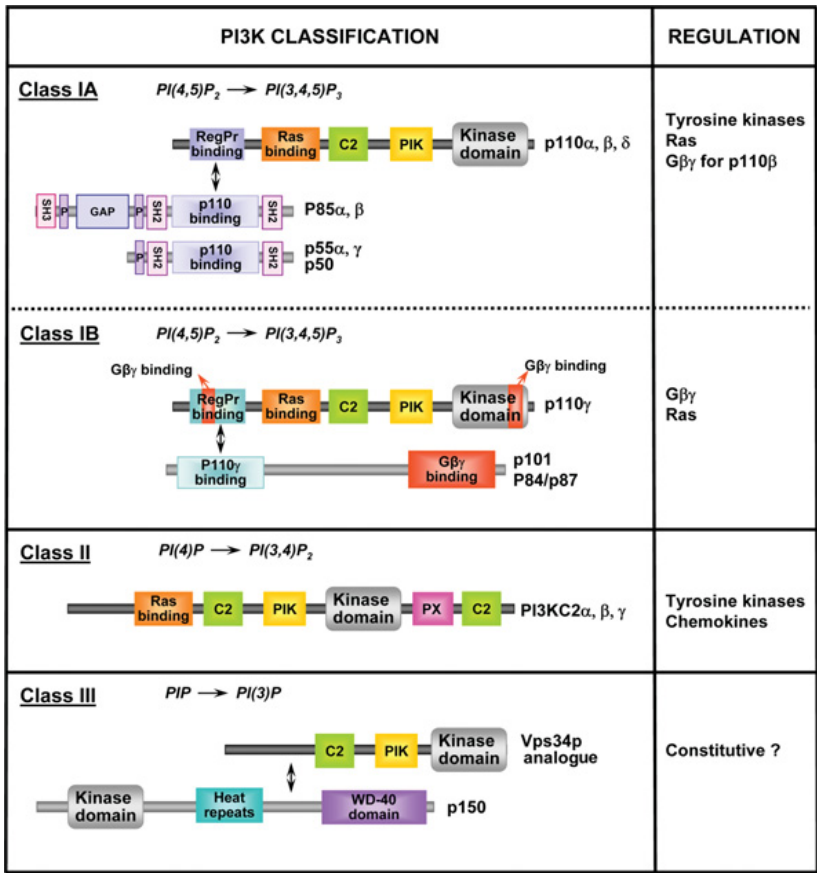

Figure 25 : Classes of PI3K and the defining features of their catalytic subunits. [15]

that it plays a role in the immune system. Indeed, the lymphocyte and mast-cell antigen receptor-dependent PI3K signaling is compromised in p110δ$^{-/-}$ mice [447]. Moreover, in p110δ$^{-/-}$ mice the production of IgM and IgG antibody is dampened suggesting an involvement in B cells themselves or in cells involved in B cells activation [448]. Finally, p110δ is very important for IL-1β, TNF, IL-6 and sIL-1Ra production by human monocyte in response to LPS and CE_{sHUT} [437].

Class IA PI3K activation is controlled by extracellular signals via receptors, or adaptors, with intrinsic protein tyrosine kinase activity. Thus, interactions between p110 and p85 (or p55; p50) subunits stabilize the catalytic subunit and inhibits its activity in resting cells. In activated cells, the recruitment of the heterodimer through Src homology 2 (SH2) domains to phosphotyrosines residues of the activated-receptor or adaptor protein, induces a proximity of p110 to its plasma membrane lipid substrates and relieves the inhibition by the regulatory subunit (Figure 26) allowing the phosphorylation of the 5'OH group of the inositol ring [18]. Furthermore, the two p85-SH2 domains, present on the C- and N-terminal of the regulatory subunit, recognize the pYXXM motif of proteins present to plasma membranes [441, 447]. Although, p110α, p110β and p110δ display high sequence homology, it was shown that the different regulatory isoforms have no preference for catalytic subunits and no redundant physiological functions. Furthermore, the specific mechanism for the attribution of regulatory isoforms to catalytic subunits remains unknown [440].

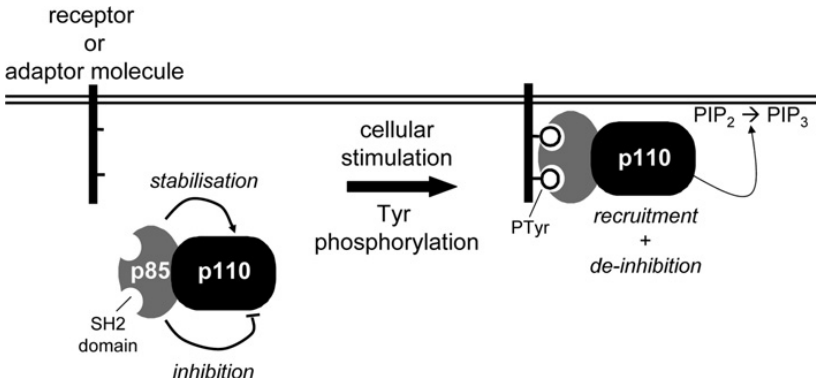

Figure 26 : The role of the p85 regulatory subunit in the regulation of class IA PI3K activity. p85 stabilizes the catalytic p110 subunit and inhibits its lipid kinase activity. Recruitment of heterodimers to activated receptors upon cellular stimulation leads to relieve of inhibition of the p110 catalytic activity. (Adapted from [18])

PI3Kγ is the only member of class IB. it is found only in mammals. It has a similar structure and function to class IA but does not have a p85 binding domain. PI3Kγ is also a heterodimer composed of one catalytic subunit, p110γ, and one regulatory subunit, p101 or p84/87 [449, 450].

The N-terminal domain of p110γ is composed of a pleckstrin homology (PH) domain which generally mediates various protein-lipid interactions but here its function is unclear [451, 452]. p110γ, whose gene is located on chromosome 7q22, is expressed mainly in leukocytes but is also found in the heart, pancreas, liver, and skeletal muscle [438, 451, 453]. In a similar manner to p110δ, p110γ expression is largely restricted to leukocytes, suggesting that this isoform plays key roles in immunity. Indeed, p110γ isoform is involved in many immune processes including inflammation, thymocyte development, T cell activation and neutrophil migration [454, 455].

p110γ activation is provided by G protein-coupled receptors (GPCRs), which represent a large family of seven transmembrane receptors that have a plethora of ligands. GPCRs agonists lead to heterotrimeric Gαβγ protein activation by exchanging the GDP to GTP into the α subunit. This activation leads to the dissociation of α subunit from the βγ dimer. Then, the free βγ dimer binds and activates p110γ through the adaptor proteins p84/p87 or p101 (see Figure 27) [21, 451, 452]. Controversially, it appears that p110β but not the other class IA can be directly activated by

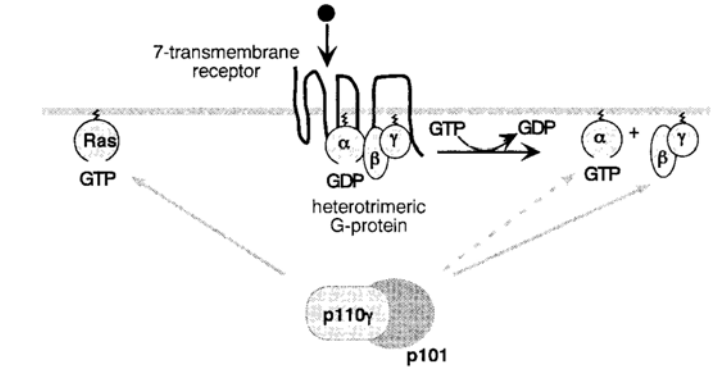

Figure 27 : Heterotrimeric G-proteins are made up of an α, β, and γ subunit, with the α subunit being the GDP/GTP binding module. In the GDP-bound form, the α subunit interacts with the β and γ subunits to form an inactive heterotrimer that binds to serpentine receptors which span the membrane seven times. Ligand binding of these receptors induces a conformational change which results in an exchange of GDP for GTP on the α subunit, inducing its dissociation from both the receptor and βγ subunit. The free α and βγ subunits then interact with and modulate the activity of their target proteins such as p110γ [21].

βγ subunit in vitro. Indeed, it was shown that p110β and p110γ can be redundantly activated upon the same GPCR stimulus, i.e. p110β bind the same βγ subunit as p110γ. Thus, p110β could provide an alternative for GPCR-linked PI3K signaling in cells where p110γ is less or not expressed. Indeed, in fibroblasts, where p110γ is not expressed, p110β activates Akt through the GPCR ligands stromal cell-derived factor [456].

Because of their particular expression and localization in leukocytes, p110δ and p110γ isoforms highlight the special interest of immunologists and pharmacological industry for the potential use as therapeutic targets.

5.2.2 Other classes of PI3K

Class II PI3K differs significantly from the PI3K class I in their mode of regulation and substrates. Indeed, PI3K class II are composed by three isoforms: PI3KC2α; PI3KC2β and PI3KC2γ with a molecular weight about 170 kDa. There is no regulatory protein described for these enzymes. However, although there is no N-terminal homology domain organization, except a C2 domain, some evidences suggest that this region in PI3KC2α binds the clathrin protein, which may function as an adaptor [457, 458]. Furthermore, the diversity of the N-terminal domains of PI3KC2α; PI3KC2β and PI3KC2γ suggest the possibility that they could be recruited by different adaptor proteins [452]. The organization of the rest of class II PI3K is a phosphotyrosines-binding motif, a catalytic domain, an helical domain, a Phox homology domain (PX), and a C-terminal C2 domain which may have a negative regulatory role on the catalytic activity (see Figure 25) [459]. PI3KC2α and PI3KC2β are ubiquitously expressed whereas PI3KC2γ is mainly found in liver. They are produced by from distinct genes [21]. Moreover, unlike PI3K class I which are localized into cytosol, the PI3K class II are mainly present at the plasma membrane. PI3K class II enzymes phosphorylate different substrates than the class I. In fact, PI3K class II use PtdIns; PtdIns(4)P and PtdIns(4;5)P2 with a strong preference for PtdIns > PtdIns(4)P >>> PtdIns(4;5)P2 in response to integrins, MCP-1,

platelets, cytokines and insulin [14, 457, 460]. The precise cellular function of class II PI3K is still unclear due to the lack of specific inhibitors to discriminate the different isoform. However, studies have shown some functions such as clathrin-mediated vesicle trafficking, neurosecretory granular exocytosis, smooth muscle cell contraction or cell migration [458, 460-462].

PI3K Class III is an homologue of the yeast vesicular protein-sorting protein Vsp34p. There is only one member of PI3K class III, Vsp34 (vacuolar protein sorting), associated with a regulatory Ser/Thr protein kinase, p150. The latter seems to be important for targeting Vsp34 to plasma membrane [463]. Vsp34 has a molecular weight of 95 kDa and displays a high homology with the catalytic subunits of other PI3K [464]. Vsp34, which is ubiquitously expressed, has a structurally uncharacterized N-terminal region followed by C2 domain, an helical domain and a kinase domain [465]. Class III can only phosphorylate the PtdIns and is likely to be responsible for the generation of a large fraction of the PtdIns(3)P. Vsp34 has an essential role in various intracellular trafficking pathways and is crucial for vesicular traffic of membrane proteins to the lysosome [466]. Recently, in addition to implication in autophagy, Vsp34 has been shown to activate mTOR. Finally, Vsp34 plays a important function in the ability of cells to respond to changes in nutrient conditions [465, 467, 468].

5.2.3 Downstream effectors of PI3K class I

Consequently to ligand receptor interactions and PI3K class I activation the amount of PtdIns(3,4,5)P3 rises rapidly, inside cell membrane. Several molecular targets have been indentified, which are translocated to plasma membrane and activated by interacting with PtdIns(3,4,5)P3 [441]. The interaction with PtdIns(3,4,5)P3 is performed through a globular protein domain of about 100 amino acids called pleckstrin-homology domains (PH). PH domain is a lipid–binding domain, which is found in a variety of intracellular proteins that act as primary effectors of the PI3K-signaling system. Indeed, PH domain binds PtdIns(3,4,5)P3

and allows the coupling of PI3K signals to downstream effector kinases such as Akt and, in turn, to activation of functional substrates. PH domain-containing proteins comprise a number of effector types including cytoskeletal proteins, signaling adapter molecules, guanine nucleotide exchange factors, tyrosine kinases and Ser-Thr kinases [21, 452]. One of the Ser/Thr kinases, Akt (or PKB), is of particular interest in class I PI3Kignaling. This kinase is expressed as three isoforms of 65 kDa: Akt1, Akt2 and Akt3 encoded by PKBα, PKBβ and PKBγ genes, respectively [469]. Akt isoforms display structural homologies: an N-terminal PH domain, a serine-threonine catalytic domain, and a small C-terminal regulatory domain [453]. Plasma membrane recruitment of Akt induces a conformational change exposing two crucial amino-acid residues for phosphorylation. Indeed, Akt is phosphorylated on Thr308 and Ser473 into the hydrophobic motif of the C-terminal tail (activation segment). Thr308 and Ser473 are phosphorylated by different kinases which were not all identified. However, Thr308 phosphorylation catalyzed by PDK1 is necessary to activate Akt and Ser473 phosphorylation increases 10 fold the activity of Akt. Indeed, Ser473 phosphorylation triggers the engagement of activation segment, promoting a more stable transition state. Then, Thr308 phosphorylation restructures the activation segment inducing a optimal comformation for kinase activity [470] The identity of the Ser473 kinase has been debated for many years and is still elusive. More than 90 kinases, called PDK2, are directly or indirectly involved in the control of Akt-Ser473 phosphorylation including Akt himself and mTORC2 [471, 472].

The number of identified Akt substrates is still increasing. Indeed, the consensus site for Akt phosphorylation consists of RXXRXXS/T motif. The knowledge of the optimal motif phosphorylated by Akt led to accelerated discovery of targets via global search of genome sequences. Thus, a certain number of protein effectors in several physiological functions were discovered such as GSK3 (cell cycle; cytokines production), rac1 (cell morphology), caspase-9 (apoptosis), mTOR, and BAD (apoptosis) (see Figure 28) [473].

5.2.4 Regulation of class I PI3K

Cell activation induces both positive and negative regulatory signaling cascades, which are crucial for cell survival and homeostasis. Thus, inhibitory signaling of PI3K pathway by degradation of PtdIns(3,4,5)P3 can be mediated by at least two phosphatase called phosphatases and tensin homology deleted on chromosome ten protein (PTEN) and SH2-containing inositol 5-phosphatase (SHIP) [474, 475].

PTEN is a dual–specific lipid phosphatase that removes the 3-phosphate on PtdIns(3,4)P2 and PtdIns(3,4,5)P3 to produce PtdIns(4)P and PtdIns(4,5)P2, respectively. Thus, PTEN inverts the catalytic function of class I PI3K [476, 477]. PTEN contains a C-terminal PDZ-binding domain and a stretch of sequences that overlap the catalytic domain which is similar to a domain within the cytoskeletal proteins tensin and auxilin [477]. It was shown that loss of PTEN predicts progression to invasive and metastatic colon cancer or tumor initiation [478, 479]. Besides lethality at E7.5, PTEN deficiency leads to a myeloproliferative disorder that progresses in to leukemia [480-482].

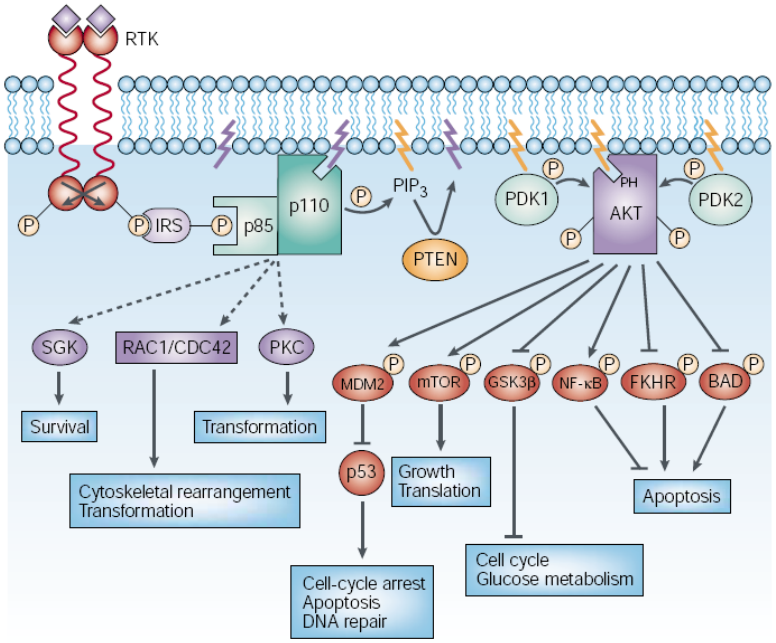

Figure 28 : PIP3 serves as a second messenger that helps to activate AKT. Through phosphorylation, activated AKT mediates the activation and inhibition of several targets, resulting in cellular growth, survival and proliferation through various mechanisms. Additionally, PI3K has been shown to regulate the activity of other cellular targets, such as the serum and glucocorticoid-inducible kinase (SGK), the small GTP-binding proteins RAC1 and CDC42, and protein kinase C (PKC), in an AKT-independent manner through poorly characterized mechanisms. The activity of these targets leads to survival, cytoskeletal rearrangement and transformation. GSK3, glycogen synthase kinase-3; NF-κB, nuclear factor of κB; PDK1/2, 3-phosphoinositide-dependent protein kinase 1/2. (Adapted from [23])

Besides lethality at E7.5, PTEN deficiency leads to a myeloproliferative disorder that progresses in to leukemia [480-482].

SHIP comprises two lipid phosphatase isoforms of 145 kDa displaying redundant activities: SHIP1 and SHIP2. SHIP removes the 5-phosphate on PtdIns(3,4,5)P3 to produce

PtdIns(3,4)P2. It contains an N-terminal SH2 domain, a 5-phosphoinositol phosphatase domain, two phosphotyrosine binding (PTB) consensus sequences and a proline-rich region at C-terminal [483]. The dephosphorylation of PtdIns(3,4,5)P3 is an important negative feedback mechanism in lymphocytes particularly in B cells. Indeed, it seems that the Akt activity is regulated through SHIP and FcγRIIB1 in B cell receptor (BCR) signaling [484]. Moreover, SHIP knockout results in an unbalanced immune response, autoimmunity development, and insulin sensitivity [485, 486].

These two alternative ways to remove phosphates on PtdIns(3,4,5)P3 lead to different types of negative regulation of PI3K and additional signal. Indeed, the absence of signal could be also an information [475].

5.2.5 Class I PI3Ks and Inflammation

Class I PI3K takes a central place in several levels in the inflammatory context. In contrast p110α and p110β that are expressed in all cells, p110γ and p110δ are preferentially expressed in leukocytes and display a wide range of involvements in inflammation. Thus, the recruitment of immune cells into the inflammatory site through chemotaxis, factors such as chemokines. The organized recruitment of inflammatory cells is a process where p110δ and p110γ play crucial roles [487]. Indeed, p110δ and p110γ regulate the capture, rolling, adhesion and transmigration of neutrophils. For instance, p110δ inhibition in endothelial cells, but not in neutrophils, increases the rolling velocity [488] whereas $p110\gamma^{-/-}$ mice show perturbations in selectin-mediated adhesion resulting in reduction of 70% of neutrophil attachment as compared to wild-type mice. Therefore, in addition with the fact that the double $p110\gamma^{-/-}\delta^{-/-}$ mice have a stronger reduction of capture and rolling, p110γ and p110δ isoforms appear to be required for efficient capture and adhesion of neutrophils by activated-endothelium. Furthermore, both isoforms are necessary to neutrophils transmigration into the inflamed tissue (Figure 29) [489]. In addition to its role in chemotaxis, p110δ is involved in cytokines

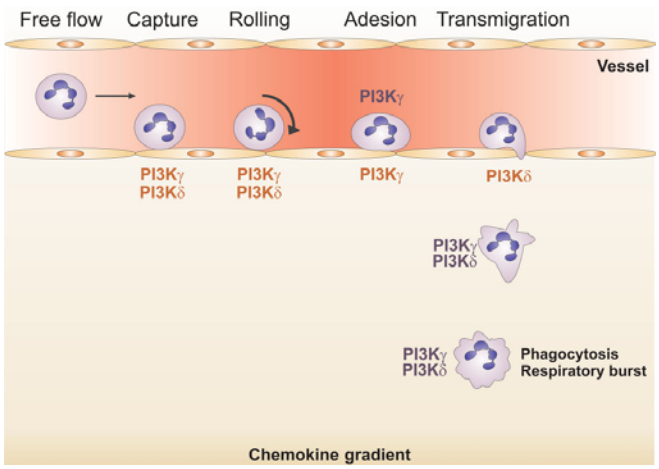

Figure 29 : PI3K signaling is involved in the multiple steps of leukocyte migration. Chemoattractant-induced exit from blood flow toward the inflammation site is a process that requires complex interactions between leukocyte and vascular endothelium. PI3K-mediated signaling plays a key role already at early stages of migration. In fact, PI3Kγ and PI3Kδ control two events mediated by selectin, the capture and the rolling, probably regulating the membrane clustering of E-selectin on the endothelial cell. Both leukocyte and endothelial PI3Kγ are also required for the successive step, the integrin-mediated firm attachment to the vessel wall. Moreover, PI3K activity was shown to be fundamental to the extravasation with PI3Kδ expressed in endothelial cells as an important mediator. (Adapted from [25])

production including IL-1β; sIL-1Ra and IL-6 in acute or chronic inflammation as well as in neutrophil migration [45, 490] whereas p110γ is activated in response to chemoattractants [491, 492].

p110δ and p110γ inhibitors have recently been investigated as new potential therapeutic targets for several diseases including systemic lupus erythematosus, RA, allergic disorders or respiratory diseases [25]. In conclusion, both isoforms are implicated in innate and adaptive immunity. However, some evidences is emerging that supports an opposite role of the two enzymes in common processes. Therefore, the benefic treatment for pathology may rise from inhibition of one isoform while other pathological conditions require a combined therapy.

5.3 Type I interferon signaling pathways

As mentioned above, type I interferon regulate a large number of biological processes. The activation of several signaling cascades by IFN receptors appears to be decisive for the generation of type I interferon-mediated biological functions and immune responses.

IFNAR1 and IFNAR2 interact with the canonical pathways associated with type I IFN signaling called Janus activated kinase (JAK)/ signal transducers and activators of transcription (STAT) pathway [493]. IFNAR1 and IFNAR2 are constitutively associated with 2 members of JAK family, namely non-receptor protein tyrosine kinase 2 (TYK2) and JAK1 [242, 257]. The firth step, initiating a signaling, is the activation of receptors by ligand-dependent

rearrangement and dimerization. Then, JAK1 and TYK 2 are activated by autophosphorylation when the receptors are triggered. The activated JAK induce the recruitment and activation of several STAT (Figure 30) [257].

STAT family is composed of seven members, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5b and STAT6 [26, 494]. In resting cells, STAT are inactive and localized in cytoplasm. They are activated, in response to extracellular signaling, by recruitment to the intracellular domain of the receptor, containing phosphotyrosines residues, through the interaction of SH2 domain present on its C-Terminal. The selectivity of STAT binding to different cytokine receptors is mediated by differences in the SH2 domain [494]. After phosphorylation by JAK, STAT form homo- and heterodimers that are translocated in to the nucleus and initiate the transcription by recognition of specific DNA consensus sequences[493]. Most STAT recognize an 8- to 10-bp 5'-TTC(N2-6)GAA-3' sequence [494, 495]. Type I IFN signaling pathway engage mainly STAT1, STAT2 and STAT3 proteins. STAT1:STAT2 dimers will associate with a member of the IFN regulatory factor (IRF), IRF9 [26]. Heterotrimer STAT1:STAT2:IRF9 (ISGF3; interferon-strimulated gene factor 9) binds to cis–acting IFN-stimulated response elements (ISRE) in order to trigger the transcription. Moreover, STAT1:STAT1 and STAT3:STAT3

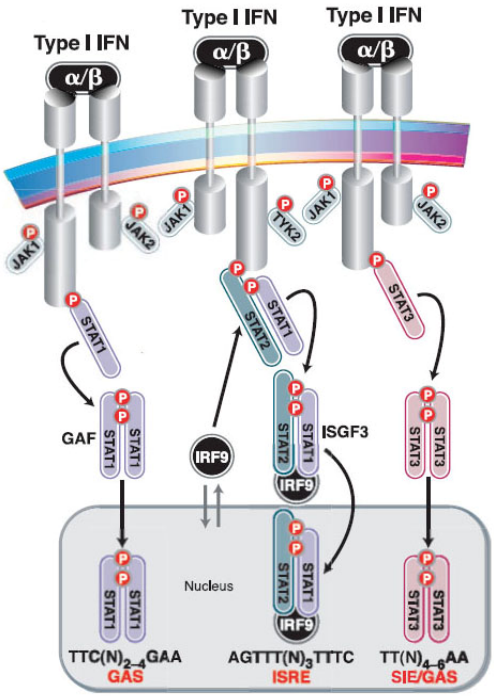

Figure 30 : Specific ligand-receptor interactions generate active transcription complexes composed of distinct STAT proteins. Type I IFNs stimulate the activity of JAK1 and TYK2 proteins, leading to STAT1or STAT2 tyrosine phosphorylation. STAT1 dimers form the Type I IFN-activated factor (GAS), which translocates to the nucleus and activates transcription from type I IFN target gene promoters containing GAS elements. The STAT2 phosphotyrosine is a docking site for latent STAT1. The activated factor ISGF3 is a heterotrimer of STAT1 and STAT2 in association with IRF9, which alone can enter the nucleus, but is retained in the cytoplasm by interactions with STAT2. Moreover, JAK1 and JAK2, could producing a phosphotyrosine docking site for STAT3. STAT3 dimers translocate to the nucleus and activate transcription from target gene promoters containing a GAS like element, sometimes referred to as the sis-inducible element (SIE). (Adapted from [26])

homodimer as well as STAT1:STAT3 heterodimer bind to sequence referred as IFNγ-activated sequence elements (GAS) (Figure 30) [247, 496-498]. Finally, recent studies have shown that the sIL-1Ra expression is STAT2:STAT6 heterodimer dependent in hepatocytes cells lines and STAT1 independent in human monocytes [330, 499].

The evidence that PI3K pathway is involved in type I IFN signaling was revealed by IFN-dependent phosphorylation of insulin receptor substrate-1 (IRS-1), which is required for its association with PI3K regulatory subunit [500]. Subsequently, different mechanisms of type I IFN-induced PI3K pathway activation have been demonstrated in various cell types [501]. Thus, STAT3 has been shown to be an adapter between IFNAR1 and p85 in HT1080 cells [502] whereas other studies showed a constitutive association between IFNAR1 and p85 in human fibrosarcoma cells [503]. The activation of PI3K seems to have important functional consequences in type I IFN transcription regulation. Indeed, PI3K pathway controls the phosphorylation of STAT1 on Ser727. However, the pharmacological inhibitor for PI3K, Ly294002 has no effects on the STAT1 translocation into the nucleus [330]. Moreover, the activation of PI3K, in type I IFN signaling is likely to be independent of TYK2 and STAT [330, 503]. PI3K emerges as major player in the generation of biological responses but the mechanisms by which these kinases regulate IFN responses remains to be defined (Figure 31) [504]. In the same time, other signaling pathways are triggered

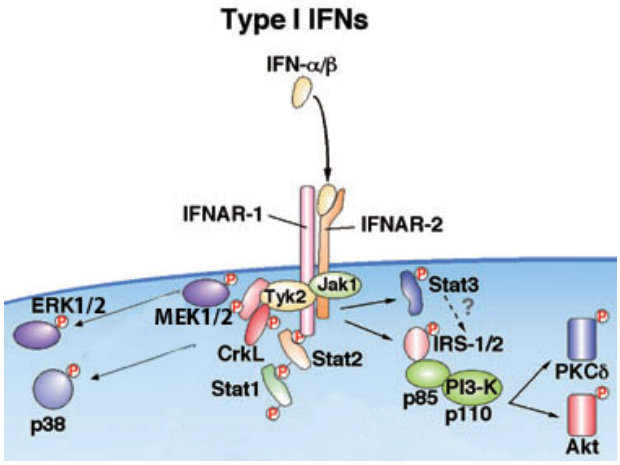

Figure 31 : We focus on type I IFN mediated signalling, by which several additional signalling pathways are activated. CrkL is recruited to Tyk2, which phosphorylates CrkL, leading to heterodimer formation. This dimer translocates into the nucleus to activate GAS-mediated gene induction. Type I IFNs also arereported to activate the PI3-kinase-mediated signalling pathway downstream of Jak1 and Tyk2, through the phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2. This pathway seems to be independent of Stats. The activated PI3-kinase consequently increases the activities of v-*akt* murine thymoma viral oncogene homologue 3 (Akt) and protein kinase C™ (PKC™). On the other hand, type I IFNs have also been shown to activate MAP kinases, namely, ERK1/2 and p38, both of which have the ability to phosphorylate Ser[727] of Stat1 *in vitro*, similar to the observation of type I IFN-activated PKC™. Regarding p38, it does not appear to be a serine kinase for Stat1 in the type I IFN signalling pathway. It remains unclear which kinase is actually responsible for the serine phosphorylation. (Adapted from [27])

in response to the ligand binding such as MAPK pathways [330, 504-506]. Indeed, it was shown that IFNβ induces the activation of ERK1/2 and p38 pathways [505, 507, 508]. Moreover, IFNβ activates ERK1/2 in a time- and cell-specific manner. The role of ERK1/2 activation by IFNβ remains unclear [509], however, it was reported that ERK pathway is implicated in STAT1 activation [510]. Furthermore, MEK1 is involved in the phosphorylation of Ser727-STAT1 as well as the Tyr701-STAT1 in IFNβ-activated monocytes [330]. In contrast, MEK1/2 inhibition has no effect on STAT1 phosphorylation in IFNα-activated cells suggesting that these pathways are triggered independently (Figure 31) [509].

There is accumulating evidence that several pathways are triggered by IFNβ and that the cooperative functions of several signaling cascades are required for the generation of responses to type I IFN. The different effectors describe above are not exhaustive. Indeed, the list of indentified IFN-regulated signaling effectors is growing [247]. Therefore, it appears that more than one signaling pathway are required for the generation of biological processes activated by type I IFN. In conclusion, several pathways are activated simultaneously or independently but they need to be interconnected and coordinated. This adds to the complexity of the IFN signaling system.

5.4 TLR4 signaling pathways

Together with CD14 and MD2, TLR4 recognizes LPS and triggers signals. LPS responsiveness is partly regulated by the amount of TLR4 present of the cell surface that is determined by the trafficking from Golgi to the plasma membrane, and the amount of TLR4 internalized within the endosome. Both signaling pathway, i.e. from plasma membrane or endosomes, involve many adaptors [511]. The cytoplasmic tail of TLR4 contains a Toll-interleukin 1 receptor (TIR) domain that allows the interaction with four downstream TIR domain-containing adaptors. Those adaptors, MyD88, TIRAP (also named Mal), TRAM, and TRIF are required to transduce the signal from TLR4 through two pathways. Thus, TLR4 is

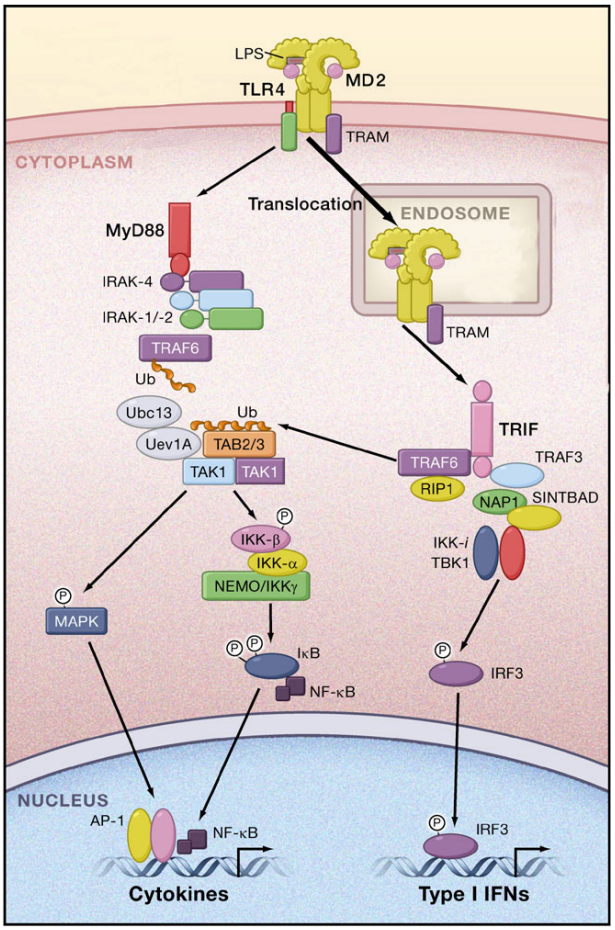

Figure 32 : LPS is recognized on the cell surface by a heterodimer of TLR4/MD2 complexes. Ligand stimulation recruits MyD88 and TIRAP to the TLR, and a complex of IRAKs and TRAF6 is subsequently formed. TRAF6 acts as an E3 ubiquitin ligase and catalyzes formation of a K63-linked polyubiquitin chain on TRAF6 itself and generation of an unconjugated polyubiquitin chain with an E2 ubiquitin ligase complex of Ubc13 and Uev1A. Ubiquitination activates a complex of TAK1 and TAB2/3 resulting in the phosphorylation of NEMO and the activation of an IKK complex. Phosphorylated IκB is degraded, and the freed NF-κB translocates to the nucleus where it drives expression of genes. Simultaneously, TAK1 activates MAP kinase cascades leading to the activation of AP-1. LPS induces translocation of TLR4 to the endosome together with TRAM. TLR4 activates TRIF-dependent signaling, which activates NF-κB and IRF3 resulting in the induction of proinflammatory cytokine genes and type I IFNs. TRAF6 and RIP1 activate NF-κB, whereas TRAF3 is responsible for phosphorylation of IRF3 by TBK1/IKK-i. NAP1 and SINTBAD are required for the activation of TBK1/IKK-i. Phosphorylated IRF3 translocates into the nucleus to induce expression of type I IFN genes. (Adapted from [4])

the unique TLR that is able to engage all four TIR domain-containing adaptors [512]. Indeed, TLR4 triggers a signal through a TIRAP-MyD88-dependent as well as through a TRAM-TRIF-dependent pathway [513].

As shown in Figure 32, at the plasma membrane, TLR4 signaling is TIRAP-MyD88-dependent. Thus, LPS activated-TLR4 recruits the adaptors TIRAP and MyD88 through the TIR domains. Then, the death domain (DD), present on MyD88, allows the interaction with IL-1 receptor-associated kinase (IRAK)-4 which, in turn, is responsible for the recruitment, activation and degradation IRAK-1/-2. IRAK family is a Ser/Thr kinase family containing an N-terminal DD. IRAK then dissociates from MyD88 and interacts with TNF receptor-associated factor (TRAF)-6. TRAF6, another adaptor is critical for the signaling MyD88-dependent upon IRAK-4 and IRAK-1. Indeed, TRAF6 is associated with ubiquitin-conjugating enzyme (UBC) 13, ubiquitin-conjugating enzyme E2 variant 1 isoform A (UEV1A) and transforming growth factor (TGF)-β-activated kinase 1 (TAK1). Together, "TRAF6 complex" catalyzes the formation of K63-

linked polyubiquitin chain on TRAF6 and generates an unconjugated free polyubiquitin chain. TAK1, TAK1-binding protein1 (TAB2) and TAB3 that are activated by the free polyubiquitin chain, phosphorylates IκB kinase (IKK)-β and activates MAPK (p38; ERK1/2; JNK). IKK-α, IKK-β and NF-κB essential modulator (NEMO) activate NF-κB, by inducing IκB degradation, allowing genes expression. AP-1, another transcription factor complex that is activated by MAPK, translocates into the nucleus to trigger cytokine genes expression (Figure 32 left pathway) [4, 511, 512, 514, 514, 515].

When TLR4 is internalized into endosomes, the signaling is TRAM-TRIF-dependent. In addition to TRAM which is localized at the plasma membrane, LPS stimulation recruits another adaptor: TRIF. TRIF contains RIP homotypic interaction motif on N-terminal mediating the interaction with RIP1. On the other hand, C-terminal of TRIF contains TRAF-binding motifs allowing the association with TRAF3 and TRAF6. TRAF3 and IKK-i associate with modulator proteins TBK1, TRAF family member-associated NF-κB (TANK), NAK-associated protein 1 (NAP1) and the TBK1 adaptor (SINTBAD). The latter complex phosphorylate IRF3 and IRF7 allowing their nuclear translocation resulting in to induction of type I IFN gene expression. However, although NAP1 and SINTBAD knockdown impair the TLR4-TRAM-TRIF-signaling, their exact function remains unclear (Figure 32 right pathway) [4, 511, 514-516].

Independently of the pathways described above, PI3K are activated in response to LPS activation [517, 518]. It was shown that p85 is constitutively bound to MyD88. However, this binding increase further upon LPS stimulation [518]. Thus, MyD88 contains an YXXM motif necessary for p85 interactions although the details of the interaction between TLR4, MyD88, and PI3K are largely unsolved [519]. The positive or negative role of PI3K in TLR4 signaling is controversial. However, the anti-inflammatory role of PI3K is supported by the observation that IL-6, IL-12, IL-1β and TNF production are increased upon PI3K inhibition [520].

Conversely, IL-10, sIL-1Ra production is decreased upon PI3K inhibition supporting a role of PI3K [521]. TLR4, MyD88 and PI3K share multiple protein interactions suggesting as potential association in signaling platform [522].

AIMS OF THE THESIS

A crucial issue arising from the cell-cell contact model is the characterization of intracellular pathways involved in triggering and controlling the production of pro- and anti-inflammatory cytokines in chronic/sterile inflammation in opposition to signaling pathways involved in acute inflammation. To address these questions, my thesis was divided in several specific aims.

1. It was previously demonstrated that cell-cell contact as well as LPS trigger the PI3K pathway for the control of sIL-1Ra and IL-1β in human monocytes [315]. The first aim was to identify the isoform of PI3K required for the production of IL-1β, TNF, sIL-1Ra, and IL-6 in human monocytes activated by T cell contact and LPS.
2. Since sIL-1Ra production in monocytes/macrophages is directly induced by IFNβ through the activation of PI3K but not STAT1 or MEK1 [330], the second aim was to identify the PI3K isoform required for sIL-1Ra production and investigate the role of MEK2 in the latter process. The results reveal an important crosstalk between PI3K and MAPK in sIL-1Ra induction in monocyte/macrophages.
3. Like IFNβ, GA is an approved treatment in MS but the mechanisms of action leading to beneficial effects remain unclear. Both IFNβ and GA display comparable therapeutic efficiency in MS patients. It was previously demonstrated that IFNβ increases the circulation level of sIL-1Ra in human monocytes [179]. Therefore, the third aim of my thesis was to investigate the potential ability of GA to modulate sIL-1Ra and IL-1β production in chronic/sterile or acute inflammation.
4. As previously stated, GA induces the production of sIL-1Ra in human monocytes [283]. However, surface receptors and intracellular signaling pathways involved in GA-induction of sIL-1Ra remain completely unknown. Thus, although the receptors of GA

remain to be identifying, the fourth aim of my thesis was to investigate the PI3K and MAPK involvements in sIL-1Ra production induce by GA.

5. Activated T cells can disseminate cell surface molecules by generating microparticles (MP) and thus, ensure “distant” cellular contact. Thus, the last aim of my thesis was to characterize the ability of MP to induce pro- and anti-inflammatory cytokines in human monocytes and whether the latter process would be modulated by HDL.

6 RESULTS

6.1 Differential Regulation of Cytokine Production by PI3Kdelta in Human Monocytes upon Acute and Chronic Inflammatory Conditions

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Differential regulation of cytokine production by PI3Kδ in human monocytes upon acute and chronic inflammatory conditions

Nicolas Molnarfi, Karim J. Brandt, Lyssia Gruaz, Jean-Michel Dayer, Danielle Burger*

Division of Immunology and Allergy, Clinical Immunology Unit, Hans Wilsdorf Laboratory, Department of Internal Medicine, University Hospital and School of Medicine, University of Geneva, 24 rue Micheli-du-Crest, CH-1211 Geneva 14, Switzerland

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ABSTRACT

Deregulated production of cytokines, including IL-1β, IL-6 and TNF plays an important role in chronic inflammation. Relevant to this condition, direct cellular contact with stimulated T cells is a potent inducer of cytokine production in human monocytes/macrophages. We previously demonstrated that PI3Ks regulate differential production of IL-1β and its specific inhibitor secreted IL-1 receptor antagonist (sIL-1Ra) by human monocytes. Here we show that in contrast with PI3Kα, β and γ, PI3Kδ accounts for most of the PI3K-dependent signaling ruling the production of IL-1β, IL-6, TNF and sIL-1Ra in monocytes activated by cellular contact with stimulated T cells (mimicked by CHAPS-solubilized membranes of stimulated T cells, CE_{sHUT}) and lipopolysaccharides (LPS); the latter stimuli being relevant to chronic/sterile and acute/infectious inflammation, respectively. Interestingly, PI3Kδ activity dampened the production of pro-inflammatory cytokines in LPS-activated monocytes, but induced it in CE_{sHUT}-activated cells. In both CE_{sHUT}- and LPS-activated monocytes PI3Kδ regulated cytokine transcript expression through the phosphorylation/inactivation of glycogen synthase kinase-3β (GSK3β). The blockade of GSK3β displayed inverse effects to those of PI3Kδ blockade. Thus, by displaying opposite functions in conditions mimicking chronic/sterile and acute/infectious inflammation, i.e., by repressing pro-inflammatory cytokine expression in LPS-activated monocytes but inducing such mediators in T cell contact-activated monocytes, PI3Kδ represents a potential therapeutic target specific to chronic/sterile inflammatory conditions.

1. Introduction

The inflammatory response aims at protecting the organism by clearing out the initial cause of cell injury (e.g., microorganisms or toxins) and the consequences of such injury (e.g., necrotic cells and tissues), and in turn at initiating mechanisms designed at repairing surrounding damaged tissues. Inflammation has to be tightly controlled in time and space to avoid detrimental developments such as those seen in sepsis and chronic inflammatory diseases including rheumatoid arthritis (RA) and multiple sclerosis (MS). It is acknowledged that the pro-inflammatory cytokines TNF, IL-1β and IL-6 play an important part in the progression of pathogenic inflammatory mechanisms (Martino et al., 2000; McInnes and Schett, 2007; Ozenci et al., 2002). To restrain inflammation, pro-inflammatory reactions are closely interconnected with counter-regulatory anti-inflammatory pathways. In the extracellular space this function is fulfilled by specific inhibitors generated by the shedding of cell surface receptors, e.g., soluble TNF receptors, soluble IL-1 receptor II and IL-1 receptor accessory protein, and the release of secreted IL-1 receptor antagonist (sIL-1Ra) that specifically inhibit IL-1 (Burger and Dayer, 2000). All these effectors are mainly produced by monocytes/macrophages, which, together with T lymphocytes, are an important part of cellular infiltrates observed in RA and MS (Gutcher and Becher, 2007; Smeets et al., 2003).

Cellular contact with stimulated T cells is acknowledged as an important mechanism to inducing cytokines in human monocytes/macrophages (Beech et al., 2006; Brennan and Foey, 2002; Burger et al., 2007). Indeed, T cell contact-mediated activation of human monocytes is as potent as lipopolysaccharides (LPS) – a prototypical stimulus of acute/infectious inflammatory conditions – to induce cytokine production (Burger, 2000). Furthermore, this mechanism is likely to be relevant in vivo since T cells isolated from RA synovial fluid constitutively display the ability to induce cytokine production in human monocytes (Brennan et al., 2002). By comparing stimuli relevant to chronic/sterile and acute/infectious inflammation, we previously demonstrated that PI3K activation was mandatory to the induction of sIL-1Ra production in human

* Corresponding author. Tel.: +41 22 372 93 76; fax: +41 22 372 93 69.
E-mail address: danielle.burger@hcuge.ch (D. Burger).

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monocytes/macrophages independently of the stimulus (Molnarfi et al., 2005; Molnarfi et al., 2007). Furthermore, we recently pinpointed that PI3Ks play a part in the differential regulation of IL-1β and sIL-1Ra production by freshly isolated blood monocytes when activated by contact with stimulated T cells or LPS (Molnarfi et al., 2007).

PI3Ks are heterodimeric enzymes that consist of a catalytic subunit (p110) and a regulatory subunit (p85) (Cantley, 2002). Class IA PI3K activation is elicited through the binding of its regulatory subunit (p85) to adaptor proteins bound to phosphotyrosine residues present in activated cellular receptors located on the plasma membrane, including TLRs (Arbibe et al., 2000; Cantley, 2002; Toker and Cantley, 1997). Class I PI3Ks synthesize the phospholipid PtdIns(3,4,5)P3 into the membranes in which they are activated. It is now acknowledged that PtdIns(3,4,5)P3 is a messenger molecule which regulates the localization and function of multiple effectors by binding to their specific pleckstrin homology (PH) domains (Hawkins et al., 2006). Transgenic and KO mouse models targeting class I PI3K signaling pathways and the development of PI3K isoform-selective inhibitors has highlighted the importance of specific isoforms of PI3K in whole-animal physiology and pathology, e.g., PI3Kα in growth and metabolic regulation, PI3Kβ in thrombosis, and PI3Kδ and γ in inflammation and asthma (Hawkins et al., 2006; Rommel et al., 2007; Wetzker and Rommel, 2004). A key physiologic mediator of class I PI3K which is recruited at the plasma membrane through its PH domain is the serine–threonine kinase Akt (Wetzker and Rommel, 2004). After recruitment, Akt is activated by phosphorylation at Ser473 and in turn phosphorylates several downstream targets of the PI3K pathway, including the constitutively active serine–threonine kinase glycogen synthase kinase-3β (GSK3β). Phosphorylation of GSK3β on Ser9 results in its inhibition (Kockeritz et al., 2006). GSK3β phosphorylates multiple substrates, including transcription factors NFATc, c-Jun, CREB, c-Myc, and HSF-1 (Doble and Woodgett, 2003) some of which being critical regulators of pro- and anti-inflammatory cytokine production (Hoeflich et al., 2000).

Since numerous studies support the premise that PI3Ks play a pivotal role in regulating the production of pro- and anti-inflammatory mediators in human monocytes/macrophages (Guha and Mackman, 2002; Tengku-Muhammad et al., 1999; Weinstein et al., 2000), the present study was undertaken to determine the respective role of PI3K isoform(s) in the regulation of production of IL-1β, sIL-1Ra, IL-6 and TNF in conditions related to acute (LPS) and chronic/sterile (T cell contact) inflammation. Because transfection techniques that had previously been used (Molnarfi et al., 2005) resulted in the loss of monocyte ability to produce IL-1β and because monocytic cell lines do not display similar responses to freshly isolated human monocytes, the technical approach involves the use of pharmacological kinase inhibitors. The usage of pharmacological inhibitor was also required because class IA PI3Ks have at least seven adaptor proteins, including p85α, p85β, and p55γ. All these splice variants make functional complexes with p110α, β and δ subunits (Vanhaesebroeck et al., 1997) and the decrease of one type of p110 subunit (in knock out mice or by RNA interference) simultaneously decreases the level of adaptor proteins, and in turn diminishes the level of other subtypes of class IA PI3Ks (Brachmann et al., 2005) therefore impairing a clear-cut identification of the isoform involved. Our results show that PI3Kδ is a key enzyme controlling the production of pro- and anti-inflammatory cytokines by human monocytes. Because PI3Kδ displays opposite effects in conditions related to acute/infectious and chronic/sterile inflammation, it represents a potential therapeutic target for chronic inflammatory diseases such as RA and MS.

2. Materials and methods

2.1. Materials

FCS, streptomycin, penicillin, L-glutamine, RPMI-1640, PBS free of Ca^{2+} and Mg^{2+} (Gibco, Paisley, Scotland); purified phytohemagglutinin (PHA) (EY Laboratories, San Marco, CA); Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden); Tri® Reagent, phorbol myristate acetate (PMA), phenylmethylsulfonyl fluoride (PMSF), endotoxin-free dimethylsulphoxide (DMSO), pepstatin A, leupeptin, iodoacetamide, neuraminidase, bovine serum albumin (BSA), polymyxin B sulfate, actinomycinD (Sigma Chemicals Co., St. Louis, MO); pure lipopolysaccharides (LPS) from *Escherichia coli* serotype EH100(Ra) was purchased from Alexis Biochemicals (San Diego, CA); and the kinase inhibitors Ly294002 and Wortmannin (LC Laboratories, http://www.lclabs.com/); compound 15e, TGX-221, and AS-604850, PI3Kα, β, and γ selective inhibitors, respectively, (Alexis Corp., Lausen, Switzerland); and SB216763 a GSK3β inhibitor (Calbiochem) were purchased from the designated suppliers. The p110δ inhibitor IC87114 was a kind gift from ICOS Corporation, Bothell, WA. All other reagents were of analytical grade or better.

2.2. Monocytes

Monocytes were isolated from blood buffy coats of healthy volunteers and provided by the Geneva Hospital blood transfusion center as previously described (Burger and Dayer, 1998).

2.3. T cells and preparation of T cell plasma membranes

HUT-78, a human T cell line (Gazdar et al., 1980), was obtained from the ATCC (Rockville, MD). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 IU/ml penicillin, and 2 mM L-glutamine (complete RPMI medium) in 5% CO_2-air humidified atmosphere at 37 °C. HUT-78 cells (2×10^6 cells/ml) were stimulated for 6 h by PHA (1 μg/ml) and PMA (5 ng/ml). Plasma membranes of stimulated HUT-78 cells and their soluble CHAPS-extract (CE_{sHUT}) were prepared as previously described (Burger et al., 2004). CE_{sHUT} displays similar activation ability toward monocytes than that of living T cells (i.e., in cocultures), fixed T cells or plasma membranes of stimulated T cells as previously described (Burger et al., 2004; Hyka et al., 2001; Jungo et al., 2001).

2.4. Cytokine production

Human monocytes (50×10^3 cells/well/200 μl) were preincubated for 60 min at the indicated concentration of kinase inhibitor and then activated for 24 h after the addition of CE_{sHUT} (3 μg/ml proteins), or LPS (100 ng/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 IU/ml penicillin, 2 mM L-glutamine (medium) containing (CE_{sHUT}) or not (LPS) 5 μg/ml polymyxin B sulfate. All conditions were carried out in triplicate. The production of cytokines was measured in culture supernatants by commercially available enzyme immunoassay: IL-1β, (Immunotech, Marseille, France), TNF, IL-6 and sIL-1Ra (Quantikine; R&D, Minneapolis, MN).

2.5. mRNA

Monocytes (3×10^6 cells/well/3 ml) were cultured in a medium containing (CE_{sHUT}) or not (LPS) 5 μg/ml polymyxin B sulfate in 6-well plates for 60 min with the specified dose of inhibitor and then activated by CE_{sHUT} (3 μg/ml proteins), or LPS (100 ng/ml) for the

indicated time. Alternatively, in transcriptional arrest experiments, 10 μg/ml actinomycin D was added 3 h after monocyte activation. Total mRNA was prepared by Tri® Reagent (Sigma) and subjected to DNase digestion. After reverse transcription, quantitative real-time duplex PCR analysis was conducted as described (Molnarfi et al., 2007). The mRNA expression levels were normalized with the expression of a housekeeping gene (18S) analyzed simultaneously. IL-6, IL-1, TNF, sIL-1Ra, and 18S probes were purchased from Applied Biosystems. All measurements were carried out in triplicates.

2.6. Western blot analysis

Human monocytes were resuspended at 8×10^6 cells/ml in medium supplemented with 10% heat-inactivated FCS and 500 μl was placed in 2-ml polypropylene tubes (Eppendorf) at 37 °C. After 1 h, inhibitors were added for 45 min and then cells were activated by CE_{sHUT} (3 μg/ml proteins), or LPS (100 ng/ml). At the indicating time, the reaction was stopped by the addition of 800 μl of ice-cold PBS and centrifugation. Total cell lysate was prepared and subjected to Western blot analysis as described previously (Molnarfi et al., 2005). Nitrocellulose membranes were probed with anti-Akt/PKB, anti-phospho Akt/PKB [pS^{473}] (Cell signaling Technology) or anti-p110δ (Calbiochem). Antibody-bound proteins were detected by the Uptilight hrp Blot Chemiluminescent substrate as described by the supplier (Uptima, Interchim, Monluçon, France).

2.7. Statistics

When required, significance of differences between groups was evaluated using Student's paired *t*-test.

3. Results

3.1. LPS and CE_{sHUT} induce the production of TNF, IL-6, IL-1β and sIL-1Ra in human monocytes

Prior to assess the role of PI3Ks in the induction of cytokine production in human monocytes, the activity of the two stimuli used in this study was ascertained. Differences were observed in the production of cytokines between different preparations of monocytes, depending on individual donors (Table 1). Neither IL-1β nor IL-6 was detected in cell supernatants in the absence of stimulus. TNF production was at the level of detection limit. Basal production of sIL-1Ra was not significantly different in the presence or absence of polymyxin B. The production of all four cytokines was enhanced by both stimuli. In accordance with previous data, CE_{sHUT} induced lower production of IL-1β, but higher production of sIL-1Ra than LPS (Molnarfi et al., 2007). Furthermore, higher production of TNF and IL-6 were observed when monocytes were activated by LPS as compared to CE_{sHUT} (Table 1). However, because high doses of CE_{sHUT} displayed cytotoxicity, a sub-optimal dose of CE_{sHUT} (3 μg/ml proteins), but an optimal dose of LPS (100 ng/ml) were used throughout this study (Molnarfi et al., 2004). Furthermore, since the production of cytokines varied greatly as a function of monocyte preparation (i.e., blood donor), the results below are presented as percentages of the cytokine production measured in the absence of inhibitor.

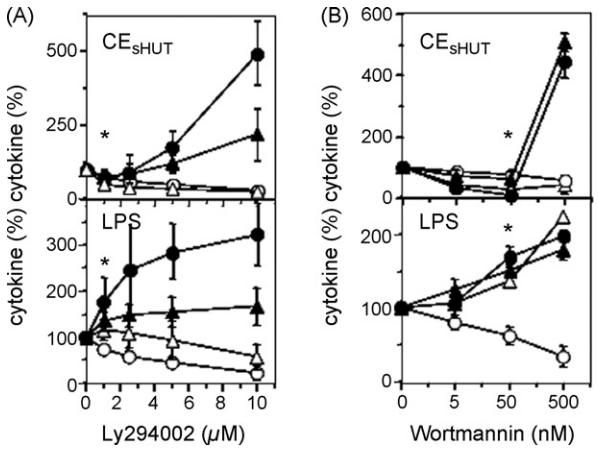

Fig. 1. PI3Ks differentially regulate cytokine production in human monocytes activated by CE_{sHUT} or LPS. Isolated monocytes (5×10^4 cells/200 μl) were preincubated for 60 min in the presence or absence of the indicated concentration of Ly294002 and wortmannin and then stimulated or not with either CE_{sHUT} (3 μg/ml proteins) or LPS (100 ng/ml) for 24 h in 96-well plates. Experiments were performed in the presence (CE_{sHUT}) or absence (LPS) of polymyxin B (5 μg/ml). Cytokine production was assessed in supernatants of triplicate cell cultures as described in Section 2. Results obtained with monocytes from six different donors are presented as mean ± S.D. of percentage of cytokine production induced by CE_{sHUT} or LPS in the absence of inhibitor. IL-1β (closed circles, ●), sIL-1Ra (open circles, ○), IL-6 (closed triangles, ▲) and TNF (open triangles, △); (*) indicate the lowest inhibitor concentration which induced a significant inhibition or increase of cytokine production, i.e., a $P \leq 0.05$.

3.2. PI3Ks differentially regulate cytokine production in monocytes activated by LPS or CE_{sHUT}

In order to assess the functions of PI3Ks in cytokine production by human monocytes, we first assessed whether and how PI3K pan-inhibitors such as wortmannin and Ly294002 would affect their production. *Per se* neither Ly294002 nor wortmannin did affect cytokine production, even at the highest concentration used (not shown). As shown in Fig. 1A, at doses near the IC_{50} (1–2 μM), Ly294002 diminished the production of the four cytokines measured in culture supernatants of monocytes activated by CE_{sHUT}. Indeed, at 1 μM Ly294002, IL-1β production was inhibited by 27.7 ± 7.7% ($P = 0.0054$), that of sIL-1Ra, by 24.6 ± 19.8% ($P = 0.0034$), that of IL-6, by 35.5 ± 17.7 ($P = 0.0277$), and that of TNF, by 49.3 ± 9.2% ($P = 0.0003$). At higher Ly294002 concentrations, the production of both IL-1β and IL-6 were enhanced while that of sIL-1Ra and TNF was further diminished. Similarly, the other PI3K pan-inhibitor, wortmannin, also displayed different effects as a function of the dose (Fig. 1B). In CE_{sHUT}-activated monocytes, low concentrations of wortmannin (5–50 nM) decreased the production of all four cytokines, although sIL-1Ra was less affected. Indeed, at 50 nM wortmannin, IL-1β production was inhibited by 88.2 ± 12.3% ($P = 0.0064$), that of sIL-1Ra,

Table 1
Cytokine production by isolated human monocytes

Stimulus	IL-1β (pg/ml)	sIL-1Ra (ng/ml)	IL-6 (ng/ml)	TNF (pg/ml)
Medium + polymyxin B	nd	0.85 ± 0.73	nd	14 ± 24
Medium	nd	1.14 ± 0.32	nd	12 ± 8
CE_{sHUT}	277 ± 140	21.42 ± 3.61	4.68 ± 0.82	577 ± 178
LPS	1373 ± 511	4.80 ± 1.15	18.53 ± 3.44	5991 ± 1666

nd: not detected.

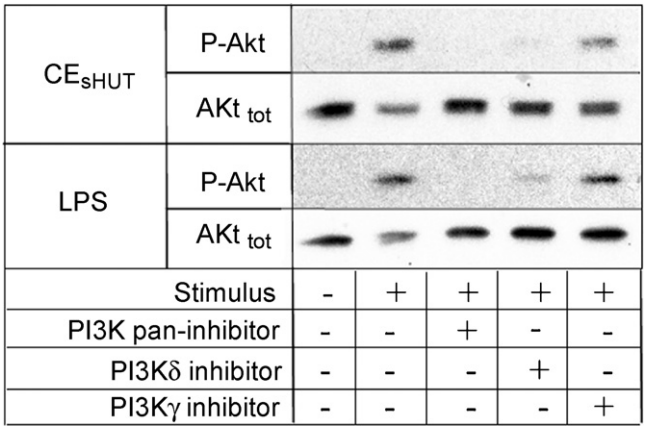

Fig. 2. CE_{sHUT} and LPS induce Akt phosphorylation via PI3Kδ activation. Isolated monocytes (4×10^6 cells/500 μl) were treated or not with the indicated dose of PI3K pan-inhibitor (10 μM Ly294002), PI3Kδ inhibitor (1 μM IC87114) and PI3Kγ inhibitor (500 nM AS-252424) for 45 min and then stimulated with either CE_{sHUT} or LPS for 60 min. Cell lysates were analyzed by Western blot as described in Section 2 with antibodies to phosphorylated Ser^{473}-Akt (P-Akt) and Akt (loading control). The presented autoradiographies are typical of three different experiments.

by 24.4 ± 3.1% ($P = 0.0052$), that of IL-6, by 43.6 ± 17.6 ($P = 0.0506$), and that of TNF, by 72.7 ± 8.6% ($P = 0.0529$). At higher concentrations, wortmannin enhanced the production of IL-1β and IL-6, while that of TNF and sIL-1Ra was still lower than that obtained in the absence of inhibitor. Together these data suggest that in conditions relevant to chronic/sterile inflammation (CE_{sHUT}), the production of TNF and sIL-1Ra was inhibited independently of the PI3K inhibitor and inhibitor dose, whereas only high doses of PI3K pan-inhibitor enhanced IL-1β and IL-6 production. In LPS-activated monocytes, Ly294002 enhanced the production of IL-1β and IL-6 but diminished that of sIL-1Ra (Fig. 1A). The production of TNF was slightly but significantly increased to reach 117.0 ± 22.0% ($P = 0.0285$) at 1 μM Ly294002 and then decreased, being inhibited by 49.1 ± 27.7% ($P = 1.6 \times 10^{-5}$) at 10 μM Ly294002 (Fig. 1A). Low doses of wortmannin poorly affected the production of pro-inflammatory cytokines, whereas sIL-1Ra production was inhibited. At concentrations higher than 50 nM wortmannin, all three pro-inflammatory cytokines were enhanced (Fig. 1B). These results demonstrate that PI3Ks were mainly involved in the dampening of pro-inflammatory cytokine production in conditions relevant to acute/infectious inflammation (LPS). Thus, PI3Ks display opposite activities in conditions mimicking acute/infectious and chronic/sterile inflammation.

3.3. Blockade of PI3Kδ but not PI3Kγ affects Akt phosphorylation in activated monocytes

We previously determined that Akt was a downstream element through which PI3Ks control IL-1β and sIL-1Ra production upon monocyte activation by either LPS or CE_{sHUT} (Molnarfi et al., 2007). This suggested the involvement of class I PI3K. Since the expression of isoforms γ and δ of class I PI3Ks is more specifically restricted to cells of the immune system, we assessed the ability of their specific inhibitors to affect Akt phosphorylation in monocytes activated by either LPS or CE_{sHUT}. As shown, in Fig. 2, both stimuli induced the phosphorylation of Akt as previously described (Molnarfi et al., 2007). Akt phosphorylation on Ser_{473} was completely abolished in the presence of the PI3K pan-inhibitor Ly294002 used as a control. PI3Kδ but not PI3Kγ blockade inhibited Akt phosphorylation, suggesting that PI3Kγ was not activated upon CE_{sHUT}- or LPS-activation of monocytes. This was confirmed by the lack of effect of the PI3Kγ inhibitor on cytokine production. Indeed, the production of IL-1β, sIL-1Ra, IL-6, and TNF was not modulated by AS-252424 (a specific PI3Kγ inhibitor) even at concentrations as high as 500 nM (data not shown). These results suggest that the effects seen with the PI3K pan-inhibitors (Fig. 1) could not be due to PI3Kγ blockade. We therefore ascertain the involvement of the three other class I PI3K isoforms, namely PI3Kδ, PI3Kα and PI3Kβ the expression of the two latter being ubiquitous, thus not restricted to immune cells.

3.4. PI3Kδ activity is critical for cytokine production in both LPS- and CE_{sHUT}-activated monocytes

In order to assess the involvement of PI3Kδ, α, and β in the control of cytokine production, monocytes were preincubated with increasing concentration of the selective inhibitor and then stimulated with either CE_{sHUT} or LPS. *Per se* none of the PI3K inhibitors used did affect cytokine production, even at the highest concentration (not shown). As depicted in Fig. 3A, IC87114 (a specific PI3Kδ inhibitor) exerted differential effects in CE_{sHUT}- and LPS-activated monocytes. Indeed, all measured cytokines were inhibited by IC87114 in a dose-dependent manner in CE_{sHUT}-activated monocytes. This reflected the results obtained with PI3K pan-inhibitors at low doses (Fig. 1). IC87114 concentrations as low as 0.5 μM diminished the production of IL-1β by 27.7 ± 7.5% ($P = 0.0024$); that of sIL-1Ra by 22.32 ± 21.49% ($P = 0.0093$); that of IL-6 by 25.2 ± 10.4% ($P = 0.0007$); and that of TNF by 27.5 ± 12.4% ($P = 0.0017$). Interestingly, the inhibition of IL-1β production reached 72 ± 8% at 10 μM IC87114, whereas that of its specific inhibitor, sIL-1Ra, reached only 35%. This suggests that IC87114 displayed anti-inflammatory properties towards the IL-1 system. In LPS-activated monocytes, sIL-1Ra production was slightly inhibited by IC87114 (around 20%) whereas that of the three pro-inflammatory cytokines was enhanced (Fig. 3A). These data confirmed results obtained with wortmannin and low doses of Ly294002 (Fig. 1), thus suggesting that PI3Kδ was mainly responsible of the differential regulation of pro- and anti-inflammatory cytokines. This was strengthened by assessing the effects of inhibitors of PI3Kα and β. As shown in Fig. 3B and C, although some variations were observed at high concentrations (i.e., concentration higher than IC_{50}), specific inhibitors of PI3Kα and β did not significantly affect cytokine production of monocytes activated by either CE_{sHUT} or LPS. This was further ascertained by the premise that none of the four cytokine transcripts was significantly affected by PI3Kα and β inhibitors (not shown). Together these results demonstrate that PI3Kδ accounts for most of the effects observed with the PI3K pan-inhibitors when monocytes were activated by LPS. This was also true for CE_{sHUT}-activated monocytes, in which IC87114 inhibited all measured cytokines, a phenomenon that was observed at concentrations of Ly294002 (1–2 μM) and wortmannin (5 and 50 nM) that were closed from the IC_{50}. This demonstrates that PI3Kδ differentially regulated the production of pro-inflammatory cytokines in conditions related to chronic/sterile and acute/infectious inflammation.

3.5. PI3Kδ controls cytokine gene transcription

In order to determine by which mechanism(s) PI3Kδ modulated the production of sIL-1Ra, IL-1β, IL-6 and TNF, the effect of IC87114 on cytokine mRNA levels and stability was assessed. In monocytes activated for 3 h by CE_{sHUT}, PI3Kδ blockade inhibited the four cytokine mRNA levels around 50% (Fig. 4A). However, after 6 h the transcription of sIL-1Ra was strongly enhanced suggesting that at that time other mechanism were set-in that overwhelmed PI3Kδ control of sIL-1Ra production. In LPS-activated monocytes, IC87114 inhibited sIL-1Ra mRNA levels by 50% but enhanced those of IL-1β, IL-6 and TNF after 3 h activation (Fig. 4B). After 6 h activa-

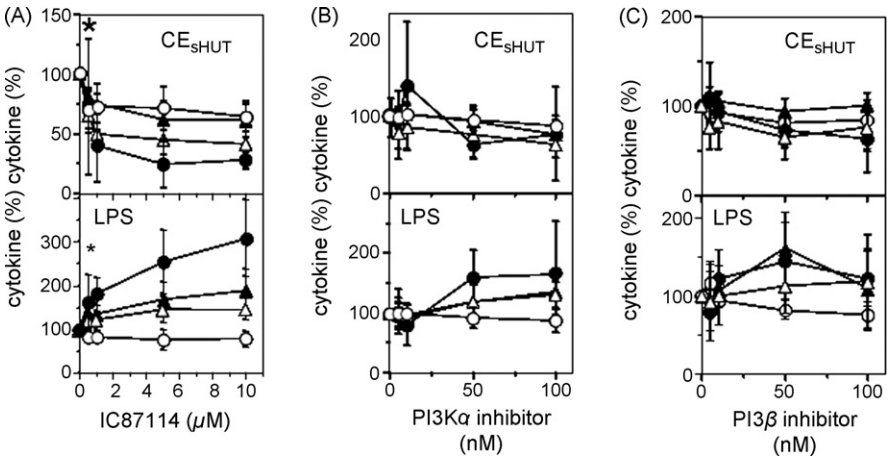

Fig. 3. Inhibitor of PI3Kδ but not PI3Kα and β inhibitors differentially regulates cytokine production in human monocytes activated by CE$_{sHUT}$ or LPS. Isolated monocytes (5×10^4 cells/200 μl) were pre-treated or not with increasing doses of IC87114 (A), PI3Kα inhibitor (B) and PI3Kβ inhibitor (C) for 60 min and then stimulated with either CE$_{sHUT}$ (3 μg/ml proteins) or LPS (100 ng/ml) in 96-well plates. After 24 h activation, Cytokine production was assessed in supernatants of triplicate cell cultures. Results obtained with monocytes from six (A) and three (B and C) different donors, respectively, are presented as mean ± S.D. of percentage of cytokine production induced by CE$_{sHUT}$ or LPS in the absence of inhibitor. IL-1β (closed circles, ●), sIL-1Ra (open circles, ○), IL-6 (closed triangles, ▲) and TNF (open triangles, △); (*) indicate the lowest inhibitor concentration which induced a significant inhibition or increase of cytokine production, i.e., a $P \leq 0.05$.

tion, sIL-1Ra mRNA level was increased in the presence or absence of IC87114, although the level of sIL-1Ra mRNA at 9 h was still 50% lower in IC87114-treated monocytes (Fig. 4B). This suggested that similarly to CE$_{sHUT}$-activated monocytes, in LPS-activated monocytes after long periods of activation, other mechanisms were set-in that prevail over PI3Kδ control. The addition of actimonycin D (a transcription inhibitor) after 3 h activation showed that IC87114 did not affect mRNA stability, neither in CE$_{sHUT}$- not in LPS-activated monocytes (Fig. 4A and B). Together these results demonstrate the opposite usage of the PI3Kδ pathway in monocytes activated upon chronic/sterile and acute/infectious inflammatory conditions. Another difference between the two stimuli resided in the stability of IL-1β mRNA. Indeed, although IL-1β mRNA displayed long-lasting stability with both stimuli, after actinomycin D addition its level decreased more rapidly in CE$_{sHUT}$- than in LPS-activated monocytes.

3.6. *PI3Kδ is present and activated in CE$_{sHUT}$- and LPS-activated human monocytes*

In order to demonstrate that PI3Kδ was actually activated by both stimuli used in this study, monocytes were activated by CE$_{sHUT}$ and LPS for 5 and 20 min, respectively. The activation time was shorter in monocytes activated by CE$_{sHUT}$ because the phosphorylation of the downstream element Akt was already observable after 5 min, reaching a persistent plateau after 20 min (Molnarfi et al., 2007). As shown in Fig. 5, PI3Kδ was present in human monocytes (total p110δ). The catalytic p110δ subunit was translocalized at the membrane upon activation by CE$_{sHUT}$ and LPS. The presence of p110δ in unactivated (time = 0 min) monocytes in the "LPS" condition was likely to be due to the absence of polymyxin in this condition, thus to a slight basal activation. The results of Fig. 5 demonstrate that PI3Kδ was activated by both chronic/sterile and acute/infectious inflammation-related stimuli.

3.7. *Regulation of cytokine production downstream PI3Kδ requires glycogen synthase kinase-3β (GSK3β) activity*

Because cytokine production is ruled by PI3Kδ at the transcriptional level in monocytes activated by either LPS or CE$_{sHUT}$, we assessed whether GSK3β, a substrate of Akt involved in nuclear factors regulation, would be implied in such mechanisms. As depicted in Fig. 6, the blockade of GSK3β by SB216763 in CE$_{sHUT}$-activated monocytes resulted in the enhancement of IL-1β (1.25 ± 0.14-fold, at 1 μM SB216763, $P = 0.018$) and IL-6 (1.28 ± 0.21-fold at 1 μM SB216763, $P = 0.011$) production, the production of TNF tending to be enhanced. The production of sIL-1Ra was significantly diminished at high (10 μM) SB216763 concentration (37.1 ± 6.7% inhibition, $P = 3.9 \times 10^{-5}$). Therefore, SB216763 displayed opposite effects to IC87114 (Fig. 3), suggesting that PI3Kδ effects were mediated through GSK3β phosphorylation/inactivation. Similarly, in LPS-activated monocytes, SB216763 slightly increased sIL-1Ra production, while reducing to some extent pro-inflammatory cytokine production (Fig. 6); statistical significance being reached at 10 μM SB216763 for IL-1β ($P = 0.003$), IL-6 ($P = 0.023$), and TNF ($P = 0.009$). Here again SB216763 displayed opposite effect to IC87114 on pro-inflammatory cytokine production although to a lesser extent. As shown in Fig. 7, the effects of GSK3β blockade were reflected at the mRNA level. Indeed, the expression of IL-1β and IL-6 mRNA was differentially modulated in CE$_{sHUT}$- and LPS-activated monocytes, being enhanced in the former and inhibited in the latter. The expression of sIL-1Ra and TNF transcripts was not significantly affected in the presence of SB216763, although TNF mRNA tended to be diminished by SB203580 in CE$_{sHUT}$- and increased in LPS-activated monocytes, respectively (Fig. 7A and B). This suggests that GSK3β controlled pro-inflammatory cytokine transcription in an opposite manner in chronic/sterile and acute/infectious inflammatory conditions.

4. Discussion

This study demonstrates that PI3Kδ differentially controls cytokine production in human monocytes activated upon chronic/sterile and acute/infectious inflammatory conditions. Indeed, under chronic/sterile inflammatory conditions mimicked by CE$_{sHUT}$, PI3Kδ participates in the up-regulation of both pro- and anti-inflammatory cytokines, whereas under acute/infectious inflammatory conditions, PI3Kδ activity dampens pro-inflammatory cytokine production, the production of sIL-1Ra being poorly up-regulated. Thus, PI3Kδ might represent an interesting specific therapeutic target in chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis, since its inhibition should diminish the production of pathologically induced pro-inflammatory cytokines without diminishing that

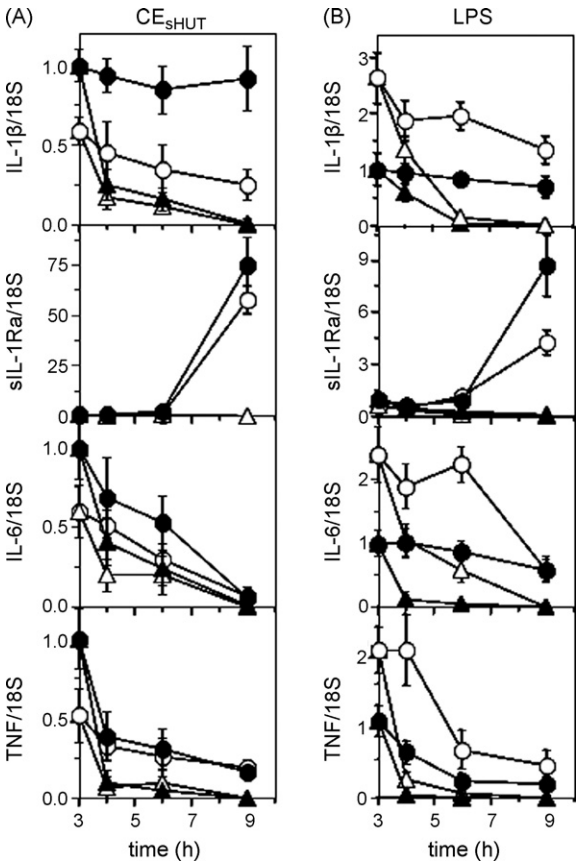

Fig. 4. Time-course of cytokine mRNA induction and stability in monocytes activated by CE$_{sHUT}$ or LPS. Isolated monocytes (3×10^6 cells/3 ml) were preincubated for 60 min in the presence (open symbols) or absence (closed symbols) of 5 μM IC87114 and then stimulated or not with either CE$_{sHUT}$ (3 μg/ml proteins) or LPS (100 ng/ml) in 6-well plates, as described in Section 2. Cells were then stimulated for 3 h before the addition (triangles) or not (circles) of 10 μg/ml actinomycin D. Cell culture was stopped at the indicated time and cytokine mRNA levels were determined by duplex quantitative real-time PCR analysis of triplicates normalized to the levels of the 18S mRNA as described in Section 2. Experiments were performed in the presence (CE$_{sHUT}$) or absence (LPS) of polymyxin B (5 μg/ml). The relative expression levels of cytokine mRNA are presented as mean ± S.D. of percentage of relative cytokine mRNA expression induced by CE$_{sHUT}$ or LPS. The value of mRNA levels in 3 h-stimulated monocytes being arbitrarily considered as 1.0. Results are representative of three distinct experiments.

induced by infectious agent, i.e., without diminishing regular defense mechanisms of the organism.

At high PI3K pan-inhibitor concentrations, the production of pro-inflammatory cytokines tends to be enhanced in both chronic/sterile and acute/infectious inflammatory condition. This is in accordance with our previous results showing that Ly294002

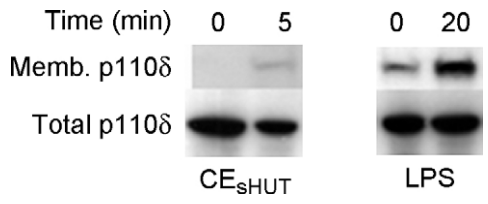

Fig. 5. PI3Kδ is expressed and activated in human monocytes. Isolated monocytes (4×10^6 cells/500 μl) were stimulated with either CE$_{sHUT}$ or LPS for the indicated time in duplicate samples. One sample was subjected to membrane preparation (memb p110δ), the other to cell lysis (total p110δ) and analyzed by Western blot as described in Section 2. The presented autoradiographies are typical of three different experiments.

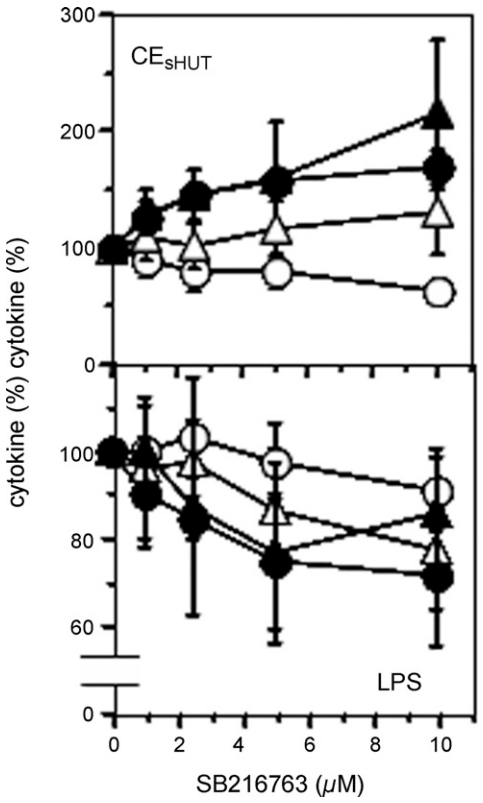

Fig. 6. GSK3β inhibition differentially regulates cytokine production in human monocytes activated by CE$_{sHUT}$ or LPS. Isolated monocytes were cultured as described in Fig. 1 with increasing doses of SB216763 (GSK3β inhibitor). Cytokine production was assessed in supernatants of triplicate cell cultures. Results obtained with monocytes from three different donors are presented as mean ± S.D. of percentage of cytokine production induced by CE$_{sHUT}$ or LPS in the absence of inhibitor. IL-1β (closed circles, ●), sIL-1Ra (open circles, ○), IL-6 (closed triangles, ▲) and TNF (open triangles, △); (*) indicate the lowest inhibitor concentration which induced a significant inhibition or increase of cytokine production, i.e., a $P \leq 0.05$.

enhanced the production of IL-1β while inhibiting sIL-1Ra transcription in both CE$_{sHUT}$- and LPS-activated monocytes (Molnarfi et al., 2007). Here we show that Ly294002 concentrations near the IC$_{50}$, i.e., 1–2 μM display similar effects to those observed with the selective PI3Kδ inhibitor. This suggests that, pan-inhibitors affect other elements involved in the control of cytokine production/secretion at concentrations higher than IC$_{50}$. Thus, caution has to be taken for the accuracy of the many studies describing PI3K involvements by using pan-inhibitors at 5–10 μM for Ly294002 or 50–500 nM for wortmannin, the latter doses being often seen in the literature. In this study, by generating dose–response curves with a panel of specific inhibitors, we demonstrate that, depending on the stimulus, PI3K inhibitors may display opposite effects as a function of their concentration. In this study, most of the effects of the PI3Kδ inhibitor were already observed at concentration ≤ 1 μM (Fig. 3A), i.e., around the IC$_{50}$ which is 0.5 μM, thus discarding most of putative unspecific effects.

The enhancement of pro-inflammatory cytokine production upon PI3K inhibition is reminiscent of a study showing that, in vivo, the pharmacological inhibition of PI3Ks (Ly294002 or wortmannin) results in increased serum levels of cytokines including IL-1β, TNF and IL-6 in septic mice (Williams et al., 2004). The differential control of pro- and anti-inflammatory factors by PI3Ks was also described in vitro in monocytes activated by *P. gingivalis* LPS, i.e., through toll-like receptor 2. In the latter system, the inhibition of PI3Ks diminished the production of the anti-inflammatory cytokine IL-10 and concomitantly enhanced that of IL-12, a pro-

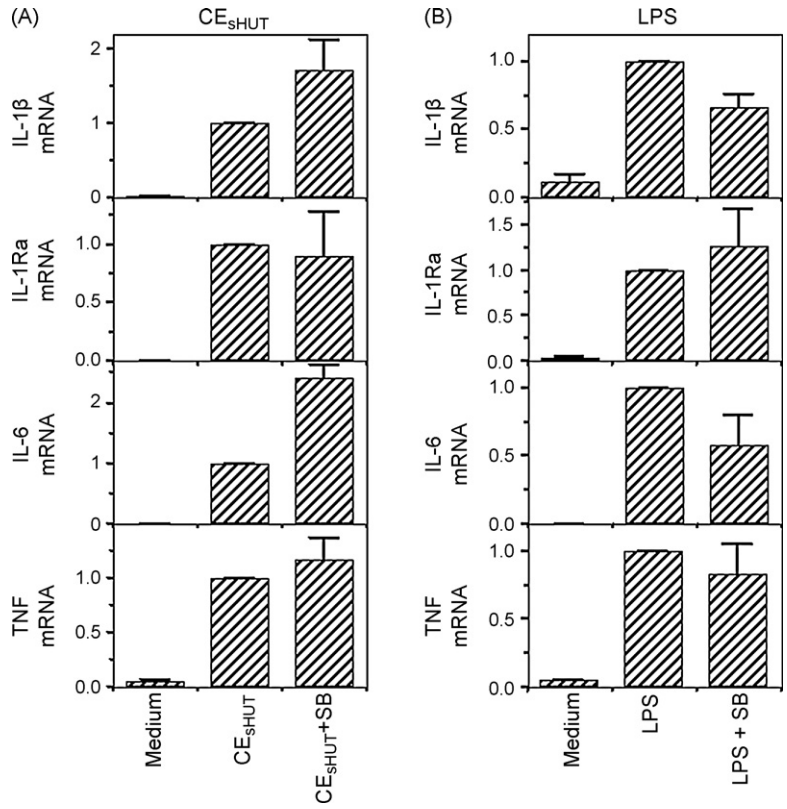

Fig. 7. GSK3β inhibition regulates cytokines mRNA expression in human monocytes activated by CE$_{sHUT}$ or LPS. Isolated monocytes (3×10^6 cells/3 ml) were preincubated for 60 min in the presence or absence of 5 μM SB203580 and then stimulated or not with either CE$_{sHUT}$ (3 μg/ml proteins) or LPS (100 ng/ml) in 6-well plates, as described in Section 2. After 3 h activation, cell culture was stopped cytokine mRNA levels were determined by duplex quantitative real-time PCR analysis of triplicates normalized to the levels of the 18S mRNA as described in Section 2. Experiments were performed in the presence (CE$_{sHUT}$) or absence (LPS) of polymyxin B (5 μg/ml). The relative expression levels of cytokine mRNA are presented as mean ± S.D. of percentage of relative cytokine mRNA expression induced by CE$_{sHUT}$ or LPS. The value of mRNA levels in the absence of inhibitor being arbitrarily considered as 1.0. Results are representative of three distinct experiments.

inflammatory cytokine (Martin et al., 2003). The repressing effects of PI3Ks on pro-inflammatory cytokine production have also been described in monocytes activated by contact with stimulated T cells. Indeed, cytokine-activated T cells and synovial T cells isolated from rheumatoid arthritis patients induce TNF production in human monocytes, the latter being enhanced upon PI3K inhibition by Ly294002 and wortmannin (Brennan et al., 2002). In contrast, IL-10 production induced by cytokine-activated T cells in macrophages was suppressed upon inhibition of PI3Ks (Foey et al., 2002). Together with the present study, the latter support anti-inflammatory functions of PI3Ks in human monocytes activated under chronic/sterile conditions. However, a foremost limitation of PI3K inhibitors, such as LY294002 and Wortmannin, as demonstrated in this study, is the broad effect of these drugs on all class I PI3K isoforms (Ly294002) and even PI3K classes (wortmannin), as well as known off-target effects on other signaling molecules (Brunn et al., 1996; Izzard et al., 1999; Meyers and Cantley, 1997; Nakanishi et al., 1992). PI3K enzymes comprise several structurally and functionally distinct classes, with class IA being responsible for most of the signaling downstream tyrosine kinase-coupled receptors such as TLR-mediated signaling (Vanhaesebroeck et al., 2001). Class IA PI3Ks are heterodimers composed of a catalytic subunit (p110α, p110β, or p110δ) and an adaptor protein (p85α, p85β, p55γ) (Vanhaesebroeck et al., 2001). Adaptor proteins may regulate any of the three p110 catalytic subunits. This implies that the decrease of one type of p110 subunit (knock out mice or RNA interference) simultaneously decreases the level of adaptor proteins, and in turn diminishes the level of other subtypes of class IA PI3Ks (Brachmann et al., 2005). This justifies the usage of selective pharmacological inhibitors to dissect class I PI3K pathway.

PI3Kδ controls cytokine transcription in both chronic/sterile and acute/infectious inflammatory conditions by inducing and dampening it down, respectively (Fig. 4). Since IC87114 inhibits the phosphorylation of Akt in both LPS and CE$_{sHUT}$-activated monocytes, it is likely that transcription control by PI3Kδ occurs through Akt activation. An important element downstream Akt that controls several transcription factors is GSK3β (Doble and Woodgett, 2003; Hoeflich et al., 2000). Our results suggest that cytokine production in human monocytes is ruled by PI3Kδ throughout the inactivation of GSK3β (Figs. 6 and 7). In accordance with the present results, GSK3β blockade was shown to differentially regulate the production of pro- (IL-1β, IL-12p40, and IL-6) and anti- (IL-10) inflammatory cytokines in human PBMC activated through ligation of toll-like receptor 4 (TLR4) by *E. coli* synthetic lipid A (Martin et al., 2005). Interestingly, upon chronic/sterile monocytes activation, GSK3β displays opposite activity to that observed upon activation by LPS (i.e., through TLR4 ligation). Indeed, in CE$_{sHUT}$-activated monocytes GSK3β activity dampens pro-inflammatory cytokine production. Once GSK3β is inactivated after PI3Kδ activation, cytokine production escapes this control. This is just the inverse mechanism that is set-in upon acute/infectious activation of monocytes.

5. Conclusions

The present study demonstrates the opposite usage of the PI3Kδ/Akt/GSK3β pathway in chronic/sterile and acute/infectious

inflammation. This is in accordance with studies showing that the PI3K/Akt signaling pathway exerts protective effects in animal models of acute/infectious inflammation (Bommhardt et al., 2004; Hirsch et al., 2000; Williams et al., 2004), but detrimental effects in models of chronic/sterile inflammation such as rheumatoid arthritis and systemic lupus (Camps et al., 2005) (Barber et al., 2005). Thus, this pathway and particularly its upstream element, PI3Kδ could be an ideal therapeutic target in inflammatory diseases as previously stated (Wymann et al., 2003). The usage of PI3Kγ specific inhibitor suppressed joint inflammation in an animal model of rheumatoid arthritis by inhibiting leukocyte recruitment to inflammatory sites (Camps et al., 2005). In the latter study, the effect of the inhibitor in acute/infectious diseases was not studied, but the mechanisms of extravasation of leukocytes are likely to be similar in both chronic/sterile and acute/infectious conditions. Here we show that the inhibition of PI3Kδ should display favorable effects in both situations, by inhibiting cytokine production upon pathologic inflammatory condition and boosting it in normal defense processes.

Acknowledgements

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6.2 A Novel MEK2/PI3Kδ Pathway Controls the Expression of IL-1 Receptor Antagonist in IFNβ-Activated Human Monocytes

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Article

A novel MEK2/PI3Kδ pathway controls the expression of IL-1 receptor antagonist in IFN-β-activated human monocytes

Karim J. Brandt, Rakel Carpintero,* Lyssia Gruaz,* Nicolas Molnarfi,† and Danielle Burger*,1*

*Division of Immunology and Allergy, Inflammation and Allergy Research Group, Hans Wilsdorf Laboratory, Department of Internal Medicine, University Hospital and Faculty of Medicine, University of Geneva, Geneva, Switzerland; and †Department of Neurology and Program in Immunology, University of California, San Francisco, California, USA

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ABSTRACT

IFN-β and sIL-1Ra play crucial roles in the regulation of innate immunity and inflammation. IFN-β, which is widely used to improve the course of relapsing, remitting multiple sclerosis, induces the production of sIL-1Ra in human monocytes through mechanisms that remain largely unknown. In this study, we identified PI3Kδ and MEK2 as key elements that control sIL-1Ra production in isolated human monocytes activated by IFN-β. Blockade of MEK2, but not of MEK1, by inhibitors and siRNA prevented IFN-β-induced PI3Kδ recruitment to the membrane, Akt phosphorylation, and sIL-1Ra production, suggesting that MEK2 acted upstream of PI3Kδ. Furthermore, ERK1/2, the only identified substrates of MEK1/2 to date, are dispensable for sIL-1Ra production in response to IFN-β stimulation. Upon IFN-β activation, MEK2 and PI3Kδ are translocated to monocyte membranes. These data suggest that MEK1 and MEK2 display different, nonredundant functions in IFN-β signaling. That neither MEK1 nor ERK1/2 play a part in this mechanism is also an unexpected finding that gives rise to a better understanding of the MAPK signaling network. Together, these findings demonstrate that IFN-β triggers an atypical MEK2/PI3Kδ signaling cascade to regulate sIL-1Ra expression in monocytes. The premise that MEK1 and MEK2 play a part in the induction of the proinflammatory cytokine, IL-1β in human monocytes provides a rationale for an alternative, IFN-β-mediated pathway to induce/enhance sIL-1Ra production and thus, to dampen inflammation. *J. Leukoc. Biol.* 88: 000–000; 2010.

Introduction

Although inflammation is an important, protective response, whose purpose is to rid the organism of infectious and toxic agents, it may be self-damaging and has to be tightly controlled in time and space by the organism. IL-1 (α and β) is a mediator of the inflammatory response, thus playing an important part in the body's defense mechanism but also in the development of pathological conditions that ultimately lead to chronic inflammation and autoinflammatory diseases [1, 2]. Although IL-1 production and its effects may be tempered to some extent by anti-inflammatory cytokines, one particularly powerful, natural inhibitor, sIL-1Ra, inhibits IL-1 inflammatory effects. Patients deficient in sIL-1Ra display autoinflammatory disorders, whose symptoms can be alleviated by treatment with rsIL-1Ra (anakinra) [3]. Treatment with anakinra is also efficient in autoinflammatory disorders as a result of mutations in the cryopyrin gene and gout [2, 4, 5]. These observations highlight the importance of sIL-1Ra in limiting the activity of IL-1 in physiological and pathological conditions in the human system. sIL-1Ra inhibits IL-1 activity by binding type I IL-1R without triggering signaling [6, 7]. As it potently inhibits the various effects of IL-1, sIL-1Ra is considered an important regulator of the inflammatory and overall immune response mediated by IL-1 [1]. Although treatment of chronic inflammatory diseases with anakinra has drawbacks as a result of its short lifespan, increased levels of sIL-1Ra were detected in multiple sclerosis patients treated with immunomodulators such as IFN-β and glatiramer acetate [8–11]. As sIL-1Ra is able to cross the blood-brain barrier [12], its enhancement in peripheral blood might be linked to the beneficial effects of IFN-β and glatiramer acetate in multiple sclerosis.

IFN-β is a type I IFN that plays an important role in the innate immune response to viral infection and is widely used to improve the clinical course of relapsing, remitting multiple sclerosis. We demonstrated previously that IFN-β induces the production of sIL-1Ra in human monocytes [13, 14] and that sIL-1Ra was an immediate, early gene downstream of IFN-β signaling [15]. Past studies have revealed that IFN-β signals mainly through the JAK/STAT1 pathway, although alternative pathways are also activated and required to induce transcription of a number of IFN-β-dependent genes [16]. These alter-

Abbreviations: IRS=insulin receptor substrate, PH=pleckstrin homology, PIP_3=phosphatidylinositol (3,4,5) trisphophate, qPCR=quantitative PCR, sIL-1Ra=secreted IL-1R antagonist, siRNA=small interfering RNA

1. Correspondence: Hans Wilsdorf Laboratory, IARG, BB-4866, University Hospital, 4 rue Gabrielle Perret-Gentil, CH-1211 Geneva 14, Switzerland. E-mail: danielle.burger@hcuge.ch

native pathways comprise PI3K and MAPK [16–18]. In this regard, we showed that the faculty of IFN-β to trigger sIL-1Ra production subsisted independently of the canonical STAT1 pathway, signaling through PI3K [15]. There are three classes of PI3Ks, of which only class I enzymes (α, β, δ, and γ isoforms) catalyze the synthesis of PI_3 in the membranes, thus eliciting the recruitment of downstream elements such as Akt/PKB via binding to the PH domain [19]. Among class I PI3Ks, PI3Kγ and -δ are expressed more specifically in leukocytes and involved in inflammatory processes [20]. We previously established that PI3Kδ is the PI3K isoform that controls sIL-1Ra production induced in human monocytes by stimuli related to acute or chronic inflammatory conditions [21]. This anti-inflammatory activity of PI3Kδ deserves attention, as specific PI3Kδ inhibitors are currently being developed for clinical use, not only in cancer, where inflammation might help to fight tumors, but also in inflammatory and/or autoimmune diseases, where inflammation contributes to part of pathogenic processes [22].

Recently, it was shown that LPS-induced production of sIL-1Ra in human monocytes is down-regulated by GSK3, downstream of PI3K, via the MAPK MEK1/2–ERK1/2 axis [23]. MEK1 and MEK2 are two Thr/Tyr dual MAPKs, whose activation is triggered by a wide variety of stimuli. ERK1 and ERK2, i.e., ERK1/2, are the only MEK1/2 substrates that have been identified to date [24]. MEK1 and MEK2 display 80% sequence homology and are ubiquitously expressed in cells and tissues [25]. Although it is commonly assumed that the two isoforms are functionally equivalent, several lines of evidence indicate that they are regulated differentially and that each may fulfill nonredundant functions [26–30]. MEK1 knockout mice display recessive lethality—their homozygous mutant embryos dying by Day 10.5 of gestation [31]. In contrast, MEK2 knockout mice are viable and fertile and show no phenotypic abnormalities [32]. Studies using RNA interference suggested that MEK1 and MEK2 are necessary for in vitro cell proliferation and that they contribute to distinct cell cycle regulatory events [33]. Activated MEK1, but not MEK2, promotes epidermal hyperplasia in transgenic mice, although both MEK proteins trigger ERK1/2 phosphorylation [34]. More recently, it was demonstrated that MEK1 and MEK2 exert distinct, nonredundant effects on the replication of herpes simplex virus type 2 [35]. In mouse macrophages activated by TNF, there is no evidence of MEK2 activation, as MEK1 is the preferred upstream kinase implicated in activation of ERK1/2 [36]. Thus, MEK1 and MEK2 may fulfill different functions depending on cell types and cellular conditions, suggesting the existence of substrates different from ERK1/2. Finally, active MEK2 may function as a regulatory scaffold promoting a crosstalk between different pathways [37].

In this study, we addressed the question of the identity of the PI3K isoform as well as the contribution of the MEK/ERK pathway to the induction of sIL-1Ra production in human monocytes activated by IFN-β. The results reveal an unforeseen pathway that requires MEK2 but not MEK1 and ERK1/2 upstream of PI3Kδ activation, which induces sIL-1Ra expression in monocytes activated by IFN-β.

MATERIALS AND METHODS

Materials

FCS, streptomycin, penicillin, L-glutamine, RPMI 1640, and PBS free of Ca^{2+} and Mg^{2+} (Gibco, Paisley, Scotland); Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden); Tri®Reagent, PMSF, endotoxin-free DMSO, LPS, and polymyxin B sulfate (Sigma Chemical Co., St. Louis, MO, USA); the inactive kinase inhibitor analog U0124 (Calbiochem-Novabiochem Corp., San Diego, CA, USA); kinase inhibitors Ly294002, PD98059, and U0126 (LC Laboratories, Woburn, MA, USA); PI3Kα, -β, and -γ selective inhibitors, compound 15e, TGX-221, and AS-604850, respectively (Alexis Corp., Switzerland); and kinase-specific Stealth RNAi™ siRNA and Stealth RNAi™ negative control duplex (Invitrogen, Carlsbad, CA, USA) were purchased from the designated suppliers. IC87114 was kindly provided by Calistoga Pharmaceuticals, Inc. (Seattle, WA, USA). All other reagents were of analytical grade or better.

Monocytes

Monocytes were isolated from buffy coats of blood from healthy donors provided by the Geneva Hospital Blood Transfusion Center (Switzerland) as described previously [38]. To avoid activation by endotoxin, 5 μg/ml polymyxin B sulfate was added in all experiments. Monocyte preparation routinely consisted of >90% $CD14^+$ cells, <1% $CD3^+$ cells, and <1% $CD19^+$ cells as assessed by flow cytometry.

sIL-1Ra production

Monocytes (5×10^4 cells/200 μl/well) were preincubated for 45 min in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 U/ml penicillin, 2 mM glutamine (medium), and 5 μg/ml polymyxin B sulfate with the indicated concentration of kinase inhibitors or DMSO as control and then activated for 24 h with 1×10^4 U/ml IFN-β. All conditions were carried out in triplicate. Culture supernatants were tested for the production of sIL-1Ra by a commercially available enzyme immunoassay (Quantikine, R&D Systems, Minneapolis, MN, USA).

mRNA quantification

Monocytes (2×10^6 cells/3 ml/well) were cultured in medium containing 5 μg/ml polymyxin B sulfate in six-well plates with the indicated inhibitors for 45 min and then activated by 1×10^4 U/ml IFN-β for 3 h. Total mRNA was prepared by Tri®Reagent (Sigma Chemical Co.), according to the provided protocol. Real-time duplex qPCR analysis was conducted after reverse transcription by SuperScript II (Invitrogen) on a TaqMan 7300 real-time qPCR system (Applied Biosystems, Foster City, CA, USA). The levels of mRNA expression were normalized against the expression of a housekeeping gene (18S) analyzed simultaneously. sIL-1Ra and 18S probes were purchased from Applied Biosystems. All measurements were conducted in triplicates.

Western blot

Human monocytes were resuspended at 6×10^6 cells/ml in medium supplemented with 5 μg/ml polymyxin B sulfate, and 500 μl was placed in 2 ml polypropylene tubes (Eppendorf) at 37°C. After 1 h, inhibitors were added for 45 min before activation with IFN-β (1×10^4 U/ml). At the indicated time, the reaction was stopped by the addition of 800 μl ice-cold PBS and centrifugation. Total cell lysate was prepared and subjected to Western blot analysis as described previously [15]. The blots were probed with the following antibodies: anti-phospho-p44/42 MAPK (Thr_{202}/Tyr_{204}), anti-p44/42 (ERK1/2), anti-phospho-Akt (Ser_{473}), anti-phospho-Akt (Thr_{308}), anti-Akt, and anti-MEK1 (Cell Signaling Technology, Beverly, MA, USA); anti-MEK2, anti-PI3Kγ, and anti-PI3Kβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-PI3Kα (Becton Dickinson, San Diego, CA, USA); anti-PI3Kδ (Calbiochem-Novabiochem Corp.); and anti-β-tubulin (Sigma Chemical Co.). Secondary HRP-conjugated goat anti-rabbit or goat anti-mouse antibodies were from Dako (Copenhagen, Denmark), and sheep anti-rabbit

antibody was from Covalab (France). Antibody-bound proteins were detected by the Uptilight HRP blot chemiluminescent substrate (Uptima, Interchim, Monluçon, France). Alternatively, secondary IR700/800-conjugated goat anti-rabbit, goat anti-mouse antibodies, or rabbit anti-goat antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA) were used, and antibody-bound proteins were detected by the Odyssey system (Li-Cor, Lincoln, NE, USA).

mRNA silencing

PI3Ks, MEK1, MEK2, and ERK1/2 were silenced with 4 μM stealth siRNA designed by the supplier (Invitrogen); 4 μM Stealth RNAi™ negative control duplex was transfected as negative (mock) control. Monocytes were transfected using a Nucleofector device and Nucleofector human monocyte kit, according to the supplier's protocol (Amaxa Lonza). Transfected monocytes ($3–6\times10^6$ cells/1.6 ml/well) were seeded into 24-well ultra-low attachment plates (Corning, Corning, NY, USA). After 48 h, transfected cells were harvested. PI3K, MEK1, MEK2, and ERK1/2 silencing was ascertained by Western blot using anti-PI3K, anti-MEK1, anti-MEK2, and anti-p42/p44 ERK1/2-specific antibodies. The capacity of silenced monocytes to produce sIL-1Ra upon IFN-β treatment was measured as described above.

Recruitment of kinases to monocyte membranes

Monocytes (10×10^6 cells/ml; 1 ml) were activated by IFN-β (1×10^4 U/ml) for 90 min. The activation was stopped by the addition of ice-cold PBS and centrifugation. Monocytes were then resuspended in 50 mM HEPES, pH 7.3, containing 150 mM NaCl, 5 mM EDTA, 1 mM $NaVO_4$, 50 mM NaF, 1 μM leupeptin, 1 μM pepstatin, and 20 μM PMSF and lysed by 10 freeze-thaw cycles. Membranes were isolated by ultracentrifugation during 45 min. The pellets were resuspended and analyzed by Western blot using anti-PI3Kδ and anti-MEK2 antibodies as described above.

Statistics

When required, significance of differences between groups was assessed using Student's paired *t* test.

RESULTS

PI3Kδ is the only PI3K isoform essential for sIL-1Ra production in human monocytes activated by IFN-β

We demonstrated previously that sIL-1Ra production in human monocytes requires the activation of PI3K but not of STAT1 in response to IFN-β activation [15]. To distinguish the respective contributions of the four class I PI3K isoforms in this mechanism, we assessed the expression of PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ in human monocytes, which expressed the four class I PI3K isoforms as demonstrated by immunoblotting analyses of cytoplasm proteins (**Fig. 1A**). IFN-β treatment led to the exclusive recruitment of PI3Kδ to monocyte membranes (Fig. 1A), indicating that this isoform plays a key role in IFN-β signaling. To investigate the role of PI3Kδ in the induction of sIL-1Ra production, we used small molecule inhibitors, class I PI3K isoform-selective. As demonstrated previously, the level of sIL-1Ra production induced by IFN-β (1×10^4 U/ml) varied greatly depending on monocyte preparation (blood donor); mean ± SD production of all experiments performed in this study ($n>30$) amounted to 6.6 ± 1.7 ng/ml sIL-1Ra. We are therefore presenting all results as percentages of sIL-1Ra production observed in the absence of kinase blockade. The

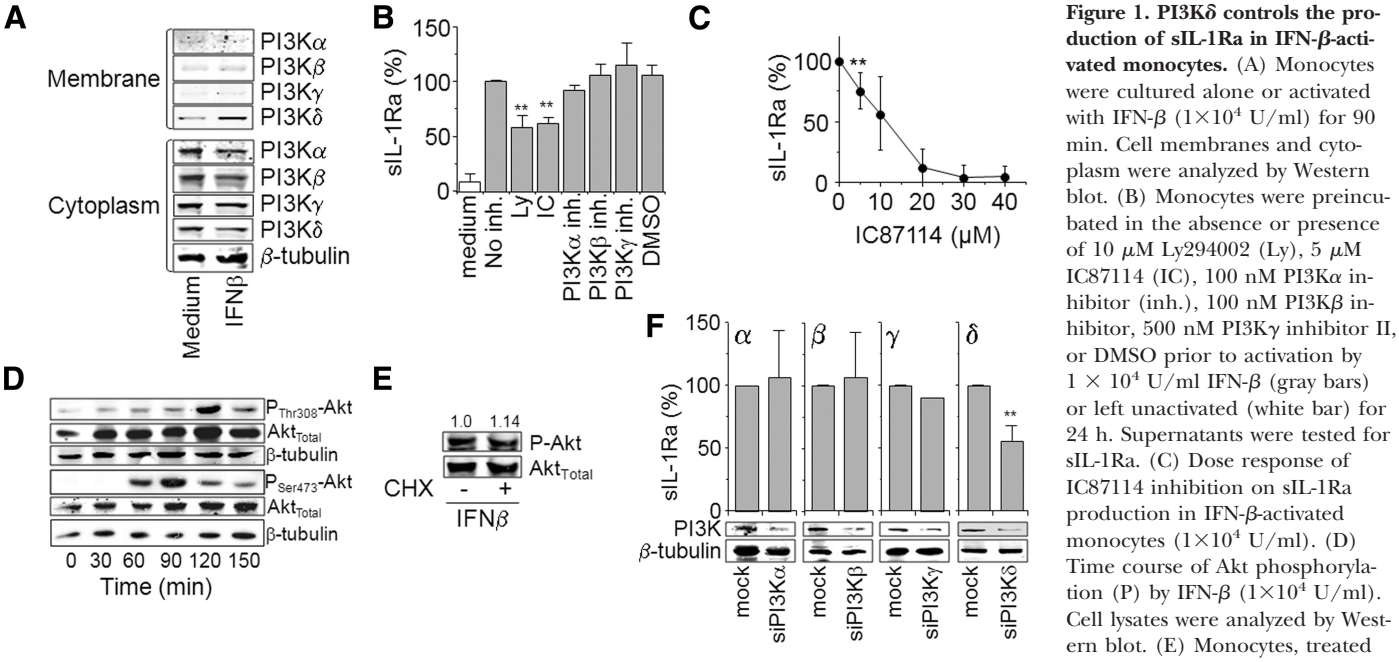

Figure 1. PI3Kδ controls the production of sIL-1Ra in IFN-β-activated monocytes. (A) Monocytes were cultured alone or activated with IFN-β (1×10^4 U/ml) for 90 min. Cell membranes and cytoplasm were analyzed by Western blot. (B) Monocytes were preincubated in the absence or presence of 10 μM Ly294002 (Ly), 5 μM IC87114 (IC), 100 nM PI3Kα inhibitor (inh.), 100 nM PI3Kβ inhibitor, 500 nM PI3Kγ inhibitor II, or DMSO prior to activation by 1×10^4 U/ml IFN-β (gray bars) or left unactivated (white bar) for 24 h. Supernatants were tested for sIL-1Ra. (C) Dose response of IC87114 inhibition on sIL-1Ra production in IFN-β-activated monocytes (1×10^4 U/ml). (D) Time course of Akt phosphorylation (P) by IFN-β (1×10^4 U/ml). Cell lysates were analyzed by Western blot. (E) Monocytes, treated with 10 μM cycloheximide (CHX) or untreated, were activated for 90 min by IFN-β (1×10^4 U/ml). Cell lysates were analyzed for Akt phosphorylation and quantified (Odyssey system, Li-Cor). (F) Monocytes were nucleofected with stealth siRNA for the indicated PI3K isoform or Stealth RNAi™ negative control duplex (mock). The efficiency of PI3K silencing (si) was assessed by Western blot. PI3K knockdown or mock-transfected monocytes were activated with IFN-β (1×10^4 U/ml) before measuring sIL-1Ra. Western blot data are representative of three separate experiments. sIL-1Ra production is presented as percentage of production in the absence of inhibitor; mean ± SD of separate experiments carried out on monocytes prepared from three different blood donors. $^{**}P < 0.01$, determined by Student's *t* test.

PI3Kδ-specific inhibitor IC87114 and the PI3K pan inhibitor Ly294002 significantly inhibited IFN-β-induced sIL-1Ra production, resulting in 33 ± 6% and 38 ± 13% inhibition, respectively (Fig. 1B). In contrast, the specific blockade of PI3Kα, PI3Kβ, and PI3Kγ activity did not affect sIL-1Ra production (Fig. 1B), suggesting that these PI3K isoforms were not required for the production of sIL-1Ra induced by IFN-β, as shown in Fig. 1A. Treatment of human monocytes with the p110δ-selective inhibitor IC87114 decreased sIL-1Ra production induced by IFN-β in a dose-dependent manner and led to the complete abrogation at a dose of 20 μM (Fig. 1C). To avoid nonspecific inhibitory effects usually observed at high inhibitor concentrations [39], we used IC87114 at a low concentration (i.e., 5 μM) in subsequent analyses.

To further define the role of PI3Kδ in IFN-β signaling, we examined the effects of PI3Kδ inhibition on activation of Akt, a downstream element of the PI3K axis. As expected, IFN-β induced the phosphorylation of Akt in monocytes in a time-dependent manner (Fig. 1D). In contrast with STAT1 [15], Akt phosphorylation was not observed in early activation times (not shown), Ser_{473} phosphorylation reached a peak at 1.5 h, and phosphorylation of Thr_{308} occurred at 2 h (Fig. 1D), suggesting a sequential phosphorylation of Akt. Although it was delayed, phosphorylation of Akt was not a result of a putative autocrine loop of an IFN-β-induced factor, as shown by the lack of effect of cycloheximide, a protein synthesis inhibitor (Fig. 1E). To validate the results obtained with IC87114, we further analyzed the role of PI3Kδ in IFN-β-induced sIL-1Ra production in human monocytes by using stealth siRNAs that target the different class I PI3K catalytic subunits. Despite achieving a marked knockdown of p110α, -β, and -γ, the IFN-β-induced production of sIL-1Ra did not differ from that observed in mock-transfected monocytes (Fig. 1F). In contrast, the knockdown of p110δ expression by 46.3 ± 18.3% reduced by 43.8% ± 11.5% sIL-Ra production by human monocytes in response to IFN-β activation. Together, these results indicate that PI3Kδ plays an essential part in controlling sIL-1Ra production by IFN-β-activated monocytes.

Activity of MEK2 but not MEK1 is sufficient to regulate the induction of sIL-1Ra production by IFN-β in human monocytes

Recent data suggest that sIL-1Ra production is regulated by mechanisms implicating MAPK MEK1/2–ERK1/2 pathway activation [23, 40]. Using pharmacological kinase inhibitors, we determined that U0126, a dual inhibitor of MEK1 and MEK2, significantly decreased the production of sIL-1Ra in response to IFN-β, reaching 48.6 ± 8.5% inhibition at a concentration of 5 μM (**Fig. 2A**). In contrast, MEK1-specific inhibitor PD98059 and U0124, the inactive analog of U0126, did not diminish sIL-1Ra production (Fig. 2A). Of note, the sIL-1Ra production induced by LPS in the amount of 17.9 ± 6.4 ng/ml (mean±SD; n=3 different monocyte preparations) was reduced to a similar extent in the presence of U0126 and PD98059, indicating that the latter molecule was active (Fig. 2B). Consequently, MEK1 activity did not contribute to the control of sIL-1Ra production in IFN-β-activated human monocytes. To avoid nonspecific inhibitory effects usually observed at high inhibitor concentrations [39], we used a low concentration of U0126 (i.e., 5 μM), which significantly inhibited sIL-1Ra production. However, IFN-β-induced sIL-1Ra production reverted to basal levels when higher doses of U0126 were used (Fig. 2C). To examine further whether MEK2 regulated the levels of sIL-1Ra mRNA, we measured the effects of MEK1 and MEK2 blockade on the expression of the sIL-1Ra transcript in response to IFN-β stimulation. As shown in Fig. 2D, U0126 treatment significantly decreased sIL-1Ra mRNA expression in IFN-β-treated monocytes, whereas PD98059 failed to affect sIL-

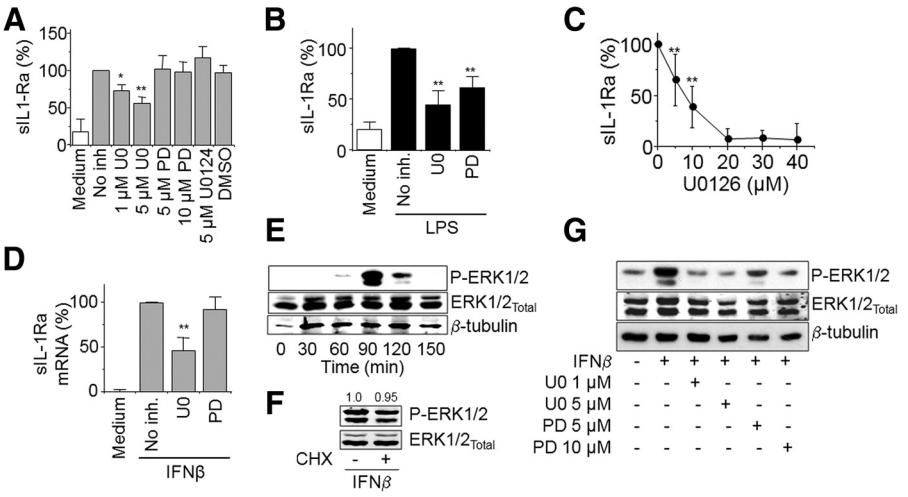

Figure 2. MEK2 but not MEK1 regulates the production of sIL-1Ra in human monocytes activated by IFN-β. (A) Monocytes were preincubated with inhibitors, U0126 (U0) and PD98059 (PD), U0124, or DMSO and were then left unstimulated (white bar) or stimulated (gray bars) with IFN-β for 24 h. (B) Monocytes were preincubated with 5 μM of the indicated inhibitor and were then left unstimulated (white bar) or stimulated (black bars) with 100 ng/ml LPS for 24 h in the absence of polymyxin B sulfate. (C) Dose response of U0126 inhibition on sIL-1Ra in IFN-β-activated monocytes (1×10^4 U/ml). (A–C) Supernatants were tested for sIL-1Ra. (D) Monocytes were treated with 5 μM of the indicated inhibitor before addition (gray bars) of IFN-β (1×10^4 U/ml) or none (white bar). After 3 h, mRNA was analyzed by qPCR. (E) Time course of ERK1/2 activation by IFN-β (1×10^4 U/ml). Cell lysates were analyzed by Western blot. (F) Monocytes, treated with 10 μM cycloheximide or untreated, were activated for 90 min by IFN-β (1×10^4 U/ml). Cell lysates were analyzed for ERK1/2 phosphorylation and quantified (Odyssey system, Li-Cor). (G) Monocytes were preincubated with U0126 or PD98059 and then activated with IFN-β (1×10^4 U/ml) for 90 min prior to immunoblotting analysis. Western blot data are representative of three separate experiments. sIL-1Ra production and mRNA data are presented as percentages of production in the absence of inhibitor; mean ± SD of separate experiments carried out on monocytes prepared from three different blood donors. *$P < 0.05$; **$P < 0.01$, determined by Student's t test.

1Ra transcript levels. Collectively, these findings indicate that MEK2 but not MEK1 was involved in the control of sIL-1Ra production in IFN-β-activated human monocytes.

To determine the precise involvement of ERK1/2, i.e., the only known substrates of MEK1/2, in sIL-1Ra production, we assessed the effects of MEK1 and/or MEK2 blockade on ERK1/2 phosphorylation. As depicted in Fig. 2E, the activation of monocytes by IFN-β induced phosphorylation of ERK1/2, which was detectable after 60 min, reached its highest level at 90 min, and reverted to a basal level after 150 min. The phosphorylation pattern of ERK1/2 was comparable with that of Akt (Ser_{473}), suggesting the coordinate phosphorylation of a PI3K downstream element and MEK1/2 substrates (Figs. 1D and 2E). Like Akt phosphorylation, although it was delayed, phosphorylation of ERK1/2 was not a result of a putative autocrine loop of an IFN-β-induced factor, as evidenced by the lack of effect of cycloheximide, a protein synthesis inhibitor (Fig. 2F). The blockade of MEK1 and/or MEK2 activity by PD98059 and U0126 significantly reduced the extent of ERK1/2 phosphorylation in response to IFN-β activation (Fig. 2G). Moreover, the fact that PD98059 inhibited ERK1/2 phosphorylation (Fig. 2G) without affecting sIL-1Ra production (Fig. 2A) was suggestive of a signaling pathway controlling IFN-β-induced sIL-1Ra production, regardless of ERK1/2 activation.

ERK1/2 activity is dispensable to MEK2-dependent regulation of IFN-β-induced sIL-1Ra production

To strengthen the data obtained with pharmacological kinase inhibitors, we analyzed the functional consequences of silencing of MEK1 and MEK2 expression on the IFN-β-induced production of sIL-1Ra. As shown by immunoblotting analysis, transfection of monocytes with MEK1 siRNA resulted in knockdown of MEK1 expression without altering MEK2 and ERK1/2 expression, whereas transfection with MEK2 siRNAs markedly knocked down MEK2 expression without affecting the expression of MEK1 and ERK1/2 (**Fig. 3A**). Similarly, siRNA-mediated knockdown of ERK1 and ERK2 lowered their protein expression without any effect on MEK1 or MEK2 expression (Fig. 3A). As shown in Fig. 3B, the silencing of MEK2 expression, although incomplete, diminished by 69 ± 23% (P=0.019) sIL-1Ra production induced by IFN-β, thus confirming the specificity of the inhibitory effect of U0126 (Fig. 2A). In contrast, knockdown of MEK1 or ERK1/2 left sIL-1Ra production unchanged (Fig. 3B). These results demonstrate that inactivation of MEK2 decreased the production of sIL-1Ra in response to IFN-β, despite the presence of high levels of MEK1 and ERK1/2, thus ruling out the involvement of MEK1 and ERK1/2 in the production of sIL-1Ra. These findings support the concept that IFN-β activates a MEK2-dependent but MEK1- and ERK1/2-independent pathway that triggers sIL-1Ra expression in monocytes.

MEK2 and PI3Kδ integrate a common pathway controlling sIL-1Ra production in IFN-β-activated human monocytes

We previously established that PI3K activation is required for optimal sIL-1Ra production in IFN-β-activated monocytes [15].

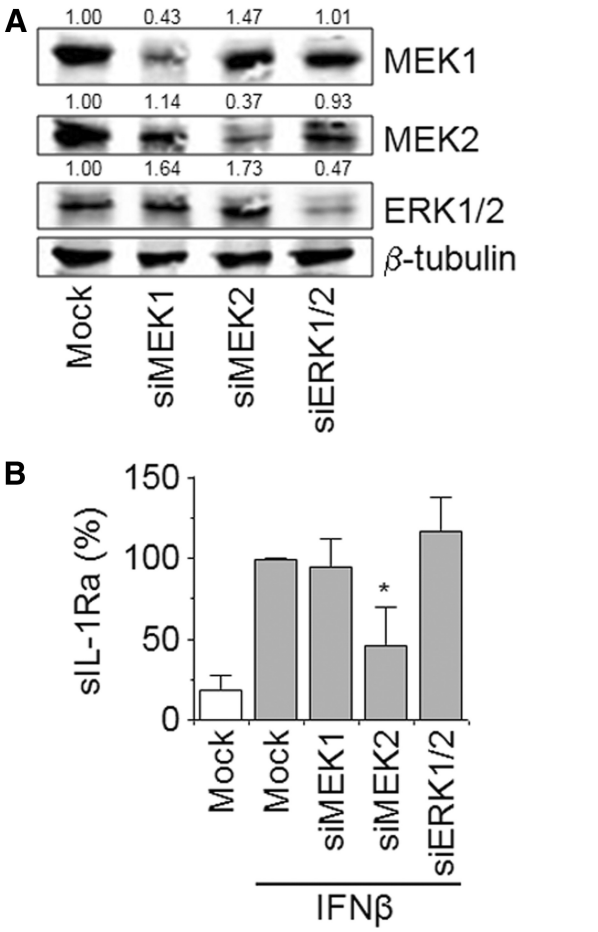

Figure 3. MEK2 controls sIL-1Ra production in IFN-β-activated monocytes via an ERK1/2-independent pathway. (A) Monocytes were nucleofected with stealth siRNA for MEK1, MEK2, ERK1, and ERK2 or Stealth RNAi™ negative control duplex (Mock). The efficiency of MEK1, MEK2, and ERK1/2 silencing was assessed by Western blot and quantified (Odyssey system, Li-Cor). (B) Knockdown monocytes were activated (gray bars) by IFN-β (1×10^4 U/ml) or left unactivated (white bar) for 24 h. Supernatants were tested for sIL-1Ra. Western blot data are representative of three separate experiments. sIL-1Ra production is presented as percentage of production in mock-nucleofected monocytes; mean ± SD of three separate experiments carried out on monocytes prepared from three different blood donors. *P = 0.019, determined by Student's t test.

The data shown herein extend these observations by illustrating that the activation of PI3Kδ and MEK2 was crucial to the control of sIL-1Ra production. To assess the sequence of PI3Kδ and MEK2 actions in the triggering of sIL-1Ra production by IFN-β, specific kinase inhibitors were used alone or in combination. PI3K pan-inhibitor (Ly294002), specific PI3Kδ inhibitor (IC87114), and dual MEK1/2 inhibitor (U0126) all proved capable of decreasing sIL-1Ra production by 40–50%. Although only low concentrations of the inhibitors were used, simultaneous addition of PI3K and MEK1/2 inhibitors did not enhance the inhibition of sIL-1Ra production (**Fig. 4A**), demonstrating that the inhibitors did not exert additional effects. These data strongly suggest that PI3Kδ and MEK2 cooperate

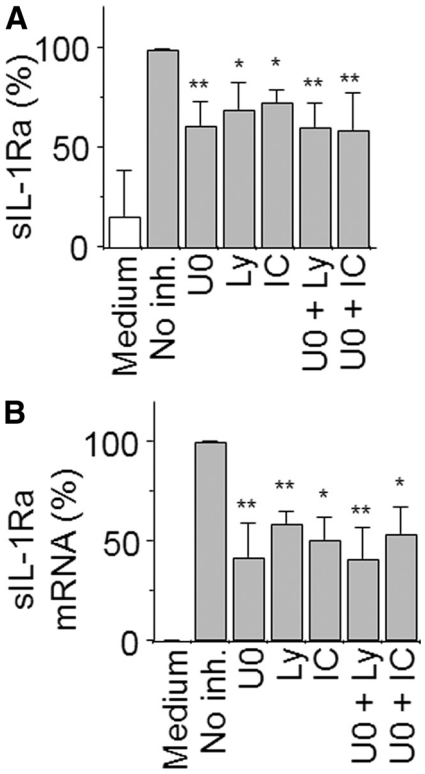

Figure 4. A MEK2/PI3Kδ pathway controls sIL-1Ra production by IFN-β-activated monocytes. (A) Monocytes were preincubated for 24 h with 5 μM U0126, 10 μM Ly294002, or 5 μM IC87114 or a mixture of indicated inhibitors prior to activation by IFN-β (1×10^4 U/ml; gray bars) or left unactivated (white bar). Supernatants were tested for sIL-1Ra production. (B) Monocytes were treated with the indicated inhibitors before addition of IFN-β (1×10^4 U/ml). After 3 h, mRNA was analyzed by qPCR. Results are presented as mean ± SD of three independent experiments, and 100% is the amount of sIL-1Ra (A) or sIL-1Ra mRNA (B) induced by IFN-β in the absence of inhibitor. $^*P < 0.05$; $^{**}P < 0.01$, determined by Student's *t* test.

sequentially in a common pathway in regulating IFN-β-induced sIL-1Ra production by monocytes. The levels of sIL-1Ra transcript expression were down-regulated upon monocyte treatment with Ly294002, IC87114, and U0126 (Fig. 4B), thus correlating with protein production. The combination of MEK1/2 and PI3K inhibitors did not result in an enhanced inhibitory effect on sIL-1Ra mRNA levels, suggesting that MEK2 and PI3Kδ are parts of a common pathway controlling sIL-1Ra expression.

MEK2 controls Akt activation

As described above (Fig. 3), the silencing of ERK1 and ERK2 expression did not affect IFN-β-induced sIL-1Ra production by human monocytes, suggesting that MEK2 activated an alternative pathway independent of ERK1/2. As MEK2 and PI3Kδ were likely to be part of the same pathway, we sought to ascertain whether MEK2 controlled the phosphorylation of Akt, i.e., a downstream element of PI3K. To assess the effect of MEK2 on Akt phosphorylation and to clarify the sequential contributions of MEK2 and PI3Kδ in the control of sIL-1Ra production, the effects of U0126 and IC87114 on Akt and ERK1/2 phosphorylation were determined. As expected, the level of Akt phosphorylation was diminished upon PI3Kδ inhibition but not when PI3Kγ was inhibited (**Fig. 5A**), which further demonstrated the crucial contribution of the PI3Kδ isoform to IFN-β signaling. IC87114 did not affect the level of ERK1/2 phosphorylation, which was strongly inhibited in the presence of U0126 (Fig. 5A). Like IC87114, U0126 markedly inhibited the phosphorylation of Akt, and the MEK1 inhibitor, PD98059, failed to affect Akt activation (Fig. 5B). We further confirmed these data using MEK1 and MEK2 knockdown monocytes. As demonstrated in Fig. 5C, the silencing of MEK2 diminished IFN-β-induced phosphorylation of Akt, and MEK1 expression remained unchanged. In contrast, the silencing of MEK1, which did not change MEK2 expression, did not affect Akt phosphorylation in IFN-β-activated monocytes (Fig. 5C). These results suggest that MEK2 controlled Akt phosphorylation.

MEK2 controls PI3Kδ activation

To determine whether MEK2 controlled phosphorylation of Akt directly or through PI3Kδ activation, the effect of U0126 on the recruitment of PI3Kδ to membranes was assessed. The amount of p110δ recruited to membranes in IFN-β-activated monocytes was diminished in the presence of U0126, demonstrating that PI3Kδ activation was dependent on MEK2 activity (**Fig. 6A**). MEK2 itself was recruited to membranes, suggesting that both enzymes were translocated upon IFN-β activation (Fig. 6A). Finally, in MEK2 knockdown monocytes but not in MEK1 knockdown monocytes, the recruitment of PI3Kδ to membranes was inhibited (Fig. 6B). Collectively, these observations indicate that IFN-β induced the corecruitment of MEK2 and PI3Kδ to monocyte membranes and in turn, PI3Kδ activation. This mechanism required the activation of MEK2, as it was inhibited by U0126. Thus, by controlling PI3Kδ activation, MEK2 controlled Akt phosphorylation on Ser_{473}.

DISCUSSION

This study highlights an unforeseen transduction pathway involving MEK2 and PI3Kδ that controls the expression and production of sIL-1Ra in human monocytes activated by IFN-β. It is noteworthy that of the four class I PI3K isoforms, only PI3Kδ contributes to sIL-1Ra production in monocytes activated by IFN-β. Importantly, MEK2 activity is essential for PI3Kδ recruitment to monocyte membranes, a crucial stage that leads to PI3Kδ activation, thus MEK2 activation is located upstream of PI3Kδ in the IFN-β signaling. Although MEK1 and MEK2 were activated and contributed to ERK1/2 activation during IFN-β stimulation, our results demonstrate that it is MEK2, but not MEK1 or ERK1/2, which plays a role in sIL-1Ra production in response to IFN-β. This might be a result of the translocation of MEK2 to monocyte membranes. To our knowledge, this is the first report of a MAPK signal transduction pathway that depends on MEK2 but not on MEK1 or ERK1/2.

According to our results, PI3Kδ is activated consecutively to IFN-β activation of human monocytes, as suggested by recruitment of the p110δ catalytic subunit to monocyte membranes.

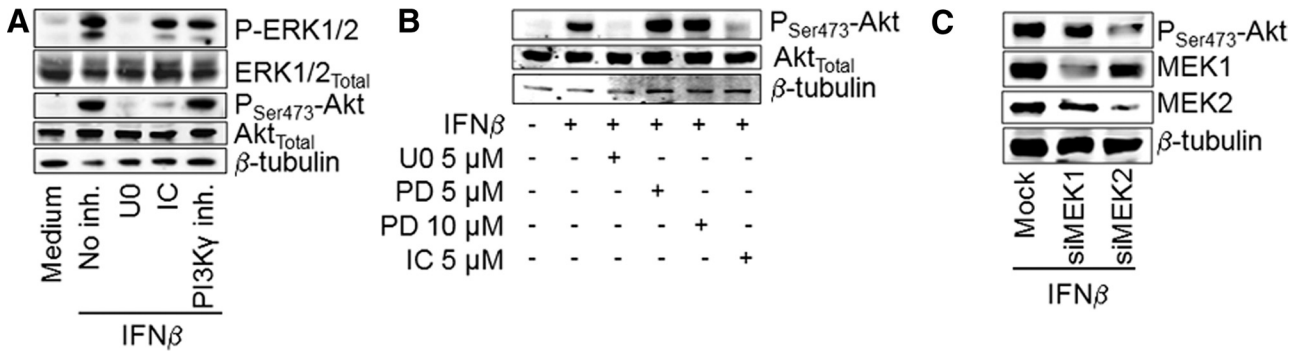

Figure 5. MEK2 regulates Akt phosphorylation. (A) Monocytes were preincubated for 90 min with 5 μM U0126, 5 μM IC87114, and 500 nM PI3Kγ inhibitor II and then activated by IFN-β (1×10^4 U/ml) or left unactivated. Cell lysates were analyzed by Western blot. (B) Monocytes were preincubated with U0126, PD98059, or IC87114 before activation by IFN-β (1×10^4 U/ml) for 90 min. Cell lysates were analyzed by Western blot. (C) Monocytes were nucleofected with stealth siRNA for MEK1 and MEK2 or Stealth RNAi™ negative control duplex (Mock) and then activated by IFN-β (1×10^4 U/ml) for 90 min. Cell lysates were analyzed by Western blot. Results are representative of three independent experiments.

Our findings indicate further that PI3Kδ is the only PI3K isoform involved in the control of sIL-1Ra production in human monocytes upon IFN-β activation. Unlike PI3Kδ, the other PI3K class I isoforms (α, β, and γ) expressed in human monocytes were not recruited to cell membranes upon activation by IFN-β, and their blockade by specific inhibitors or by siRNA-mediated knockdown did not affect sIL-1Ra production. Our current data further support the premise that PI3Kδ activation is a prerequisite to the optimal secretion of sIL-1Ra in monocytes under various stimulatory conditions [21]. Indeed, we reported recently that PI3Kδ accounts for most of the PI3K-dependent signals giving rise to the production of sIL-1Ra in monocytes activated by cellular contact with stimulated T cells and LPS—the latter stimuli relevant to chronic/sterile and acute/infectious inflammation, respectively [21]. Interestingly, although PI3Kδ activity is required for sIL-1Ra induction, regardless of the type of stimulus, it lowers the production of proinflammatory cytokines in LPS-activated monocytes but induces it in T cell contact-activated monocytes. Although the isoform was not identified, PI3K was also implicated in IL-4-dependent sIL-1Ra production in db/+ mouse macrophages [41], thus highlighting the importance of PI3K signaling in the control of anti-inflammatory cytokine production by monocytes/macrophages. In addition to regulating the balance between pro- and anti-inflammatory cytokine production, PI3Kδ differentially regulates β1 and β2 integrin-mediated adhesion and spreading of human monocytes and modulates their diapedesis [42]. Thus, the data presented herein extend these observations by showing that PI3Kδ is likely to be a key element in regulating inflammatory effector functions of human monocytes.

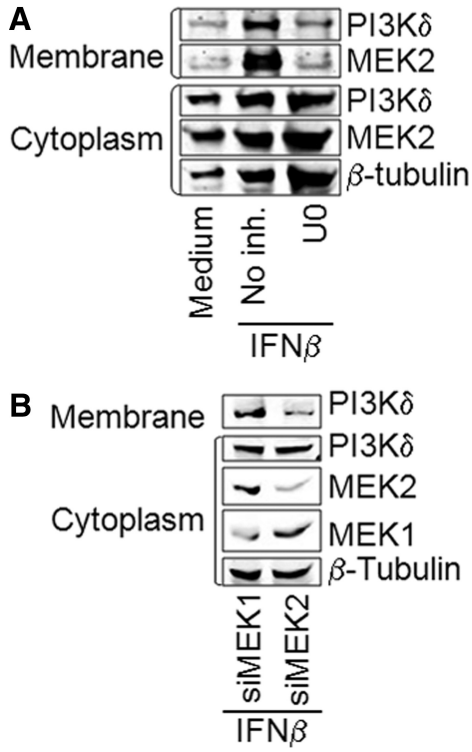

Figure 6. MEK2 controls PI3Kδ recruitment to membranes. (A) Monocytes were preincubated with 5 μM U0126 and then left unstimulated or stimulated by IFN-β (1×10^4 U/ml) for 90 min. Membrane and cytoplasm fractions were analyzed for the presence of the PI3Kδ catalytic subunit and MEK2. (B) Monocytes were nucleofected with stealth siRNA for MEK1 and MEK2 and then activated by IFN-β (1×10^4 U/ml) for 90 min. Cell membrane and cytoplasm fractions were analyzed by Western blot. Results are representative of three independent experiments.

The present study establishes that MEK2 but not MEK1 activity is required for optimal production of sIL-1Ra. Although it is rare in the literature that a distinction is made between MEK1 and MEK2 activities, recent studies have shown that MEK1 and MEK2 may fulfill different functions depending on cell types and cellular conditions [29, 30, 43]. In mouse macrophages, it was MEK1 to be preferentially phosphorylated upon activation by TNF [36], suggesting the necessity for a specific MEK isoform depending on the inflammatory conditions. More recently, MEK1 and MEK2 functions proved to be nonexchangeable in growth factor-induced ERK phosphorylation [44]. Furthermore, growing evidence points to MEK1 and

MEK2 playing individual parts in the regulation of several cellular processes, as reviewed recently [45], implying that MEKs may differentially phosphorylate or regulate substrates other than ERK1/2. Indeed, MEK2 may regulate protein activities through its newly described dynamic regulatory scaffold function that is subject to its phosphorylation but not to the activation of ERK1/2 [37]. This hypothesis is strengthened by the translocation of MEK2 and PI3Kδ to the membrane fraction. Consequently, although ERK1/2 are still the only identified substrates of MEK1/2 to date, the present data suggest that MEK2 signaling occurs via a pathway that diverges from the canonical Ras/Raf/MEK/ERK cascade upstream to the level of ERK1/2.

Although the precise mechanism of PI3Kδ activation by MEK2 remains elusive, we provide evidence that MEK2, like PI3Kδ, was located in monocyte membranes—the translocation of both kinases diminished in the presence of U0126. This observation is reminiscent of a recent study showing that activated MEK2 may act as a regulatory scaffold promoting crosstalk between ERK and another pathway to regulate cell migration [37]. The present results demonstrate that MEK2 activity intervenes upstream of PI3Kδ activation, resulting in the recruitment of the latter to the monocyte membranes. However, whether MEK2 directly interacts with and activates PI3Kδ remains to be determined [46, 47].

Class I PI3Ks are activated by the binding of the p85 subunit to a phosphorylated Tyr residue within a YXXM motif of another protein [48]. The latter can be a receptor or an adaptor, e.g., PI3Ks are activated by TLR2 via the direct interaction with p85 [49], whereas TLR4, which does not contain a YXXM motif, needs the MyD88 adaptor to interact with PI3Ks [50]. The type I IFNR lacks a YXXM motif, thus suggesting the existence of an adaptor protein that binds p85 to activate PI3Kδ in accordance with previous studies (for review, see ref. [17]). Several proteins might carry out the adaptor function, including IRS family members, STAT3 and Gab proteins [17, 51, 52]. Although PI3K activation requires interaction with IRS1/2 downstream of IFN-α-mediated activation, Akt was not activated [53], in contrast with the present results. We demonstrated previously that STAT3 is not implicated in sIL-1Ra production by IFN-β in human monocytes [15]. Here, we demonstrate that MEK2 activation occurs upstream of PI3Kδ to induce sIL-1Ra production in IFN-β-activated monocytes. MEK2 lacks a YXXM motif, ruling out a classical interaction between MEK2 and p85, and consequently, between MEK2 and PI3Kδ. It can thus be hypothesized that MEK2 might interact with an unidentified receptor or an adaptor likely to recruit PI3Kδ through YXXM interactions. This function might be achieved by members of the Gab family, which have been shown to activate PI3K and MEK pathways [51]. MEK2, which fulfills regulatory scaffold functions [37], contains a proline-rich region between residues 266 and 315 that differs from MEK1 [25]. This region might represent a potential, alternative interaction site for the Src homology 3 domain of PI3Kδ [46] and in turn, might support the interaction of PI3Kδ with a receptor/adaptor containing a YXXM motif. Finally, the PI3Kδ catalytic subunit (p110δ) contains a proline-rich region and a basic region leucine zipper-like domain, which constitute unique, potential protein–protein interaction modules [47]. The identification of the adaptor protein(s) and/or pathway(s) that link the type I IFNR to MEK2 and in turn, to PI3Kδ activation should shed light on the molecular mechanism by which MEK2 regulates IFN-β-induced Akt phosphorylation and sIL-1Ra production. The involvement of the PI3K/Akt pathway downstream of IFN-β activation of human monocytes has been reported previously [54]. However, to our knowledge, this is the first time that MEK2 is defined as an upstream element in this pathway.

The recruitment of Akt to the plasma membrane through the binding of its PH domain to PIP_3, generated by class I PI3K, i.e., PI3Kδ in the present setting, is a prerequisite to its phosphorylation [55]. The phosphorylation of Akt on Thr_{308} and Ser_{473} is required for optimal activity [56, 57], with Ser_{473} phosphorylation stabilizing the active form of the enzyme [58]. Our data suggest that MEK2 activity is required for Akt phosphorylation at Ser_{473}, which occurs within the same timeframe as ERK1/2 phosphorylation in IFN-β-stimulated monocytes, and phosphorylation of Akt Thr_{308} was observed later. 3-Phosphoinositide-dependent protein kinase-1 phosphorylates Akt on Thr_{308} [59], and the identity of the kinase(s) that phosphorylate(s) Ser_{473} has been debated for several years [55, 56]. More than 90 kinases are involved directly or indirectly in the control of Akt-Ser_{473} phosphorylation [60]. Our data suggest that MEK2 activity is required for Akt phosphorylation on Ser_{473} through PI3Kδ activation in IFN-β-stimulated monocytes. Together, the present results demonstrate that IFN-β-mediated, MEK2-dependent, late PI3Kδ activation results in sequential phosphorylation of Akt—first on Ser_{473} and then on Thr_{308} residues.

Our study also emphasizes the importance of a functional crosstalk between MEK2 and PI3Kδ pathways to regulate sIL-1Ra production. To our knowledge, this is the first time that the regulation of a PI3K/Akt pathway by MEK is described, although numerous studies dealt with the regulation of the MEK/ERK pathway by PI3K pathway elements [23, 61–65]. We thus propose a model in which MEK2 and PI3Kδ contribute to sIL-1Ra production by functioning as parts of a common signaling pathway. This model is supported by the premise that the blockade/silencing of MEK2 inhibits the recruitment/activation of the catalytic subunit of PI3Kδ to the membranes as well as Akt phosphorylation. In contrast with the present results, GSK3, a downstream PI3K/Akt effector, was shown recently to curtail ERK1/2 activity, leading to the down-regulation of sIL-1Ra production in LPS-activated monocytes [23]. It is thus likely that the type of stimulus will determine the involvement of ERK1/2 activation in triggering sIL-1Ra expression.

IFN-β is an important immunomodulator administered to treat the relapsing-remitting form of multiple sclerosis. IFN-β enhances the circulating levels of sIL-1Ra in multiple sclerosis patients, a mechanism that is likely to be relevant to its therapeutic efficacy [8–10]. We previously established that in addition to the direct induction of sIL-1Ra in monocytes, IFN-β restrains the production of the proinflammatory cytokine IL-1β in monocytes activated under conditions related to chronic inflammation [14]. In such conditions, PI3Kδ differen-

tially regulates IL-1β and sIL-1Ra expression; it enhances sIL-1Ra production and inhibits that of IL-1β [21]. It should be emphasized that in contrast to PI3Kδ, MEK1 and MEK2 play a part in the induction of the proinflammatory cytokine and its antagonist [40], therefore, providing a rationale for an alternative pathway for IFN-β to induce/enhance sIL-1Ra production that will dampen inflammation.

Our results reveal that IFN-β activates a noncanonical MEK2/PI3Kδ signaling cascade to regulate sIL-1Ra expression in monocytes. The evidence that neither MEK1 nor ERK1/2 play a part in this mechanism is also a new and unforeseen finding that contributes to a better understanding of the MAPK signaling network. In conclusion, our study reveals an original signaling pathway of IFN-β in human monocytes that does not require ERK1/2 but is driven by MEK2 and PI3Kδ.

AUTHORSHIP

N.M. and D.B. contributed equally to this work and thus, are coprincipal investigators. K.J.B., N.M., and D.B. conceived of and designed the experiments. K.J.B., R.C., and L.G. performed the experiments. K.J.B., N.M., and D.B. analyzed the data. D.B. wrote the paper.

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6.3 Glatiramer Acetate Increases IL-1 Receptor Antagonist but Decreases T cell-Induced IL-1beta in Human Monocytes and Multiple Sclerosis

Burger D, Molnarfi N, Weber MS, Brandt KJ, Benkhoucha M, Gruaz L, Chofflon M, Zamvil SS, Lalive PH.

Glatiramer acetate increases IL-1 receptor antagonist but decreases T cell-induced IL-1β in human monocytes and multiple sclerosis

Danielle Burger[a,1], Nicolas Molnarfi[a,b], Martin S. Weber[b,c], Karim J. Brandt[a], Mahdia Benkhoucha[d], Lyssia Gruaz[a], Michel Chofflon[e], Scott S. Zamvil[b], and Patrice H. Lalive[d,e,f]

[a]Division of Immunology and Allergy, Clinical Immunology Unit (Hans Wilsdorf Laboratory), Department of Internal Medicine, University Hospital and Faculty of Medicine, University of Geneva, 1211 Geneva, Switzerland; [b]Department of Neurology and Program in Immunology, University of California, San Francisco, CA 94143; [c]Department of Neurology, Technische Universität München, D-80538 Munich, Germany; [d]Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, 1211 Geneva, Switzerland; and [e]Department of Neurosciences, Division of Neurology, Neuroimmunology Laboratory, and [f]Department of Genetics and Laboratory Medicine, Laboratory Medicine Service, University Hospital of Geneva, 1211 Geneva, Switzerland

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Mechanisms of action as well as cellular targets of glatiramer acetate (GA) in multiple sclerosis (MS) are still not entirely understood. IL-1β is present in CNS-infiltrating macrophages and microglial cells and is an important mediator of inflammation in experimental autoimmune encephalitis (EAE), the MS animal model. A natural inhibitor of IL-1β, the secreted form of IL-1 receptor antagonist (sIL-1Ra) improves EAE disease course. In this study we examined the effects of GA on the IL-1 system. In vivo, GA treatment enhanced sIL-1Ra blood levels in both EAE mice and patients with MS, whereas IL-1β levels remained undetectable. In vitro, GA per se induced the transcription and production of sIL-1Ra in isolated human monocytes. Furthermore, in T cell contact-activated monocytes, a mechanism relevant to chronic inflammation, GA strongly diminished the expression of IL-1β and enhanced that of sIL-1Ra. This contrasts with the effect of GA in monocytes activated upon acute inflammatory conditions. Indeed, in LPS-activated monocytes, IL-1β and sIL-1Ra production were increased in the presence of GA. These results demonstrate that, in chronic inflammatory conditions, GA enhances circulating sIL-1Ra levels and directly affects monocytes by triggering a bias toward a less inflammatory profile, increasing sIL-1Ra while diminishing IL-1β production. This study sheds light on a mechanism that is likely to participate in the therapeutic effects of GA in MS.

experimental autoimmune encephalitis | cellular contact | inflammation | autoimmune disease

Glatiramer acetate (GA; copolymer-1; Copaxone) is composed of a mixture of synthetic peptides of 50 to 90 aa randomly composed of L-glutamic acid (E), L-lysine (K), L-alanine (A), and L-tyrosine (Y). Initially developed to mimic the myelin basic protein, a major component of the myelin sheath, and to induce experimental autoimmune encephalitis (EAE), GA unexpectedly inhibited EAE in both rodents and monkeys (1). In subsequent clinical trials, GA reduced relapse rate and progression of disability in patients with relapsing–remitting multiple sclerosis (MS; RRMS) leading to its approval in 1995 (2).

A number of investigations in MS and EAE addressed the immunological basis of GA clinical effects. However, the mechanisms of GA action are still elusive. Initial investigations attributed most GA activity to a preferential Th2-polarization of myelin-specific T cells, thus focusing on its effects on the adaptive immune response (3). However, recent reports indicated that GA treatment also exerts immunomodulatory activity on cells of the monocytic lineage, i.e., monocytes/macrophages and dendritic cells (4–9). For instance, monocytes from GA-treated patients with MS secrete less IL-12 and TNF in response to LPS stimulation compared with monocytes from healthy controls and untreated patients with MS (4). Accordingly, dendritic cells and monocytes isolated from GA-treated patients produce more anti-inflammatory IL-10 and less pro-inflammatory IL-12 (5, 9). Furthermore, GA promotes the development of anti-inflammatory type II monocytes in EAE, accompanied by induction of regulatory T cells and increased secretion of both IL-10 and TGF-β (10).

IL-1β is a pleiotropic pro-inflammatory cytokine whose production is tightly controlled at several levels (11). Indeed, as recently reviewed, there are several roadblocks to the release of IL-1β beginning with the transcription of the *IL1B* gene and ending with the exit of the active cytokine from the cell. In the extracellular space, IL-1β activity is mainly ruled by the secreted IL-1 receptor antagonist (sIL-1Ra), which binds type I IL-1 receptor without triggering signals (12). As it potently inhibits the various effects of IL-1, sIL-1Ra is considered an important regulator of the inflammatory and overall immune response mediated by IL-1 (13). Because of its extreme efficacy as a pro-inflammatory mediator, if these intracellular and extracellular roadblocks are not enough to limit IL-1β activity, it may also be reduced by the preferential binding to the cell surface or soluble form of type II IL-1 receptor, preventing it from triggering the signal-transducing type I receptor (11). Finally, the facilitation of IL-1β processing by the caspase 1 inflammasome through ATP activation of the $P2X_7$ receptor can also be viewed as a potential roadblock to the activity of IL-1β (11).

IL-1β is mainly produced upon activation of cells of the monocytic lineage. In chronic/sterile immuno-inflammatory diseases, the factors triggering pro-inflammatory cytokine production are still elusive. T cells may exert a pathological effect through direct cellular contact with monocytes/macrophages, inducing massive up-regulation of IL-1β and TNF (14). Besides triggering pro-inflammatory cytokine production, contact-mediated activation of monocytes induces the production and/or shedding of cytokine inhibitors such as sIL-1Ra and soluble receptors of IL-1 and TNF (15). The importance of T cell contact-mediated activation of monocytic cells in MS was further demonstrated in vitro in co-cultures of T cells and microglial cells (16, 17).

In MS, IL-1β is mainly expressed by microglial cells and infiltrating monocyte/macrophages throughout the white matter and in acute lesions (18). This assertion was further confirmed in EAE studies. Indeed, dark agouti rats treated with sIL-1Ra during the induction of EAE, or after adoptive transfer with myelin antigen-primed lymph node cells, develop milder signs of

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[1]To whom correspondence should be addressed. E-mail: danielle.burger@hcuge.ch.

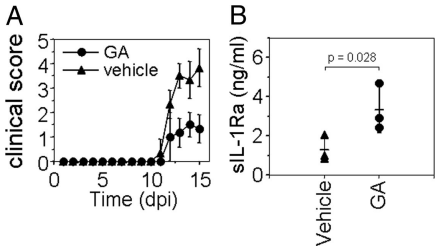

Fig. 1. sIL-1Ra levels are elevated in sera of EAE mice treated with GA. (*A*) GA ameliorates EAE. C57BL/6 mice were injected s.c. daily with GA (150 μg) or vehicle (PBS solution) 7 d before immunization with 10 μg myelin oligodendrocyte glycoprotein 35–55 peptide (dpi, day post-immunization). (*B*) EAE mice treated (GA) or not (vehicle) were killed at disease peak and their serum analyzed for IL-1β and sIL-1Ra content. IL-1β was not detected (not shown).

the disease (19). sIL-1Ra delivered by non-replicative HSV-1 vectors in EAE C57BL/6 mice delays disease onset and decreases disease severity (20). In addition, IL-1α/β double deficient (IL-1$^{-/-}$) mice exhibit significant resistance to EAE induction with reduction in disease severity, whereas IL-1Ra$^{-/-}$ mice are highly susceptible to EAE induction in the absence of pertussis toxin administration (21). These observations demonstrate that the IL-1/IL-1Ra system is crucial for autoantigen-specific T cell induction in mice and that sIL-1Ra efficiently blocks IL-1β effects and ameliorates EAE disease course (19–22).

In this study we addressed the question of the effects of GA on IL-1 system in vivo and in vitro. The results show that GA-treatment increases the circulating levels of sIL-1Ra in both EAE mice and patients with MS. This is reflected in vitro by the direct effect of GA on human blood monocytes. Indeed, GA induces the production of the cytokine inhibitor sIL-1Ra and diminishes the production of IL-1β in conditions related to chronic inflammation, i.e., in monocytes activated by direct contact with stimulated T cells.

Results

sIL-1Ra Serum Levels Are Elevated in GA-Treated EAE Mice. To assess whether GA-treatment affected sIL-1Ra levels in the MS animal model, EAE was induced in mice treated either with GA or PBS solution (i.e., vehicle). As shown in Fig. 1*A*, EAE severity was reduced in GA-treated mice, as previously demonstrated (10). At peak disease, mouse sera were analyzed for levels of sIL-1Ra and IL-1β. IL-1β was not detectable in any of the sera (not shown). However, sIL-1Ra was significantly elevated in mice treated with GA (3,336 ± 1,190 pg/mL sIL-1Ra, mean ± SD) compared with animals that received vehicle as a control (1,296 ± 657 pg/mL sIL-1Ra; Fig. 1*B*). This demonstrates that GA-treatment enhanced sIL-1Ra concentration in EAE mouse serum.

sIL-1Ra Levels Are Elevated in Sera of Patients with MS Treated with GA. sIL-1Ra circulating levels in MS have been shown to vary as a function of clinical status and treatment, so we examined whether GA-treatment would affect sIL-1Ra levels in patients with MS. IL-1β and sIL-1Ra levels were measured in sera of patients with RRMS treated with GA or untreated, and in healthy controls (Table 1). IL-1β was not detectable in any of the sera. As shown in Fig. 2, sIL-1Ra was significantly increased in serum of patients treated with GA (434 ± 265 pg/mL sIL-1Ra) whereas there was no significant difference between untreated patients (218 ± 60 pg/mL sIL-1Ra) and healthy controls (188 ± 65 pg/ml sIL-1Ra). This demonstrates that GA treatment enhances sIL-1Ra in the serum of patients with MS.

GA Differentially Regulates IL-1β and sIL-1Ra Production in Human Monocytes. To assess whether GA per se would affect the IL-1 system in human monocytes, freshly isolated human monocytes were activated by increasing doses of GA. The production of sIL-1Ra was enhanced by GA in a dose-dependent manner, reaching a plateau at 25 μg/mL (Fig. 3*A*). The latter dose was used for the in vitro experiments described later. Noticeably, GA did not induce IL-1β production, demonstrating that GA triggers an anti-inflammatory bias in human monocyte cytokine production.

To confirm that GA affected the IL-1 system, we assessed its effect on human monocytes activated upon chronic/sterile and acute/infectious inflammatory conditions as mimicked by direct cellular contact with stimulated T cells and LPS, respectively. Studies of cell-cell interactions such as those occurring in T cell contact activation of human monocytes are usually complicated by the simultaneous presence of at least 2 viable cell types. To obviate this problem, and possible interferences caused by the

Table 1. Clinical characteristics of patients with MS and healthy controls

Clinical category	Sex	Age (y)	Disease duration (y)	EDSS	GA treatment duration (mo)
Healthy controls					
1	F	31	—	—	—
2	F	34	—	—	—
3	F	36	—	—	—
4	M	29	—	—	—
5	M	24	—	—	—
6	F	24	—	—	—
7	F	30	—	—	—
8	F	35	—	—	—
9	F	44	—	—	—
10	M	40	—	—	—
Mean ± SD	—	32.7 ± 6.4	—	—	—
Untreated RRMS					
1	F	45	17	3.0	—
2	F	31	10	1.5	—
3	M	15	1	1.5	—
4	F	23	1	2.0	—
5	F	41	6	7.0	—
6	F	36	3	2.0	—
7	F	34	5	2.0	—
8	M	27	3	1.5	—
9	F	31	10	1.0	—
10	F	46	6	2.5	—
11	M	41	6	4.0	—
Mean ± SD	—	33.6 ± 9.6	6.2 ± 4.7	2.5 ± 1.7	—
GA-treated RRMS					
1	M	39	6	2.5	56
2	F	33	7	2.0	18
3	M	26	8	1.5	60
4	F	45	15	1.5	27
5	F	32	11	2.0	13
6	F	27	4	0	44
7	M	23	2	1.0	29
8	M	39	9	2.0	36
9	F	25	5	1.0	19
Mean ± SD	—	32.1 ± 7.6	7.4 ± 3.9	1.5 ± 0.8	33.6 ± 16.8

EDSS: Expanded Disability Status Score; RRMS: relapsing-remitting multiple sclerosis; GA: glatiramer acetate. Data expressed at time of sampling.

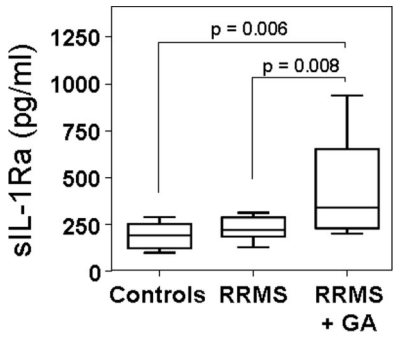

Fig. 2. sIL-1Ra levels are elevated in sera of patients with MS treated with GA. The levels of sIL-1Ra and IL-1β were measured in sera of patients with RRMS treated with GA or not treated, and age-matched healthy controls, as described in Table 1. IL-1β was under the detection limit (15 pg/mL) in all individuals. There was no significant difference between healthy controls and untreated patients with RRMS. Results are presented as a box plot (GraphPad Prism 4).

fact that target cells are potentially phagocytic, isolated membranes from stimulated HUT-78 cells were solubilized with CHAPS and used as a stimulus, referred to as CE_{sHUT} (23). As shown in Fig. 3*B*, GA enhanced the production of sIL-1Ra in monocytes activated by CE_{sHUT} and LPS in a similar manner, and GA-induced sIL-1Ra production was additive to that triggered by CE_{sHUT} or LPS. In contrast, the production of IL-1β induced by CE_{sHUT} was inhibited by GA, whereas LPS-induced production of IL-1β was enhanced in the presence of GA (Fig. 3*C*). These observations suggest that GA displays opposite effects on signaling events downstream of LPS and CE_{sHUT}.

GA Affects the Expression of Cytokine Transcripts. To assess whether GA affected the production of cytokines at the transcriptional

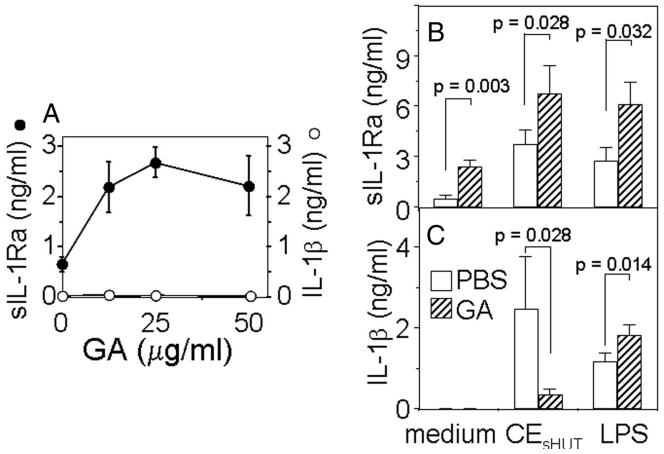

Fig. 3. GA differentially regulates IL-1β and sIL-1Ra production in human monocytes. (*A*) Monocytes (5×10^4 cells/200 μL/well; 96-well plates) were activated with the indicated dose of GA. After 48 h, supernatants were harvested and the production of IL-1β (open circles) and sIL-1Ra (filled circles) were measured in triplicate wells and represented as mean $\pm$ SD. Results are representative of 3 different experiments. (*B*) Monocytes (5×10^4 cells/200 μL/well; 96-well plates) were preincubated for 1 h with or without 25 μg/mL GA and then cultured for 48 h in the presence or absence of CE_{sHUT} (1 μg/mL) or LPS (100 ng/mL). sIL-1Ra was measured in culture supernatants (mean $\pm$ SD, $n = 3$ different experiments). (*C*) Monocytes (5×10^4 cells/200 μL/well; 96-well plates) were preincubated for 1 h with or without 25 μg/mL GA and then cultured for 48 h in the presence or absence of CE_{sHUT} (6 μg/mL) or LPS (100 ng/mL). IL-1β was measured in culture supernatants (mean $\pm$ SD, $n = 3$ different experiments, i.e., monocytes prepared from 3 different blood donors).

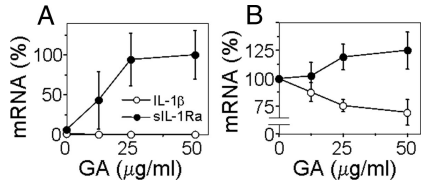

Fig. 4. GA affects sIL-1Ra and IL-1β mRNA in both CE_{sHUT}-activated and resting monocytes. (*A*) Monocytes (2×10^6 cells/well/3 mL) were preincubated for 1 h with the indicated dose of GA and then cultured for 3 h in the presence of CE_{sHUT} (6 μg/mL). Total mRNA was isolated and analyzed by duplex real-time quantitative PCR (see *Materials and Methods*) for the presence of IL-1β (open circles) and sIL-1Ra (filled circles) transcripts. Results are presented as percentage of mRNA expression level, 100% being transcript expression measured after 3 h of monocyte activation by CE_{sHUT} in the absence of GA; mean $\pm$ SD of 3 different experiments. (*B*) Monocytes (2×10^6 cells/well/3 mL) were activated for 18 h with the indicated dose of GA. Total mRNA was isolated and analyzed by duplex real-time quantitative PCR (see *Materials and Methods*) for the presence of IL-1β (open circles) and sIL-1Ra (filled circles) transcripts. Results are presented as percentage of mRNA expression level, 100% being the transcript level at 50 μg/mL GA; mean $\pm$ SD of 3 different experiments, i.e., monocytes prepared from 3 different blood donors.

level, monocytes were incubated for 1 h with increasing doses of GA and then activated by CE_{sHUT} or not activated. As shown in Fig. 4*A*, GA, in the absence of other stimuli, induced the expression of sIL-1Ra transcript in a dose-dependent manner, whereas that of IL-1β was not induced. When monocytes were activated by CE_{sHUT}, GA diminished IL-1β mRNA expression by 30% whereas it enhanced sIL-1Ra mRNA expression by 25% in monocytes activated by CE_{sHUT} (Fig. 4*B*), corroborating the effects of GA on protein production (Fig. 3). Together, these data suggest that GA displays opposite activity toward IL-1 system members in monocytes/macrophages by directly inducing sIL-1Ra expression and production and by modulating both IL-1β and sIL-1Ra expression/production induced by CE_{sHUT}.

Discussion

The present study sheds light on mechanisms by which GA might exert beneficial effects in MS. GA treatment enhances sIL-1Ra blood levels in patients with MS and in EAE mice. This is likely to be the consequence of the direct triggering effect of GA on monocytic production of sIL-1Ra. In addition, GA diminishes monocytic IL-1β production induced by direct contact with stimulated T cells. Thus, through different mechanisms, GA dampens IL-1β activity, which correlates with disease severity.

Recent insights derived from studies on the mechanism of action of GA show a pivotal role of monocytes in the modulation of the immune system and highlight the importance of these cells as a target for pharmacologic intervention in autoimmune diseases (4–10, 24). These results suggest that GA might be useful in autoimmune diseases other than MS, as suggested by its beneficial effect in animal models of autoimmune diseases such as uveoretinitis (25) and inflammatory bowel disease (26), and graft rejection (27), whereas its efficacy has not been demonstrated in animal models of systemic lupus erythematosus (28) and collagen-induced arthritis (29).

The premise that GA enhances sIL-1Ra levels in treated patients with MS is reminiscent of observations made with another immunomodulator used in MS. Indeed, IFNβ also increases circulating serum levels of sIL-1Ra in patients with MS (30). Interestingly, with both immunomodulators, the circulating levels of sIL-1Ra are doubled in treated patients compared with untreated individuals. Together these observations suggest that the enhancement of sIL-1Ra might be relevant to therapeutic effects of both GA and IFNβ. Indeed, sIL-1Ra is transported and expressed into the CNS, where it could inhibit the pro-

inflammatory activities of IL-1β, whose expression is increased in MS lesions (18, 31). The efficiency of sIL-1Ra treatment was demonstrated in EAE animals, in which it results in delayed and milder disease (19, 22). Besides, polymorphisms encoded within the IL-1 gene cluster were associated with MS (32). In particular, mild/moderate disease has been correlated to allele 2 of the IL-1Ra gene (*IL1RN*) variable number of tandem repeats genotype, which favors the production of sIL-1Ra (12, 33). In addition, families displaying high innate IL-1β/sIL-1Ra ratio are at increased risk to have a relative who develops MS (34). Together, these studies reinforce the potential clinical benefit of GA to selectively induce sIL-1Ra secretion by monocytes in MS, as demonstrated in the present study. Of note, direct treatment with the commercially available form of sIL-1Ra (Anakinra) may represent an alternative treatment for MS, although its short lifespan once injected in humans may limit its efficacy (13). Nevertheless, as demonstrated here, the enhancement of intrinsic production of sIL-1Ra might be a mediator of the beneficial effects of GA in MS.

Most studies have focused on the effects of GA treatment on the adaptive immune system, in particular on T cells. Recent data favor the view that primary immune modulation of APC directs T cell immune modulation as a secondary step. Indeed, in mice lacking mature B and T cells, the GA treatment effect on monocytes is unbowed as indicated by an anti-inflammatory "type II" cytokine shift (10). This indicates that GA does not require T cells or T cell products to alter monocytic cytokine production. Furthermore, adoptive transfer of highly purified, GA-induced type II monocytes into mice with EAE triggers T cell immune modulation and ameliorates the disease course of recipient mice. However, MHC II-deficient type II monocytes were unable to mediate this effect on T cells or disease severity (10). Taken together, these observations indicate that in vivo GA treatment exerts a direct effect on APC, which rules T cell immune modulation as the effector arm of GA clinical benefit in CNS autoimmune disease. The present study confirm the direct effect of GA on cells of the monocytic lineage by demonstrating that it down-regulates T cell contact-induced IL-1β production and directly triggers the production of sIL-1Ra. Thus, GA directly affects both the antigen presentation and cytokine production of monocytic cells.

Direct cellular contact with stimulated T cells is a major pathway for the production of IL-1β and TNF in monocytes/macrophages under sterile conditions (17, 35, 36). Indeed, contact-mediated activation of monocytes/macrophages by stimulated T lymphocytes is as potent as optimal doses of LPS to inducing IL-1β and TNF production in monocytes (37, 38). This model was recently used to assess the potency of kinase inhibitors in acute and chronic inflammatory conditions (39). It is thus likely that this mechanism is highly relevant to the pathogenesis and persistence of chronic/sterile inflammation in diseases such as MS. The effect of GA on cytokine production induced by contact with stimulated T cells in human microglial cells was previously demonstrated (16). Stimulated T cells that were pretreated with GA induced lower levels of TNF, IL-1β, and IL-6 in human microglial cells and phorbol 12-myristate 13-acetate (PMA)/IFNγ-treated U937 monocytic cells. However, in the latter study, GA was absent during microglial cell activation, implying that GA rather inhibited the ability of T cells to activate cytokine production by cells of the monocytic lineage. The present study demonstrates that GA influences cytokine production by acting directly on human monocytes. Together, these studies suggest that GA affects the activation stage of both T cells and monocytes/macrophages to diminish contact-induced pro-inflammatory cytokine production.

GA displays opposite effects on monocytes activated by LPS and T cell contact. Indeed, in contrast with CE_{sHUT}-activated monocytes, the production of the pro-inflammatory cytokine IL-1β was up-regulated when cells were activated by LPS. This result is in agreement with a previous study showing that GA enhances the production of IL-1β in the human monocytic cell line THP-1 when activated by LPS (40). Thus, GA displays opposite effects on cytokine production by monocytes activated upon acute/infectious (i.e., LPS) and chronic/sterile (i.e., CE_{sHUT}) inflammatory conditions. Therefore, the use of LPS as an in vitro stimulus should be used with caution to mimic inflammatory conditions when chronic/sterile inflammatory diseases are investigated (39).

In conclusion, this study demonstrates that GA directly affects monocytes/macrophages by triggering the production of the anti-inflammatory cytokine sIL-1Ra. As sIL-1Ra can be both transported through the blood–brain barrier and induced within the CNS, it might exert immunomodulatory effects in both systemic and CNS compartments. In the latter, GA may also dampen the production and activity of IL-1β. Finally, the present data strengthen recent demonstrations that, in addition to the modulation of the adaptive immune system, GA significantly affects the innate immune system.

Materials and Methods

Patients. Patients and healthy volunteers were recruited at the University Hospital of Geneva in accordance with institutional guidelines, and approval of the local ethical committee was obtained. Blood was drawn from 10 healthy controls, 11 untreated patients with RRMS, and 9 GA-treated patients with RRMS (Table 1). Sex, age, clinical score, and disease duration were matched between groups. All enrolled patients had definite RRMS according to revised McDonald criteria (41). At the time of blood sampling, GA-treated patients received 20 mg of GA s.c. daily for at least 1 year, with mean treatment duration of 33.6 ± 16.8 months (Table 1). None of the patients were receiving an immunomodulatory or immunosuppressive drug in addition to GA. Patients from the untreated group did not receive any immunosuppressive or immunomodulatory drug for at least 6 months preceding the study.

EAE Induction and GA Treatment. EAE was induced in 6 C57BL/6 mice using 10 μg myelin oligodendrocyte glycoprotein 35–55 peptide in complete Freund adjuvant. After immunization and 48 h later, mice received an i.v. injection of 200 ng pertussis toxin. Mice were scored as follows: 0, no symptoms; 1, decreased tail tone; 2, mild monoparesis or paraparesis; 3, severe paraparesis; 4, paraplegia and/or quadriparesis; and 5, moribund condition or death. Mice received daily s.c. injections of 150 μg GA suspended in PBS solution ($n = 3$) or PBS solution alone ($n = 3$) starting 7 days before EAE induction as described elsewhere (42). All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Materials. FCS, streptomycin, penicillin, L-glutamine, RPMI-1640, and PBS solution free of Ca^{2+} and Mg^{2+} were purchased from Gibco; purified phytohemagglutinin from EY Laboratories; Lymphoprep from Axis-Shield; PMA, polymyxin B sulfate, and mouse anti-β-tubulin antibody from Sigma; and GA from Sanofi-Aventis. Other reagents were of analytical grade or better.

Monocytes. Peripheral blood monocytes were isolated from buffy coats of blood of healthy volunteers as previously described (43). To avoid activation by endotoxin, polymyxin B sulfate (2 μg/mL) was added in all solutions during isolation procedure.

T Cell Stimulation and Membrane Isolation. HUT-78, a human T cell line, was obtained from the American Type Culture Collection. Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/mL streptomycin, 50 IU/mL penicillin, and 2 mM L-glutamine in 5% CO_2-air humidified atmosphere at 37 °C. HUT-78 cells (2×10^6 cells/mL) were stimulated for 6 h by phytohemagglutinin (1 μg/mL) and PMA (5 ng/mL). Plasma membranes of stimulated HUT-78 cells were prepared as previously described and solubilized in 8 mM CHAPS (23, 44). CHAPS extract of membranes of stimulated HUT-78 cells was referred as to CE_{sHUT}. Previous studies demonstrated that fixed, stimulated HUT-78 cells, plasma membranes of the latter

cells, and CE_{sHUT} display similar ability to induce cytokine production in human monocytes (15). To activate monocytes, CE_{sHUT} was used at either 1 μg/mL or 6 μg/mL proteins as previously determined (38, 44).

Cytokine Production. Monocytes (5×10^4 cells/well/200 μL) were activated with the indicated stimulus in RPMI medium 1640 supplemented with 10% heat-inactivated FCS, 50 μg/mL streptomycin, 50 U/mL penicillin, 2 mM L-glutamine, and 5 μg/mL polymyxin B sulfate in 96-well plates and cultured for 48 h. The production of sIL-1Ra and IL-1β was measured in culture supernatants and patients' serum by commercially available enzyme immunoassay: IL-1β (Immunotech) and sIL-1Ra (Quantikine; R&D Systems). IL-1β and sIL-1Ra concentrations in serum of patients with RRMS and healthy controls were determined by triplicate measurements of the same sample.

mRNA Quantification. Monocytes (2×10^6 cells/well/3 mL) were cultured in 6-well plates with the indicated stimulus for 3 h or 18 h. Total RNA was isolated and analyzed by quantitative real-time PCR as previously described (23).

Statistics. When required, significance of differences between groups was evaluated using the Student *t* test.

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6.4 Glatiramer Acetate Triggers PI3Kδ/Akt and MEK/ERK Pathways to Induce IL-1 Receptor Antagonist in Human Monocytes

Rakel Carpintero, Karim J. Brandt, Lyssia Gruaz, Nicolas Molnarfi, Patrice H. Lalive, and Danielle Burger.

Glatiramer acetate triggers PI3Kδ/Akt and MEK/ERK pathways to induce IL-1 receptor antagonist in human monocytes

Rakel Carpintero[a], Karim J. Brandt[a], Lyssia Gruaz[a], Nicolas Molnarfi[b], Patrice H. Lalive[c,d,e], and Danielle Burger[a,1]

[a]Division of Immunology and Allergy, Hans Wilsdorf Laboratory, Inflammation and Allergy Research Group, Department of Internal Medicine, University Hospital and Faculty of Medicine, University of Geneva, 1211 Geneva, Switzerland; [b]Department of Neurology and Program in Immunology, University of California, San Francisco, CA 94143; [c]Department of Clinical Neurosciences, Division of Neurology, Neuroimmunology Laboratory, and [d]Department of Genetics and Laboratory Medicine, Laboratory Medicine Service, University Hospital of Geneva, 1211 Geneva, Switzerland; and [e]Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, 1211 Geneva, Switzerland

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Glatiramer acetate (GA), an immunomodulator used in multiple sclerosis (MS) therapy, induces the production of secreted IL-1 receptor antagonist (sIL-1Ra), a natural inhibitor of IL-1β, in human monocytes, and in turn enhances sIL-1Ra circulating levels in MS patients. GA is a mixture of peptides with random Glu, Lys, Ala, and Tyr sequences of high polarity and hydrophilic nature that is unlikely to cross the blood–brain barrier. In contrast, sIL-1Ra crosses the blood–brain barrier and, in turn, may mediate GA anti-inflammatory activities within the CNS by counteracting IL-1β activities. Here we identify intracellular signaling pathways induced by GA that control sIL-1Ra expression in human monocytes. By using kinase knockdown and specific inhibitors, we demonstrate that GA induces sIL-1Ra production via the activation of PI3Kδ, Akt, MEK1/2, and ERK1/2, demonstrating that both PI3Kδ/Akt and MEK/ERK pathways rule sIL-1Ra expression in human monocytes. The pathways act in parallel upstream glycogen synthase kinase-3α/β (GSK3α/β), the knockdown of which enhances sIL-1Ra production. Together, our findings demonstrate the existence of signal transduction triggered by GA, further highlighting the mechanisms of action of this drug in MS.

multiple sclerosis | immunomodulation | inflammation | Copaxone | signal transduction

Secreted IL-1 receptor antagonist (sIL-1Ra) is a natural IL-1 inhibitor that competes with the agonist for the binding to IL-1 type I receptor to which it binds without inducing signal transduction (1, 2). Because it potently inhibits the various effects of IL-1, sIL-1Ra is considered an important regulator of the inflammatory and overall immune response mediated by IL-1 (3, 4). The importance of sIL-1Ra in the maintenance of cytokine homeostasis in humans was recently demonstrated (5). IL-1β is a pleiotropic proinflammatory cytokine the production of which is tightly controlled (6). Because IL-1β is expressed by microglial cells and infiltrating monocyte/macrophages throughout the white matter in and around the lesions in acute multiple sclerosis (MS) (7), it is likely to play an important part in MS pathogenesis. This assertion was confirmed in multiple studies performed in experimental autoimmune encephalomyelitis (EAE), i.e., an MS animal model. Indeed, rats treated with sIL-1Ra develop milder signs of EAE (8). sIL-1Ra delivered by nonreplicative HSV-1 vectors in EAE C57BL/6 mice delays disease onset and decreases disease severity (9). In addition, IL-1α/β double deficient mice exhibit significant resistance to EAE induction with reduction in disease severity, although IL-1Ra$^{-/-}$ mice are highly susceptible to EAE induction in the absence of pertussis toxin administration (10). These observations demonstrate that the IL-1/IL-1Ra system is crucial for autoantigen-specific T-cell induction in mice, and that sIL-1Ra can efficiently block IL-1β effects and can ameliorate EAE disease course (8–11). Moreover, IL-1β is essential for the differentiation of human and mouse IL-17–producing T helper cells (12, 13), corroborating the reduced production of IL-17 in IL-1$^{-/-}$ EAE mice and its increase in IL-1Ra$^{-/-}$ EAE mice (10). In both IL-1$^{-/-}$ and IL-1Ra$^{-/-}$ mice, the production of IFNγ by T cells followed that of IL-17, suggesting the involvement of the IL-1 system in the polarization of both TH1 and TH17 subsets (10). Overall, these studies suggest that alterations of the IL-1/IL-1Ra system are implicated in the development of autoimmunity within the CNS.

Glatiramer acetate (GA; Copolymer-1, Copaxone) is a mixture of synthetic peptides of 50–90 amino acid randomly composed of Glu, Lys, Ala, and Tyr. GA is an approved drug for treatment of relapsing remitting MS (14). However, the mechanisms of GA actions are still elusive. Mainly based on studies in EAE, GA activity was attributed to perturbations in T-cell reactivity to antigen, thus focusing on its effects on the adaptive immune response. Nevertheless, increasing number of reports indicate that GA treatment also exerts immunomodulatory activity on cells of the myelo-monocytic lineage, i.e., monocytes/macrophages and dendritic cells (15–23). These observations suggest that GA might be useful in autoimmune diseases other than MS, as suggested by its beneficial effect in animal models of uveoretinitis (24), inflammatory bowel disease (25), and graft rejection (26).

Like IFNβ, another immunomodulator used in MS therapy (27, 28), GA induces sIL-1Ra production in isolated human monocytes, an activity reflected by the enhancement of sIL-1Ra levels in the blood of GA-treated MS patients and EAE mice (29). Thus, both GA and IFNβ, which display comparable therapeutic effects (30), act on sIL-1Ra levels that might be a common mode of action in MS treatment. The ability of circulating sIL-1Ra to cross the blood–brain barrier (31) indicates that it may inhibit the proinflammatory activities of IL-1β into the CNS, a mechanism particularly important in regard to GA, the high polarity and hydrophilic nature of which are likely to impede CNS penetration (32). Therefore, sIL-1Ra might mediate part of the beneficial antiinflammatory effects of GA into the CNS. Increasing evidence of the direct effects of GA on monocytes strengthens the hypothesis that it exerts immunomodulatory effects mainly in the periphery and not directly in the CNS (32).

The induction of sIL-1Ra by GA in human monocytes suggests the triggering of intracellular pathways leading to gene tran-

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The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. E-mail: danielle.burger@hcuge.ch.

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scription. In monocytes activated under acute/infectious or chronic/sterile inflammatory conditions, the production of sIL-1Ra depends on the activation of PI3Kδ (33), highlighting an important role of this kinase independently of the stimulus. In addition, both MAPK and glycogen-synthase kinase 3 (GSK3) were shown to play a part in the control of sIL-1Ra production (34, 35). Here, we address the question of GA-induced signaling pathways that lead to sIL-1Ra production in monocytes. The results show that the induction of sIL-1Ra production by GA occurs through pathways comprising PI3Kδ, Akt, MEK1/2, ERK1/2, and GSK3.

Results

GA Triggers PI3K/Akt and MEK/ERK Pathways in Monocytes. PI3Ks and extracellular signal-regulated kinase-1/2 (ERK1/2) have been shown to contribute to the induction of sIL-1Ra in monocytes. To assess whether GA was able to turn on PI3K/Akt and MAPK pathways, monocytes were activated by an optimal concentration of GA (25 μg/mL) that was previously determined (29). GA induced the phosphorylation of Akt and ERK1/2, i.e., downstream elements of PI3Ks and MEK1/2, both kinases being coordinately phosphorylated, reaching a maximum at 2 h (Fig. 1*A*). Transduction signals were directly induced by GA, as shown by the phosphorylation of ERK1/2 after 2 h activation in the presence or absence of the protein synthesis inhibitor cycloheximide (Fig. 1*B*). These results suggest that GA, per se, induced signal transduction in monocytes and did not operate via an autocrine or paracrine loop.

PI3Kδ Is the PI3K Isoform Involved in the Induction of sIL-1Ra Production. Because PI3Kδ controls sIL-1Ra induction in monocytes activated by several stimuli, we first assessed its activation in response to GA treatment. The catalytic subunit of PI3Kδ was localized in membrane fraction of monocytes exposed to GA (Fig. 2*A*), demonstrating the activation of PI3Kδ by GA. To ascertain the involvement of PI3Kδ in sIL-1Ra production and rule out involvement of other class I PI3K isoforms, we assessed the effects of both specific PI3K knockdown by siRNA and blockade by pharmacological inhibitors. The PI3K pan-inhibitor Ly294002 suppressed GA-induced production of sIL-1Ra (Fig. 2*B*), suggesting that PI3K pathway activation controlled sIL-1Ra production induced by GA. Specific blockade of PI3Kα, PI3Kβ, and PI3Kγ activity with optimal doses of inhibitors did not affect sIL-1Ra production. In contrast, IC87114, a specific inhibitor of PI3Kδ, reduced GA-induced sIL-1Ra production to basal level (Fig. 2*B*), suggesting that PI3Kδ controlled sIL-1Ra production in response to GA. The results obtained with kinase inhibitors were confirmed by the silencing of the different class I PI3K

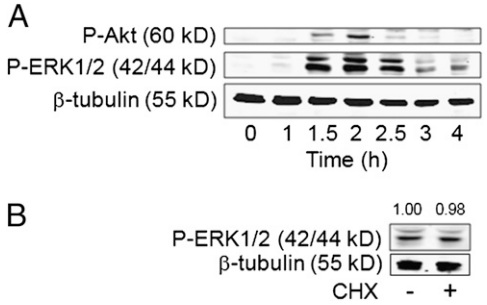

Fig. 1. GA triggers both PI3K and MAPK pathways in human monocytes. (*A*) Monocytes were stimulated by GA. After the indicated time, cell lysates were subjected to Western blot analysis with the indicated antibody. (*B*) Monocytes were preincubated for 30 min in the presence or absence of 10 μM cycloheximide (CHX) before activation by GA for 2 h. Cell lysates were subjected to Western blot analysis.

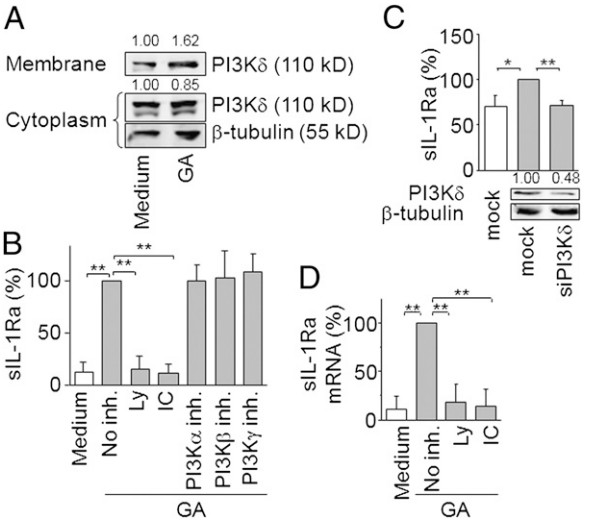

Fig. 2. PI3Kδ controls sIL-1Ra induction by GA in human monocytes. (*A*) Monocytes were stimulated by GA. Cell membrane and cytoplasm fractions were analyzed by Western blot. (*B*) Monocytes were preincubated or not preincubated with 10 μM Ly294002 (Ly), 5 μM IC87114 (IC), 50 nM PI3Kα inhibitor, 50 nM PI3Kβ inhibitor, or 500 nM PI3Kγ inhibitor as indicated. Cells were activated with GA (gray columns) or left unactivated (white column). Production of sIL-1Ra was measured in supernatants and presented as percentage of production observed in the absence of inhibitor (no inh. = 100% = 1989 ± 867 pg/mL sIL-1Ra). (*C*) Monocytes were nucleofected with stealth siRNA for PI3Kδ or negative control (mock). PI3Kδ silencing was assessed by Western blot (*Lower*). PI3Kδ knockdown or mock transfected monocytes were activated (gray columns; 100% = 1806 ± 634 pg/mL sIL-1Ra) or not activated (white column; sIL-1Ra concentration = 1173 ± 696 pg/mL) with GA and sIL-1Ra measured in culture supernatants. (*D*) Monocytes were treated with 10 μM Ly294002 (Ly) or 5 μM IC87114 (IC) before activation (gray columns) by GA or left unactivated (white column). sIL-1Ra mRNA was analyzed by qPCR and presented as percentage of transcript level induced by GA in the absence of inhibitor. Results are either representative of three experiments (Western blots) or mean ± SD of three independent experiments carried out with monocytes isolated from three different individuals. $^{**}P < 0.01$, $^{*}P < 0.05$ as determined by Student *t* test.

catalytic subunits in monocytes. As previously shown (36), transfection of monocytes with siRNA markedly increased the basal production of sIL-1Ra, i.e., from 465 ± 239 pg/mL to 1,173 ± 696 pg/mL. However, GA clearly enhanced sIL-1Ra production in mock-transfected monocytes reaching 1,806 ± 634 pg/mL, i.e., a significant 1.54-fold enhancement ($P < 0.01$). In contrast to PI3Kα, PI3Kβ, and PI3Kγ silencing (Fig. S1*A*), the specific knockdown of PI3Kδ abolished GA-induced production of sIL-1Ra (Fig. 2*C*), indicating a major role of this PI3K isoform in GA-induced sIL-1Ra production. The involvement of PI3Kδ was further confirmed by the exquisite sensitivity of GA-induced sIL-1Ra production to Ly294002 and IC87114; the production of sIL-1Ra reverting to basal levels at 2.5 μM of either inhibitor (Fig. S1 *B* and *C*). To ascertain that PI3Kδ controlled the steady state levels of sIL-1Ra mRNA, we measured the effect of specific inhibitors on sIL-1Ra transcript expression in response to GA-stimulation. Both PI3K pan-inhibitor and PI3Kδ specific inhibitor decreased sIL-1Ra mRNA expression to basal level in GA-treated monocytes (Fig. 2*D*). Together, these results identify PI3Kδ as the PI3K isoform controlling sIL-1Ra expression in GA-activated monocytes.

GA Triggers Production of sIL-1Ra through a PI3Kδ/Akt Pathway. To assess the contribution of Akt downstream PI3Kδ in the control of sIL-1Ra production in GA-activated monocytes, the phosphorylation of Akt was first ascertained in GA-activated monocytes in the presence of PI3K and PI3Kδ inhibitors. The GA-

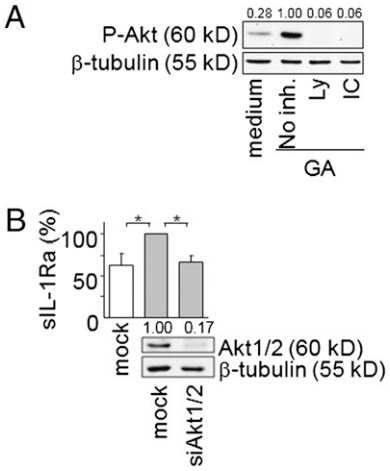

Fig. 3. GA triggers the production of sIL-1Ra through a PI3Kδ/Akt pathway. (*A*) Monocytes were preincubated with or without 10 μM Ly294002 (Ly) or 5 μM IC 87114 (IC) before stimulation by GA. Cell lysates were subjected to Western blot analysis. (*B*) Monocytes were nucleofected with stealth siRNA for Akt1/2 or negative control (mock). The efficiency of Akt1/2 silencing was assessed by Western blot (*Lower*); a representative experiment out of 3 is presented. Akt1/2 knockdown or mock transfected monocytes were GA activated (gray columns; 100% = 2174 ± 467 pg/mL sIL-1Ra) or left unactivated (white column; sIL-1Ra concentration = 1,346 ± 467 pg/mL). sIL-1Ra was measured in culture supernatants and presented as described in legend of Fig. 2*B*. Data are mean ± SD of three independent experiments. *$P < 0.05$ as determined by Student *t* test.

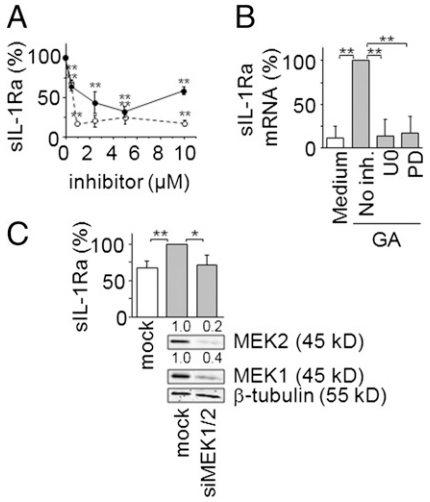

Fig. 4. MEK1/2 controls GA-induced sIL-1Ra production. (*A*) Monocytes were preincubated in the absence or presence of increasing concentration of U0126 (○) or PD98059 (●) before the addition of GA. sIL-1Ra production was measured in supernatants. Results are presented as described in Fig. 2*B* (no inh. = 100% = 2177 ± 837 pg/mL sIL-1Ra). (*B*) Monocytes were treated with 5 μM U0126 (U0) or 5 μM PD98059 (PD) before addition (gray columns) or no addition (white column) of GA. mRNA was isolated and analyzed by real-time qPCR. Results are presented as in Fig. 2*D*. (*C*) Monocytes were nucleofected with stealth siRNA for MEK1 and MEK2 or negative control (mock). Efficiency of MEK1/2 silencing was assessed by Western blot (*Lower*), and sIL-1Ra production by MEK1/2 knocked-down or mock transfected monocytes activated (gray columns; 100% = 1933 ± 656 pg/mL sIL-1Ra) or not activated (white column; sIL-1Ra concentration = 1211 ± 547 pg/mL) by GA was measured in culture supernatants. Results are presented as mean ± SD of three independent experiments. **$P < 0.01$, *$P < 0.05$ as determined by Student *t* test.

induced phosphorylation of Akt was inhibited to similar extent when monocytes were pretreated with the PI3K pan-inhibitor Ly294002 or the PI3Kδ specific inhibitor IC87114 (Fig. 3*A*). These results indicated that PI3Kδ controlled Akt phosphorylation in monocytes activated by GA. To confirm the implication of Akt in the control of sIL-1Ra production, we evaluated the effect of siRNA specific for Akt1/2 on sIL-1Ra production induced by GA in monocytes. The silencing of Akt knocked down its expression by 83% (Fig. 3*B*). Concomitantly, GA-induced production of sIL-1Ra was reduced to basal level (Fig. 3*B*). Collectively, these observations indicate that PI3Kδ/Akt pathway is mandatory to the induction of sIL-1Ra production by GA-activated monocytes.

MEK1, MEK2, and ERK1/2 Control GA-Induced sIL-1Ra Production. We next investigated the role of the MEK/ERK pathway in GA-induced sIL-1Ra production, as GA induced the phosphorylation of ERK1/2 in monocytes (Fig. 1). The MEK1 specific inhibitor, PD980159, and the MEK1 and MEK2 dual inhibitor, U0126, diminished sIL-1Ra production induced by GA in a dose–response manner (Fig. 4*A*). The inhibition of sIL-1Ra production by U0126 was more pronounced than that observed in the presence of PD98059 at all doses. More particularly, sIL-1Ra production was abolished in the presence of 1 μM U0126, whereas basal levels of sIL-1Ra were hardly reached in the presence of PD98059. These data suggest that both MEK1 and MEK2 take part in the control of sIL-1Ra production in response to GA. To optimize the inhibition of sIL-1Ra production after GA induction, a concentration of 5 μM of either inhibitor was used in the experiments described below (unless otherwise stated). Both MEK1 and MEK1/2 inhibitors diminished sIL-1Ra mRNA levels (Fig. 4*B*), suggesting that these kinases participate in the control of sIL-1Ra transcript steady state levels in response to GA activation. The results obtained with the kinase inhibitors were confirmed by silencing MEK1 and MEK2 in monocytes. Transfection of monocytes with MEK1 and MEK2 siRNA significantly diminished the expression of both kinases, as demonstrated by Western blot analysis (Fig. 4*C*). As observed above (Figs. 2*C* and 3*B*), the production of sIL-1Ra was significantly enhanced following nucleofection of monocytes with siRNA. However, the silencing of MEK1/2 resulted in the inhibition of sIL-1Ra production induced by GA that reverted to basal levels (Fig. 4*C*). To address the involvement of ERK1/2, the canonical substrates of MEK1/2, in the control of sIL-1Ra production, the kinases were silenced by specific stealth siRNA. The transfection of monocytes with ERK1/2 siRNA strongly reduced protein expression of each kinase (Fig. 5). The silencing of ERK1/2 abolished GA-induced

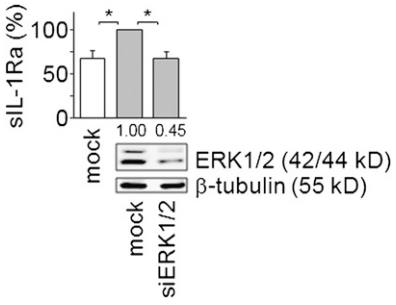

Fig. 5. ERK1/2 controls GA-induced sIL-1Ra production. Monocytes were nucleofected with stealth siRNA for ERK1/2 or negative control (mock). Efficiency of ERK1/2 silencing was assessed by Western blot (*Lower*); one representative experiment among three is presented. ERK1/2 knocked-down or mock-transfected monocytes were activated (gray columns; 100% = 2,334 ± 556 pg/mL sIL-1Ra) or not activated (white column; sIL-1Ra concentration = 1,555 ± 767 pg/mL) with GA and sIL-1Ra measured in culture supernatants. Results are presented as mean ± SD of three independent experiments; *$P <$ 0.05 as determined by Student *t* test.

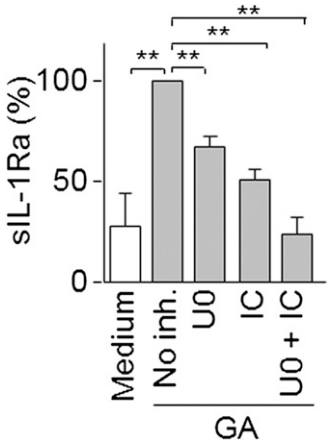

Fig. 6. PI3Kδ and MEK1/2 are part of two different pathways controlling sIL-1Ra production. (*A*) Monocytes were preincubated in the absence or presence of 0.5 μM U0126 (U0, MEK1/2 inhibitor), 0.5 μM IC87114 (IC, PI3Kδ inhibitor) or a mixture of both inhibitors (U0 + IC). Cells were then activated (gray columns) or not activated (white column) by GA. Production of sIL-1Ra was measured in harvested supernatants. Results are presented as described in Fig. 2*B* (no inh. = 100% = 2720 ± 1726 pg/mL sIL-1Ra). Data are mean ± SD of three experiments carried out with monocytes prepared from blood of three different donors. $^{**}P < 0.01$ as determined by Student *t* test.

production of sIL-1Ra (Fig. 5). Together the results of Figs. 4 and 5 demonstrate the implication of the MEK/ERK pathway in sIL-1Ra production downstream GA-activation of monocytes.

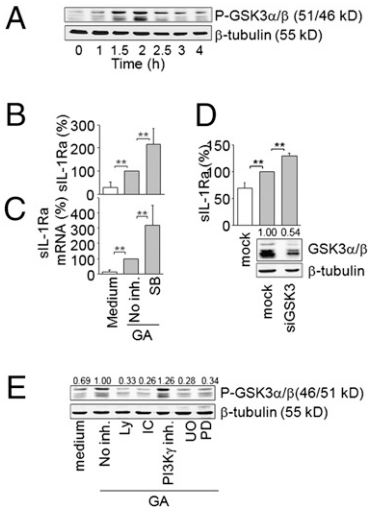

Fig. 7. GSK3α/β controls GA-induced sIL-1Ra production. (*A*) Monocytes were stimulated by GA for indicated time, and cell lysates were subjected to Western blot analysis. (*B* and *C*) Monocytes were preincubated for 45 min in the presence or absence of 10 μM SB216763 (SB) and then cultured in the absence (white columns) or presence (gray columns) of GA. (*B*) Production of sIL-1Ra was measured in harvested supernatants (no inh. = 100% = 2020 ± 636 pg/mL sIL-1Ra) and (*C*) mRNA analyzed by real-time quantitative PCR; results are presented as described in Fig. 2 *B* and *D*, respectively. (*D*) Monocytes were nucleofected with stealth siRNA for GSK3α and GSK3β (siGSK3) or negative control (mock). Efficiency of GSK3α/β silencing was assessed by Western blot (*Lower*). sIL-1Ra was measured in culture supernatants of GSK3α/β knocked-down or mock-transfected monocytes activated (gray columns; 100% = 2174 ± 467 pg/mL sIL-1Ra) or not activated (white column; sIL-1Ra concentration = 1,344 ± 676 pg/mL) by GA. (*E*) Monocytes were preincubated in the absence or presence of 10 μM Ly294002 (Ly); 5 μM IC87114 (IC); 500 nM PI3Kγ inhibitor, 5 μM U0126 (U0), or 5 μM PD98059 (PD) before activation by GA. Cell lysates were subjected to Western blot analysis. $^{**}P < 0.01$ as determined by Student's *t* test.

PI3Kδ/Akt and MEK/ERK Are Part of Two Parallel Pathways Controlling sIL-1Ra Production. To determine whether PI3Kδ and MEK1/2 integrated a common pathway to regulate sIL-1Ra production in response to GA in monocytes, we assessed the effect of inhibitors in combination or alone. Because signals induced by GA were exquisitely sensitive to kinase inhibitors, we used suboptimal concentrations of inhibitors as determined by dose–response curves (Fig. S1*C* and Fig. 4*A*), i.e., 0.5 μM IC87114 and 0.5 μM U0126. At such inhibitor concentrations, we observed a decrease of sIL-1Ra production by 45 ± 5% and 69 ± 6% with PI3Kδ and MEK1/2 inhibitor, respectively (Fig. 6). When both IC87114 and U0126 were combined, the GA-induced production of sIL-1Ra was abolished, reaching basal level. These results suggest that the PI3Kδ/Akt and the MEK/ERK pathways act in parallel to regulate sIL-1Ra production in monocytes activated by GA.

PI3Kδ/Akt and MEK/ERK Pathways Converge on GSK3α/β to Control GA-Induced sIL-1Ra Production. GSK3 is a constitutively active Ser-Thr kinase, whose phosphorylation downstream PI3K/Akt or MAPK pathway results in its inhibition (37, 38). To assess the contribution of GSK3 to GA-induced sIL-1Ra production by monocytes, the phosphorylation of GSK3 at Ser21 for GSK3α and Ser9 for GSK3β was first analyzed. Likewise Akt and ERK1/2 phosphorylation (Fig. 1*A*), the phosphorylation of GSK3α/β was induced by GA, reaching a maximum at 2 h (Fig. 7*A*). To evaluate the involvement of GSK3 in the control of sIL-1Ra production, monocytes were pretreated with the GSK3 inhibitor SB216763 before activation by GA. When monocytes were pretreated with SB216763, the production of sIL-1Ra induced by GA increased 2.15- ± 0.69-fold (Fig. 7*B*). Concomitantly, pretreatment of monocytes with SB216763 enhanced sIL-1Ra transcript expression in response to GA (Fig. 7*C*), indicating that active GSK3α/β repressed sIL-1Ra production in GA-activated monocytes. To confirm results obtained with the inhibitor, we examined the effect of siRNA-mediated GSK3α/β silencing on GA-induced sIL-1Ra production. The silencing of GSK3α/β reduced its expression by 46% (Fig. 7*D*) and further increased the induction of sIL-1Ra production by 1.95- ± 0.05-fold after monocyte stimulation by GA (Fig. 7*D*). To assess whether GSK3 inactivation was controlled by the PI3Kδ/Akt or the MEK/ERK pathway, we tested the effects of PI3K and MEK inhibitors on GSK3α/β phosphorylation. Inhibitors of both pathways decreased GSK3α/β phosphorylation, whereas the PI3Kγ inhibitor was ineffective (Fig. 7*E*). These data demonstrate that activation of the PI3Kδ/Akt and MEK/ERK pathways by GA converge on GSK3 and repress its activity to control sIL-1Ra expression in monocytes.

Discussion

This study demonstrates that GA triggers at least two different parallel pathways (PI3Kδ/Akt and MEK/ERK) that converge on GSK3 to promote sIL-1Ra production in human monocytes (Fig. S2). PI3Kδ is activated in monocytes exposed to GA, as suggested by the recruitment of p110δ catalytic subunit at monocyte membranes. Our data further indicate that PI3Kδ is the only PI3K isoform involved in the control of sIL-1Ra production in monocytes upon GA-activation. PI3Kδ controls sIL-1Ra production through the activation of its downstream element, Akt, which phosphorylates GSK3 and turns off its repressive activity. In addition, both MEK1 and MEK2 control sIL-1Ra production through the activation of their canonical substrates ERK1/2 and GSK3. The PI3Kδ/Akt and MEK/ERK pathways are likely to act in parallel upstream GSK3, although the present results do not rule out other cross-talks. The demonstration that GA, which is a mixture of peptides with different sequences of Glu, Lys, Ala, and Tyr, induces signal transduction leading to the production of sIL-1Ra, strongly suggests that monocytes express a specific receptor/sensor that binds/recognizes GA or a part of its peptides.

The existence of a specific GA sensor/receptor in monocytes is sturdily suggested by our observations demonstrating the induction of signal transduction pathways. In vitro, GA was shown to interact with empty, recombinant MHC class II molecules, competing with the binding of myelin basic protein into the peptide-binding groove (39). GA also interacts promiscuously with MHC class II molecules at the surface of living APCs (40). The latter study demonstrates that binding of GA to MHC class II molecules occurred very rapidly after GA application, i.e., GA binding at time = 0 (80%) was not different from that at time = 20 h. This contrasts with the present results showing delayed signal transduction with maximum Akt and ERK1/2 phosphorylation at 2 h. Because signal transduction induced by ligation of MHC class II molecules by antibodies, their natural ligand (LAG-3), or superantigens occurs within minutes (41–43), it is likely that GA signaling is mediated by other receptors/sensors. Moreover, contrasting with GA effects, IL-1β production is induced in monocytes upon ligation of MHC class II by antibodies, F(ab) from the latter, and superantigens (42, 44–46). As GA induces sIL-1Ra but not IL-1β production in human monocytes (29), it is unlikely that MHC class II molecules may act as GA receptors to induce sIL-1Ra production. Furthermore, the demonstration that in MHC class II-deficient mice, GA induces type II monocyte differentiation favoring the production of anti-inflammatory cytokines, strongly indicates that GA may affect monocyte function in the absence of MHC class II molecules (47). In addition to MHC class II, GA has been shown to interact with Mac-1 (CD11b/CD18 integrin) (48). Ligation of β2-integrins by either CD23 or antibodies induces the production of IL-1β in human monocytes that depends on the early (2–5 min) phosphorylation of ERK1/2 (49). Therefore, alike MHC class II molecules, Mac-1 is unlikely to act as the GA receptor on monocytes involved in sIL-1Ra induction.

Our results further support the premise that PI3Kδ activation is a key requirement to optimal secretion of sIL-1Ra in monocytes upon various stimulatory conditions. Indeed, we recently reported that PI3Kδ accounts for the PI3K-dependent signaling ruling the production of sIL-1Ra in monocytes activated by lipopolysaccharides (LPS), i.e., a prototypical stimulus of acute inflammation, and by contact with stimulated T cells, i.e., a stimulus likely to reflect chronic inflammatory conditions (33). Interestingly, although PI3Kδ activity is required to sIL-1Ra induction independently of the stimulus, it dampens the production of proinflammatory cytokines in LPS-activated monocytes, but induces such production in T-cell contact-activated monocytes (33). Thus, PI3Kδ is likely to be a key element in the regulation of inflammatory effector functions of monocytes.

The activation of both MEK1 and MEK2 is required to optimal production of sIL-1Ra, which is controlled by their downstream substrates, ERK1/2. Of note, GA requires the activation of PI3Kδ/Akt and MEK/ERK pathways, which act in parallel to regulate sIL-1Ra production. Indeed, when suboptimal doses of inhibitors of either MEK1/2 or PI3Kδ were used, only 45–69% of inhibition of GA-induced sIL-1Ra production was observed. However, when inhibitors were combined, the induction of sIL-1Ra production was abolished, indicating that both pathways independently controlled sIL-1Ra following GA-activation. Because Akt, ERK1/2, and GSK3α/β were phosphorylated within the same time frame, i.e., 2 h after GA-activation, the two PI3Kδ/Akt and MEK/ERK pathways are likely to be concomitantly activated, both leading to GSK3 phosphorylation. Although PI3Kδ is used by other sIL-1Ra inducers, there are different factors ruling sIL-1Ra production downstream the different stimuli. In LPS-activated monocytes the production of sIL-1Ra is regulated via the PI3K pathway by GSK3, which acts upstream the MEK1/2/ERK1/2 pathway (34). Our current data are in agreement with these findings, demonstrating that GSK3 phosphorylation dampens sIL-1Ra production in GA-activated monocytes, although we identify GSK3 as a downstream effector of the MEK1/2/ERK1/2 pathway. GSK3 is a constitutively active Ser-Thr kinase whose phosphorylation downstream PI3K/Akt or MAPK pathway results in its inhibition (37, 38). GSK3 phosphorylates/inactivates multiple substrates, including transcription factors that are critical regulators of pro- and antiinflammatory cytokine production (50). In accordance with the present results, previous studies have demonstrated that active GSK3β negatively regulates the production of antiinflammatory cytokines while concurrently upregulating production of proinflammatory cytokines (51).

We previously demonstrated that GA increases the circulating levels of sIL-1Ra in GA-treated MS patients and EAE mice (29). This observation corroborates the triggering of sIL-1Ra production by GA in isolated blood monocytes. Furthermore, in T-cell contact-activated monocytes, i.e., a mechanism relevant to chronic inflammation, GA strongly diminishes the expression of IL-1β and enhances expression of sIL-1Ra, contrasting with the effect of GA in monocytes activated upon acute inflammatory conditions (29). Similar effects were observed on elements of the IL-1 system in IFNβ-activated monocytes (52). Taken together, these findings suggest that both immunomodulators used in MS treatment, i.e., GA and IFNβ, affect the IL-1 system in comparable ways.

To conclude, our results demonstrate that GA signals through two parallel signaling pathways, i.e., the PI3Kδ/Akt and MEK/ERK cascades, which activities converge to phosphorylate GSK3α/β and result in the induction of sIL-1Ra production in monocytes. In addition to the direct activation of signal transduction by GA, this study strongly suggests the existence of a specific receptor/sensor of GA in human monocytes.

Materials and Methods

Details on materials and methods are given in *SI Materials and Methods*.

Monocytes. Peripheral blood monocytes were prepared as described elsewhere (53).

sIL-1Ra Production, mRNA Quantification, and mRNA Silencing. sIL-1Ra production, mRNA quantification, and mRNA silencing were carried out as previously described (29).

Western Blot Analysis. Western blot analyses were carried out as described previously (54).

Translocation of PI3Kδ at Monocyte Membranes. Monocytes (10×10^6 cells/mL; 1 mL) were activated by GA (25 μg/mL) for 2 h. Their membranes were isolated by ultracentrifugation and were subjected to Western blot analysis.

Statistical Analysis. When required, significance of differences between groups was evaluated using a Student paired *t* test.

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Supporting Information

Carpintero et al. 10.1073/pnas.1009443107

SI Materials and Methods

Materials. The following materials were purchased from the designated suppliers: FCS, streptomycin, penicillin, L-glutamine, RPMI-1640, PBS free of Ca^{2+} and Mg^{2+} (Gibco); Lymphoprep (Axis-Shield); TriReagent, endotoxin-free dimethylsulphoxide (DMSO), phenylmethylsulfonyl fluoride (PMSF), polymyxin B sulfate (Sigma Chemical), GA (Sanofi-Aventis); PI3K pan-inhibitor Ly294002, MEK1/2 inhibitor, U0126 and MEK1 inhibitor, PD98059 (LC Laboratories); PI3Kα, β and γ selective inhibitors, compound 15e, TGX-221 and AS-604850, respectively (Alexi Corp.); and GSK3α/β inhibitor, SB216763 (Sigma Chemical); Kinase specific Stealth RNAi siRNA and Stealth RNAi negative control duplex (Invitrogen). IC87114 was kindly provided by Calistoga Pharmaceuticals. Other reagents were of analytical grade or better.

Monocytes. Peripheral blood monocytes were isolated from buffy coats of blood of healthy volunteers provided by the Geneva Hospital Blood Transfusion Center as previously described (1). In accordance with the ethical committee of the Geneva Hospital, the blood bank obtained informed consent from the donors, who are thus informed that part of their blood will be used for research purposes. To avoid activation by endotoxin, polymyxin B sulfate (5 μg/mL) was added in all solutions during isolation procedure and in all experiments. Monocyte purity routinely consisted of >90% CD14+ cells, <1% CD3+ cells, and <1% CD19+ cells as assessed by flow cytometry.

sIL-1Ra Production. Monocytes (5×10^4 cells/200 μL/well) were preincubated for 45 min in RPMI medium 1640 supplemented with 10% heat-inactivated FCS, 50 μg/mL streptomycin, 50 U/mL penicillin, 2 mM L-glutamine, and 5 μg/mL polymyxin B sulfate (medium) in 96-well plates at the indicated concentration of kinase inhibitors or DMSO, and then activated 48 h with 25 μg/mL GA. The latter concentration was previously determined as optimal (2). All conditions were carried out in triplicate. The production of sIL-1Ra was measured in culture supernatants by commercially available enzyme immunoassay (Quantikine, R&D). All experiments were carried out with monocytes isolated from at least three different blood donors. DMSO used as a control for inhibitors did not display any effects.

mRNA Quantification. Monocytes (2×10^6 cells/3 mL/well) were cultured in six-well plates in medium containing 5 μg/mL polymyxin B sulfate for 45 min with the indicated inhibitor or DMSO and then activated by 25 μg/mL GA for 18 h. Total mRNA was prepared by TriReagent (Sigma) according to the provider protocol. Quantitative real-time duplex PCR analysis was conducted on a TaqMan 7300 quantitative real time PCR system (Applied Biosystems) after reverse transcription by SuperScript II (Invitrogen). The levels of mRNA expression were normalized with the expression of a housekeeping gene (18S) analyzed simultaneously. sIL-1Ra and 18S probes were purchase form Applied Biosystems. All measurements were conducted in triplicates. DMSO used as a control for inhibitors did not display any effects.

Western Blot Analysis. Human monocytes were resuspended at 6×10^6 cells/mL in medium supplemented with 5 μg/mL polymyxin B sulfate and 500 μL was placed in 2-mL polypropylene tubes (Eppendorf) at 37 °C for 1 h before addition of kinase inhibitors or DMSO for 45 min. Cells were then activated with 25 μg/mL GA. At the indicated time, the activation was stopped by the addition of 800 μL ice-cold PBS and underwent centrifugation. Total cell lysates were prepared and subjected to Western blot analysis as described previously (3). Nitrocellulose membranes were probed with anti–phospho-p44/42 MAP Kinase (Thr202/Tyr204), anti-p44/42 (ERK 1/2), anti–phospho-Akt (Ser_{473}), anti-Akt, anti–phospho-GSK3α/β (Ser21/9), anti-MEK 1, anti GSK3α and anti-GSK3β (Cell Signaling Technology), anti-PI3Kδ (Calbiochem), anti-PI3Kα (BD), anti–β-tubulin (Sigma), and anti-MEK2, anti-PI3Kβ, and anti-PI3Kγ (Santa Cruz). Secondary IR700/800 conjugated goat antirabbit or goat antimouse antibodies (Rockland) were used, and antibody-bound proteins were detected and quantified with an Odyssey system (Li-Cor). DMSO used as a control for inhibitors did not display any effects.

mRNA Silencing. PI3Ks, MEK1, MEK2, ERK1/2, Akt, and GSK3α/β were silenced with 4 μM stealth siRNA or Stealth RNAi negative control duplex (mock) designed by the provider (Invitrogen). Monocytes were transfected using Nucleofector device and Nucleofector human Monocyte kit according to the provider protocol (Amaxa). Transfected monocytes (6×10^6 cells/1.6 mL/well) were seeded into 24-well ultra-low attachment plates (Corning). After 48 h, transfected cells were harvested. PI3Ks, MEK1/MEK2, ERK1/2, Akt, and GSK3α/β silencing was ascertained by Western blot using anti-PI3Kα, anti-PI3Kβ, anti-PI3Kγ, anti-PI3Kδ, anti-MEK1, anti-MEK2, anti-p44/42 ERK1/2, anti-Akt, and anti-GSK3α/β–specific antibodies. The ability of silenced monocytes to produce sIL-1Ra upon GA-treatment was measured as described above.

Translocation of PI3Kδ at Monocyte Membranes. Monocytes (10×10^6 cells/mL; 1 mL) were activated by GA (25 μg/mL) for 2 h. The activation was stopped by the addition of ice-cold PBS and centrifugation. Monocytes were then resuspended in 50 mM Hepes (pH 7.3) containing 150 mM NaCl, 5 mM EDTA, 1 mM $NaVO_4$ 50 mM NaF, 1 μM leupeptin, 1 μM pepstatin ,and 20 μM PMSF and lyzed by 10 freeze–thaw cycles. Membranes were isolated by ultracentrifugation at 100,000 × *g* for 45 min. The pellets were resuspended in sample buffer and subjected to Western blot analysis with anti-PI3Kδ as described above.

Statistical Analysis. When required, significance of differences between groups was evaluated using the Student paired *t* test.

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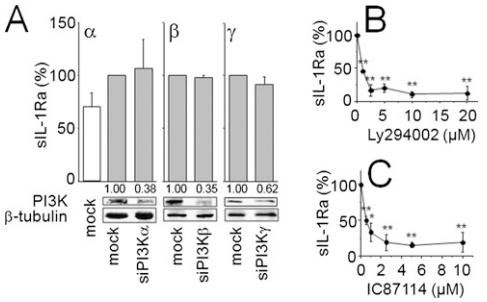

Fig. S1. PI3Kδ controls sIL-1Ra induction by GA in human monocytes. (*A*) Monocytes were nucleofected with stealth siRNA for the indicated PI3K isoform or negative control (mock). PI3K silencing was assessed by Western blot (*Lower*). PI3K knocked-down or mock-transfected monocytes were activated (gray columns; 100% = 1806 ± 634 pg/mL sIL-1Ra) or not activated (white column; sIL-1Ra concentration = 1173 ± 696 pg/mL) with GA and sIL-1Ra measured in culture supernatants. (*B* and *C*) Monocytes were preincubated with increasing concentration of Ly294002 (*B*) or IC87114 (*C*) before the addition of GA. sIL-1Ra production was measured in harvested supernatants and presented as percentage of sIL-1Ra production induced by GA in the absence of inhibitor (100% = 2347 ± 956 pg/mL sIL-1Ra). Results are presented as mean ± SD of three independent experiments carried out with monocytes isolated from three different individuals. $^{**}P < 0.01$ and $^{*}P < 0.05$ as determined by Student *t* test.

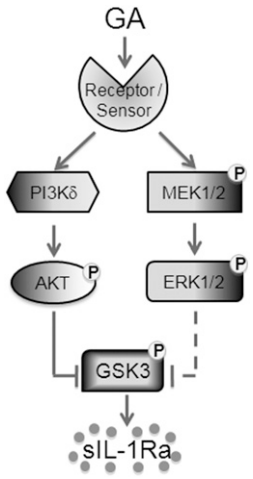

Fig. S2. Model of how GA activates PI3Kδ/Akt and MEK/ERK pathways to induce sIL-1Ra production. GA is recognized by a receptor (cell surface) or a sensor (inside the cell) that transduces signal via activation of both the PI3Kδ/Akt and MEK/ERK pathways. The two pathways then converge to phosphorylate/inactivate GSK3, resulting in the induction of sIL-1Ra production in monocytes.

6.5 Stimulated T Cells Generate Microparticles, which Mimic Cellular Contact Activation of Human Monocytes: Differential Regulation of Pro- and Anti-Inflammatory Cytokine Production by High-density Lipoproteins

Scanu A, Molnarfi N, Brandt KJ, Gruaz L, Dayer JM, Burger D.

Stimulated T cells generate microparticles, which mimic cellular contact activation of human monocytes: differential regulation of pro- and anti-inflammatory cytokine production by high-density lipoproteins

Anna Scanu,[1] Nicolas Molnarfi, Karim J. Brandt, Lyssia Gruaz, Jean-Michel Dayer, and Danielle Burger[2]

Division of Immunology and Allergy, Clinical Immunology Unit, Department of Internal Medicine, University Hospital and School of Medicine, Geneva, Switzerland

Abstract: Imbalance in cytokine homeostasis plays an important part in the pathogenesis of chronic inflammatory diseases such as multiple sclerosis and rheumatoid arthritis. We demonstrated that T cells might exert a pathological effect through direct cellular contact with human monocytes/macrophages, inducing a massive up-regulation of the prototypical proinflammatory cytokines IL-1β and TNF. This mechanism that might be implicated in chronic inflammation is specifically inhibited by high-density lipoproteins (HDL). Like many other stimuli, besides proinflammatory cytokines, the contact-mediated activation of monocytes induces the production of cytokine inhibitors such as the secreted form of the IL-1 receptor antagonist (sIL-1Ra). The present study demonstrates that stimulated T cells generate microparticles (MP) that induce the production of TNF, IL-1β, and sIL-1Ra in human monocytes; the production of TNF and IL-1β but not that of sIL-1Ra is inhibited in the presence of HDL. The results were similar when monocytes were stimulated by whole membranes of T cells or soluble extracts of the latter. This suggests that MP carry similar monocyte-activating factors to cells from which they originate. Thus, by releasing MP, T cells might convey surface molecules similar to those involved in the activation of monocytes by cellular contact. By extension, MP might affect the activity of cells, which are usually not in direct contact with T cells at the inflammatory site. Furthermore, this study demonstrates that HDL exert an anti-inflammatory effect in nonseptic activation of human monocytes, not only by inhibiting the production of IL-1β and TNF but also, by leaving sIL-1Ra production unchanged. *J. Leukoc. Biol.* 83: 921–927; 2008.

Key Words: *IL-1β · TNF · IL-1Ra · inflammation · acute-phase reactants*

INTRODUCTION

Imbalance in cytokine homeostasis plays an important part in the pathogenesis of chronic inflammatory diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA). This suggests that the mechanisms ruling the production of proinflammatory cytokines, including TNF and IL-1β and their inhibitors, i.e., soluble receptors and secreted IL-1 receptor antagonist (sIL-1Ra), escape normal controls. TNF and IL-1β are mainly produced upon activation of monocytes/macrophages. In immunoinflammatory diseases, in the absence of an infectious agent (i.e., in nonseptic conditions), the nature of the factors triggering TNF and IL-1β production is still elusive. We previously demonstrated that direct cellular contact with stimulated T cells induces the massive up-regulation of IL-1 and TNF in monocytes/macrophages [1]. Besides triggering proinflammatory cytokine production, contact-mediated activation of monocytes induced the production and/or shedding of cytokine inhibitors such as sIL-1Ra and soluble receptors of IL-1 and TNF [2–5]. Most T cell types, including T cell clones, freshly isolated T lymphocytes, and T cell lines such as HUT-78 cells, induce IL-1 and TNF in monocytes/macrophages [6]. Furthermore, depending on T cell type and T cell stimulus, direct cell–cell contact with stimulated T lymphocytes can induce different patterns of products in monocytes/macrophages, as reviewed in refs. [1, 7, 8], suggesting that multiple ligands and counter-ligands are involved in the contact-mediated activation of monocytes/macrophages. In some cases, an imbalance in the production of proinflammatory versus anti-inflammatory cytokines has been observed. Indeed, TH1 cell clones preferentially induce IL-1β rather than sIL-

[1] Current address: Section of Rheumatology, Department of Clinical and Experimental Medicine, University of Padova, Via Giustiniani, 2, 35128 Padova, Italy.

[2] Correspondence: Clinical Immunology Unit, BB-4866, University Hospital, 24 rue Micheli-du-Crest, CH-1211 Geneva 14, Switzerland. E-mail: danielle.burger@hcuge.ch

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1Ra production, and cytokine-stimulated T lymphocytes induce TNF production and fail to trigger that of IL-10 [4, 9].

The production of IL-1β and TNF by monocytes upon contact activation by stimulated T cells is inhibited by high-density lipoprotein (HDL)-associated apolipoprotein A-I (apo A-I) [10], a "negative", acute-phase protein. In contact-mediated activation of monocytes, HDL-associated apo A-I exerts most of its inhibitory activity by interfering with monocyte-activating factors at the surface of T cells, thus decreasing the production of IL-1β and TNF [10]. In vivo, apo A-I is retained in the perivascular regions of RA-inflamed synovium, i.e., in T cell-rich regions [11].

Upon chronic inflammation, after extravasation, most T cells remain in the perivascular region, and other infiltrating cells, such as monocytes/macrophages and neutrophils, have to cross the perivascular layer of T cells and in turn, make contact with the latter cells before penetrating further into the target tissue. Consequently, direct cell–cell contact with T cells is less frequent outside perivascular regions. However, cells can disseminate cell surface molecules by generating microparticles (MP) and thus, ensure "distant" cellular contact. MP are fragments (0.1–0.8 μm diameter) shed from the plasma membrane of stimulated or apoptotic cells. As MP lose membrane polarity, phosphatidylserine residues are exposed at their surface, which in turn, can bind Annexin V [12]. Having long been considered inert debris reflecting cellular activation or damage, MP are now acknowledged as cellular effectors involved in cell–cell crosstalk [13, 14]. Indeed, they harbor membrane proteins as well as bioactive lipids implicated in a variety of fundamental processes and thus, constitute a disseminated pool of bioactive effectors [15]. MP are found in the circulation of healthy subjects [16–18], but their numbers are increased in various pathological conditions, such as thrombotic or infectious diseases [17, 19–23]. Elevated MP have also been reported in chronic inflammatory diseases [12, 24, 25], including RA [26–29] and MS [25, 30–33]. In MS, MP are present in patients' plasma, although cerebrospinal fluid has, to our knowledge, not been investigated for the presence of MP. In RA synovial fluid, MP are abundant and modulate fibroblast-like synoviocyte activity in vitro [28, 29]. As most cell types can generate MP [29, 34], the latter process might represent a way for T cells to disseminate cellular contact from a distance. This study was undertaken to assess whether MP generated by stimulated T cells could induce cytokine production in human monocytes and whether the latter process would be modulated by HDL.

MATERIALS AND METHODS

Materials

FCS, streptomycin, penicillin, L-glutamine, RPMI-1640, PBS free of Ca^{2+} and Mg^{2+}, and TRIzol™ reagent (Gibco, Paisley, Scotland); purified PHA (EY Laboratories, San Marco, CA, USA); Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden); PMA, PMSF, polymyxin B sulfate, TriReagent, and BSA (Sigma Chemical Co., St. Louis, MO, USA); and Annexin V-FITC (BD Biosciences, San Jose, CA, USA) were purchased from the designated suppliers. Other reagents were of analytical grade or better.

Monocytes

Peripheral blood monocytes were isolated from buffy coats of blood of healthy volunteers as described previously [10]. To avoid activation by endotoxin, polymyxin B (2 μg/ml) was added to all solutions during the isolation procedure.

Isolation of peripheral blood T lymphocytes

Peripheral blood T lymphocytes were prepared as described previously [35]. T lymphocytes (4×10^6 cells/ml) were stimulated or not for 48 h with PHA (1 μg/ml) and PMA (5 ng/ml), as described [36]. MP were isolated from cell supernatants as described below. Proteins were determined by the method of Bradford [37].

T cell stimulation and membrane isolation

The human T cell line HUT-78 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 IU/ml penicillin, and 2 mM L-glutamine in a 5% CO_2 air-humidified atmosphere at 37°C. HUT-78 cells (2×10^6 cells/ml) were stimulated for 6 h by PHA (1 μg/ml) and PMA (5 ng/ml). Plasma membranes of stimulated (msHUT) or unstimulated (musHUT) HUT-78 cells were prepared as described previously [38]. Isolated membranes were solubilized in 8 mM CHAPS as described previously [39]. CHAPS extracts of msHUT and musHUT were referred to as CE_{sHUT} and CE_{usHUT}, respectively. Proteins were measured by the method of Bradford [37].

Isolation of MP

MP were isolated from culture supernatants of HUT-78 cells and peripheral blood T lymphocytes. Briefly, cell supernatant was obtained after cell removal by centrifugation at 800 g for 5 min. Supernatant was then centrifuged at 7000 g for 5 min to discard large debris. After additional centrifugation at 20,000 g for 45 min, MP were washed twice in PBS, resuspended in PBS, and measured for protein content [37]. Annexin V-FITC binding and MP concentration (number) were determined by flow cytometry (FACSCalibur, BD Biosciences), according to ref. [12]. An event discrimination threshold was set on the side-scatter channel at the lowest channel allowed, and a size gate was set using 0.8 μm latex beads (Sigma Chemical Co.). RNA and DNA content in MP was estimated by measuring OD at 260 nm after isolation with TriReagent as described by the supplier [39]. When kept at 4°C upon sterile conditions in PBS, isolated MP were stable for at least 3 weeks in terms of their ability to induce cytokine production in human monocytes.

Isolation of HDL

Human serum HDL were isolated according to Havel et al. [40] as described previously [10]. Proteins were measured by the method of Bradford [37]. To optimize the inhibitory effect of HDL, they were always added together with the stimulus.

Cytokine production and measurement

Monocytes (5×10^4 cells/well/200 μl) were activated with the indicated stimulus in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 U/ml penicillin, 2 mM L-glutamine, and 5 μg/ml polymyxin B sulfate (medium) in 96-well plates and cultured for 48 h unless stated otherwise. The production of sIL-1Ra, IL-1β, and TNF was measured in culture supernatants by a commercially available enzyme immunoassay: IL-1β (Immunotech, Marseille, France), TNF, and sIL-1Ra (Quantikine, R&D Systems, Minneapolis, MN, USA).

mRNA quantification

Monocytes (2.1×10^6 cells/well/3 ml) were cultured in six-well plates with the indicated stimulus for 3 h. Total RNA was isolated and analyzed by real-time PCR as described previously [39].

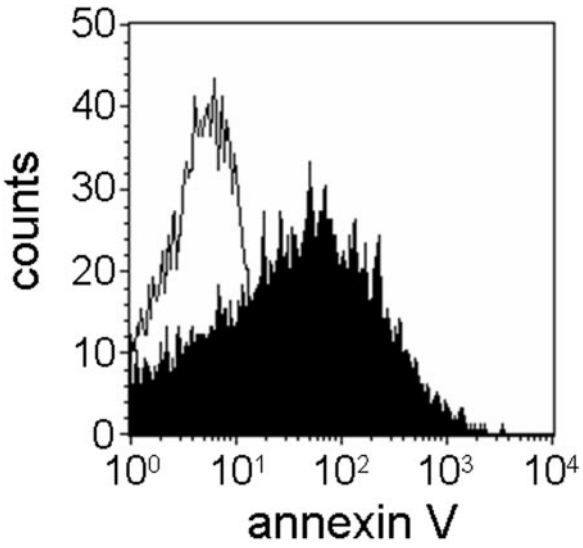

Fig. 1. Phosphatidylserine residues are exposed at the surface of MP generated by T cells. MP generated by msHUT-78 cells were labeled or not with Annexin V-FITC and analyzed by flow cytometry. Fluorescence intensity (*x*-axis) versus MP count (*y*-axis) is shown. Open histogram, MP that were not incubated with Annexin V-FITC; filled histogram, MP that bind Annexin V-FITC.

RESULTS

Characterization of MP generated by T cells

MP generated by HUT-78 cells and isolated from cell culture supernatant were subjected to flow cytometry analysis. MP from stimulated HUT-78 and unstimulated HUT-78 cells displayed similar size characteristics with diameters between 0.1 and 0.8 μm. MP bound Annexin V-FITC (**Fig. 1**), demonstrating that phosphatidylserine was exposed at their surface. Stimulated HUT-78 cells produced $11.5 \pm 2.3 \times 10^6$ MP/10^6 cells, whereas unstimulated HUT-78 cells produced $0.07 \pm 0.03 \times 10^6$ MP/10^6 cells; i.e., approximately 200-fold less MP were produced by unstimulated HUT-78 as compared with stimulated HUT-78 cells. Similar results were obtained with peripheral blood T lymphocytes. Indeed, stimulated T lymphocytes generated $1.6 \pm 0.4 \times 10^6$ MP/10^6 cells, whereas unstimulated T lymphocytes generated $0.03 \pm 0.02 \times 10^6$ MP/10^6 cells. Comparable amounts of proteins were measured in MP from stimulated HUT-78 and unstimulated HUT-78 cells and peripheral blood T lymphocytes reaching 19.6 ± 4.8 μg protein/10^6 MP. Total RNA in MP generated by stimulated HUT-78 cells reached 34.3 ± 15.5 μg/mg proteins, i.e., 0.7 ± 0.3 μg RNA/10^6 MP. This suggests that MP were indeed closed vesicles able to protect RNA from degradation by RNases. IL-1β and IL-1Ra were not detected in MP produced by stimulated HUT-78 or unstimulated HUT-78 cells. TNF, which is present in membranes isolated from HUT-78 cells [36], was detected in MP from stimulated HUT-78 and unstimulated HUT-78 cells, reaching 455 ± 37 and 267 ± 45 pg/mg proteins, respectively. Furthermore, DNA was below the detection limit, thus amounting to <3 ng/mg proteins in MP from stimulated HUT-78 and unstimulated HUT-78 cells. This demonstrates that only few apoptotic bodies were present amongst MP generated by stimulated T cells.

MP from stimulated T lymphocytes induce monocytic cytokine production that is modulated by HDL

To assess whether MP would induce cytokine production, human monocytes were activated by increasing doses of MP from stimulated and unstimulated T lymphocytes in the presence or absence of an optimal concentration of HDL. As shown in **Figure 2**, MP generated by stimulated T cells induced the production of TNF, IL-1β, and sIL-1Ra in human monocytes in a dose-dependent manner. In contrast, MP generated by unstimulated T lymphocytes did not affect cytokine production. The production of IL-1β and TNF induced by MP from stimulated T lymphocytes was inhibited in the presence of HDL, whereas the production of sIL-1Ra remained unchanged. This suggests that MP carry activating factors that induce the production of cytokines in human monocytes. As the generation of MP by T lymphocytes from human peripheral blood in sufficient amounts to activate monocytes required a large number of T lymphocytes, the monocytic cell line HUT-78 cell was used

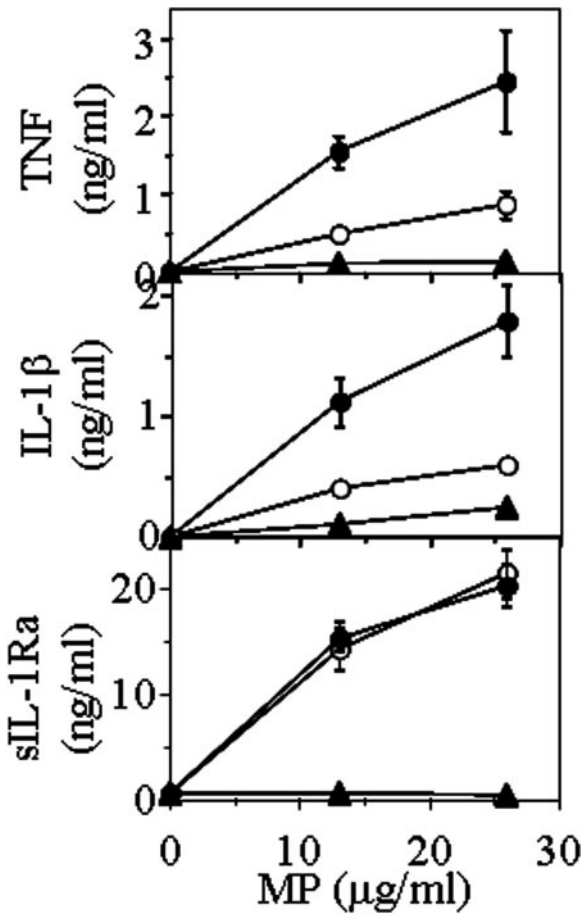

Fig. 2. MP generated by stimulated peripheral blood T lymphocytes display a cytokine-inducing capacity that is differentially affected by HDL. Monocytes (5×10^4 cells/200 μl/well; 96-well plates) were activated for 48 h with increasing doses of MP isolated from stimulated (circles) or unstimulated (triangles) T lymphocyte culture supernatants. Cells were activated in the presence (○) or absence (●) of 0.2 mg/ml HDL. TNF, IL-1β, and sIL-1Ra were measured in culture supernatants of triplicates and represented as mean ± SD. Results are representative of three different experiments.

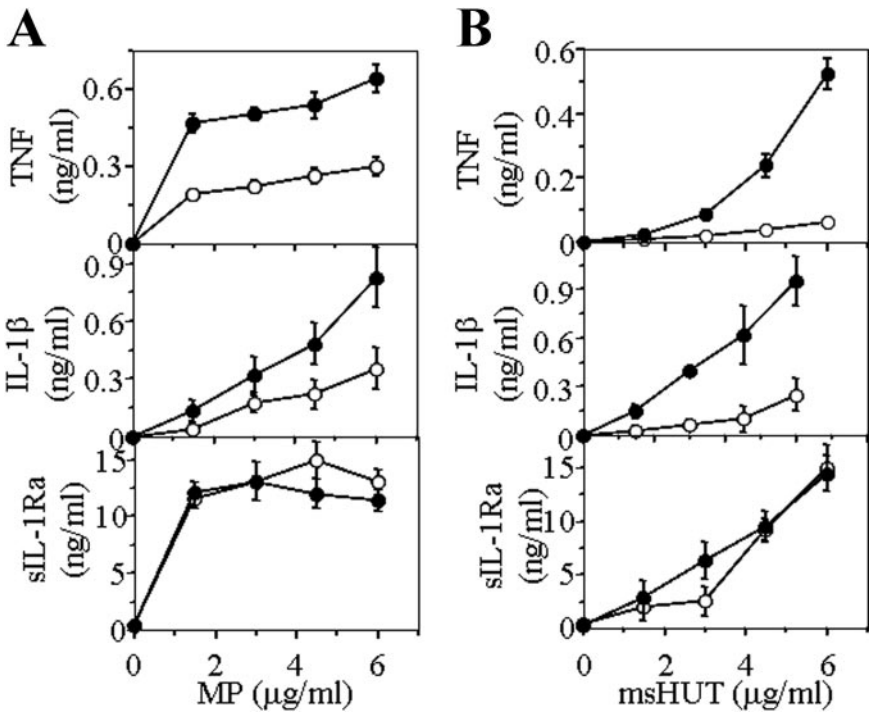

Fig. 3. MP generated by stimulated HUT-78 cells display a similar cytokine-inducing capacity to whole plasma membrane preparation. Monocytes (5×10^4 cells/200 μl/well; 96-well plates) were activated for 24 h with increasing doses of MP isolated from stimulated HUT-78 cell culture supernatants (A) or membranes isolated from the same HUT-78 cells (B). Cells were activated in the presence (○) or absence (●) of 0.2 mg/ml HDL. The production of sIL-1Ra, IL-1β, and TNF was measured in cell supernatants of triplicate cultures. Results are presented as percentage of cytokine production. (A and B) Representative experiments out of three are presented.

to further study cytokine induction in human monocytes by MP from stimulated T cells.

To ascertain that the monocyte-activating capacity of MP would be similar to that of T cell plasma membranes, i.e., cellular contact, MP and plasma membranes were isolated from the same HUT-78 cells that had been stimulated for 6 h (i.e., a period that had previously been tested for optimal contact-activating capacity). As shown in **Figure 3A**, MP generated by stimulated HUT-78 cells induced the production of cytokines in human monocytes to a similar extent as MP from stimulated T lymphocytes. MP-induced production of sIL-1Ra was not affected in the presence of HDL, whereas that of IL-1β and TNF was inhibited (Fig. 3A). Comparable results were obtained when stimulated HUT was used as a stimulus (Fig. 3B). Here again, the production of sIL-1Ra was not affected by HDL, whereas that of IL-1β and TNF, serving as controls, was strongly inhibited. Similar levels of cytokines were induced by MP and msHUT, although the inhibition of TNF and IL-1β production by HDL was more efficient when monocytes were activated by msHUT than by MP (Fig. 3, A and B). This might be a result of the differential accessibility of activating factors in plasma membrane preparation and MP. Together, these results imply that similar monocyte-activating factors were present at the surface of MP and the cells by which they were generated.

HDL did not inhibit contact-mediated induction of sIL-1Ra in monocytes

We previously demonstrated that HDL inhibit the production of proinflammatory cytokines induced by membranes isolated from stimulated T lymphocytes or msHUT-78 cells, but the effect of HDL on sIL-1Ra production has not been assessed so far. As shown in **Figure 4**, msHUT but not musHUT significantly induced the production of TNF, IL-1β, and sIL-1Ra in isolated blood monocytes. The production of sIL-1Ra remained unchanged, whereas that of proinflammatory cytokines used as controls was inhibited by HDL (Fig. 4). We previously demonstrated that the effect of membranes isolated from stimulated T lymphocytes or stimulated HUT-78 cells was not a result of their particulate form, as a similar cytokine-inducing ability was observed when soluble extract of membranes was used to activate monocytes. We thus assessed the effect of HDL on cytokine production induced by soluble extracts from membranes of HUT-78 cells. As shown in Figure 4, solubilized membranes of HUT-78 (CE_{sHUT} and CE_{usHUT}) displayed similar activity to whole membranes, although CE_{sHUT} tended to induce four- to tenfold more IL-1β and two- to threefold less sIL-1Ra than msHUT, depending on monocyte preparation, i.e., depending on individual blood donors. HDL did not inhibit sIL-1Ra production induced by CE_{sHUT} on monocytes but decreased the production of TNF and IL-1β (Fig. 4). These results demonstrate that HDL display anti-inflammatory activity by affecting pro- and anti-inflammatory cytokine balance.

HDL do not inhibit the expression of sIL-1Ra mRNA in contact-activated monocytes

HDL inhibit T cell contact-mediated induction of IL-1β and TNF at the transcriptional level. As cytokine production is controlled at several levels, the effect of HDL on sIL-1Ra mRNA expression was assessed to rule out the possibility that a putative inhibition of sIL-1Ra expression was compensated by the release of intracellular pools of IL-1Ra. As shown in **Figure 5A**, HDL did not inhibit sIL-1Ra mRNA expression in CE_{sHUT}-activated monocytes, whereas IL-1β and TNF mRNA expression was inhibited. That the lack of sIL-1Ra inhibition was not a result of the release of intracellular pools was further confirmed, as CE_{sHUT}-induced, cell-associated IL-1Ra levels were not diminished in the presence of HDL. The measurement

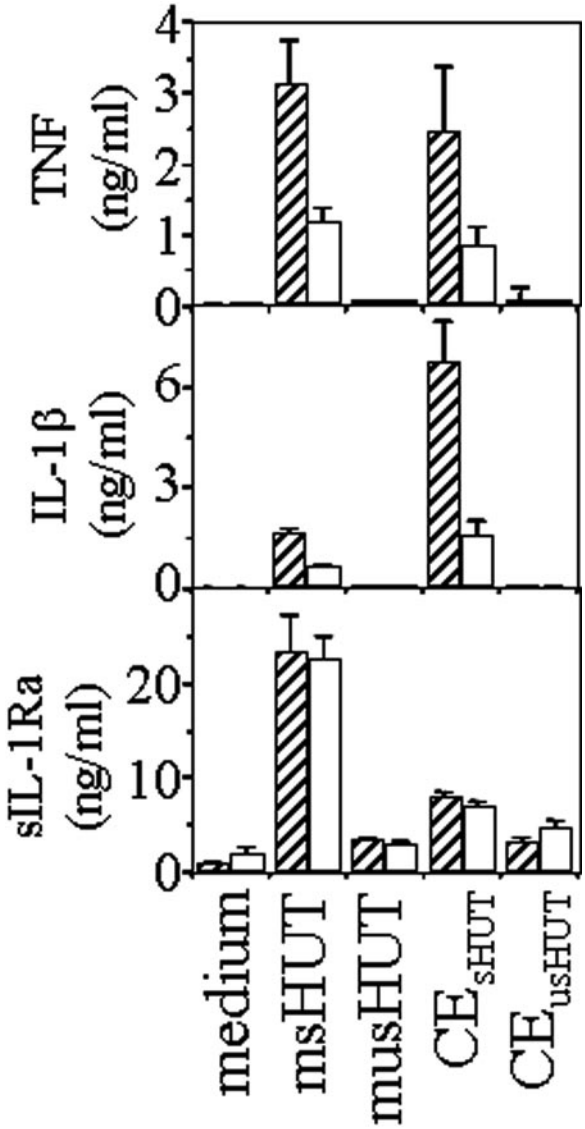

Fig. 4. HDL do not inhibit sIL-1Ra production in human monocytes upon contact-mediated activation by stimulated T cells. Isolated human blood monocytes (5×10^4 cells/200 μl/well; 96-well plates) were activated for 24 h with msHUT (6 μg/ml), musHUT (6 μg/ml), CE_{sHUT} (7 μg/ml), and CE_{usHUT} (7 μg/ml), as indicated, in the presence (open bars) or absence (hatched bars) of HDL (0.2 mg/ml proteins). sIL-1Ra, IL-1β, and TNF were measured in culture supernatants of triplicates and represented as mean ± SD. Results are representative of three different experiments.

of IL-1β and TNF production (assessed as controls) showed that the induction of both cell-associated and secreted IL-1β and TNF was inhibited by HDL (Fig. 5, B and C). These results suggest that sIL-1Ra was induced in monocytes by factor(s) whose activity is not affected by HDL.

DISCUSSION

The present study demonstrates that stimulated T cells generate MP, which mimic the activation of human monocytes mediated by cellular contact. The fact that HDL display similar effects on cytokine production when monocytes were activated by MP, msHUT, or CE_{sHUT}—i.e., they inhibited the production of IL-1β and TNF without affecting that of sIL-1Ra—suggests that similar activating factors are present on T cell surface and the MP they generate. Furthermore, the fact that HDL inhibit proinflammatory cytokine production while leaving the production of sIL-1Ra unchanged stresses the anti-inflammatory properties of HDL [8].

Our previous studies have shown that direct contact with stimulated T lymphocytes is an important pathway to induce proinflammatory cytokines and their inhibitors in human monocytes and that this mechanism might account for the unbalanced production of cytokines that drives uncontrolled inflammation in chronic/sterile inflammatory diseases such as MS and RA [1, 7, 8, 41]. MP from stimulated HUT-78 cells and peripheral blood T lymphocytes display comparable activity with isolated cell membranes, suggesting that similar molecules are present in MP and at the surface of stimulated T cells. Thus, in addition to adjacent monocytes/macrophages, stimulated T cells could make contact with distant monocytes/macrophages (e.g., macrophage-like synoviocytes in RA pannus-lining layer) through the release of MP. Furthermore, as contact with T cell plasma membranes or fixed, stimulated T cells triggers the production of pro- and anti-inflammatory products in fibroblasts (fibroblast-like synoviocytes and dermal fibroblasts), endothelial cells, and neutrophils [35, 38, 42–44], MP might remotely convey activating factors from T cells to such cells and participate in the pathogenic mechanisms of chronic inflammation. For instance, in RA, T cells are rare or absent from the lining layer of the pannus, where fibroblast-like synoviocytes are located. In the latter cells, direct contact with stimulated T cells or MP they generate induces an unbalanced production of matrix metalloproteases and their inhibitors [29, 38, 45]. In the absence of neighboring T cells, this activity might be conveyed by T cell MP. Similarly, in active, chronic MS lesions, where T cells are more abundant at the edge of lesions, unlike macrophages, which are more frequent at the lesion center [46], the generation of MP by activated T cells might ensure remote contact-mediated activation of monocytes/macrophages from the edge to the center of the lesion and in turn, maintain the activation stage of monocytes/macrophages.

We previously showed that HDL inhibit the production of IL-1β and TNF production by human monocytes and the monocytic cell line THP-1 by affecting the induction of cytokine transcription by msHUT [10]. This is further confirmed here by quantitative real-time PCR in monocytes activated by CE_{sHUT}. In contrast, neither the production nor the mRNA expression of sIL-1Ra was affected by the presence of HDL, demonstrating that the ratio of sIL-1Ra to IL-1β production was enhanced by HDL, stressing even more their anti-inflammatory nature. These results also demonstrate that several types of monocyte-activating factors are present at the surface of stimulated T cells—HDL inhibiting those responsible for the production of proinflammatory cytokines such as IL-1β and TNF.

MP may display pro- or anti-inflammatory activities [14]. In this study, MP released by stimulated T cells displayed inflammatory effects on isolated monocytes by inducing not only the production of the prototypical proinflammatory cytokines,

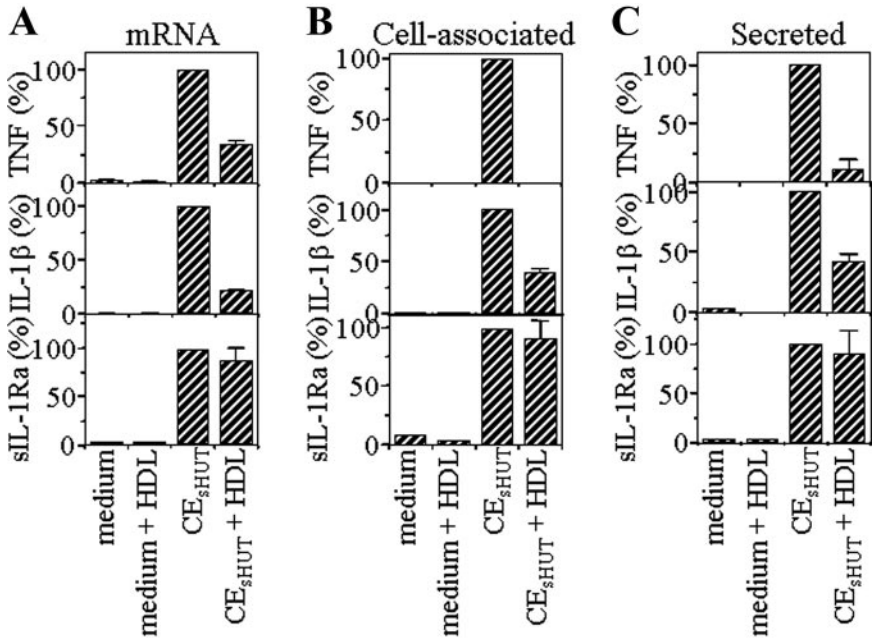

Fig. 5. Expression of sIL-1Ra mRNA is not affected by HDL. (A) Isolated monocytes (2×10^6 cells/well/3 ml; six-well plates) were activated or not by CE_{sHUT} (7 μg/ml) in the presence or absence of 0.2 mg/ml HDL. After 3 h, cells were harvested, and total RNA was analyzed by real-time PCR (see Materials and Methods). Results are presented as mean ± SD of three different experiments, 100% being considered as the level of cytokine mRNA induced by CE_{sHUT} in the absence of HDL. (B and C) Monocytes (5×10^4 cells/200 μl/well; 96-well plates) were activated by CE_{sHUT} in the presence or absence of 0.2 mg/ml HDL. After 24 h, the supernatants were harvested and monocytes solubilized in 200 μl PBS containing 1% Nonidet P-40. Cyokines were measured in culture supernatants (B) and cell lysate (C) to determine the production of secreted and cell-associated cytokine, respectively. Results are presented as mean ± SD of three different experiments, 100% being considered as the level of cytokine induced by CE_{sHUT} in the absence of HDL.

IL-1β and TNF, but also, the secretion of sIL-1Ra, a specific inhibitor of IL-1 [47]. Thus, similarly to membranes of T cells or fixed T cells, MP induced the production of pro- and anti-inflammatory mediators. This production is driven toward a less-inflammatory pattern in the presence of HDL. The generation of MP might also be a way for the cell to remove detrimental molecules from its surface. However, surface proteins carried by MP still display activity. This suggests that MP generation by T cells does not aim at removing surface factors from the cell surface but rather, at generating vectors likely to convey signals between distant cells.

In conclusion, this study demonstrates that MP generated by T cells carry similar molecules to the cells from which they originate, as they are able to activate the production of cytokines in human monocytes, and HDL affect this mechanism by inhibiting the production of proinflammatory cytokines IL-1β and TNF but not that of the anti-inflammatory cytokine sIL-1Ra. Consequently, through the generation of MP, stimulated T cells may ensure distant contact-mediated activation of cells, which are not located in their proximity at the inflammatory site.

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6.6 HDL Interfere with the Binding of T Cell Microparticles to Human Monocytes and Inhibit Pro-Inflammatory Cytokine Production

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HDL Interfere with the Binding of T Cell Microparticles to Human Monocytes to Inhibit Pro-Inflammatory Cytokine Production

Rakel Carpintero[1], Lyssia Gruaz[1], Karim J. Brandt[1], Anna Scanu[2], Dorothée Faille[3], Valery Combes[3], Georges E. Grau[3], Danielle Burger[1]*

1 Hans Wilsdorf Laboratory, Inflammation and Allergy Research Group, Division of Immunology and Allergy, Department of Internal Medicine, Faculty of Medicine and University Hospital, University of Geneva, Geneva, Switzerland, **2** Department of Clinical and Experimental Medicine, University of Padova, Padova, Italy, **3** Department of Pathology, University of Sydney, Camperdown, Australia

Abstract

Background: Direct cellular contact with stimulated T cells is a potent mechanism that induces cytokine production in human monocytes in the absence of an infectious agent. This mechanism is likely to be relevant to T cell-mediated inflammatory diseases such as rheumatoid arthritis and multiple sclerosis. Microparticles (MP) generated by stimulated T cells (MP_T) display similar monocyte activating ability to whole T cells, isolated T cell membranes, or solubilized T cell membranes. We previously demonstrated that high-density lipoproteins (HDL) inhibited T cell contact- and MP_T-induced production of IL-1β but not of its natural inhibitor, the secreted form of IL-1 receptor antagonist (sIL-1Ra).

Methodology/Principal Findings: Labeled MP_T were used to assess their interaction with monocytes and T lymphocytes by flow cytometry. Similarly, interactions of labeled HDL with monocytes and MP_T were assessed by flow cytometry. In parallel, the MP_T-induction of IL-1β and sIL-1Ra production in human monocytes and the effect of HDL were assessed in cell cultures. The results show that MP_T, but not MP generated by activated endothelial cells, bond monocytes to trigger cytokine production. MP_T did not bind T cells. The inhibition of IL-1β production by HDL correlated with the inhibition of MP_T binding to monocytes. HDL interacted with MP_T rather than with monocytes suggesting that they bound the activating factor(s) of T cell surface. Furthermore, prototypical pro-inflammatory cytokines and chemokines such as TNF, IL-6, IL-8, CCL3 and CCL4 displayed a pattern of production induced by MP_T and inhibition by HDL similar to IL-1β, whereas the production of CCL2, like that of sIL-1Ra, was not inhibited by HDL.

Conclusions/Significance: HDL inhibit both MP_T binding to monocytes and the MP_T-induced production of some but not all cytokines, shedding new light on the mechanism by which HDL display their anti-inflammatory functions.

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* E-mail: danielle.Burger@hcuge.ch

Introduction

An unbalanced cytokine homeostasis plays an important part in the pathogenesis of chronic inflammatory diseases. This suggests that the mechanisms ruling the production of pro-inflammatory cytokines, their inhibitors, and inhibitory mechanisms escape normal controls. IL-1β is a prototypical pro-inflammatory cytokine whose involvement in immuno-inflammatory diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA) is well established. In the absence of an infectious agent (*i.e.*, in non-septic conditions), the nature of the factors triggering the production of the prototypical pro-inflammatory cytokines, TNF and IL-1β, is still elusive. In chronic inflammatory diseases of autoimmune etiology, T cells and monocytes/macrophages infiltrate the target tissue. In animal models of MS and RA, the transfer of T cells isolated from diseased animals induces the disease in healthy animals, strongly suggesting that T cells play a pathogenic role [1,2]. It is now acknowledged that direct cellular contact with stimulated T cells induces the massive up-regulation of IL-1 and TNF in human monocytes/macrophages [3–5]. Besides triggering pro-inflammatory cytokine production, contact-mediated activation of monocytes also induces the production and/or shedding of cytokine inhibitors such as the secreted form of IL-1 receptor antagonist (sIL-1Ra), and soluble receptors of IL-1 and TNF [6–9]. Once stimulated, most T cell types, including T cell clones, freshly isolated T lymphocytes, and T cell lines such as HUT-78 cells, induce the production of IL-1β and TNF in monocytes/macrophages [10]. Furthermore, depending on T cell

type and T cell stimulus, direct cellular contact with stimulated T lymphocytes can induce different patterns of products in monocytes/macrophages (reviewed in [3,4,11]), suggesting that multiple ligands and counter-ligands are involved in the contact-mediated activation of monocytes/macrophages. This premise strengthened by observations showing that TH1 cell clones preferentially induce IL-1β rather than sIL-1Ra production, and cytokine-stimulated T lymphocytes induce TNF production while failing to trigger that of IL-10 [8,12]. Therefore, cellular contact with stimulated T cells can induce an imbalance in the production of pro-inflammatory versus anti-inflammatory cytokines, reflecting that observed in chronic inflammatory diseases.

By generating microparticles (MP) cells can disseminate cell surface molecules and thus ensure "distant" cellular contact. MP are fragments (0.1–1 μm diameter) shed from the plasma membrane of stimulated or apoptotic cells. Having long been considered inert debris reflecting cellular activation or damage, MP are now acknowledged as cellular effectors involved in cell-cell crosstalk [13]. Indeed, MP display membrane proteins as well as bioactive lipids implicated in a variety of fundamental processes and thus constitute a disseminated pool of bioactive effectors [14]. MP are present in the circulation of healthy subjects, and their numbers increase upon various pathological conditions [15]. Elevated MP have also been reported in chronic inflammatory diseases [16–18] including RA [19–22] and MS [18,23–26]. Although present in patients' plasma, MS cerebrospinal fluid has, to our knowledge, not been investigated for the presence of MP. In RA synovial fluid, MP are abundant and modulate fibroblast-like synoviocyte activity *in vitro* [21,22,27,28]. We recently demonstrated that MP generated by stimulated T cells can activate monocytes to produce cytokines similarly to membranes or solubilized membranes of stimulated T cells [29]. Furthermore, T cell contact-induced production of IL-1β and TNF in monocytes is specifically inhibited by high-density lipoproteins (HDL)-associated apolipoprotein A–I (apo A–I) [30], a "negative" acute-phase protein. HDL may infiltrate the inflamed tissue to counteract T cell contact-induce monocytes activation [31]. Furthermore, microarray analysis demonstrated that direct contact with stimulated T cells induces the expression of genes mostly related to inflammatory pathways but different from those induced under acute/infectious inflammatory conditions (e.g., induced by lipopolysaccharides), and that HDL inhibit the expression of pro rather than anti-inflammatory molecules [32]. For instance, in contrast to the production of IL-1β, HDL do not inhibit that of sIL-1Ra [29]. However, the mechanism by which HDL affect cytokine production in monocytes is still elusive. In this study we used MP to assess their interaction with monocytes and the effects of HDL. The results show that MP generated by stimulated T cells bind monocytes but not T lymphocytes and that HDL inhibit the interaction of MP_T with monocytes. Therefore, HDL may inhibit cytokine production in human monocytes by interfering with the binding of the activating factor(s) at the surface of stimulated T cells to receptor(s) at the surface of monocytes.

Results

Characterization of microparticles generated by stimulated HUT-78 cells (MP_T)

We previously demonstrated that MP generated by stimulated HUT-78 cells (here referred to as MP_T) display similar monocyte activating ability to MP generated by stimulated blood T lymphocytes [29]. In the present study we used MP_T to avoid variations often observed between T lymphocytes from different blood donors. Prior to assessing the ability of MP_T to activate human monocytes, we determined their physicochemical characteristics. As demonstrated by electron microscopy, MP_T are round particles with heterogeneous sizes displaying diameters between 0.1 and 0.8 μm, although most of MP_T were of small size (Fig. 1A). Flow cytometry analysis of MP_T preparation shows that particles between 0.1 and 0.8 μm bound annexin V (Fig. 1B) demonstrating that phosphatidylserine was exposed at their surface, thus defining them as microparticles. To assess the quality of MP_T preparations, we tested their ability to activate IL-1β and sIL-1Ra production in isolated monocytes. As previously described [29], MP isolated from unstimulated HUT-78 cells did not affect the production of cytokines in human monocytes (data not shown). We previously determined that the production of both IL-1β and sIL-1Ra was induced in a dose-response manner by MP_T, the production of sIL-1Ra reaching a plateau at 1 μg/ml proteins of MP_T while that of IL-1β was still increasing at 6 μg/ml proteins of MP_T [29]. Here we used an intermediate dose, 3 μg/ml proteins of MP_T, which induced the production of both IL-1β and sIL-1Ra in monocytes (Fig. 1C). MP_T-induced IL-1β production was inhibited in the presence of 0.2 mg/ml HDL, *i.e.*, a concentration that was determined to be optimal [30]. In contrast, sIL-1Ra production was not significantly affected by HDL, suggesting that different pathways or surface molecules were involved in the induction of the latter molecules. These results demonstrate that MP_T were able to activate monocytes and confirmed previous results suggesting that HDL inhibited only a part of factors induced by contact with stimulated T cells or MP_T [29,32].

MP_T specifically bind and activate human monocytes

Since direct cellular contact with stimulated T cells is required to induce cytokine production in monocytes [33], we

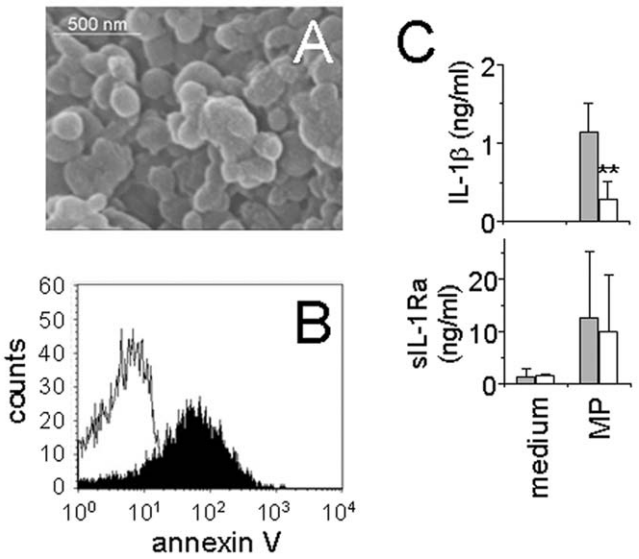

Figure 1. Characterization of microparticles generated by stimulated HUT-78 cells (MP_T). (A) Scanning electron microscopy of isolated MP_T. Scale bar = 500 nm. (B) Flow cytometry analysis of the binding of FITC-annexin V to MP_T. (C) Monocytes (5×10^4 cells/200 μl/well; 96-well plates) were activated by MP_T (3 μg/ml) for 24 h in the presence (empty columns) or absence (grey columns) of HDL (0.2 mg/ml proteins). Cell culture supernatants were measured for the presence of the indicated cytokines. Results are expressed as mean ± SD of 3 experiments carried out with monocytes isolated from 3 individual donors. ** $p<0.01$, as determined by paired student t test.
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sought to assess whether MP_T were able to durably interact with monocytes. To this aim, we assessed the binding of green PKH67-labelled MP_T to $CD14^+$ monocytes by flow cytometry. A large part of $CD14^+$ monocytes (62.7%) bound MP_T (Fig. 2A). Non-specific MP_T binding to or fusion with target cell membranes was ruled out since MP_T did not bind $CD3^+$ cells, *i.e.*, lymphocytes (Fig. 2B). This suggests that MP_T specifically interacted with monocytes. Furthermore, MP isolated from supernatants of unstimulated HUT-78 cells did not bind to $CD14^+$ monocytes (data not shown), further suggesting that the binding of MP_T to monocytes occurred through molecules expressed at the surface of stimulated T cells but not on unstimulated cells. A fraction of $CD14^+$ monocytes (21.4%) bound MP from TNF-activated endothelial cells but were not induced to produce IL-1β (Figs. 2C and 2D). Indeed, only MP_T triggered the production of IL-1β in human monocytes, whereas MP generated from activated platelets or endothelial cells were inefficient, even at concentrations 4- to 5-fold higher than that of MP_T (Fig. 2D). Together these results suggest that only MP_T were able to bind and activate monocytes to produce IL-1β.

HDL inhibit MP_T interactions with human monocytes

Because HDL inhibited IL-1β production in MP_T-activated monocytes, we assessed whether they would interfere with MP_T binding to monocytes. As shown in Fig. 3A, the binding of MP_T (12 μg/ml) to monocytes was inhibited in the presence of 0.2 mg/ml HDL. The binding of PKH67-labelled MP_T was dose-dependent and reached a plateau between 12 and 24 μg/ml protein, *i.e.*, around 1×10^6 MP/ml (Fig. 3B). HDL inhibited the binding of MP_T to monocytes by 30±12% between 3 and 24 μg/ml MP_T (Fig. 3B). This observation suggests that HDL inhibit IL-1β production by interfering with the binding of the activating factor to its receptor on monocytes.

HDL bind MP_T

To determine whether HDL interacted with the activating factor on MP_T or to its monocytic receptor, the binding of FITC-HDL to monocytes and MP from both stimulated and resting HUT-78 cells was assessed by flow cytometry. FITC-HDL bound $CD14^+$ monocytes to some extent, a small enhancement of fluorescence intensity being observed (Fig. 4A), confirming previous results [30]. In contrast, FITC-HDL bound MP_T to a great extent (Fig. 4B) suggesting that HDL might inhibit monocyte activation by primarily interacting with the activating factor(s) at the surface of MP_T, *i.e.*, at the surface of stimulated T cells. Interestingly, FITC-HDL only slightly interacted with MP isolated from unstimulated T cells (Fig. 4C), indicating that HDL bound to molecules that were only expressed on stimulated T cells. Together these results show that HDL are likely to inhibit the production of cytokines in monocytes activated by MP_T by competing with the monocyte receptor(s) for binding the activating factor.

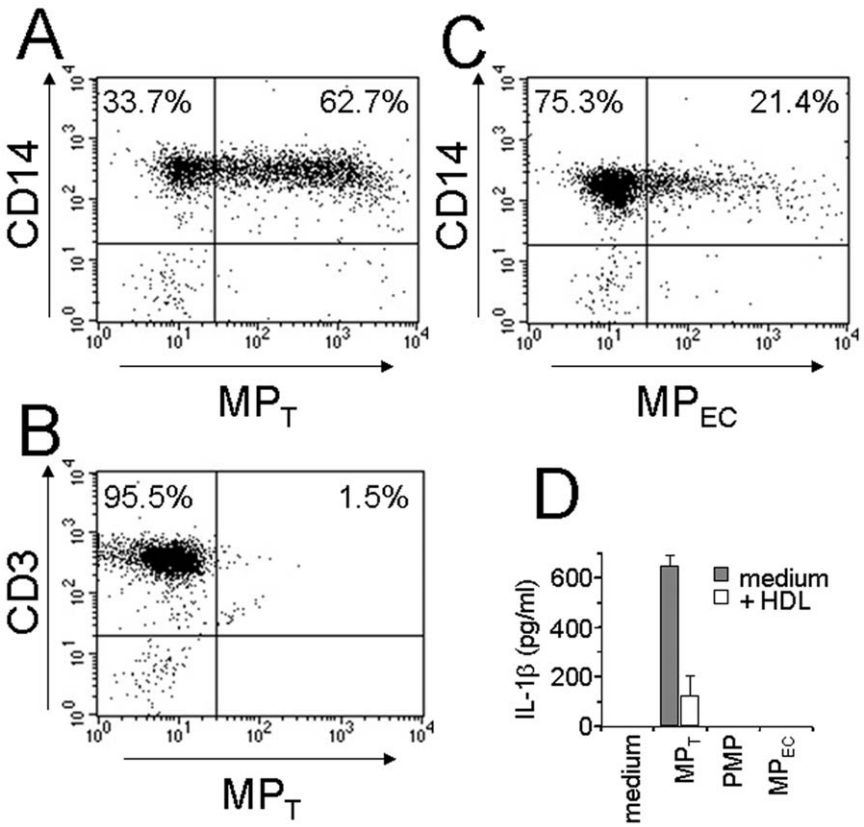

Figure 2. MP_T specifically bind and activate human monocytes. (A–C) The binding of PKH67-labelled MP from different cellular sources to isolated human monocytes and T lymphocytes was assessed by flow cytometry. Binding of MP_T (12 μg/ml) to $CD14^+$ monocytes (A) and $CD3^+$ T lymphocytes (B). (C) Binding of endothelial cell MP (MP_{EC}; 12 μg/ml) to $CD14^+$ monocytes. (D) Monocytes (5×10^4 cells/well/200 μl/well; 96-well plates) were activated by 3 μg/ml MP_T, 14 μg/ml activated endothelial cells (MP_{EC}) and 14 μg/ml activated platelets (PMP) in the presence (empty columns) or absence (grey columns) of 0.2 mg/ml HDL. IL-1β was measured in culture supernatants after 24 h incubation. Results are expressed as mean ± SD of triplicates.

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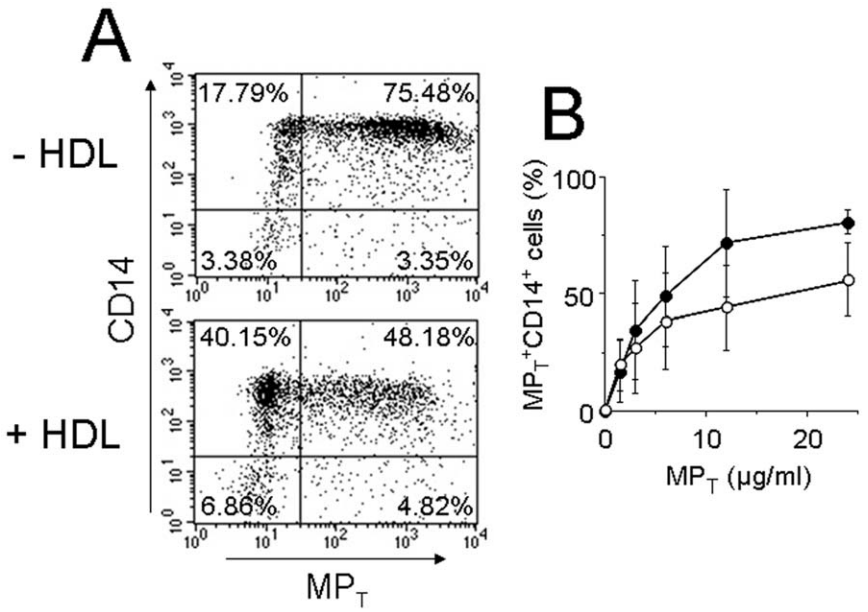

Figure 3. HDL inhibit the binding of MP_T to human monocytes. The binding of PKH67-labelled MP_T to $CD14^+$ monocytes in the presence or absence of HDL was measured by flow cytometry. (A) Representative binding of PKH67-labelled MP_T (12 µg/ml proteins) to $CD14^+$ monocytes in the presence or absence of 0.2 mg/ml HDL (as indicated). (B) Flow cytometry measurement of the binding of increasing concentration of PKH67-labelled MP_T to $CD14^+$ monocytes in the absence (closed circles) or presence (empty circles) of 0.2 mg/ml HDL. The percentage ± SD of $MP_T^+CD14^+$ monocytes (upper right panel) in 3 different experiments is presented.
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HDL inhibit MP_T-induced cytokine and chemokine production in human monocytes

HDL are not a general inhibitor of T cell contact-activation of human monocytes [32]. Indeed, HDL preferentially inhibited the expression of factors with a pro-inflammatory profile, as exemplified by IL-1β, in the present study, whilst they did not affect the expression of anti-inflammatory factors, exemplified here by sIL-1Ra. To extend this observation to the effect of HDL on MP_T-induced cytokine production in human monocytes, we assessed the effects of HDL on a range of cytokines and chemokines induced by MP_T in human monocytes. As shown in Fig. 5, in addition to that of IL-1β and sIL-1Ra, MP_T induced the

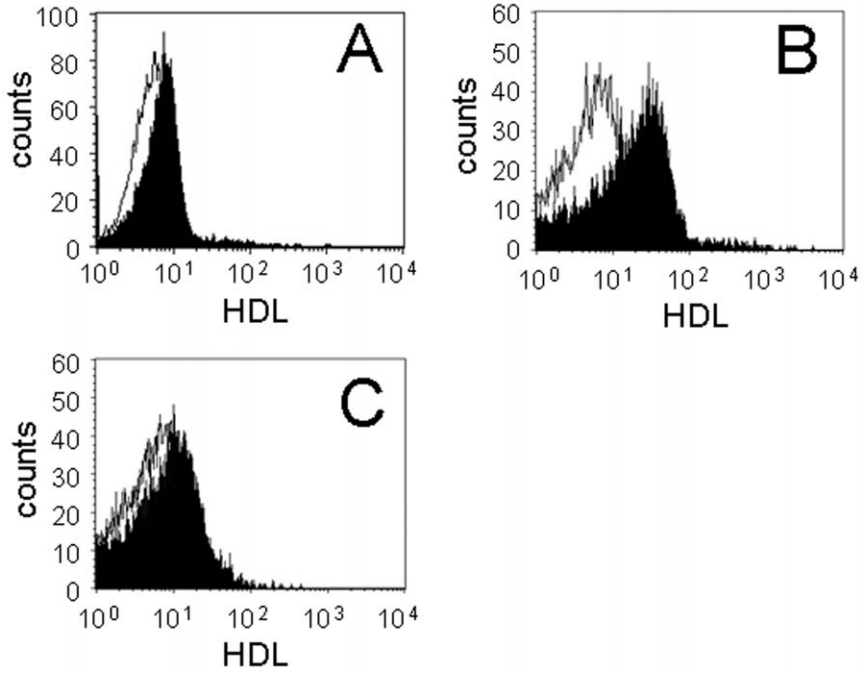

Figure 4. HDL interaction with MP_T. The binding of FITC-HDL to monocytes (A), MP_T (B) and MP from unstimulated HUT-78 cells (C) was analyzed by flow cytometry. Results are representative of 3 different experiments.
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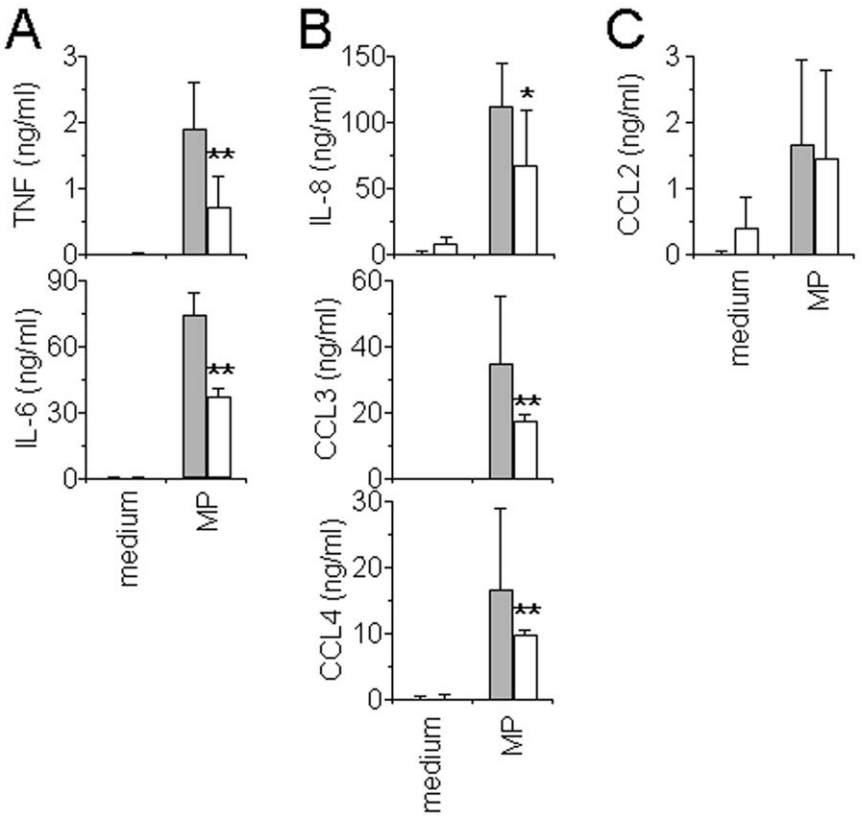

Figure 5. Modulation of cytokine production by HDL in MP_T-activated monocytes. Monocytes (5×10^4 cells/200 µl/well; 96-well plates) were activated by MP_T (6 µg/ml) for 24 h in the presence (empty columns) or absence (grey columns) of HDL (0.2 mg/ml proteins). Cell culture supernatants were measured for the presence of the indicated cytokines. Results are expressed as mean ± SD of 3 experiments carried out with monocytes isolated from 3 individual donors. * $p<0.05$; ** $p<0.01$, as determined by paired student t test.
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production of the prototypical pro-inflammatory cytokines TNF and IL-6, and the chemokines IL-8, CCL2, CCL3 and CCL4. The production of pro-inflammatory cytokines was inhibited in the presence of HDL (Fig. 5A) suggesting that they were induced by a similar activating factor as the one inducing IL-1β production. This was also true for chemokines (Fig. 5B), with the exception of CCL2 (Fig. 5C), whose production was not affected by HDL similarly to that of sIL-1Ra. By comparison with results obtained in monocytes activated by CE_{sHUT} [32], the present data demonstrate that MP_T indeed displayed similar activity as soluble extracts of membranes isolated from stimulated HUT-78 cells, *i.e.*, CE_{sHUT}. Furthermore they strengthen results of Fig. 4 demonstrating that different surface molecules were involved in monocyte activation, part of them being inhibited through interaction with HDL.

Discussion

This study reveals that MP_T specifically interact with monocytes to trigger cytokine and chemokine production. MP_T-monocyte interaction is inhibited by HDL which are likely to bind the activating factor(s) on MP_T, in turn inhibiting pro-inflammatory cytokine and chemokine production in monocytes. Interestingly, the production of sIL-1Ra and CCL2 was not inhibited in the presence of HDL confirming previous results [29,32] and suggesting that different factors at the surface of stimulated T cells and MP_T are involved in the induction of pro- and anti-inflammatory factors in monocytes.

Although studies showed that MP from endothelial cells and platelets could induce the expression of adhesion molecules and tissue factor-dependent procoagulant activity in the monocytic cell line THP-1 [34,35], activation of freshly isolated monocytes is not a general characteristic of MP in terms of induction of cytokine production. Indeed, MP generated by activated endothelial cells and platelets do not induce IL-1β production in monocytes. However, a small percentage of monocytes do bind MP from endothelial cells, demonstrating that MP interaction with monocytes is not exclusively due to interactions between activating factors at the surface of MP_T and receptors/counter-ligands on monocytes, but may occur through adhesion molecules likely to be present on the surface of all MP as demonstrated in MP generated by endothelial cells and neutrophils [36,37]. This suggests that the binding of MP to target cells may occur through multiple ligands and counter-ligands. It is likely to be the case for MP_T, since only part of their binding to monocytes is inhibited in the presence of HDL indicating that interactions occur through ligands different from the IL-1β activating factor(s). Partial inhibition of MP_T binding to monocytes by HDL is also reflected by the inhibition of the production of a part of cytokines and chemokines induced by MP_T (see Fig. 5), suggesting the involvement of activating factors which do not bind and therefore are not inhibited by HDL as exemplified by sIL-1Ra and CCL2 in the present study.

HDL do not represent a universal inhibitor of monocyte activation since they inhibit the production of only particular factors induced by contact with MP_T. Indeed, among the cytokines and chemokines which production is induced in monocytes upon

contact with MP_T, sIL-1Ra and CCL2 are not inhibited by HDL. These results are reminiscent of previous data showing that the production of sIL-1Ra, CCL2, and other factors that mainly display anti-inflammatory functions, is not inhibited by HDL upon activation by CE_{sHUT} [32]. Indeed, HDL mainly inhibit pro-inflammatory pathways induced by contact with stimulated T cells. CCL2 which is a major monocyte chemoattractant is far to be a prototypical pro-inflammatory factor. Indeed, CCL2 influences T cell immunity in that it induces a bias towards TH2 polarization [38]. Because chronic inflammatory diseases such as MS and RA in which T cell contact is likely to play a pathogenic part are mediated by TH1 and TH17, the production of CCL2 by monocytes/macrophages might be considered as an attempt to revert T cell polarization to a less inflammatory phenotype [39]. Besides, the premise that the activation of cytokine production by CE_{sHUT} and MP_T is similarly inhibited by HDL, confirms that MP_T and stimulated T cells exhibit similar surface molecules. In agreement with this observation, multiple studies have shown that MP express similar surface proteins to the cell they originate from (reviewed in [40]). Since HDL bind activating factor(s) at the surface of stimulated T cells and MP_T, it is likely that different molecules on T cells activate monocytes to secrete cytokines and chemokines; the activity of some/one of them being inhibited by HDL.

In conclusion, this study demonstrates that stimulated T cells and MP_T express surface factor(s) that bind monocytes and in turn induce cytokine production. Both MP_T binding and the MP_T-induced production of some but not all cytokines are inhibited by HDL, suggesting that different factors at the surface of T cells and MP_T trigger the production of cytokines. Although the identity of the activating factors remains elusive, the premise that it displays tight interactions with monocytes and HDL may provides clues as to its identification.

Materials and Methods

Ethics statement

Buffy coats of blood of healthy donors were provided by the Geneva Hospital Blood Transfusion Center. In accordance with the ethical committee of the Geneva Hospital, the blood bank obtained informed consent from the donors, who are thus informed that part of their blood will be used for research purposes.

Materials

Fetal calf serum (FCS), streptomycin, penicillin, L-glutamine, RPMI-1640 and PBS free of Ca^{2+} and Mg^{2+} (Gibco, Paisley, Scotland); purified phytohaemagglutinin (PHA) (EY Laboratories, San Marco, CA); Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden); phorbol myristate acetate (PMA), phenylmethylsulfonyl fluoride (PMSF), polymyxin B sulfate, amphiphilic cell linker dye kit (PKH67), calcium ionophore A23187, human TNF, and bovine serum albumin (Sigma Chemicals Co., St. Louis, MO); and annexin V-FITC, PE-labeled anti-human CD14, and anti-human CD3 (BD Biosciences) were purchased from the designated suppliers. Other reagents were of analytical grade or better.

Blood monocytes and T lymphocytes

Peripheral blood monocytes and T lymphocytes were isolated from buffy coats of blood of healthy volunteers as previously described [30]. In order to avoid activation by endotoxin, polymyxin B (2 μg/ml) was added to all solutions during the monocyte isolation procedure.

T cell stimulation and Isolation and labeling of microparticles (MP)

The human T cell line HUT-78 was purchased from the ATCC (Rockville, MD). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 U/ml penicillin and 2 mM L-glutamine in 5% CO_2-air humidified atmosphere at 37°C. HUT-78 cells (2×10^6 cells/ml) were stimulated for 6 h with PHA (1 μg/ml) and PMA (5 ng/ml) as previously described [41,42]. MP were isolated from culture supernatants of HUT-78 cells as previously described [29]. MP isolated from supernatants of stimulated HUT-78 cells were referred to as MP_T. As previously demonstrated, MP_T display similar cytokine induction ability as MP generated by stimulated T lymphocytes isolated from human blood [29]. Total RNA in MP_T reached 35.2 ± 17.5 μg/mg proteins, i.e., 0.7 ± 0.4 μg RNA/10^6 MP_T. This suggests that MP_T were indeed closed vesicles able to protect RNA from degradation by RNases. IL-1β and sIL-1Ra were not detected in MP_T or MP from unstimulated HUT-78 cells. DNA was below the detection limit, thus amounting to <3 ng/mg proteins in MP_T, suggesting that no or few apoptotic bodies were present amongst MP_T. Alternatively, MP were isolated from culture supernatants of human brain endothelial cells activated with TNF (MP_{EC}) and human blood platelets activated with the ionophore A23187 (PMP) as described previously [16,43]. Isolated MP were counted and their protein content measured as described [29]. MP preparations contained 19.7 ± 4.2 μg proteins/10^6 MP independently of the cellular origin confirming previous results [29]. MP were labeled with a green fluorescent amphiphilic cell linker dye kit (PKH67, Sigma) as described elsewhere [43].

Scanning electron microscopy (SEM)

MP_T were centrifuged (20,000 g, for 45 min) and the pellet fixed with 2% glutaraldehyde (Sigma) in 0.1 M sodium cacodylate, pH 7.4. The fixed MP_T were treated with 1% osmium tetroxide (Sigma) in 0.1 M cacodylate buffer prior to dehydration in increasing concentrations of ethanol (30 to 100%). MP_T were then critical-point dried, sputter-coated with gold, and observed under a Cambridge Stereoscan 260 scanning electron microscope.

Isolation, labeling and immobilization of HDL

Human serum HDL were isolated according to Havel et al. [44]. When required, HDL were labeled with fluorescein isothiocyanate (FITC-HDL) as previously described [30]. The binding of FITC-HDL to cells and MP_T was analyzed by direct flow cytometry on a flow cytometer (FACSCalibur, BD) as previously described [30].

Cytokine production and measurement

Monocytes (5×10^4 cells/well/200 μl) were activated with the indicated stimulus in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 U/ml penicillin, 2 mM L-glutamine and 5 μg/ml polymyxin B sulfate (medium) in 96 well plates and cultured for 24 h unless stated otherwise. When required, monocytes (2×10^6 cells/well/1 ml) were pre-activated by MP_T (6 μg/ml) in 24-well Ultra Low Attachment plates (Corning). After the indicated time, cells were harvested, washed in PBS and then activated as described above. The production of cytokines was measured in culture supernatants by commercially available enzyme immunoassay: IL-1β (Beckman Coulter Inc.), other cytokines and chemokines (Quantikine, R&D, Minneapolis, MN).

MP binding to target cells

Monocytes or T lymphocytes (2×10^5 cells/well/200 µl) were incubated for 3 h at 37°C with the indicated concentration of PKH67-labelled MP in round bottom polypropylene 96-well plates. After washing with PBS containing 2% heat inactivated human AB serum, 1% BSA and 0.1% NaN_3, cells were incubated with PE-labeled anti-human CD14 (monocytes) or anti-human CD3 (T lymphocytes) antibodies for 20 min. After thorough washing, cells were analyzed by flow cytometry (FACSCalibur, BD). Buffers used for flow cytometry analysis were subjected to filtration (Stericup 0.22 µm, Millipore) to discard interferences with small debris.

Statistics

When required, significance of differences between groups was evaluated using Student's paired *t* test.

Author Contributions

Conceived and designed the experiments: RC GEG DB. Performed the experiments: RC LG KJB AS DF VC. Analyzed the data: RC VC GEG DB. Contributed reagents/materials/analysis tools: DF VC GEG. Wrote the paper: DB.

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7 DISCUSSION

The results presented in this thesis have been already debated in the "discussion section" of each paper or manuscript. Nevertheless, I have chosen to bring a special focus in the present chapter on the roles of PI3Kδ, as they emerge from my different studies as an important topic in cytokines homeostasis.

This thesis work emphasizes the networking of intracellular signaling pathways in human monocytes in response to different stimuli. The signaling pathways are often described as very linear and static, yet this representation is inappropriate. A correct description would stress that the transduction pathways are extraordinarily branched, regulated and dynamic. Hence, while it is important to recognize that the transduction pathways are broadly organized in networks, highly specific signaling cascades are associated with particular stimuli. Once activated, a transduction pathway is further capable to operate through various signaling elements, which contribution may differ in the regulation of a specific target. Consequently, while being activated, a part of a transduction cascade triggered by a stimulus may contribute to the regulation of one specific target but not to another. As highlighted in this thesis, the individual transduction cascade that is dependent on both the nature of the stimulus implicated and the targets may therefore ensure an optimally adapted response in a single system.

Data indicate that the PI3K family provides crucial signals for many cellular processes. In particular, class I PI3Ks play a central part in inflammation. In contrast to p110α and p110β that are ubiquitously expressed, p110γ and p110δ are preferentially expressed in leukocytes and display a wide range of activities in immunity, including, among others, the capture, rolling, adhesion, and transmigration of neutrophils. Further studies implicate p110δ activity in cytokine production and the contribution of p110γ in response to chemoattractants [45, 491, 492]. In this context, we have demonstrated that the production of sIL-1Ra, IL-1β, IL-6 and TNF is dependent on p110δ activity in response to LPS and CE_{sHUT}, stimuli mimicking monocyte

activation in acute and chronic inflammatory conditions, respectively [437]. We reported that p110δ inhibition diminished the production of all four cytokines in monocytes stimulated with CE_{sHUT}. In contrast, we observed that p110δ blockade reduced the production of sIL-1Ra but increased that of pro-inflammatory cytokines in response to LPS. These observations are reminiscent from previous studies from our laboratory indicating that IFNβ, an immunomodulatory cytokine used in MS treatment, induces sIL-1Ra production through a mechanism requiring the PI3K pathway but not the STAT1 or MEK1 cascades. In the present thesis, we further demonstrate that IFNβ controls sIL-1Ra production through a pathway involving p110δ and MEK2, but not ERK1/2. These results are consistent with the observation that ERK2 interacts with STAT1 in IFNβ-treated cells [510]. Altogether, our studies strongly suggest that p110δ is a key enzyme in sIL-1Ra production in human monocytes, regardless of the stimulus implicated. Interestingly, we have shown that glatiramer acetate (GA), another MS treatment, induces sIL-1Ra production through a mechanism requiring p110δ, MEK1 and MEK2. These observations suggest that p110δ activity is necessary for both IFNβ and GA to trigger sIL-1Ra production, while the contribution of MEK1 is differentially implicated along with the specific trigger. It should be emphasized that in addition to PI3Kδ, MEK1 and MEK2 activities contribute to the induction of both sIL-1Ra and the pro-inflammatory cytokines in response to CE_{sHUT} [315], providing therefore a rationale for an alternative pathway for IFNβ to induce/enhance sIL-1Ra production in order to dampen inflammation.

Multiple sclerosis (MS) is primarily an inflammatory disorder of the central nervous system (CNS) in which focal lymphocytic and monocytic infiltration leads to myelin and axon damages. It is increasingly recognized that the innate immune system plays a central role in the immunopathogenesis of MS, a disease characterized by an important release of pro- and anti-inflammatory cytokines [173, 174]. Extensive evidence indicates that both IL-1 and sIL-1Ra

are produced within the CNS by several cell types, including resident microglial cells and infiltrating monocyte/macrophages. Although the presence of IL-1β has not been demonstrated in the normal CNS, increased levels of IL-1β have been measured in MS brain lesions [523]. Data further indicate that the production of IL-1β and sIL-1Ra may differ depending on the stage of disease [129] and that the circulating sIL-1Ra levels correlate with MS activity [176]. Moreover, we recently demonstrated that GA treatment increased sIL-1Ra levels in MS blood patients and in mice with EAE.

It is now recognized, that, in addition to $CD8^+$ and T helper 1 (Th1) cells, Th17 cells may be important in inducing CNS autoimmunity. Data indicate that the development of EAE is suppressed in IL-$17^{-/-}$ mice [364, 365] and a growing body of evidence suggests a pathogenic role for Th17 in MS. Importantly, several studies have shown that IL-1β is essential for the differentiation of IL-17-producing human and murine Th cells [524, 525]. Furthermore, data indicate that IL-17 production is reduced in IL-$1\alpha/\beta^{-/-}$ EAE mice and increased in IL-$1Ra^{-/-}$ EAE mice [526]. Overall, these data suggest that abnormal T cell activation caused by the imbalance of the IL-1/sIL-1Ra system may be responsible for the development of CNS autoimmunity. Thus, sIL-1Ra could have a dual role in both innate and adaptive immunity. On one hand, sIL-1Ra contributes in reducing the IL-1-mediated inflammatory state inside the CNS; sIL-1Ra being produced by infiltrating cells from the periphery crossing the blood brain barrier (BBB), whose permeability is enhanced by IL-1β, and/or resident cells. By its capacity to increase the permeability of the BBB, IL-1β indirectly participates to the anti-inflammatory effects of sIL-1Ra. On the second hand, in the periphery sIL-1Ra may contribute to impair the differentiation of Th17 cells mediated by IL-1. Thus, it is more the relative levels of sIL-1Ra and IL-1β rather than the absolute levels of each cytokine that represent an important therapeutic strategy for MS treatment. In conclusion, our data and those from others suggest a beneficial role for sIL-1Ra in MS pathology. Furthermore, the signaling pathway (*i.e.* p110δ)

is critical for sIL-1Ra production in response to either immunomodulatory drugs (*e.g.* IFNβ and GA) or cell-cell activation (CE_{sHUT}). Thus, the beneficial effects of IFNβ and GA in MS could arise from the property of both drugs to be potent and long lasting inducers of sIL-1Ra.

Rheumatoid arthritis (RA) can be considered as a "cytokine deregulation disease" leading to cartilage and bone destruction in synovial joints. Joint damage is mediated in part by IL-1β and TNF [182], which are capable to induce the production of proteases, *e.g.,* matrix metalloproteinases (MMPs), but also PGE_2. Data indicate that the rheumatoid synovium contains a high concentration of IL-1β and sIL-1Ra in patients with RA or in mice with collagen-induced arthritis (CIA) [190]. Moreover, the injection of IL-1 into rat knee joints results into the development of features related to chronic arthritis [187] while the injection of neutralizing antibodies against IL-1 reverses synovial inflammation. In contrast, it has been reported that antibodies against sIL-1Ra exacerbate LPS-induced arthritis in rabbit [155] and that sIL-1Ra-deficient mice exhibit a more severe form of CIA [188]. As in EAE, recent data indicate that Th17 cells could be critically responsible for the induction and the maintenance of chronic inflammatory course in RA [366, 527]. While data indicate that the endogenous production of sIL-1Ra is not sufficient to abrogate cartilage degradation [192], clinical trials of recombinant human sIL-1Ra in RA have demonstrated decreased radiologic damages, pain and mononuclear cell infiltration [191]. All these observations suggest that the IL-1 system is a potential target to control the pathological inflammation of synovial joints. However, due to the short lifespan of recombinant sIL-1Ra in the circulation, the treatment of RA with sIL-Ra was discarded in favor of administration of monoclonal antibodies against TNF. However, the perspective to control the endogenous production of sIL-1Ra, and therefore p110δ activity with pharmacological compounds may represent an attractive alternative treatment in RA (at least in TNF therapy–resistant RA patients). More particularly, the control of endogenous

production of sIL-1Ra should overcome issues with his short lifespan and also avoid the daily injection of recombinant sIL-1Ra.

Leukocytic class I PI3Ks, and in particular the p110δ isoform, represent promising targets in the development of novel treatments for autoimmune and inflammatory diseases. Class I PI3Ks are involved in innate and adaptive immunity and p110δ in particular is required for the lymphocyte capture, rolling, and transmigration [25]. In adaptive immunity, p110δ is involved in B as well as Th1, Th2, Th17 and Treg lymphocyte differentiation and maturation [447]. In addition, accumulating evidence suggests that p110δ is involved in cytokine production in human monocytes. We demonstrated that under chronic inflammatory conditions, *i.e* CE_{sHUT}, p110δ participates in the up-regulation of both pro- and anti-inflammatory cytokines, whereas under acute/infectious inflammatory conditions, *i.e*. LPS, p110δ activity dampens pro-inflammatory cytokine production, the production of sIL-1Ra being poorly up-regulated. Our data indicate that IFNβ triggers sIL-1Ra production through a MEK2/p110δ-dependent, but MEK1 and ERK1/2-independent pathway. We previously reported that IFNβ is capable to up-regulate the production of sIL-1Ra and concomitantly to down-regulate IL-1β and TNF production in CE_{sHUT}-activated human monocytes, while upon LPS stimulation, IFNβ increases the production of sIL-1Ra, IL-1β and TNF [277]. Our data further indicate that LPS activation requires MEK1 to trigger sIL-1Ra production (unpublished data). Consequently, upon LPS stimulation, the production of sIL-1Ra is likely to result from the synergic contribution of p110δ, MEK2 and MEK1, which activation is mediated in part via an autocrine/paracrine loop of IFNβ. The scenario is more complex regarding the production of pro-inflammatory cytokines, such as IL-1β or TNF in response to LPS-stimulation, as well as following monocyte activation through CE_{sHUT}. Our observations suggest that p110δ activation enhances the production of both pro- and anti-inflammatory cytokines in CE_{sHUT}-activated monocytes whereas co-stimulation with IFNβ specifically decreases the production

of pro-inflammatory cytokines only. Moreover, our data indicate that p110δ has a repressive function on pro-inflammatory cytokines production in LPS-activated monocytes while co-stimulation with IFNβ enhances the production of IL-1β and TNF. Therefore, one can hypothesize that the action of IFNβ on pro-inflammatory cytokine production in response to LPS or CE_{sHUT} is not associated to its ability to activate p110δ. It is however apparent that p110δ activation contributes to sIL-1Ra production in monocytes regardless of the nature of the stimulus.

7.1 Conclusion

PI3K are required for a plethora of immune and non-immune processes. In particular, p110δ represents to my opinion an interesting therapeutic target in chronic inflammatory autoimmune diseases such as RA and MS, since its inhibition might reduce the production of pathologically induced pro-inflammatory cytokines without reducing that induced by infectious agents.

As illustrated in this thesis, a specific transduction element, *i.e* p110δ, could be part of different signaling cascades, such as those induced by IFNβ, LPS and cell-cell contact, but its activation could have opposite effects on specific targets. These observations provide a clear example of a finely regulated signaling pathway. This regulation is provided through the highly branched and dynamic interactions between the members of a same pathway, such as Akt, or within members of different pathway, such as MAPK and PI3Ks. Thus, to define a complex network of signaling pathways, one has first to elucidate those pathways at a basic level, taking care of the specificities of each of them, before transposing them into a higher level of complexity. By this mean only, one can have a better and correct understanding of the signal transduction networking.

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