Immunité innée, balance th1/th17 et précurseurs musculaires dans les myopathies inflammatoires

Anne Tournadre

N° d'ordre 247-2010
Année 2010

THESE DE L'UNIVERSITE DE LYON

Délivrée par

L'UNIVERSITE CLAUDE BERNARD LYON 1

ECOLE DOCTORALE INTERDISCIPLINAIRE SCIENCES - SANTE

DIPLOME DE DOCTORAT

(arrêté du 7 août 2006)

Discipline : Immunologie

soutenue publiquement le 19 Novembre 2010

par

TOURNADRE Anne

IMMUNITE INNEE, BALANCE TH1/TH17 ET PRECURSEURS MUSCULAIRES DANS LES MYOPATHIES INFLAMMATOIRES

Directeur de thèse : Pr Pierre MIOSSEC

JURY : Pr Jacques BIENVENU
Pr Romain GHERARDI
Pr Pierre MIOSSEC
Pr Alexander SO

A Guillaume et Romain,

Remerciements

Je remercie tout particulièrement le Professeur Pierre MIOSSEC pour m'avoir accueilli dans son laboratoire et dans son équipe tout en soutenant l'idée d'une collaboration entre Lyon et Clermont-Ferrand, puis pour avoir accepté de me guider et de m'initier à la pensée et à l'écriture scientifique. J'espère avoir répondu à ses attentes.

Je remercie les Professeurs Romain GHERARDI, Alexander SO et Jacques BIENVENU, pour avoir accepté de participer à l'évaluation de cette thèse et pour l'intérêt qu'ils ont porté à mon travail.

Je remercie le Professeur Jean-Michel RISTORI pour m'avoir fermement soutenue dés l'initiation de cette démarche scientifique et ce tout au long de la réalisation de ce travail. J'ai l'occasion aujourd'hui de lui exprimer toute ma gratitude pour m'avoir accueillie dans son service et me permettre de poursuivre une carrière hospitalière.

Je remercie Vanina LENIEF, qui m'a fortement aidé dans la réalisation des techniques de culture cellulaire avec compétence et efficacité, et sans qui je n'aurai pu aller au bout. Je la remercie également pour l'ambiance de travail agréable qu'elle a contribué à créer au sein du groupe.

Je remercie le Pr Pierre DECHELOTTE, le Dr Anne-Marie BEAUFRERE, ainsi que l'ensemble du personnel du laboratoire d'anatomie pathologique du CHU de Clermont-Ferrand (en particulier Christelle PICARD) pour m'avoir ouvert les portes de leur laboratoire, pour leur accueil toujours chaleureux, leur disponibilité et pour leur aide technique.

Un des aspects enrichissants du métier de chercheur est sans nul doute lié aux discussions, aux rencontres, aux collaborations, qui au fil des années se nouent avec des personnes souvent différentes mais toujours enrichissantes. Je tiens donc à remercier de manière non exhaustive : Martin SOUBRIER, Jean-Jacques DUBOST, Sandrine MALOCHET-GUINAMAND, Arnaud HOT, Assia ELJAAFARI-DANY, Hubert MAROTTE, Ling TOH, Saloua ZRIOUAL, Fanny TURREL-DAVIN, Julie RACINET, Marie-Angélique CAZALIS, Véronique BARBALAT, Frédéric REYNIER, Alexandre PACHOT, Bruno MOUGIN.

RESUME en français

Cette thèse, consacrée aux myopathies inflammatoires, démontre le rôle dans les maladies auto-immunes des Toll-like récepteurs (TLRs), véritable passerelle entre immunité innée et adaptative, et plus spécifiquement dans le muscle, le rôle fondamental de la cellule musculaire elle-même. Après une présentation globale des myopathies inflammatoires et des différents aspects immunopathologiques, la réponse immunitaire adaptative est abordée en rapportant notamment dans le muscle des myopathies inflammatoires une accumulation de cellules dendritiques matures, et la présence des lymphocytes Th1 et Th17, avec un profil prépondérant Th1. L'implication de l'immunité innée est démontrée *in vivo* par l'expression musculaire des TLR3 et 7, et des C-type lectin récepteurs, spécifique des myopathies inflammatoires. *In v itro*, l'activation de la voie TLR3 induit la production par les cellules musculaires d'IL6, de la βchémokine CCL20, contribuant au recrutement et à la différentiation des cellules dendritiques et lymphocytes T, et de l'IFNβ qui participe à la surexpression des antigènes HLA de classe I. Les mécanismes de régulation impliquent une balance cytokinique Th1 et Th17. Finalement, l'importance des précurseurs musculaires immatures est soulignée. Contrairement au tissu musculaire normal, une surexpression des antigènes HLA de classe I, des TLRs, des auto-antigènes et de l'IFNβ, par les précurseurs musculaires immatures, est caractéristique des myopathies inflammatoires. Le rôle central de ces cellules musculaires immatures à potentiel de régénération pourrait expliquer un défaut de réparation associé au processus auto-immun de destruction musculaire.

TITRE en anglais

Innate immune system, Th1/Th17 balance and immature myoblast precursors in inflammatory myopathies

RESUME en anglais

This thesis, devoted to the inflammatory myopathies, is demonstrating the potential role in autoimmune disorders of Toll-like receptors (TLRs), gateway between innate and adaptive immune system, and more specifically in muscular diseases the fundamental role of muscle cell it-self. After the presentation of the general clinical features and the immunopathology of inflammatory myopathies, the adaptive immune response is the subject of the second part, demonstrating the abnormal accumulation of mature dendritic cells in myositis muscle, and the presence of Th1 and Th17 cells with a predominant Th1 profile. Innate immune system is next investigated, demonstrating the overexpression of TLR3 and 7 and of C-type lectin receptors characteristic of inflammatory myopathies. *In vitro* , stimulation of the TLR3 pathway in human myoblasts induces the production of IL6 and of the βchemokine CCL20, which in turn participate to the differentiation and the migration of T cells and dendritic cells, and of IFNβ which contributes to HLA class I up-regulation. The expression of TLR3 is differentially regulated by Th1 and Th17 cytokines. Finally, this work strongly implicates immature myoblast precursors in the pathogenesis of inflammatory myopathies. In contrast to normal muscle tissue, myositis tissue is characterized by the overexpression of HLA class I antigens, TLR3 and TLR7, myositis autoantigens, and IFNβ, all observed in immature myoblast precursors. By focusing damage onto those cells accomplishing repair, a feed-forward loop of tissue damage is induced and could explain the defective repair in muscle in addition to the autoimmune attack.

DISCIPLINE

Ecole Doctorale Interdisciplinaire Sciences – Santé
Biologie, médecine et santé
Immunologie

MOTS-CLES

Myopathie inflammatoire, polymyosite, dermatomyosite, précurseurs musculaires, immunité innée, Toll-like récepteurs, interféron type I, cytokines.

Inflammatory myopathy, polymyositis, dermatomyositis, immature myoblast precursors, innate immunity, Toll-like receptors, type I interferon, cytokines.

INTITULE ET ADRESSE DE L'U.F.R. OU DU LABORATOIRE

Unité de recherche EA 4130, Immunogénomique et inflammation
Hôpital Edouard Herriot Pavillon P
5 Place d'Arsonval
69437 Lyon Cedex 04

Publications incluses dans la thése

La contribution spécifique de la candidate aux travaux de recherche présentés est spécifiée avant chaque article.

Table des matières

Chapitre I

Introduction générale.

Les myopathies inflammatoires sont des affections chroniques du muscle squelettique aboutissant à un processus de destruction musculaire. Elles ont en commun des manifestations cliniques, dominées par un déficit musculaire proximal, et la présence dans le muscle d'un infiltrat inflammatoire mononuclée. Bien que d'étiologie inconnue, l'association d'une cytotoxicité locale médiée par les lymphocytes T, d'une surexpression des antigènes HLA de classe I à la surface des fibres musculaires et d'autoanticorps, plaident pour une origine auto-immune [1]. Des caractéristiques cliniques et notamment la présence de signes cutanés, histologiques et immunopathologiques permettent de distinguer les polymyosites (PM), dermatomyosites (DM) et myosites à inclusions (IBM).

L'atteinte musculaire de la DM est considérée comme une vascularite alors qu'un dysfonctionnement de l'immunité cellulaire dirigé contre la fibre musculaire caractérise la PM. Cette dichotomie est en grande partie issue des observations histopathologiques opposant la DM, dont l'infiltrat inflammatoire musculaire constitué de lymphocytes B et T CD4+ prédomine dans les régions périvasculaires, à la PM où les lymphocytes T CD8+ et les macrophages siègent au sein du tissu musculaire dans l'endomysium [1]. Néanmoins, cette classification reste limitée par un chevauchement clinique et histologique, par l'absence de critères diagnostic internationaux validés et par l'observation d'un profil inflammatoire commun. En effet, la présence dans l'infiltrat inflammatoire musculaire à la fois des PM et des DM, des deux cytokines proinflammatoires, l'IFNγ et l'IL17, associées à la migration, la différentiation et la maturation des cellules dendritiques [2], suggère l'implication des lymphocytes T activés Th1, Th17 et des cellules dendritiques dans le processus de destruction musculaire. La surexpression à la surface des fibres musculaires des antigènes HLA de classe I, absents dans le tissu musculaire normal, est une autre des caractéristiques communes des myopathies inflammatoires et pourrait participer à leur physiopathologie [3]. L'identification ces dernières années d'autoanticorps spécifiques des myosites et associés à de nouvelles entités cliniques [4], a également permis d'améliorer la compréhension des mécanismes physiopathologiques. Ainsi, les autoantigènes correspondants, fortement exprimés dans le tissu musculaire inflammatoire des PM et DM, participent à la migration des cellules immunitaires [5,6].

Outre le rôle de l'immunité adaptative, plusieurs travaux récents retrouvant une signature interféron (IFN) type I suggèrent un rôle de l'immunité innée dans la physiopathologie des myopathies inflammatoires [7,8]. Les récepteurs de l'immunité innée,

Pattern Recognition Receptors (PRR), sont le lien entre l'immunité innée et adaptative. Présents à la surface des cellules dendritiques, ainsi que de multiples variétés cellulaires (cellules de l'immunité innée, cellules épithéliales, fibroblastes), leur activation par des motifs moléculaires microbiens conservés au cours de l'évolution va induire la production de cytokines et de chémokines capables d'activer et de recruter les cellules dendritiques et les cellules de l'immunité adaptative. Des travaux récents, démontrant la possibilité d'une activation par des ligands endogènes, suscitent un nouvel intérêt pour ces récepteurs dans la physiopathologie des maladies auto-immunes [9-12].

Enfin, la cellule musculaire elle-même peut contribuer aux mécanismes physiopathologiques. *In vitro* , elle est capable de produire cytokines proinflammatoires et chémokines impliquées dans le recrutement et la différentiation des cellules dendritiques et des lymphocytes T [13]. *In vivo* , les PM et les DM sont caractérisées par un processus de destruction et de nécrose musculaire, mais également par la présence d'un processus de régénération qui va s'avérer non fonctionnel puisqu'aboutissant à une perte musculaire.

L'objectif de ce travail est de préciser les interactions possibles entre les cellules effectrices de l'immunité adaptative (lymphocytes T et cellules dendritiques), les récepteurs de l'immunité innée et la cellule musculaire elle-même cible de la réponse immunitaire. Après avoir évoqué les caractéristiques épidémiologiques, cliniques, thérapeutiques puis immunopathologiques des myopathies inflammatoires au cours des chapitres II et III, nous aborderons plus spécifiquement dans les chapitres IV et V, le rôle des différents acteurs du processus inflammatoire : cellules dendritiques, chémokines et cytokines, et récepteurs de l'immunité innée. Enfin, le rôle central des cellules musculaires et plus particulièrement des précurseurs musculaires sera détaillé dans le chapitre VI.

Chapitre II

Les myopathies inflammatoires en clinique.

Sommaire

II-a. Epidémiologie et classification.

La fréquence des PM et DM reste mal établie. En fonction des critères diagnostic choisis, les estimations oscillent entre 0.6 et 1 pour 100000 [1]. La DM est la plus fréquente des myopathies inflammatoires. Affectant préférentiellement la femme, elle peut toucher à la fois les adultes et les enfants. La PM est plus rare, et la myosite à inclusions est la plus fréquente chez les hommes de plus de 50 ans.

En 1975, Bohan et Peter proposaient des critères pour le diagnostic des PM et des DM sur la base des éléments suivants [14] : (1) Atteinte symétrique et progressive des muscles proximaux des ceintures et des fléchisseurs de nuque avec ou sans dysphagie ou atteinte des muscles respiratoires (2) Présence à la biopsie musculaire de foyers de nécrose et de phagocytose des fibres musculaires associés à des foyers de régénération, et à un infiltrat inflammatoire constitué essentiellement de cellules mononuclées (3) Elévation des enzymes musculaires sériques (4) Etude électromyographique (EMG): potentiels polyphasiques de courte durée, activité de fibrillation de repos, réduction de la durée et des amplitudes des contractions et recrutement temporospatial. Seule la présence de signes cutanés caractéristiques permettait de distinguer la DM. Les patients étaient ainsi classés en PM, DM, PM/DM associées aux cancers, et PM/DM associées aux autres connectivites. A l'opposé, une seconde approche, à la fois diagnostic et physiopathologique, s'appuyant uniquement sur les caractéristiques histopathologiques de la biopsie musculaire pour distinguer PM, DM et myosite à inclusions, s'est secondairement développée [15]. Néanmoins, les PM et DM ainsi définies sur des seuls critères histologiques semblent rares, et dans une étude rétrospective de cohorte portant sur 165 myosites (myosite à inclusions exclues), la biopsie musculaire seule ne permet le diagnostic de PM que dans 9 cas et de DM dans 27 cas, laissant une majorité de myosites inclassées [16]. Pour le diagnostic de myosite à inclusions, des critères diagnostic combinant critères cliniques et histologiques ont été établis par Griggs *et al.* en 1995 [17]. Du fait d'une grande hétérogénéité clinique, histopathologique et immunologique, la classification des myopathies inflammatoires en PM, DM et myosite à inclusions, s'est enrichie de nouvelles entités dont les myosites nécrosantes autoimmunes, les myosites non spécifiques et les myosites de chevauchement [18, 19]. Un cancer peut être associé, en particulier dans les dermatomyosites et les myosites nécrosantes autoimmunes. L'identification d'autoanticorps spécifiques des myosites a également permis de caractériser de nouvelles entités, dont le syndrome des antisynthétases ou les myosites à anti-signal

recognition particle (SRP) [4]. Faute de critères internationaux validés et de consensus, la plupart des études portant sur les myopathies inflammatoires s'appuient encore sur la classification originale proposée par Bohan et Peter [14] bien que les critères diagnostic qui en découlent restent très controversés.

II-b Caractéristiques cliniques.

La symptomatologie débute le plus souvent de façon progressive par l'apparition d'un déficit musculaire proximal et symétrique responsable d'une fatigabilité musculaire ou de difficultés à réaliser les activités quotidiennes. Les muscles axiaux, en particulier les extenseurs de nuque, peuvent être atteints réalisant alors un tableau de « tête tombante ». Les douleurs musculaires sont rares. Dans les formes sévères et aigües, des troubles de la déglutition ou une atteinte des muscles respiratoires peuvent être présents. La symptomatologie pulmonaire peut être liée à une insuffisance respiratoire restrictive par déficit des muscles respiratoires, aux troubles de la déglutition dans le cadre d'une pneumopathie d'inhalation ou à une pneumopathie interstitielle. L'atteinte du muscle cardiaque n'est pas rare, de dépistage difficile et probablement sous-estimée.

Les dermatomyosites se distinguent par la présence de signes cutanés caractéristiques. L'érythème violacé s'associe fréquemment à un œdème et siège à la face pour réaliser l'érythème lilacé héliotrope des paupières, à la paroi thoracique antérieure réalisant l'érythéme en V du décolleté ou à la partie postérieure du cou et des épaules. Les papules de Gottron, érythémateuses ou violacées, surélevées, siégeant aux faces d'extension des articulations des mains ou des grosses articulations, sont pathognomoniques. Un autre signe cutané très évocateur est l'érythème périunguéal douloureux à la pression qui peut être associé aux mégacapillaires visibles en capillaroscopie. Les calcifications ou calcinoses sous-cutanées et des tissus mous sont surtout notées chez l'enfant et sont responsables d'un handicap fonctionnel et esthétique. Le syndrome de Raynaud et l'hyperkératose fissurée des mains ou « mains de mécaniciens », associés à une pneumopathie interstitielle, à une polyarthrite, et à l'anticorps anti-Jo1 dirigé contre l'histidyl tRNA synthétase, définissent le syndrome des antisynthétases. L'association d'une DM à une autre connectivite définit un syndrome de chevauchement qui représente 10 à 20 % de l'ensemble des myosites [1]. La sclérodermie, le syndrome de Gougerot-Sjögren, le lupus erythémateux disséminé, la polyarthrite rhumatoïde

sont les affections le plus souvent rencontrées. Lorsque les signes cutanés sont au premier plan et que l'atteinte musculaire n'est pas symptomatique, la DM est dite amyopathique.

A l'inverse des DM au cours desquelles le diagnostic clinique est orienté par l'association de signes cutanés aux signes musculaires, la présentation des PM est peu spécifique, et le diagnostic doit exclure les myopathies secondaires acquises (toxiques, endocriniennes, infectieuses), les pathologies neuromusculaires, et les myopathies congénitales, dystrophiques ou métaboliques. Comme la DM, elle peut être associée à d'autres maladies auto-immunes (Lupus, Syndrome de Gougerot-Sjögren, polyarthrite rhumatoïde…).

Les myosites à inclusions sont sporadiques ou rarement héréditaires, se manifestant le plus souvent chez l'homme de plus de 50 ans. Elles se distinguent par une atteinte musculaire lentement progressive, asymétrique pouvant affecter les muscles distaux, notamment les fléchisseurs des doigts et poignets, par l'absence d'autoanticorps et surtout par leur résistance aux corticoïdes et aux immunosuppresseurs qui font poser la question d'une maladie initialement dégénérative [20].

II-c Diagnostic.

Le diagnostic clinique sera confirmé en s'aidant d'examens biologiques (dosage sérique des enzymes musculaires et recherche d'autoanticorps), de l'EMG et surtout de la biopsie musculaire dont les résultats seront détaillés au cours du chapitre III.

Les signes biologiques sont surtout marqués par une élévation des enzymes musculaires, retrouvée dans 75 à 85 % des cas et témoignant d'une souffrance et d'une lyse musculaire. Les CPK (créatine-phospho-kinases) sont les plus spécifiques, aspartate et alanine aminotransférases, lactate déshydrogénases, et aldolases sont également augmentées. Il peut s'y associer un syndrome inflammatoire non spécifique. Des autoanticorps spécifiques ou associés aux myosites sont notés dans 50-60 % des cas [4]. Il s'agit le plus souvent d'anticorps dirigés contre des antigènes cytoplasmiques, dont les plus connus sont les anticorps antisynthétases ciblant les aminoacyl-tRNA synthétases (anti-Jo1, anti-PL7, anti-PL12, anti-EJ, anti-JS, anti-KS, anti-OJ), et l'anticorps anti-signal recognition particle (SRP) qui s'associe à une forme aigüe et sévère de myosite caractérisée sur la biopsie musculaire par

de nombreuses fibres musculaires nécrotiques sans infiltrat inflammatoire, une atteinte cardiaque et/ou pulmonaire et une mauvaise réponse aux corticoïdes. Il peut également s'agir d'autoanticorps dirigés contre des antigènes nucléaires, parmi lesquels l'anticorps anti-Mi2 spécifique des DM, ou les anticorps anti-PM-Scl et anti-KU qui s'associent aussi aux sclérodermies et syndromes de chevauchement.

L'électromyogramme permet de différencier l'atteinte musculaire pure (syndrome myogène), de l'atteinte des fibres nerveuses ou de la jonction neuromusculaire. L'étude de la conduction nerveuse dans les atteintes primitives musculaires est normale par définition. L'étude de la contraction musculaire au repos révèle une activité musculaire spontanée riche avec une activité de fibrillation de repos abondante. Lors de la contraction volontaire, des anomalies secondaires au déficit de fibres musculaires peuvent être observées, avec notamment une réduction de la durée et des amplitudes des contractions mais aussi un recrutement temporospatial des fibres musculaires donnant l'aspect d'un tracé trop riche par rapport à la contraction développée.

La biopsie musculaire est l'examen crucial du diagnostic. Néanmoins, elle peut être source d'erreur, du fait d'une mauvaise interprétation ou d'une atteinte segmentaire et focale. Le diagnostic repose sur la mise en évidence d'un processus inflammatoire primitif, excluant les myosites toxiques, nécrosantes ou associées aux dystrophies (dystrophinopathies, dysferlinopathies) et nécessite pour cela une congélation des prélèvements afin d'effectuer une analyse immunoenzymatique et immunohistochimique. Une relecture de la biopsie musculaire peut s'avérer nécessaire et il faut savoir reconsidérer le diagnostic initial en cas d'échec thérapeutique avant d'intensifier inutilement le traitement. L'IRM musculaire peut aider au diagnostic en guidant le prélèvement sur les zones inflammatoires.

II-d Traitement.

L'hétérogénéité des myopathies inflammatoires, leur rareté et l'absence de critères d'évaluation internationaux validés rendent difficile la réalisation d'essais contrôlés et randomisés. L'évaluation de l'efficacité des traitements peut également s'avérer difficile en raison de leurs effets secondaires et notamment du risque de myopathie cortisonique associée [21]. De ce fait, la stratégie thérapeutique est largement empirique s'appuyant la plupart du

temps sur des études ouvertes non contrôlées ou sur des séries d'observations rapportées dans la littérature [22].

Le traitement repose sur les corticoïdes. Ils sont associés à un traitement immunosuppresseur en cas d'échec ou de corticodépendance, mais également de plus en plus souvent d'emblée dans les formes sévères et dans un but d'épargne cortisonique bien que la supériorité d'un traitement en première ligne par immunosuppresseur ne soit actuellement pas démontrée en l'absence d'étude contrôlée. Méthotrexate, azathioprine, et plus rarement ciclosporine, sont les traitements immunosuppresseurs utilisés. L'utilisation des immunoglobulines intraveineuses (IVIG), limitée par leur coût, est réservée aux dermatomyosites réfractaires où leur efficacité est démontrée contre placebo, aux myosites s'accompagnant de troubles de la déglutition, ou en cas de contre-indication aux immunosuppresseurs conventionnels. L'effet des IVIG pourrait dépendre de la voie médiée par les lymphocytes Th17 [23]. En cas d'échec à un traitement de seconde ligne, le plus souvent méthotrexate et corticoïdes, il faut savoir reconsidérer le diagnostic initial avant d'envisager soit l'association d'immunosuppresseurs (méthotrexate-azathioprine), soit de nouvelles options thérapeutiques parmi lesquelles le mycophénolate mofétil et les biothérapies, en particulier le rituximab [24]. Les antiTNFα doivent être utilisés avec prudence en raison du risque d'aggravation musculaire paradoxale [22]. Le traitement des pneumopathies interstitielles sévères et évolutives repose sur le cyclophosphamide, le tacrolimus, la ciclosporine, ou le mycophénolate mofetil, en association avec la corticothérapie [22].

Les myosites à inclusion, à l'opposé des DM et PM, répondent rarement aux corticoïdes. Les immunosuppresseurs conventionnels, et en particulier le méthotrexate, sont inefficaces tout comme les IVIG qui peuvent néanmoins apporter un bénéfice transitoire en cas de trouble de la déglutition [25]. Les résultats de l'alemtuzumab (CAMPATH 1-H) dans la myosite à inclusion sont encourageants [26].

Les corticoïdes ont révolutionné le pronostic de ces maladies, permettant d'obtenir une rémission initiale chez 30 à 90 % des patients [22]. Mais, l'évolution est le plus souvent chronique et polycyclique avec un taux de rechute élevé nécessitant un traitement au long cours, associant dans la plupart des cas corticothérapie et immunosuppresseurs. La prise en charge des patients doit donc également inclure des mesures visant à lutter contre le déficit

musculaire qui est autant lié à la maladie qu'à la corticothérapie. L'exercice physique adapté doit être conseillé pour prévenir et améliorer la perte musculaire [27]. Les carences nutritionnelles doivent être corrigées. La corticothérapie nécessite la prévention du risque de chute et de fracture ostéoporotique incluant des mesures éducatives et une prise en charge de l'environnement.

Tournadre A, Dubost JJ, Soubrier M. Treatment of inflammatory muscle disease in adults. Joint Bone Spine. 2010;77:390.

Contribution spécifique de la candidate :

- Revue de la littérature et écriture de l'article.
- Réponses aux commentaires des rapporteurs.

Joint Bone Spine 77 (2010) 390–394

Available online at ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com

Review

Treatment of inflammatory muscle disease in adults

Anne Tournadre*, Jean-Jacques Dubost, Martin Soubrier

Service de rhumatologie, hôpital G.-Montpied, CHU Clermont-Ferrand, 58, boulevard Montalembert, BP 69, 63003 Clermont-Ferrand cedex 1, France

ARTICLE INFO

Article history:
Accepted 4 January 2010
Available online 2 June 2010

Keywords:
Inflammatory muscle disease
Polymyositis
Dermatomyositis
Inclusion body myositis

ABSTRACT

Inflammatory muscle disease is a term used to designate a heterogeneous group of autoimmune diseases of unknown etiology whose main target is the skeletal muscle. Clinical, histological, and immunopathological criteria are classically used to distinguish polymyositis, dermatomyositis, and inclusion body myositis. Major obstacles to controlled therapeutic trials in patients with inflammatory muscle diseases include the heterogeneity of these diseases, their low prevalence, and the absence of validated evaluation criteria. To date, no such trials are available and the treatment is therefore largely empirical. Glucocorticoids are usually given first. Methotrexate is then added in the event of resistance to, or dependency on, glucocorticoid therapy. Methotrexate may be used from the outset in patients with severe disease in an attempt to decrease the glucocorticoid requirements. However, no controlled trials have been conducted to determine whether first-line methotrexate therapy improves outcomes. Intravenous immunoglobulin infusion is a costly treatment option that is reserved for the following situations: refractory dermatomyositis, based on a trial showing superiority over a placebo; myositis with impaired swallowing; and patients with contraindications to immunosuppressants. In patients who fail second-line treatment, which usually consists of methotrexate plus a glucocorticoid, the diagnosis should be carefully reappraised before other treatment options are considered. These options include methotrexate plus azathioprine and recently introduced drugs such as mycophenolate mofetil and rituximab. Caution is in order when considering TNFα antagonist therapy, as cases of paradoxical exacerbation of the muscle involvement have been reported.

1. Introduction

The term "inflammatory muscle disease" designates a group of chronic skeletal muscle disorders that share a number of features including prominent proximal muscle weakness and an inflammatory mononuclear-cell infiltrate within the muscle. The first two inflammatory muscle diseases to be described were polymyositis and dermatomyositis, which were distinguished based on a number of clinical features, most notably the presence of skin lesions in dermatomyositis [1]. Inclusion body myositis (IBM) is a usually sporadic and occasionally inherited disease whose features include asymmetrical muscle involvement, possible involvement of the distal limb muscles, absence of autoantibodies in typical cases and, most importantly, unresponsiveness to glucocorticoid and immunosuppressant therapy suggesting that the initial abnormality may be a degenerative process [2].

The heterogeneity of the clinical, histological, and immunological features has led to the introduction within the group of inflammatory muscle diseases of new entities such as autoimmune necrotizing myositis, nonspecific myopathy, and overlap myopathy [3,4]. A malignancy may be present, most notably in patients with dermatomyositis or autoimmune necrotizing myositis. The identification of autoantibodies specific of myositis has also led to the characterization of new forms of inflammatory muscle disease. Anti-Jo1 antibodies to histidyl tRNA synthetase are the most common autoantibodies and are associated with polymyositis or, less often dermatomyositis, potentially serious interstitial lung disease, polyarthritis, Raynaud's phenomenon, and hyperkeratosis with fissuring over the fingers, a combination known as antisynthetase syndrome [5]. Among other autoantibodies specific of myositis, antibody to the signal recognition particle (anti-SRP) is associated with acute severe disease and with the presence in muscle biopsies of numerous necrotized muscle fibers but no inflammatory infiltrate [6]. Cardiac and/or pulmonary involvement and an inadequate response to glucocorticoid therapy predict a poor outcome.

The heterogeneity of inflammatory muscle diseases, their low prevalence, and the absence of internationally validated evaluation criteria constitute major obstacles to the conduct of randomized controlled therapeutic trials. Another challenge is the possible occurrence of side effects, such as glucocorticoid-induced myopathy, which may complicate the efficacy assessments. Consequently, the treatment of inflammatory muscle disease is largely empirical and rests chiefly on uncontrolled open-label studies or published case series.

* Corresponding author. Tel.: +33 473 751 488; fax: +33 473 751 489.
E-mail address: atournadre@chu-clermontferrand.fr (A. Tournadre).

1297-319X/$ – see front matter © 2010 Société française de rhumatologie. Published by Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.jbspin.2010.04.007

2. Glucocorticoid therapy

No randomized controlled trials comparing glucocorticoid therapy to a placebo in patients with inflammatory muscle disease are available. Nevertheless, glucocorticoids are used as first-line therapy, a strategy that has radically improved patient outcomes, decreasing mortality far below the previous 50% rate [7]. There is nearly universal agreement that oral prednisone 1 mg/kg/day should be given at the acute phase. Once a clinical remission is achieved and the creatine phosphokinase (CPK) level returns to normal, usually after at least 4 weeks, the prednisone dosage can be tapered. Intravenous methylprednisolone bolus therapy may be considered in patients with acute severe disease or impaired swallowing. This treatment modality has been described chiefly in patients with juvenile dermatomyositis [8,9]. In adults, an open-label trial compared methylprednisolone (500 mg/day for 3 consecutive days once a week for 3 to 9 weeks plus oral prednisolone 1 mg/kg/day in the intervals between boluses) in 11 patients (eight with polymyositis and three with dermatomyositis) to oral prednisolone alone (1 mg/kg/day for 2 months) in 14 patients (10 with polymyositis and four with dermatomyositis) [10]. The rate of complete sustained remissions was higher in the methylprednisolone group (10/11 versus 6/14), in which the muscle enzyme levels returned to normal more rapidly. Overall, the initial remission rate with glucocorticoid therapy ranges from 30 to 90%, depending on whether partial or complete remissions are considered, and about 40% of patients are able to discontinue their treatment after 3 to 5 years [11]. Nevertheless, most patients experience intermittent flare-ups or chronic progression of their disease, and the relapse rate is high [4,7,12,13]. Add-on immunosuppressant therapy should be considered promptly in patients with steroid-dependent or steroid-resistant disease. IBM responds less well to glucocorticoid therapy and, overall, to immunosuppressants, compared to polymyositis and dermatomyositis.

3. Conventional immunosuppressants

Methotrexate, azathioprine and, more rarely, cyclosporine, are used as second-line drugs in patients with steroid-resistant or steroid-dependent inflammatory muscle disease. In addition, these drugs are being increasingly used from the outset in combination with glucocorticoid therapy. Overall, they seem to have similar efficacy with an about 70% response rate in retrospective cohort studies of patients having failed glucocorticoid therapy [11].

The only prospective randomized study of first-line immunosuppressant therapy was conducted with azathioprine (2 mg/kg/day) plus prednisolone (60 mg/day) compared to prednisolone alone (60 mg/day) in 16 patients with polymyositis [14]. After 3 months of treatment, muscle performance was nonsignificantly better in the azathioprine group.

Methotrexate can be given orally, subcutaneously, or intramuscularly, in a weekly dosage of 7.5 to 25 mg. There is no scientific proof that adding methotrexate to glucocorticoid therapy for the first-line treatment of polymyositis and dermatomyositis improves patient outcomes. In children with dermatomyositis, first-line methotrexate plus glucocorticoid therapy decreases the glucocorticoid requirements by 50% [15]. First-line methotrexate plus glucocorticoid therapy is currently recommended in some forms of inflammatory muscle disease characterized by a chronic course or by a poor response to glucocorticoids [4]. A European controlled randomized trial of first-line methotrexate plus glucocorticoid therapy versus glucocorticoid therapy alone is under way in adults with polymyositis or dermatomyositis (Prometheus, http://clinicaltrials.gov). The results may help to determine the optimal first-line strategy in these two diseases. In a 48-week placebo-controlled trial of methotrexate in 44 patients with IBM, methotrexate was not better than the placebo in terms of muscle performance and the only significant difference between the two groups was a lower CPK level with methotrexate [16]. Two trials in patients with refractory polymyositis or dermatomyositis showed no significant differences in efficacy across methotrexate (7.5–15 mg/week), azathioprine (2.5 mg/kg/day), and cyclosporine (3–3.5 mg/kg/day), combined with oral glucocorticoid therapy, although the safety profile was better with methotrexate than with azathioprine [17,18]. In patients who fail to respond to glucocorticoid therapy combined with a conventional immunosuppressant, the addition of a second immunosuppressant may be considered. In a trial of 30 patients with steroid-resistant polymyositis or dermatomyositis, most of whom had received methotrexate and/or azathioprine, combined methotrexate (25 mg/week) and azathioprine (150 mg/day) therapy induced a response in 53% of patients [19].

Mycophenolate mofetil (2 g/day) is among the therapeutic options for patients who fail methotrexate or azathioprine therapy. This immunosuppressant is widely used not only in organ transplant recipients, but also in patients with autoimmune diseases, most notably lupus nephropathy. Mycophenolate mofetil inhibits the proliferation of B cells and T cells. Data from several case-series including a total of about 40 patients with refractory polymyositis or dermatomyositis support the efficacy of mycophenolate mofetil, as muscle performance and muscle enzymes improved in nearly 80% of patients [20–27]. Mycophenolate mofetil has also been reported to improve autoimmune interstitial lung disease [28]. However, safety concerns have been raised by a report of 10 patients with dermatomyositis treated with mycophenolate mofetil, among whom three had opportunistic infections, including one that proved fatal [24].

Leflunomide was found effective in six patients with unresponsiveness or intolerance to conventional immunosuppressants, including four with dermatomyositis and two with polymyositis [29–31].

The use of cyclophosphamide is limited by toxicity. Cyclophosphamide therapy should be considered only in patients with progressive interstitial lung disease [32,33].

4. Intravenous immunoglobulins

In a placebo-controlled trial in patients with steroid-resistant dermatomyositis, intravenous immunoglobulins (IVIg) were effective in a dosage of 2 g/kg [34]. An uncontrolled open-label trial of IVIg in patients with polymyositis resistant to glucocorticoids and conventional immunosuppressants showed that six monthly courses of IVIg improved muscle performance in 70% of patients [35]. IVIg combined with mycophenolate mofetil was effective and well tolerated in seven patients with polymyositis or dermatomyositis [36]. IVIg may be helpful in patients with polymyositis or dermatomyositis responsible for severe swallowing impairments that fail to respond to glucocorticoid therapy [37]. The therapeutic effect of IVIg may be mediated by the Th17 lymphocyte pathway [38].

In patients with IBM, two placebo-controlled trials found no significant difference with IVIg, although swallowing muscle performance improved more in the IVIg group [39]. Nevertheless, given the poor response of IBM to glucocorticoid therapy and conventional immunosuppressants, IVIg may provide a transient improvement in patients with dysphagia.

In patients with steroid-resistant dermatomyositis or polymyositis or with IBM responsible for severe dysphagia, the French Agency for Healthcare Product Safety (AFSSAPS) authorizes the use of IVIg (http://www.afssaps.fr/Dossiers-thematiques/

Tarification-a-l-activite-T2A-medicaments/Immunoglobulines/(offset)/8#paragraph_2242).

5. Targeted immunotherapy

5.1. TNFα antagonists and IL-1 receptor antagonist

Numerous immunohistochemical and molecular biology studies suggest a role for the proinflammatory cytokines TNFα and IL-1 in the pathophysiology of inflammatory muscle disease [40]. All the clinical trials done to evaluate TNFα antagonists used an uncontrolled open-label design. Conflicting results were obtained with both the monoclonal antibody infliximab and the soluble receptor etanercept. In a retrospective study of eight patients with refractory polymyositis or dermatomyositis, including six given etanercept and two infliximab, muscle strength improved in six patients [41]. Dramatic improvements were reported after four infliximab infusions in five patients with juvenile dermatomyositis [42]. Nevertheless, several recent open-label studies suggest that infliximab or etanercept therapy may exacerbate the muscle symptoms [43–45]. Thus, in five patients with dermatomyositis who received etanercept therapy, the muscle weakness was either unchanged or exacerbated and the muscle enzyme levels increased [43]. Of 13 patients (five with polymyositis, four with dermatomyositis, and four with IBM) given infliximab for 14 weeks, only three experienced improvements [45]. Furthermore, the main improved areas were quality of life, joint symptoms, and overall physician assessments. Muscle strength testing scores failed to improve. In three patients (two with dermatomyositis and one with polymyositis), exacerbation of the clinical and laboratory signs of muscle involvement required discontinuation of infliximab therapy [45]. In nine patients with IBM treated with etanercept, the results did not support a therapeutic effect [46]. Although no controlled trials are available, the existing data suggest that the benefits provided by TNFα antagonist therapy in polymyositis and dermatomyositis may be modest, with improvements in less than half the cases. Furthermore, the possibility of muscle disease exacerbation indicates a need for caution when using TNFα antagonists. This exacerbation may be mediated by aberrant activation of the type I interferon pathway, which has been implicated in the pathogenesis of inflammatory muscle disease [45,47].

The IL-1 receptor antagonist anakinra was effective in a patient with antisynthetase syndrome, particularly in improving the fever and polyarthritis [48].

5.2. Rituximab

The chimerical monoclonal anti-CD20 antibody rituximab has been proved effective in several autoimmune diseases mediated by B cells, including rheumatoid arthritis (RA). Rituximab targets the B cell, which may explain that it acts not only on autoantibody-producing cells, but also on antigen presentation and interactions with T cells. Rituximab holds promise for the treatment of polymyositis and dermatomyositis. In all, 38 patients with polymyositis or dermatomyositis refractory to conventional immunosuppressants have been treated with rituximab, usually according to the schedule recommended for RA (two 1-g infusions 2 weeks apart). The results are available as uncontrolled open-label trial reports or anecdotal case reports [49–61]. A response was obtained in 29 of the 38 patients. Of the nine nonresponders [56,61], three had final diagnoses of IBM, muscle dystrophy, and lymphoma, respectively [61]. The rituximab response rate was highest in patients with dermatomyositis ($n = 16$), autoantibody myositis (including six patients with antisynthetase antibodies), and anti-SRP antibodies, although five patients with polymyositis and no detectable autoantibodies responded also. Rituximab improved the muscular, cutaneous, pulmonary, and cardiac manifestations. A multicenter Phase II, prospective trial in patients with refractory myositis and antibodies to Jo1 and SRP is under way (FORCE, http://clinicaltrials.gov, NCT00774462).

6. Plasmapheresis

A randomized controlled trial in patients with polymyositis or dermatomyositis found no evidence that plasmapheresis was effective [62].

7. Treatment of interstitial lung disease

The prevalence of interstitial lung disease is very high among patients with inflammatory muscle disease who undergo routine evaluation using imaging studies or lung function tests. In a recent prospective study of 23 patients with polymyositis or dermatomyositis followed up for 35 months, these tests found interstitial lung disease in 78% of cases [63]. Conventional immunosuppressant and glucocorticoid therapy is usually effective in controlling interstitial lung disease. Thus, lung function test results improve or stabilize in 33 and 39% of patients, respectively. Nevertheless, acute or subacute progressive interstitial lung disease, which is a feature in 28% of patients, predicts poor patient outcomes and is sometimes rapidly fatal. Cyclophosphamide, tacrolimus, cyclosporine, or mycophenolate mofetil, in combination with glucocorticoids, may be appropriate in this situation, as single or combination-drug therapy [28,32,33,64–66].

8. Treatment of the cutaneous involvement

The skin lesions may evolve independently from the muscle symptoms. Patients with isolated refractory skin lesions may benefit from sunlight protection, topical glucocorticoids, oral hydroxychloroquine or, rarely, topical tacrolimus [11].

9. Nonpharmacological treatments

Inflammatory muscle disease usually runs a chronic course that requires long-term treatment, usually with a combination of glucocorticoids and immunosuppressants. Therefore, the long-term treatment strategy should include measures designed to improve the muscle weakness, which is related both to the disease and to the glucocorticoid therapy. Appropriate physical activity should be recommended to prevent and/or correct the muscle loss related to the disease or glucocorticoids [67,68]. In two studies, exercise reconditioning programs produced improvements in patients with polymyositis or dermatomyositis, without exacerbating the histological signs of inflammation [69,70].

Nutritional deficiencies should be corrected. Long-term glucocorticoid therapy requires appropriate measures aimed at decreasing the risk of falls and osteoporotic fractures, including patient education and elimination of environmental hazards.

10. What is the best treatment strategy?

In patients with polymyositis or dermatomyositis, systemic glucocorticoid therapy should be used first. First-line combination therapy with a glucocorticoid and an immunosuppressant has not been proved effective to date but may deserve to be considered rapidly in patients with severe disease and as a glucocorticoid-sparing strategy. In patients with steroid-resistant or steroid-dependent disease, methotrexate is the preferred immunosuppressant drug, based on its better safety profile com-

pared to other immunosuppressants. The results of an ongoing trial can be expected to better define the indications of first-line methotrexate therapy. In patients with steroid-resistant dermatomyositis, IVIg therapy may be considered. IVIg therapy has been proved effective but is costly and requires repeated intravenous infusions. IVIg therapy may be helpful in patients with dermatomyositis or polymyositis who have either steroid-resistant swallowing impairments or contraindications to immunosuppressant or glucocorticoid therapy. In patients who fail second-line therapy, the diagnosis should be reappraised to avoid unnecessary treatment intensification. The muscle biopsy slides should be reexamined for evidence of IBM, which often responds poorly to various medications; metabolic muscle disease; mitochondrial disease; and, above all, specific forms of muscle dystrophy such as the dysferlinopathies [71] characterized by major muscle enzyme elevation and, in some cases, by inflammatory infiltrates in biopsy specimens. Glucocorticoid-induced myopathy [72] and an underlying malignancy should be considered also. When a malignancy is found, its treatment may lead to the resolution of the paraneoplastic muscle disease. If all these differential diagnoses are ruled out, combined methotrexate and azathioprine therapy [19] or one of the newer drugs such as mycophenolate mofetil or rituximab may deserve to be considered.

In contrast to dermatomyositis and polymyositis, IBM rarely responds to glucocorticoid therapy. Conventional immunosuppressants, particularly methotrexate, are not effective. Similarly, IVIg therapy has little effect in this disease, although swallowing impairments may be transiently improved. Cricopharyngeal myotomy may be offered to patients with impaired swallowing [73]. Alemtuzumab (CAMPATH 1-H) has produced promising results in patients with IBM [74].

Conflict of interest

The authors have no conflicts of interest to declare.

References

[1] Bohan A, Peter JB. Polymyositis and dermatomyositis. N Engl J Med 1975;292:344–7.
[2] Benveniste O, Dubourg O, Herson S. La myosite à inclusions. Rev Rhum 2008;75:151–6.
[3] Hoogendijk JE, Amato AA, Lecky BR, et al. 119th ENMC international workshop: trial design in adult idiopathic inflammatory myopathies, with the exception of inclusion body myositis 10–12 October 2003, Naarden, The Netherlands. Neuromuscul Disord 2004;14:337–45.
[4] Troyanov Y, Targoff IN, Tremblay JL, et al. Novel classification of idiopathic inflammatory myopathies based on overlap syndrome features and autoantibodies: analysis of 100 French Canadian patients. Medicine (Baltimore) 2005;84:231–49.
[5] Imbert-Masseau A, Hamidou M, Agard C, et al. Antisynthetase syndrome. Joint Bone Spine 2003;70:161–8.
[6] Sordet C, Goetz J, Sibilia J. Contribution of autoantibodies to the diagnosis and nosology of inflammatory muscle disease. Joint Bone Spine 2006;73:646–54.
[7] Sultan SM, Ioannou Y, Moss K, et al. Outcome in patients with idiopathic inflammatory myositis: morbidity and mortality. Rheumatology 2002;41:22–6.
[8] Laxer RM, Stein LD, Petty RE. Intravenous pulse methylprednisolone treatment of juvenile dermatomyositis. Arthritis Rheum 1987;30:328–34.
[9] Seshadri R, Feldman BM, Ilowite N, et al. The role of aggressive corticosteroid therapy in patients with juvenile dermatomyositis: a propensity score analysis. Arthritis Rheum 2008;59:989–95.
[10] Matsubara S, Sawa Y, Takamori M, et al. Pulsed intravenous methylprednisolone combined with oral steroids as the initial treatment of inflammatory myopathies. J Neurol Neurosurg Psychiatry 1994;57:1008.
[11] Iorizzo LJ, Jorizzo JL. The treatment and prognosis of dermatomyositis: an updated review. J Am Acad Dermatol 2008;59:99–112.
[12] Philipps BA, Zilko P, Garlepp MJ, et al. Frequency of relapses in patients with polymyositis and dermatomyositis. Muscle Nerve 1998:1668–72.
[13] Bronner IM, van der Meulen MF, de Visser M, et al. Long-term outcome in polymyositis and dermatomyositis. Ann Rheum Dis 2006;65:1456–61.
[14] Bunch TW, Worthington JW, Combs JJ, et al. Azathioprine with prednisone for polymyositis. A controlled, clinical trial. Ann Intern Med 1980;92(3):365–9.
[15] Ramanan AV, Campbell-Webster N, Ota S, et al. The effectiveness of treating juvenile dermatomyositis with methotrexate and aggressively tapered corticosteroids. Arthritis Rheum 2005;52:3570–8.
[16] Badrising UA, Maat-Schieman ML, Ferrari MD, et al. Comparison of weakness progression in inclusion body myositis during treatment with methotrexate or placebo. Ann Neurol 2002;51:369–72.
[17] Vencovský J, Jarosová K, Machácek S, et al. Cyclosporine A versus methotrexate in the treatment of polymyositis and dermatomyositis. Scand J Rheumatol 2000;29:95–102.
[18] Miller J, Walsh Y, Saminaden S, et al. Randomized double blind trial of methotrexate and steroids compared with azathioprine and steroids in the treatment of idiopathic inflammatory myopathy. J Neurol Sci 2002;199(Suppl.):S53.
[19] Villalba L, Hicks JE, Adams EM, et al. Treatment of refractory myositis: a randomized crossover study of two new cytotoxic regimens. Arthritis Rheum 1998;41:392–9.
[20] Gelber AC, Nousari HC, Wigley FM. Mycophenolate mofetil in the treatment of severe skin manifestations of dermatomyositis: a series of 4 cases. J Rheumatol 2000;27:1542–5.
[21] Hervier B, Masseau A, Mussini JM. Long-term efficacy of mycophenolate mofetil in a case of refractory antisynthetase syndrome. Joint Bone Spine 2009;76:575–6.
[22] Schneider C, Gold R, Schäfers M, et al. Mycophenolate mofetil in the therapy of polymyositis associated with a polyautoimmune syndrome. Muscle Nerve 2002;25:286–8.
[23] Majithia V, Harisdangkul V. Mycophenolate mofetil (CellCept): an alternative therapy for autoimmune inflammatory myopathy. Rheumatology (Oxford) 2005;44:386–9.
[24] Rowin J, Amato AA, Deisher N, et al. Mycophenolate mofetil in dermatomyositis: is it safe? Neurology 2006;66:1245–7.
[25] Edge JC, Outland JD, Dempsey JR, et al. Mycophenolate mofetil as an effective corticosteroid-sparing therapy for recalcitrant dermatomyositis. Arch Dermatol 2006;142:65–9.
[26] Pisoni CN, Cuadrado MJ, Khamashta MA, et al. Mycophenolate mofetil treatment in resistant myositis. Rheumatology (Oxford) 2007;46:516–8.
[27] Caramaschi P, Volpe A, Carletto A, et al. Long-standing refractory polymyositis responding to mycophenolate mofetil: a case report and review of the literature. Clin Rheumatol 2007;26:1795–6.
[28] Saketkoo LA, Espinoza LR. Experience of mycophenolate mofetil in 10 patients with autoimmune-related interstitial lung disease demonstrates promising effects. Am J Med Sci 2009;337:329–35.
[29] Lange U, Piegsa M, Müller-Ladner U, et al. Anti-Jo-1 antibody positive polymyositis–successful therapy with leflunomide. Autoimmunity 2006;39:261–4.
[30] Boswell JS, Costner MI. Leflunomide as adjuvant treatment of dermatomyositis. J Am Acad Dermatol 2008;58:403–6.
[31] Sangle VS, Sangle SR, D'Cruz DP. Leflunomide as a remission-maintaining therapy in difficult-to-treat dermatomyositis. Ann Rheum Dis 2008;67:723.
[32] Kameda H, Nagasawa H, Ogawa H, et al. Combination therapy with corticosteroids, cyclosporin A, and intravenous pulse cyclophosphamide for acute/subacute interstitial pneumonia in patients with dermatomyositis. J Rheumatol 2005;32:1719–26.
[33] Yamasaki Y, Yamada H, Yamasaki M, et al. Intravenous cyclophosphamide therapy for progressive interstitial pneumonia in patients with polymyositis/dermatomyositis. Rheumatology (Oxford) 2007;46:124–30.
[34] Dalakas MC, Illa I, Dambrosia JM, et al. A controlled trial of high-dose intravenous immune globulin infusions as treatment for dermatomyositis. N Engl J Med 1993;329:1993–2000.
[35] Cherin P, Pelletier S, Teixeira A, et al. Results and long-term follow-up of intravenous immunoglobulin infusions in chronic, refractory polymyositis: an open study with thirty-five adult patients. Arthritis Rheum 2002;46:467–74.
[36] Danieli MG, Calcabrini L, Calabrese V, et al. Intravenous immunoglobulin as add on treatment with mycophenolate mofetil in severe myositis. Autoimmun Rev 2009;9:124–7.
[37] Marie I, Hachulla E, Levesque H, et al. Intravenous immunoglobulins as treatment of life threatening esophageal involvement in polymyositis and dermatomyositis. J Rheumatol 1999;26:2706–9.
[38] Tournadre A, Porcherot M, Chérin P, et al. Th1 and Th17 balance in inflammatory myopathies: interaction with dendritic cells and possible link with response to high-dose immunoglobulins. Cytokine 2009;46:297–301.
[39] Dalakas MC. Intravenous immunoglobulin in autoimmune neuromuscular diseases. JAMA 2004;291:2367–75.
[40] Chevrel G, Granet C, Miossec P. Contribution of tumour necrosis factor alpha and interleukin (IL) 1beta to IL6 production NF-kappaB nuclear translocation, and class I MHC expression in muscle cells: in vitro regulation with specific cytokine inhibitors. Ann Rheum Dis 2005;64:1257–62.
[41] Efthimiou P, Schwartzman S, Kagen LJ. Possible role for tumour necrosis factor inhibitors in the treatment of resistant dermatomyositis and polymyositis: a retrospective study of eight patients. Ann Rheum Dis 2006;65:1233–6.
[42] Riley P, McCann LJ, Maillard SM, et al. Effectiveness of infliximab in the treatment of refractory juvenile dermatomyositis with calcinosis. Rheumatology (Oxford) 2008;47:877–80.
[43] Iannone F, Scioscia C, Falappone PC, et al. Use of etanercept in the treatment of dermatomyositis: a case series. J Rheumatol 2006;33:1802–4.

[44] Hengstman GJ, De Bleecker JL, Feist E, et al. Open-label trial of anti-TNF-alpha in dermato- and polymyositis treated concomitantly with methotrexate. Eur Neurol 2008;59:159–63.
[45] Dastmalchi M, Grundtman C, Alexanderson H, et al. A high incidence of disease flares in an open pilot study of infliximab in patients with refractory inflammatory myopathies. Ann Rheum Dis 2008;67:1670–7.
[46] Barohn RJ, Herbelin L, Kissel JT, et al. Pilot trial of etanercept in the treatment of inclusion-body myositis. Neurology 2006;66(Suppl. 1):S123–124.
[47] Eloranta ML, Barbasso Helmers S, Ulfgren AK, et al. A possible mechanism for endogenous activation of the type I interferon system in myositis patients with anti-Jo-1 or anti-Ro 52/anti-Ro 60 autoantibodies. Arthritis Rheum 2007;56:3112–24.
[48] Furlan A, Botsios C, Ruffatti A, et al. Antisynthetase syndrome with refractory polyarthritis and fever successfully treated with the IL-1 receptor antagonist, anakinra: a case report. Joint Bone Spine 2008;75:366–7.
[49] Levine TD. Rituximab in the treatment of dermatomyositis: an open-label pilot study. Arthritis Rheum 2005;52:601–7.
[50] Lambotte O, Kotb R, Maigne G, et al. Efficacy of rituximab in refractory polymyositis. J Rheumatol 2005;32:1369–70.
[51] Chiappetta N, Steier J, Gruber B. Rituximab in the treatment of refractory dermatomyositis. J Clin Rheumatol 2005;11:264–6.
[52] Noss EH, Hausner-Sypek DL, Weinblatt ME. Rituximab as therapy for refractory polymyositis and dermatomyositis. J Rheumatol 2006;33:1021–6.
[53] Arlet JB, Dimitri D, Pagnoux C, et al. Marked efficacy of a therapeutic strategy associating prednisone and plasma exchange followed by rituximab in two patients with refractory myopathy associated with antibodies to the signal recognition particle (SRP). Neuromuscul Disord 2006;16:334–6.
[54] Brulhart L, Waldburger JM, Gabay C. Rituximab in the treatment of antisynthetase syndrome. Ann Rheum Dis 2006;65:974–5.
[55] Dinh HV, McCormack C, Hall S, et al. Rituximab for the treatment of the skin manifestations of dermatomyositis: a report of 3 cases. J Am Acad Dermatol 2007;56:148–53.
[56] Chung L, Genovese MC, Fiorentino DF. A pilot trial of rituximab in the treatment of patients with dermatomyositis. Arch Dermatol 2007;143:763–7.
[57] Mok CC, Ho LY, To CH. Rituximab for refractory polymyositis: an open-label prospective study. J Rheumatol 2007;34:1864–8.
[58] Touma Z, Arayssi T, Kibbi L, et al. Successful treatment of cardiac involvement in dermatomyositis with rituximab. Joint Bone Spine 2008;75:334–7.
[59] Tournadre A, Amarger S, Joly P, et al. Polymyositis and pemphigus vulgaris in a patient: successful treatment with rituximab. Joint Bone Spine 2008;75:728–9.
[60] Feist E, Dörner T, Sörensen H, et al. Longlasting remissions after treatment with rituximab for autoimmune myositis. J Rheumatol 2008;35:1230–2.
[61] Sultan SM, Ng KP, Edwards JC, et al. Clinical outcome following B cell depletion therapy in eight patients with refractory idiopathic inflammatory myopathy. Clin Exp Rheumatol 2008;26:887–93.
[62] Miller FW, Leitman SF, Cronin ME, et al. Controlled trial of plasma exchange and leukapheresis in polymyositis and dermatomyositis. N Engl J Med 1992;326:1380–4.
[63] Fathi M, Vikgren J, Boijsen M, et al. Interstitial lung disease in polymyositis and dermatomyositis: longitudinal evaluation by pulmonary function and radiology. Arthritis Rheum 2008;59:677–85.
[64] Ochi S, Nanki T, Takada K, et al. Favorable outcomes with tacrolimus in two patients with refractory interstitial lung disease associated with polymyositis/dermatomyositis. Clin Exp Rheumatol 2005;23:707–10.
[65] Guglielmi S, Merz TM, Gugger M, et al. Acute respiratory distress syndrome secondary to antisynthetase syndrome is reversible with tacrolimus. Eur Respir J 2008;31:213–7.
[66] Kotani T, Makino S, Takeuchi T, et al. Early intervention with corticosteroids and cyclosporin A and 2-hour postdose blood concentration monitoring improves the prognosis of acute/subacute interstitial pneumonia in dermatomyositis. J Rheumatol 2008;35:254–9.
[67] Horber FF, Scheidegger JR, Grunig BE, et al. Evidence that prednisone-induced myopathy is reversed by physical training. J Clin Endocrinol Metab 1985;61:83–8.
[68] Wiendl H. Idiopathic inflammatory myopathies: current and future therapeutic options. Neurotherapeutics 2008;5:548–57.
[69] Alexanderson H, Dastmalchi M, Esbjornsson-Liljedahl M, et al. Benefits of intensive resistance training in patients with chronic polymyositis or dermatomyositis. Arthritis Rheum 2007;57:768–77.
[70] de Salles Painelli V, Gualano B, Artioli GG, et al. The possible role of physical exercise on the treatment of idiopathic inflammatory myopathies. Autoimmun Rev 2009;8:355–9.
[71] Nguyen K, Bassez G, Krahn M, et al. Phenotypic study in 40 patients with dysferlin gene mutations: high frequency of atypical phenotypes. Arch Neurol 2007;64:1176–82.
[72] Tournadre A, Soubrier M. Le rhumatologue face à une myopathie cortisonique. Rev Rhum 2008;75:157–61.
[73] Oh TH, Brumfield KA, Hoskin TL, et al. Dysphagia in inflammatory myopathy: clinical characteristics, treatment strategies, and outcome in 62 patients. Mayo Clin Proc 2007;82:441–7.
[74] Dalakas MC, Rakocevic G, Schmidt J, et al. Effect of Alemtuzumab (CAMPATH 1-H) in patients with inclusion-body myositis. Brain 2009;132:1536–44.

Chapitre III

Bases immunopathologiques des myopathies inflammatoires.

Sommaire

III.a. Développement normal de la fibre et du tissu musculaire.

La fibre musculaire squelettique contient dans son cytoplasme de nombreuses myofibrilles qui supportent la contraction musculaire grâce à l'interaction des protéines des myofilaments (myosine, α-actine, troponine, tropomyosine). Un changement de conformation de l'actine et de la myosine après hydrolyse de l'ATP permet le déplacement des myofilaments. L'énergie est fournie sous forme d'ATP par les nombreuses mitochondries du tissu musculaire et la créatine constitue le stock énergétique après sa phosphorylation par la créatine kinase. Ainsi, en cas de besoin une réaction inverse permet de reconstituer l'ATP. Plusieurs types de myofibrilles peuvent être identifiés par des méthodes histochimiques. Ainsi les études enzymatiques distinguent les fibres rouges de contraction lente, riches en myoglobine et en mitochondries, des fibres blanches de contraction rapide riches en glycogène. L'étude de l'activité ATPase différencie les fibres de type I lentes des fibres de type II rapides qui se subdivisent en IIA et IIB en fonction de la sensibilité à différents pH.

La régénération musculaire dépend de cellules quiescentes plaquées sous la membrane plasmique, les cellules satellites, véritables cellules souches musculaires dont le nombre décroît avec l'âge. Ces progéniteurs musculaires, caractérisés par l'expression des gènes *Pax3/Pax7,* sont activés par la libération de facteurs mitogènes suite à une lésion musculaire (exercice, traumatisme ou processus pathologique), et expriment ainsi différents facteurs de transcription, dont Myf5 et MyoD, permettant l'entrée en cycle de prolifération et la génération de cellules musculaires immatures, les myoblastes [28] (Figure 1). Secondairement, les myoblastes, sous l'influence de facteurs de différentiation dont la myogenin, vont se différentier en myocytes puis myotubes, et fusionner pour former les fibres musculaires matures [28] (Figure 1). Des données *in vitro* indiquent que parmi les cellules satellites activées, *Pax7+/MyoD+*, certaines vont perdre l'expression MyoD et retourner à l'état quiescent pour reconstituer le stock initial [28]. De nombreux facteurs et signaux régulent les cellules satellites incluant les voies de signalisation Notch et Wnt, le fibroblast growth factor (FGF), l'insulin growth factor (IGF), l'hepatocyte growth factor (HGF), NF-kB, le monoxyde d'azote (NO) et la myostatin appartenant à la famille du transforming growth factor (TGF)-β [28]. En condition anormale ou pathologique, ce processus de régénération peut être non fonctionnel, soit du fait d'une capacité de régénération insuffisante au regard de l'importance de la destruction, soit du fait d'un défaut de différentiation en fibres matures. Les marqueurs biologiques des cellules satellites sont répertoriés dans le tableau 1 [28]. La

plupart sont des molécules de surface (récepteurs ou molécules d'adhésion) également présentes à la surface d'autres types cellulaires, CD56 (NCAM), étant la plus utilisée. Parmi les marqueurs récents, Pax7 semble le plus intéressant, puisque spécifique des cellules satellites et présent sur l'ensemble des sous-types cellulaires.

Figure 1 [28]. Développement de la cellule musculaire.

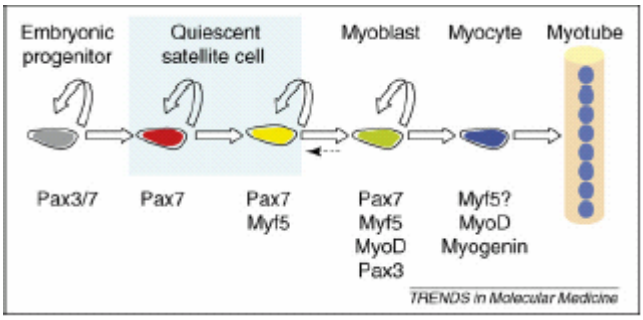


Tableau 1 [28]. Marqueurs biologiques des cellules satellites.

Table 1. Satellite cell markers

Marker	Expression Species[a]	Q	A	D	Function
Cell surface					
c-met	m, h	+	+	+	HGF receptor (reviewed in [1])
Caveolin-1	m	+	–	–	Cell-cycle arrest [43,44]
CD34	m	+	+	+	Unknown, isotype switching during activation (reviewed in [1])
CTR	m	+	–	–	Regulation of quiescence [41]
CXCR4/SDF-1[b]	m, h	+	+	+	Migration [52,102,103]
ErbB receptor	m	–	+	+	Anti-apoptotic [46]
Igsf4a	m	+	+	–	Unknown [104]
Integrin α_7	m, h	+	+	+	ECM signaling, fusion [104,105]
Integrin β_1	m, h	+	+	+	ECM signaling, fusion [106,107]
M-cadherin	m, h	+	+	+	Anchoring (reviewed in [1])
Necdin	m	–	+	+	Promotes differentiation [45]
Megf10	m	+/–	+	–	Regulation of quiescence [108]
NCAM	m, h	+	+	+	Adhesion (reviewed in [1])
Neuritin-1	m	+	+	–	Unknown [104]
p75NTR/BDNF[c]	m	+	+	–	Inhibition of differentiation [109]
Pb99	m	+	+	–	Unknown [104]
SM/C-2.6	m	+	+	–	Unknown [110]
Sphingomyelin	m	+	–	–	Cell-cycle entry [42]
Syndecan 3/4	m	+	+	+	ECM signaling [111]
TcRβ	m	+	+	–	Unknown [104]
VCAM-1/VLA-4[d]	m	+	+	+	Myoblast fusion [104,112]
Transcription factor					
Foxk1	m	+	+	+	Proliferation or cell cycle [113,114]
HoxC10	m	+	+	+	Unknown [104]
Lbx1	m	–	+	+	Forcing activated cells to quiescence [47]
Myf5	m, h	+	+	+	Myogenic commitment and transient amplification [18,115]
MyoD	m, h	–	+	+	Activation and myogenic differentiation (reviewed in [1])
Msx1	m	+	–	–	Inhibition of differentiation [116,117]
Pax3	m	+/–	+/–	–	Multiple roles (see text)
Pax7	m, h	+	+	–	Multiple roles (see text)
Sox8/9	m	+	+	–	Inhibition of differentiation [118]
Other					
Desmin	m, h	+/–	+	+	Cytoskeleton [40,119]
Myostatin/ACVR2	m, h?	+	+	+	Inhibit satellite cell activation and muscle growth [73,120]
Nestin	m, h	+	–	–	Cytoskeleton, nuclear organization? [40,104,121]

Abbreviations: A, activated (cycling) satellite cell; ACVR2, activitin receptor type 2; CTR, calcitonin receptor; D, differentiating myoblast; ECM, extracellular matrix; Q, quiescent satellite cell.

[a]Species column only includes mouse (m) and human (h).

[b]CXCR4/SDF-1: stromal derived factor 1 (SDF-1) is a ligand for CXCR4 receptor.

[c]p75NTR/BDNF: p75NTR is a neurotrophin receptor for BDNF (brain-derived neurotrophic factor).

[d]VCAM-1/VLA-4: very late antigen-4 (VLA-4) or integrin $\alpha_4\beta_1$ is a receptor for vascular cell adhesion molecule 1 (VCAM-1).

III-b Caractéristiques histopathologiques du tissu musculaire inflammatoire.

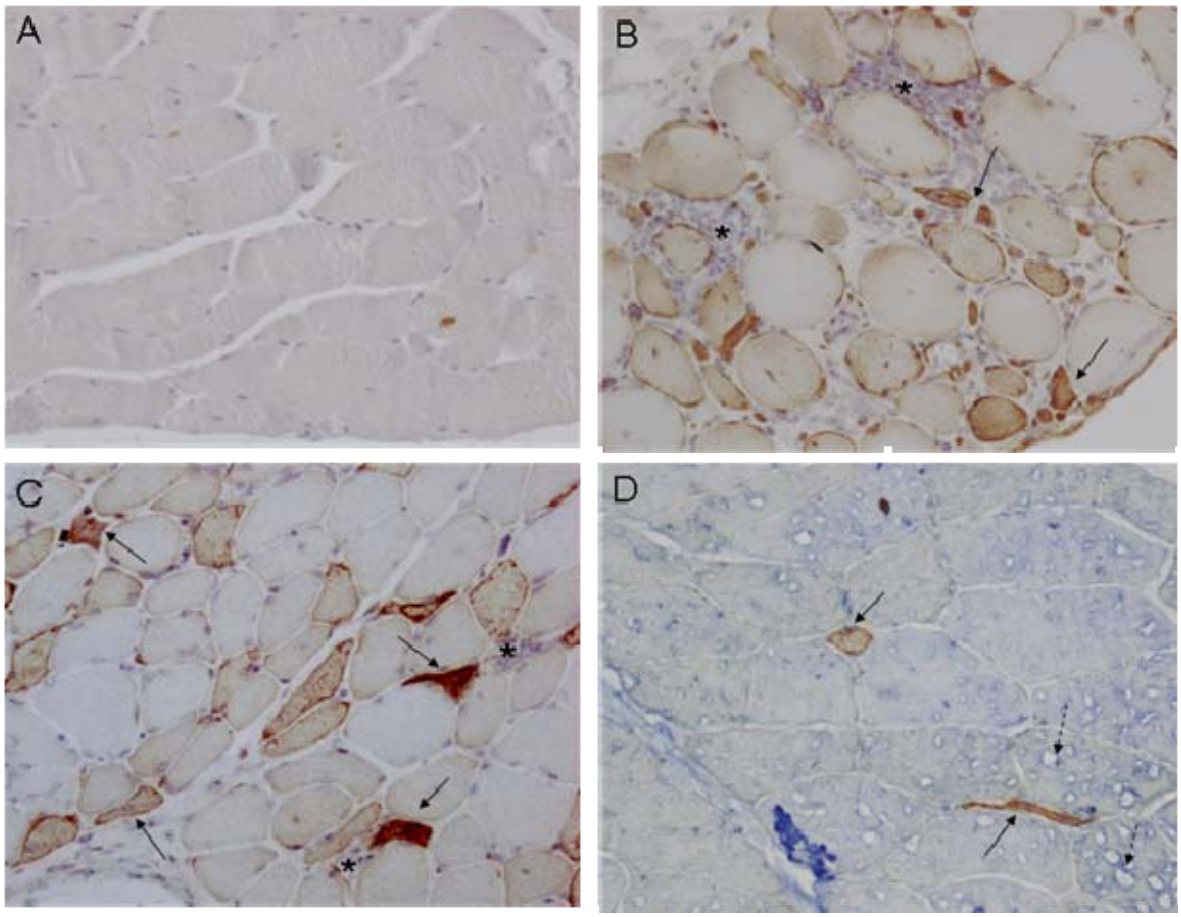
Le tissu musculaire des myopathies inflammatoires est caractérisé par des signes de destruction associant des fibres musculaires de taille irrégulière avec noyau centralisé en cours de dégénération, de nécrose, de phagocytose, mais également par des signes régénération, dont la présence de petites fibres musculaires irrégulières exprimant le marqueur CD56 (Figure 2). Il s'y associe un infiltrat inflammatoire constitué de cellules mononuclées et une atteinte de la microvascularisation (Figure 2). La présence, la localisation et la distribution de ces différents éléments caractérisent chaque forme de myopathie inflammatoire.

Dans la DM, l'infiltrat inflammatoire, composé de lymphocytes T CD4+ et de lymphocytes B, siège dans le périmysium et les régions périvasculaires. Des anomalies vasculaires associant une hyperplasie endothéliale, l'expression endothéliale de cytokines proinflammatoires [29], la présence de microthrombi et de dépôts de complément dans les capillaires, entrainent une réduction du nombre des capillaires qui contribue à l'atrophie périfasciculaire quasi-pathognomonique.

Dans la PM, l'infiltrat inflammatoire constitué de lymphocytes T CD8+ et de macrophages se localise dans l'endomysium, autour des fibres musculaires pour envahir des fibres saines non nécrotiques exprimant les antigènes HLA de classe I. La démonstration *in vitro* d'une cytotoxicité dirigée contre les myotubes des lymphocytes T obtenus à partir de muscle de PM [30], et l'expansion clonale de lymphocytes T autoréactifs dans le muscle de PM [31], suggèrent un processus auto-immun dirigé contre un auto-antigène musculaire encore non identifié.

Lorsque des vacuoles bordées contenant des dépôts amyloïdes et des inclusions éosinophiles sont identifiées au sein du cytoplasme des fibres musculaires en association avec un infiltrat inflammatoire endomysial, le diagnostic de myosite à inclusions peut être retenu [17].

Figure 2. Caractéristiques immunopathologiques du tissu musculaire normal (A), de PM (B), de DM (C), et de myopathie non inflammatoire métabolique (D) après immunomarquage par un anticorps anti-CD56 (marquage marron) x200.



Le tissu musculaire normal (A) est caractérisé par l'absence d'infiltrat inflammatoire, par des fibres musculaires de taille régulière, et par l'absence du marqueur de régénération CD56.
Les PM (B) et DM (C) sont caractérisées par la présence d'infiltrats inflammatoires mononuclées (*) et par de nombreuses fibres musculaires immatures en cours de régénération, de petite taille, irrégulières, et CD56 positives (flèches pleines).
Le tissu musculaire non inflammatoire des myopathies métaboliques (Glycogénose type V ou maladie de Mac Ardle) (D) est caractérisé par l'absence d'infiltrat inflammatoire, par la présence de rares fibres musculaires en cours de régénération CD56 positives (flèches pleines), et par de nombreuses fibres vacuolaires témoignant de la surcharge en glycogéne (flèches en pointillés).

III-c Rôle de la surexpression musculaire du CMH de classe I.

Contrairement au tissu musculaire des myopathies inflammatoires, les fibres musculaires matures du muscle sain n'expriment pas les antigènes du complexe majeur d'histocompatibilité (CMH) de classe I ou II [32, 33]. Les mécanismes conduisant à la surexpression des antigènes HLA de classe I à la surface des fibres musculaires au cours des myopathies inflammatoires restent mal expliqués. En effet, la surexpression du HLA de classe I pourrait résulter soit d'une réponse non spécifique liée au processus de régénération musculaire lui-même, soit au contraire d'un processus pathologique inflammatoire spécifique. De façon intéressante, les données *in vitro* montrent que le niveau d'expression du CMH de classe I à la surface des cellules musculaires est régulé durant les différents stades de différentiation et peut également être modulé par le statut inflammatoire du milieu de culture. Ainsi, de façon constitutionnelle, les myoblastes expriment faiblement les antigènes du CMH de classe I ou II. Ce niveau d'expression décroît au cours de la différentiation en myotubes puis en fibre musculaire [34]. En créant des conditions proinflammatoires par addition dans le milieu de culture de cytokines IL1, TNFα, IFNγ, l'expression du CMH de classe I à la surface cellulaire peut être augmentée [35].

La surexpression du HLA de classe I à la surface des fibres musculaires de patients atteints de PM ou de DM est une des caractéristiques histologiques majeures [32], qui associée à une cytotoxicité musculaire médiée par les lymphocytes T [30], est fortement évocatrice d'un processus auto-immun à l'origine de la destruction musculaire. Néanmoins, l'absence de corrélation systématique entre le degré d'inflammation tissulaire et la sévérité de l'atteinte histologique ou de l'atteinte clinique [36, 37], et des observations de modifications structurales du tissu musculaire en dehors de la présence d'un infiltrat inflammatoire [32, 38, 39], peuvent suggérer la contribution d'un processus non immunologique en plus du mécanisme de présentation antigénique soutenu par les molécules du CMH. L'hypothèse d'une toxicité directe des antigènes du CMH de classe I dans les maladies auto-immunes a été initialement suggérée dans le diabète, les pathologies démyélinisantes du système nerveux et les dysthyroïdies, en utilisant un modèle de souris transgéniques surexprimant le CMH de classe I uniquement dans les cellules cibles qui normalement n'expriment que très peu ces antigènes [40, 41, 42]. Des souris transgéniques chez qui la surexpression du CMH de classe I est obtenue uniquement dans le muscle squelettique, développent une myosite auto-immune très proche des myosites auto-immunes humaines, caractérisée par un déficit musculaire et

une amyotrophie, une augmentation des enzymes musculaires, des anomalies histologiques associant dégénération-régénération musculaire, infiltrat inflammatoire mononucléé, et des autoanticorps de type anti-histidyl-tRNA synthétase [3]. L'accumulation des molécules de CMH de classe I dans le réticulum endoplasmique responsable d'un stress cellulaire et d'une activation de la voie NF-κB, pourrait expliquer une toxicité musculaire directe de la surexpression du CMH de classe I [43]. En retour, l'activation de la voie NF-κB peut induire l'expression endogène des molécules du CMH de classe I et des gènes cibles proinflammatoires (cytokines, récepteurs des cytokines, molécules d'adhésion, chémokines), amplifiant ainsi le processus. Elle peut également inhiber l'expression de MyoD et compromettre ainsi la différentiation musculaire [44].

Le second mécanisme par lequel les molécules du CMH de classe I peuvent être responsables d'une toxicité musculaire est la présentation antigénique aux lymphocytes T cytotoxiques. Cette hypothèse est confortée par les observations histopathologiques des PM, au cours desquelles les fibres musculaires non nécrotiques surexprimant à leur surface les antigènes HLA de classe I sont envahies par des lymphocytes T cytotoxiques CD8+ [32]. La capacité des cellules musculaires à activer des lymphocytes T naïfs par la présentation antigénique nécessite de capturer l'antigène, d'exprimer les molécules HLA ainsi que les molécules d'adhésion et de costimulation indispensables à la bonne interaction du complexe cellule présentatrice d'antigène-lymphocyte T. En exprimant les molécules HLA et les molécules d'adhésion sous conditions inflammatoires, la cellule musculaire peut fournir le premier signal correspondant à l'interaction HLA-peptide-récepteur des lymphocytes T (TCR). A l'inverse du tissu musculaire normal, les fibres musculaires des PM et DM expriment les molécules d'adhésion ICAM-1, et l'expression du récepteur correspondant LFA-1α est retrouvée à la surface des cellules inflammatoires mononucléées [33, 45, 46]. I*n vitro,* l'expression d'ICAM-1 peut être induite à la surface des myoblastes et myotubes après stimulation par l'IL1, le TNFα et l'IFNγ [35, 47, 48, 49]. De plus, les myoblastes dont l'expression d'ICAM-1 et d'HLA-DR a été induite après stimulation par IFNγ, peuvent présenter de manière fonctionnelle un antigène à des lymphocytes T CD4+ [47]. Pourtant, l'expression des molécules de costimulation, CD80 (B7-1) et CD86 (B7-2), normalement présentes à la surface des cellules présentatrices d'antigènes activées et nécessaires à l'interaction avec les lymphocytes T via les ligands CD28 et CTLA-4, ne sont pas détectées, ni *in vitro* après stimulation par des cytokines proinflammatoires à la surface des myoblastes,

ni *in vivo* à la surface des fibres musculaires des PM et DM [50]. Deux molécules de costimulation apparentées à la famille B7 et dénommée BB-1 et B7-H1, sont exprimées à la surface des fibres musculaires des PM et DM alors qu'elles sont absentes dans le tissu musculaire normal ou non inflammatoire [51, 52]. Il apparait donc que si la cellule musculaire ne possède pas toutes les molécules de surface lui permettant de fonctionner comme une véritable cellule présentatrice d'antigène, elle peut néanmoins interagir de façon limitée avec le lymphocyte T.

III-d Signature interféron type I dans les myopathies inflammatoires.

Des travaux récents suggèrent une contribution du système IFN type I dans la pathogénie des myopathies inflammatoires. La présence des cellules dendritiques plasmacytoïdes, source importante d'IFN de type I, est rapportée dans l'infiltrat inflammatoire musculaire des PM, DM (formes adulte et juvéniles) et IBM [7, 53, 54, 55], en particulier chez les patients avec autoanticorps de type anti-Jo1 ou anti-SSA/SSB [7]. D'autre part, l'expression des gènes et protéines induits par les IFN de type I α/β est augmentée dans le tissu musculaire et le sang périphérique de patients atteints de PM et DM [7, 8, 53, 56], et est corrélée à l'activité de la maladie [8, 56]. Une exacerbation de l'activité IFN type I pourrait être impliquée dans les mécanismes d'aggravation décrits après traitement des myopathies inflammatoires par anti-TNFα [57]. L'ensemble de ces données suggèrent donc un rôle potentiel de la voie IFN type I dans la physiopathologie des myopathies inflammatoires. La production locale d'IFN de type I pourrait en retour favoriser la surexpression des molécules du CMH de classe I [58].

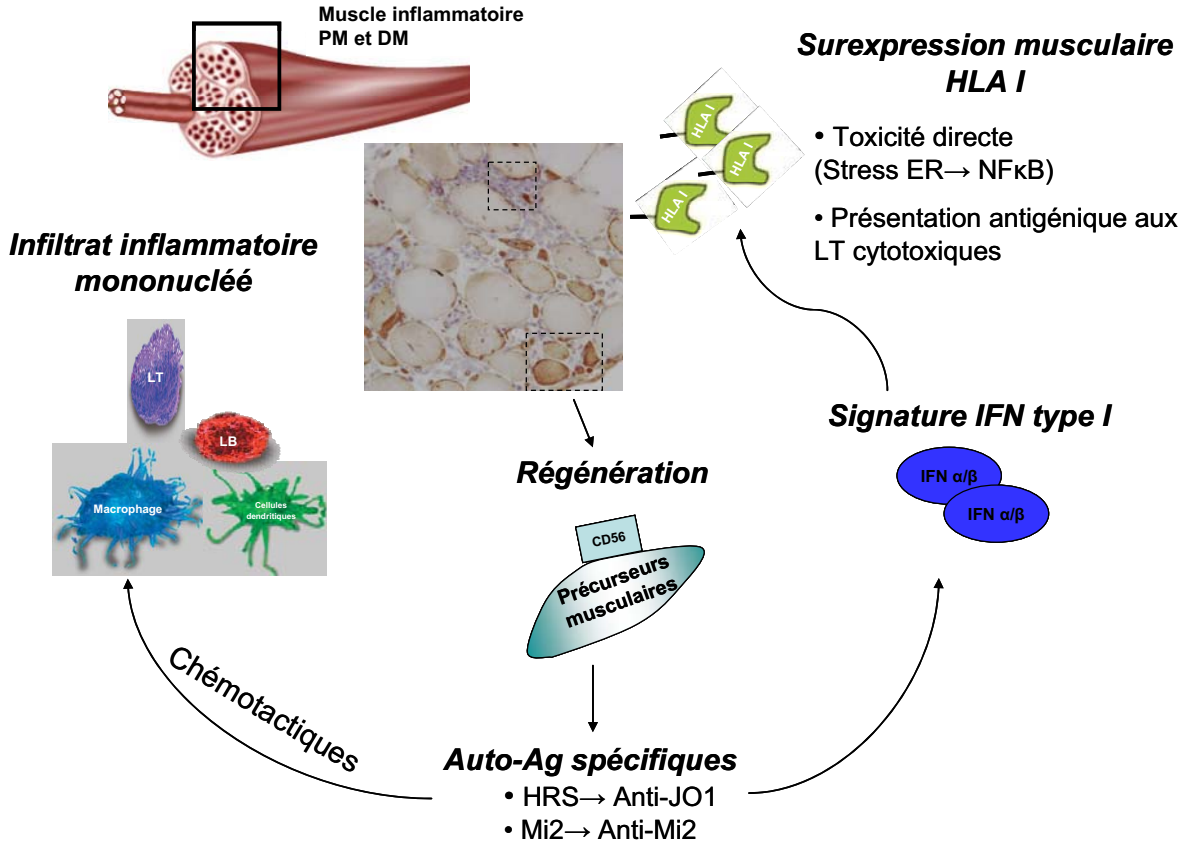
III-e Rôle physiopathologique des autoantigènes.

Les autoanticorps spécifiques des myosites, notamment les anticorps anti-synthétases, parmi lesquels la cible antigénique la plus fréquemment retrouvée est l'histidyl-tRNA synthétase pour l'anti-Jo1, et les anticorps anti-Mi2 dirigés contre un complexe peptidique impliqué dans les mécanismes de transcription, représentent plus que des marqueurs biologiques associés à des phénotypes cliniques. Ils peuvent contribuer à la compréhension des mécanismes physiopathologiques. Ainsi, plusieurs études rapportant une corrélation entre le taux sérique d'anti-Jo1 et des marqueurs d'activité de la maladie musculaire, pulmonaire ou articulaire, supportent un lien pathogénique entre ces autoanticorps et le processus

inflammatoire tissulaire [59, 60, 61]. De plus, l'expression tissulaire des autoantigènes spécifiques des myosites, histidyl-tRNA synthétase et Mi2, est fortement augmentée dans le muscle des PM et DM alors qu'elle est faiblement observée dans le muscle normal [5]. L'expression de Mi2, dont l'autoanticorps est spécifique des DM, elles-mêmes fréquemment associées aux cancers, est augmentée uniquement dans le muscle des DM et dans les tumeurs malignes du sein et du poumon, alors qu'elle est absente dans le muscle des PM ainsi que dans le tissu mammaire ou pulmonaire normal [5]. D'autre part, l'étude en immunohistochimie de biopsies musculaires de PM et DM montre que l'expression de l'autoantigène histidyl-tRNA synthétase est localisée aux cellules musculaires immatures en cours de régénération, CD56 positives, exprimant également fortement les antigènes HLA de classe I [5]. *In vitro* , l'expression des autoantigènes histidyl-tRNA synthétase et Mi2 est régulée au cours de la différentiation, et diminue lors de la fusion des myoblastes en myotubes [5]. L'expression des autoantigènes spécifiques des myosites ne semble donc pas ubiquitaire mais plutôt restreinte au tissu musculaire en cours de régénération ou à certains tissus tumoraux. Il est également démontré que l'épitope principal reconnu par les anticorps anti-Jo1 diffère des épitopes reconnus en présence de l'antigène histidyl-tRNA synthétase purifié ce qui suggère un rôle important de l'environnement tissulaire et inflammatoire dans la genèse de la réponse immunitaire [59]. La génération d'épitopes antigéniques uniques rendant compte de la production d'autoanticorps peut s'expliquer par un niveau élevé d'autoantigènes libérés dans le tissu cible et par des altérations de conformation de ces derniers après clivage enzymatique [62]. La plupart des autoantigènes rencontrés dans les maladies autoimmunes doivent leur immunogénicité au clivage par la protéase granzyme B contenue dans les granules des lymphocytes T cytotoxiques. Les autoantigènes cibles des PM et DM sont également des substrats de granzyme B [62] et les épitopes antigéniques ainsi générés, par leur potentiel chémotactique, pourraient participer à l'initiation et la propagation de la réponse autoimmune en recrutant des cellules inflammatoires et des cellules présentatrices d'antigènes dans le tissu musculaire cible [6]. En effet, l'histidyl- et l'asparaginyl- tRNA synthétases, toutes 2 cibles antigéniques dans les PM et DM (anti-Jo1 et anti-KS), ont la capacité d'induire la migration des lymphocytes T CD4+ et CD8+, des monocytes activés, et des cellules dendritiques immatures *in vitro* , alors que l'aspartyl- et la lysyl-tRNA synthétases non autoantigéniques ne sont pas chémotactiques [6]. Ces propriétés chémoattractantes sont médiées par les récepteurs aux chémokines CCR3 et CCR5 présents à la surface des cellules mononuclées de l'infiltrat inflammatoires des PM et DM [6]. D'autre part, les anticorps anti-Jo1 contenus dans le sérum de patients atteints de PM et DM, en association avec des cellules

nécrotiques, ont la capacité d'induire la production *in v itro* d'IFNα [7] suggérant que les autoanticorps spécifiques des myosites pourraient servir de passerelles entre le système immunitaire inné et adaptatif, conduisant alors à une rupture de tolérance et au processus autoimmun de destruction musculaire.

Figure 3. Principales caractéristiques immunopathologiques du tissu musculaire inflammatoire dans les polymyosites (PM) et dermatomyosites (DM).



Le tissu musculaire des polymyosites (PM) et dermatomyosites (DM) est caractérisé par la présence d'un infiltrat inflammatoire mononucléé constitué de macrophages, de lymphocytes T et B et de cellules dendritiques. La prédominance d'un type cellulaire et la localisation endomysiale ou périmysiale permet de distinguer PM et DM. Des signes de régénération s'associent aux signes de destruction. Les autoantigènes spécifiques des myosites fortement exprimés dans les cellules musculaires immatures en cours de régénération favorisent le recrutement des cellules inflammatoires. Les autoanticorps correspondants contribuent à l'activation de la voie IFN type I et à la signature IFN type I observée dans les PM et DM. Le tissu musculaire des PM et DM est également caractérisé par une surexpression des antigènes HLA de classe I à la surface des fibres musculaires, qui peut contribuer aux mécanismes pathogéniques via une toxicité directe (stress du réticulum endoplasmique) ou indirecte (présentation antigénique aux lymphocytes T cytotoxiques).

Chapitre IV

Cellules dendritiques, chémokines et cytokines dans les myopathies inflammatoires.

Sommaire

IV-a Les cellules dendritiques.

Les cellules dendritiques, véritables sentinelles du système immunitaire, jouent un rôle central dans le développement de l'immunité innée et adaptative. Ce sont les plus efficaces des cellules présentatrices d'antigènes. Elles sont nécessaires à l'initiation du processus immunitaire en permettant l'activation des lymphocytes T naïfs, la prolifération des lymphocytes T mémoires et des lymphocytes B. Elles contrôlent également la nature, l'intensité et la qualité de la réponse immunitaire [63], et sont donc responsables du processus de tolérance périphérique [64]. Elles circulent du sang périphérique vers les tissus où elles peuvent capturer les antigènes, puis vont migrer vers les organes lymphoïdes pour activer les lymphocytes T naïfs. Après activation, soit directement par des composants microbiens soit via les effecteurs de l'immunité innée, et durant leur maturation et migration, les cellules dendritiques subissent des changements morphologiques et phénotypiques qui les font passer d'un statut de cellules capables de capter un antigène à celui de cellules capables de présenter un antigène et d'activer les lymphocytes T. Ces changements comprennent l'expression à leur surface des molécules de costimulation CD40, CD80, CD86 et la translocation des molécules du HLA de classe II du compartiment intracellulaire à la surface cellulaire [63, 64]. Les cellules dendritiques vont également produire différentes chémokines et cytokines pour attirer les effecteurs du système immunitaire dans le tissu cible et permettre leur différentiation et leur polarisation [65]. L'activation des cellules dendritiques dépend de stimuli dérivés d'agents microbiens et des cellules nécrotiques, des cellules de l'immunité innée et de l'immunité adaptative [65]. Les agents microbiens peuvent ainsi être reconnus par des récepteurs de l'immunité innée présents à la surface des cellules dendritiques, parmi lesquels les Toll-like récepteurs (TLRs) et les C-type lectin récepteurs (CLRs), via des motifs moléculaires conservés au cours de l'évolution et appelés pathogen-associated molecular patterns (PAMPs). Les cytokines telles que l'IL1, le TNFα, le GM-CSF, en association avec l'IL4, vont contribuer à la maturation des cellules dendritiques [63]. Deux types de cellules dendritiques, avec des activités biologiques différentes, sont identifiés en fonction de leur évolution à partir des progéniteurs hématopoïétiques: les cellules dendritiques myéloïdes et les cellules dendritiques plasmacytoïdes. Les cellules dendritiques myéloïdes sont les plus puissantes des cellules présentatrices d'antigènes. Les cellules dendritiques plasmacytoïdes se caractérisent elles par leur forte capacité à sécréter de l'IFN de type I en réponse à une stimulation virale.

Dans le tissu musculaire des myopathies inflammatoires, des cellules dendritiques à la fois immatures, CD1a positives, et matures, DC-LAMP positives, sont détectées au sein de l'infiltrat inflammatoire périvasculaire des DM et endomysial des PM [2, 23, 66]. Une analyse quantitative en immunohistochimie montre que les cellules dendritiques immatures, CD1a positives, sont plus rarement observées que les cellules dendritiques matures, DC-LAMP positives [23], et que l'accumulation de cellules dendritiques matures, quantifiée par le ratio de cellules positives DC-LAMP/CD1a, est plus importante dans les PM que dans les DM [2]. La présence dans l'infiltrat inflammatoire des biopsies musculaires de PM et DM de la chémokine CCL20, impliquée dans la migration des cellules dendritiques immatures, en l'absence des chémokines CCL19 et CCL21 impliquées dans le recrutement des cellules dendritiques matures, suggère un processus de maturation local expliquant l'accumulation de cellules dendritiques matures [2]. Les 2 sous-types de cellules dendritiques, myéloïdes et plasmacytoïdes, sont présents dans les biopsies musculaires de myopathies inflammatoires, malgré une répartition qui semble différer entre PM et DM [53, 54, 55]. Ainsi, dans les PM, les cellules dendritiques présentes dans l'infiltrat inflammatoire sont le plus souvent des cellules dendritiques myéloïdes [55], mais des cellules dendritiques plasmacytoïdes peuvent également être observées en particulier chez les patients avec autoanticorps anti-Jo1 ou anti-SSA [7]. Dans les DM de l'adulte et de l'enfant, la majorité des cellules dendritiques est constituée de cellules dendritiques plasmacytoïdes matures regroupées en amas dans les régions périvasculaires et périmysiales [53, 54, 55]. De façon intéressante, ces cellules dendritiques plasmacytoïdes matures expriment également le marqueur CD4 et leur présence pourrait donc en partie expliquer la forte accumulation de cellules CD4 positives qui caractérise l'infiltrat inflammatoire musculaire des DM [53]. A l'inverse du tissu musculaire inflammatoire, le tissu musculaire normal ne contient que de très rares cellules dendritiques plasmacytoïdes, dispersées et immatures, servant probablement de sentinelles immunitaires [54]. L'accumulation anormale de cellules dendritiques myéloïdes et plasmacytoïdes matures dans le tissu musculaire des myopathies inflammatoires témoigne donc d'une réaction immunitaire en cours, susceptible d'aboutir au processus de destruction musculaire. La présence au sein du tissu musculaire des PM et DM des cellules dendritiques plasmacytoïdes, source importante d'IFNα, pourrait contribuer à la signature interféron type I retrouvée dans les myopathies inflammatoires [7, 8], et renforce le rôle potentiel de l'immunité innée dans la physiopathologie de ces affections autoimmunes.

IV-b Rôle des chémokines dans la migration des cellules de l'immunité.

Les chémokines sont des protéines de petite taille, produites par les leucocytes, les cellules dendritiques, les cellules mésenchymateuses et endothéliales, et qui ont des propriétés chémoattractantes en induisant la migration des cellules immunitaires vers les tissus cibles. En fonction de la position de leur résidu cystéine, on peut distinguer les chémokines CXC, dont la plus connue est l'IL8, qui induisent la migration des polynucléaires neutrophiles, et les chémokines CC ou β chémokines, qui attirent principalement les lymphocytes et les monocytes [67]. Les cellules dendritiques jouent un rôle central dans l'initiation et la propagation de la réponse immune. Les chémokines qui participent à leur migration tissulaire sont donc d'un intérêt particulier. De nombreuses β chémokines telles que monocyte chemotactic protein (MCP)-3, MCP-4, macrophage inflammatory protein CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES (regulated on activation, normal T cell expressed and secreted) ont démontré *in vitro* des propriétés chémoattractantes pour les cellules dendritiques [68]. La réponse des cellules dendritiques aux différentes chémokines va varier durant le processus de maturation. Ainsi, les cellules dendritiques immatures répondent aux β chémokines CCL3/MIP-1α, CCL5/RANTES et CCL20/MIP-3α alors que les cellules dendritiques matures perdent leur sensibilité à ces chémokines et vont répondre aux chémokines CCL19/MIP-3β et CCL21 [68, 69, 70]. Dans le tissu synovial de patients atteints de polyarthrite rhumatoïde (PR), CCL20 et son récepteur CCR6 agissent sur la migration des cellules dendritiques immatures alors que CCL19, CCL21 et leur récepteur CCR7 sont impliqués dans l'accumulation de cellules dendritiques matures et ainsi la formation de centres germinatifs au sein de la synovite rhumatoïde [69].

Dans les myopathies inflammatoires, plusieurs β chémokines (CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES) et leurs récepteurs respectifs (CCR2, CCR1, CCR5) sont détectés dans le tissu musculaire [71]. La présence de CCL20 associée à son récepteur CCR6 est rapportée dans le muscle des PM et DM, au sein de l'infiltrat inflammatoire des régions endomysiales ou périvasculaires, alors qu'elle est absente dans le tissu musculaire normal [2]. La co-localisation en immunohistochimie, à la fois dans les PM et DM, de cellules dendritiques immatures CD1a positives et de cellules exprimant CCL20 suggère un lien entre le recrutement des cellules dendritiques immatures et la production de la β chémokine CCL20 [2]. A l'inverse, l'expression de CCL19, CCL21 et du récepteur CCR7, impliqués dans la migration des cellules dendritiques matures, est rarement détectée ce qui

laisse suggérer un processus de maturation local expliquant l'accumulation de cellules dendritiques matures [2].

Tournadre A, Miossec P. Chemokines and dendritic cells in inflammatory myopathies. *Ann Rheum Dis.* 2009;68:300-4.

Contribution spécifique de la candidate:

- Revue de la littérature et écriture de l'article.
- Réponses aux commentaires des rapporteurs.

Chemokines and dendritic cells in inflammatory myopathies

A Tournadre,[1,2] P Miossec[1]

[1] Department of Immunology and Rheumatology, University of Lyon and Hospices Civils de Lyon, Lyon, France;
[2] Department of Rheumatology, Hospital Gabriel Montpied, Clermont-Ferrand, France

Correspondence to:
Professor P Miossec, Clinical Immunology Unit, Department of Immunology and Rheumatology, Hospital Edouard Herriot, 69437 Lyon Cedex 03, France;
pierre.miossec@univ-lyon1.fr

Accepted 5 September 2008

ABSTRACT

This review focuses on the contribution of the local production of chemokines and cytokines and of dendritic cells (DC) to the pathogenesis of inflammatory myopathies. DC are the most efficient professional antigen-presenting cells (APC), which are critical for the development of innate and adaptive immune responses. Chemokines are important mediators of the immune response as they regulate leucocyte recruitment to tissue and play a key role in inflammatory diseases by acting on T-cell and DC migration. Recent advances indicate that the muscle cell itself could participate in the inflammatory process. Furthermore, the T-helper (Th) type 1 and Th17 proinflammatory cytokines, present in myositis samples, are associated with the migration, differentiation and maturation of inflammatory cells and allow a network of interactions between all the components of the immune response. An understanding of such interactions is essential because it can lead to therapeutic applications.

Inflammatory myopathies are a group of chronic muscle disorders of unknown origin, which have in common clinical symptoms such as muscle weakness and an inflammatory infiltrate in muscle tissue. Although no specific target antigens have been identified, the autoimmune origin of the disease is supported by the association with autoantibodies, major histocompatibility complex (MHC) class I overexpression in muscle cells and evidence of a T-cell-mediated cytotoxicity.[1] Based on clinical, histological and immunopathological features, inflammatory myopathies can be divided into the two main subtypes: dermatomyositis and polymyositis. Dermatomyositis is considered a CD4-driven disease resulting in a microangiopathy affecting skin and muscle, whereas polymyositis is considered a CD8-driven disease in which muscle is the primary target of the immune attack.[1] However, this classification remains limited by the clinical and histological overlap between all myositis and the lack of internationally validated and agreed criteria. The use of the diagnostic criteria of Bohan and Peter[2] and/or histopathological criteria[1] to distinguish polymyositis from other myopathies is still controversial.[3] Moreover, immunohistochemistry studies in polymyositis and dermatomyositis muscle have shown a common profile of dendritic cells (DC), T helper (Th) type 1 and Th17 cytokines.[4]

DC are central to the development of innate and adaptive immune responses. Both mature and immature DC have been identified in the lymphocytic infiltrates of dermatomyositis and polymyositis, reflecting an ongoing immune-mediated reaction.[4] The mechanisms underlying the sustained recruitment of these cells to muscle tissue involve the chemokine system related to mononuclear cell migration and antigen presentation. This review focuses on recent advances in the interactions between T cells, DC and the chemokine and cytokine systems, primarily in polymyositis and dermatomyositis.

HISTOLOGICAL CHARACTERISTICS OF INFLAMMATORY MYOPATHIES

In inflammatory myopathies, histological characteristics show evidence of tissue destruction with degenerating and regenerating muscle fibres, variation in fibre size and centralised myonuclei. In contrast to normal muscle tissue, increased MHC class I expression on the surface of muscle fibres is a common feature of inflammatory myopathy subsets.[5] Normal muscle is poorly infiltrated by immune cells. Criteria for the diagnosis of inflammatory myopathies include an inflammatory infiltrate in affected muscle tissue.[6] In polymyositis, endomysial CD8 T cells and macrophages surround and invade non-necrotic muscle fibres expressing MHC class I antigens. In dermatomyositis, mononuclear cells consisting of perimysial CD4 T cells and B cells predominate in perivascular regions and MHC class I expression predominates on perifascicular fibres. Dermatomyositis is distinguishable from polymyositis by the presence of perifascicular atrophy, which probably reflects hypoperfusion caused by the focal depletion of capillaries. Vascular changes result from the deposition of complement membrane attack complexes in capillaries.[1] Involvement of the microvasculature in inflammatory myopathies is also suggested by phenotypical changes of the capillaries both in dermatomyositis and polymyositis, with a strong expression of proinflammatory cytokines by endothelial cells.[7]

DC IN INFLAMMATORY MYOPATHIES

DC are the most efficient professional antigen-presenting cells (APC). They play a unique role in initiating immunity through the activation of naive T cells, and support local immune responses by the generation and the proliferation not only of CD4 helper T cells but also of CD8 cytotoxic T lymphocytes. During maturation, DC undergo changes in phenotype by the upregulation of B7 molecules and of MHC class II antigens. At the same time, DC move from an antigen-capturing cell into an APC capable of activating antigen-specific lymphocytes. Cytokines, such as IL-1, tumour necrosis factor (TNF) α, granulocyte macrophage colony-stimulating factor, combined with IL-4 contribute to DC maturation.[8] DC are

also responsible for the establishment of peripheral tolerance.[9] Two main types of DC that mediate distinct biological outcomes are distinguished: myeloid DC and plasmacytoid DC. Myeloid DC are potent APC of the adaptive immunity that stimulates lymphocytes capable of highly specific immune responses. Plasmacytoid DC play an important role in innate immunity and specifically in the control of viruses through their ability to produce large amounts of type I interferons (IFNα, IFNβ).

In dermatomyositis and polymyositis, both mature DC, defined as CD1a-positive cells and immature DC, defined as DC-LAMP-positive cells, are localised in perivascular infiltrates in dermatomyositis samples and in infiltrates surrounding or invading muscle fibres in polymyositis.[4] The mature/immature DC ratio is higher in polymyositis, suggesting a relative accumulation of more mature DC in polymyositis. Both plasmacytoid and myeloid DC are observed in dermatomyositis and polymyositis samples despite a different ratio.[10–12] In the muscle of patients with dermatomyositis and juvenile dermatomyositis, the majority of these plasmacytoid DC are mature cells found in perimysial and perivascular areas.[10 11] In normal control muscles, few immature plasmacytoid DC that probably serve as the sentinels of host defence are also detected. Immature plasmacytoid DC are scattered throughout the tissue, whereas mature plasmacytoid DC are within the foci of lymphocytic infiltrates.[11] In dermatomyositis infiltrates, the greater relative number of CD4 cells compared with polymyositis infiltrates could be explained by the presence of many plasmacytoid DC expressing CD4.[10] In polymyositis muscle, although most DC are myeloid DC,[12] plasmacytoid DC are also observed, in particular in patients with anti-Jo-1 and anti-SSA autoantibodies.[13] The contribution of the type I interferon system in inflammatory myopathies is suggested by the presence of plasmacytoid DC, the major cellular source of IFNα, and by an upregulation of IFNα/β inducible genes in blood[14] and IFNα/β inducible proteins in muscle tissue.[13]

Taken together, these data demonstrate the abnormal accumulation of DC in inflammatory myopathy muscle, which reflects an ongoing immune-mediated reaction that may lead to further muscle damage. In both dermatomyositis and also polymyositis, the accumulation of plasmacytoid cells and type I interferon-inducible protein and gene overexpression suggests a role of the innate immune system in addition to the adaptive system. The type I interferon system may be activated by immune complexes composed of autoantibodies to RNA-binding proteins, such as anti-Jo1 and necrotic cell material.[13] The local production of IFNα could then contribute to the increased MHC class I expression that may play a role in the pathogenesis of inflammatory myopathies.[15 16]

ROLE OF CHEMOKINES AND CYTOKINES IN INFLAMMATORY MYOPATHIES

Chemokines and migration of immune cells

Chemokines are small chemoattractant proteins produced by leucocytes, DC, mesenchymal cells and endothelial cells. They induce cell migration to target tissues. Chemokines are subdivided into different groups according to the position of cysteine residues.[17] Among them, CXC chemokines such as IL-8 mainly attract neutrophils to inflammatory sites, whereas CC chemokines or β chemokines act mainly on lymphocytes and monocytes. A strong expression of various β chemokines (CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES) and their receptors (CCR2, CCR1, CCR5) has been reported in inflammatory myopathies.[18] DC migratory properties are critical to initiate immune responses. The expression of chemokines that play a role in DC migration is thus of particular interest. The β chemokine CCL20/macrophage inflammatory protein-3α is involved in DC migration.[19] CCL20 and its receptor CCR6 control the migration of immature DC in rheumatoid arthritis synovium.[19 20] CCL19, CCL21 and their associated receptor CCR7 contribute to the accumulation of mature DC in T-cell-rich areas of lymphoid organs[21] and to the formation of germinal centres in rheumatoid arthritis synovium.[20] In dermatomyositis and polymyositis, but not in normal muscle sections, CCL20 and its associated receptor CCR6 are detected in the perivascular or lymphocytic infiltrates.[4] The close association between CCL20-producing cells and immature CD1a+ DC found by immunohistochemistry suggests a link between the accumulation of immature DC and the expression of CCL20.[4] CCL19 and CCL21, known to control the migration of mature DC, are rarely expressed in dermatomyositis and polymyositis and their associated receptor CCR7 is absent. In adult polymyositis, CCR7 expression is not reported, whereas this has been reported in juvenile dermatomyositis.[22] The presence of mature DC despite a low expression of the CCL19,CCL21/CCR7 chemokine system suggests a role for CCL20 in the migration of immature DC and argues for a local DC maturation in adult inflammatory myopathies. However, in contrast to previous studies, Tateyama *et al*,[23] combining the results of immunohistochemistry and reverse transcriptase PCR, conclude that the CCL19,CCL21/CCR7 chemokine system is present in polymyositis but is not constitutively expressed in normal muscle. CCR7 is expressed by approximately 60% of endomysial CD8 T cells that surround the non-necrotic muscle fibres. CCL19 is detected mainly on muscle fibres in proximity to CCR7+ mononuclear cells, on vessels and some mononuclear cells. CCL21 is expressed on rare infiltrating mononuclear cells, muscle fibres or vessels. Taken together, these data suggest that β chemokines may be involved in the recruitment and local maturation of T cells and DC in muscle tissue taking part in the pathogenesis of inflammatory myopathies.

Interactions with cytokines and chemokines

In inflammatory myopathies, most studies have shown a strong expression of pro-inflammatory cytokines mainly produced by activated macrophages, monocytes and DC.[8 24–27] These cytokines, in particular IL-1 and TNFα, participate in mononuclear cell recruitment by inducing the broad expression of chemokines and by the upregulation of adhesion molecules on endothelial cells. In myositis tissue, endothelial cells support the migration of mononuclear cells to the perimysial and endomysial spaces through the expression of adhesion molecules (interstitial cell adhesion molecule type 1 (ICAM-1), vascular adhesion molecule type 1), which bind to T-cell and macrophage integrins (leucocyte function antigen type 1 (LFA-1), very large antigen type 4, Mac-1).[28] Although debated, most studies have suggested a main Th1 response in inflammatory myopathies. In polymyositis and dermatomyositis, IFNγ is frequently detected by reverse transcriptase PCR or by immunohistochemistry[8 24 29] and IL-18, a cytokine driving both Th1 differentiation and IFNγ production, is overexpressed in muscle biopsies from patients with dermatomyositis and polymyositis.[30] Both macrophages and DC localised in perimysial or endomysial areas of dermatomyositis and polymyositis samples are the main producers of IL-18, and IL-18R is highly expressed in endothelial cells and CD8 T cells.[30] IL-18 stimulates the production of different chemokines, including CCL2/MCP-1, which attracts

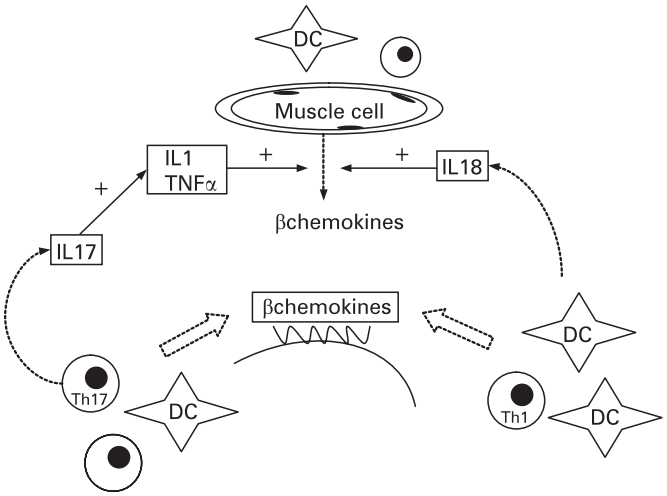


Figure 1 Dendritic cells and interactions with chemokines and cytokines in myositis. Chemokines produced by leucocytes, dendritic cells (DC), mesenchymal cells and endothelial cells are detected in polymyositis and dermatomyositis lymphocytic infiltrates. They induce cell migration and are involved in the recruitment and local maturation of T cells and DC in muscle tissue. IL-1 and tumour necrosis factor (TNF) α participate in the mononuclear cell recruitment by increasing a broad expression of chemokines. In synergy with IL-12, IL-18 produced by DC stimulates the production of IFNγ and chemokines. IL-17 increases TNF/IL-1β-induced secretion of chemokines (CCL-20 in particular) by muscle cells, which in turn are chemoattractant for T helper (Th) type 17 cells.

monocytes, memory T lymphocytes and plasmacytoid DC.[31] Activation of the IL-18/IL-18R pathway may promote the migration of inflammatory cells within muscle.

The Th1/Th2 concept has now to be revisited to take into account the recently described Th17 cells.[32 33] A synergistic effect on IL-6 production between IL-1, TNFα and IL-17 has been demonstrated in myoblasts, and IL-17 increases the production of IL-1 and TNFα suggesting a regulatory role of IL-17.[34] Immunostaining of dermatomyositis and polymyositis biopsies has shown a moderate expression of IL-17 in the T-lymphocyte-rich areas, whereas normal muscle is negative.[35] Both immature and mature DC subsets are associated with the presence of IL-17 and IFNγ-producing cells in polymyositis and dermatomyositis muscle.[4] In functional studies, IL-17 in combination with IL-1β strongly increases IL-6 production by human myoblasts.[35] IL-17 increases in a synergistic fashion the inducing effect of IL-1β on the secretion of CCL20 by myoblasts.[35] CCL20 is in turn a chemotactic factor for Th17 cells. Muscle fibre itself may also be implicated in the recruitment of leucocytes and DC by the local production of β chemokines induced by IL-1β and IL-17.[35 36]

MUSCLE FIBRE AS AN ACTOR OF THE IMMUNE RESPONSE

Cytokines and muscle cells

In addition to being implicated in the recruitment of DC by the local production of β chemokines, human myoblasts constitutively produce low levels of IL-6 and transforming growth factor (TGF) β, which are increased in a dose-dependent manner after stimulation with proinflammatory cytokines (IL-1α, IL-1β, TNFα) and with IFNγ.[37–39] IL-6 can act on T-cell and plasma cell differentiation and could play a role in the early stage of muscle cell differentiation.[40] Its exact effect remains unclear, with a catabolic response for some authors[41] or an anabolic response for others.[42] In addition, the combination of IL-6 and TGFβ has an inhibitory effect on regulatory T-cell function. Such inhibition may contribute to chronic inflammation in inflammatory myopathies. In inflammatory myopathies, increased MHC class I expression on the surface membrane of muscle fibres is a common feature in contrast to normal mature muscle fibres that express neither MHC class I nor MHC class II molecules.[5 37] In vitro, MHC class I expression can be induced on muscle cells by cytokines (IL-1α, IL-1β, TNFα, IFNγ) and by the chemokine CCL3/MIP-1α, whereas TGFβ reduces its expression.[39] As mice with MHC class I overexpression develop myositis, muscle MHC class I upregulation could affect muscle function, contributing to the pathogenesis of inflammatory myopathies.[16]

Myositis autoantigens as chemoattractants

Myositis autoantigens (Mi-2, histidyl tRNA synthetase (Jo1)) may play a role in the migration of immune cells to muscle tissue. They are found at high levels in myositis muscle, particularly in regenerating muscle cells, whereas they are expressed at low levels in normal muscle.[43] Furthermore, autoantigenic aminoacyl-tRNA synthetases (histidyl and asparaginyl tRNA synthetases) induce the migration of CD4 and CD8 lymphocytes and immature DC.[44] Combined together, these results suggest that myositis autoantigens, released from damaged muscle cells during the cell death process, have chemoattractant properties leading to an amplification of a specific immune response.[45] In addition, the role of autoantigens in myositis pathogenesis is suggested by the striking association between distinct clinical phenotypes and myositis associated autoantibodies, which could lead to a new inflammatory myopathy classification based on autoantibody profiles.[46]

Muscle cells as APC

The ability of human myoblasts to activate naive T cells by presenting antigen depends on their ability to capture antigen, to express MHC molecules and to express adhesion and costimulatory molecules that promote efficient APC–T-cell interactions. Muscle cells provide the first signal between MHC-peptide–T-cell receptors, by expressing MHC antigens and adhesion molecules under proinflammatory conditions. IL-1, TNFα and IFNγ induce a strong expression of ICAM-1 on cultured myoblasts and myotube surface, whereas under basal conditions, neither human cultured myoblasts nor normal muscle fibres express adhesion molecules.[39 47–49] In polymyositis and dermatomyositis, ICAM-1 expression is increased on muscle fibres and its receptor LFA-1α on mononuclear cells in contrast to controls.[28 37 50] In vitro, IFNγ induced myoblasts expressing HLA-DR and ICAM-1 can act as functional APC in the context of HLA-DR to antigen-specific CD4 T cells.[47] However, B7.1(CD80)/B7.2(CD86) costimulatory molecules, normally expressed on activated professional APC and required for the interaction with T-cell ligands (CTLA-4 and CD28), are not detected on cultured myoblasts even after proinflammatory cytokine stimulation or in muscle biopsies from inflammatory myopathies.[51] However, low levels of CTLA4 and CD28 are observed on muscle fibres from inflammatory myopathies but not in control muscle biopsies.[52] BB-1 and B7-H1, which are other B7 family members with regulatory functions contributing to T-cell costimulation, are expressed on muscle fibres from inflammatory myopathies but not from non-inflammatory and non-myopathic controls.[53 54] At this stage, it is unclear to what extent muscle cells are able to activate naive T cells, but there are probably complex interactions between infiltrated immune

cells and muscle cells, in which muscle cells probably act as "limited APC" rather than professional APC.

THERAPEUTIC APPLICATIONS

Such knowledge may provide ideas for the development of new immunomodulatory therapy. As TNFα and IL-1 participate in the pathogenesis of inflammatory myopathies, cytokine inhibitors could be used to control disease. In vitro, their soluble receptors decrease IL-6 production by muscle samples, down-regulate nuclear factor kappa B translocation induced by TNFα and IL-1β and inhibit MHC class I expression.[55] Previous results from limited uncontrolled and off-label studies on cytokine inhibitors in inflammatory myopathies were conflicting.[56–59] However, recent data suggest that anti-TNF treatment, in particular infliximab, could worsen muscle inflammation by activating the type I IFN system.[58 59] As TNFα, IL-1 and IL-17 have synergistic effects in vitro, IL-17 may represent a new target for the treatment of polymyositis and dermatomyositis. In ex-vivo models of synovial inflammation and bone destruction, combination therapy by soluble receptors of TNFα, IL-1 and IL-17 increase the inhibitory effects on IL-6 production and matrix degradation.[60] These experimental results suggest that IL-17 inhibitors could be used to control synovial inflammation and possibly inflammatory myopathies.

B-cell-targeted therapy using a CD20 monoclonal antibody (rituximab) appears to be a promising therapeutic approach in inflammatory myopathies, with beneficial effects in open-label studies and case reports.[61 62] Acting on B cells that are also APC is a way to disrupt interactions with T cells. Recent advances in biological therapies that are targeting T-cell activation may represent new therapeutic options. Abatacept (CTLA4Ig) selectively modulates the costimulatory pathway of T-cell activation by preventing the engagement of CD80/CD86 on APC with CD28 on T cells. In patients with rheumatoid arthritis, selective modulation of costimulation with abatacept is effective to reduce chronic inflammation.[63] As muscle cells can participate in inflammatory myopathy pathogenesis as limited APC by expressing adhesion and costimulatory molecules distinct from B7.1 and B7.2, acting on the costimulatory pathway may be a new therapeutic option.

CONCLUSIONS

Although a recent concept suggests that muscle cell itself may participate in the immune process, DC are at this time the only professional APC able to initiate the immune adaptive or innate response. In this context, chemokine and cytokine networks play a key role in the migration and maturation of T cells and DC to target tissues. Myositis autoantigens and muscle cell itself may be involved in the recruitment of leucocytes and DC by the local production of chemokines (see fig 1 for a summary). The accumulation of class I MHC during regeneration and activation of the endoplasmic reticulum pathway may induce the release of myositis autoantigens from apoptotic cells.[43 64] Leucocytes attracted through the production of proinflammatory, Th1 and Th17 cytokines promote the migration, differentiation and proliferation of T cells and DC. In addition to DC, muscle cells can participate in the immune process by acting as limited APC. The inflammatory environment sustained by inflammatory cells and muscle cells themselves induces changes in muscle cell metabolism that can lead to damage. All these inflammatory conditions create a self-sustaining autoimmune loop. Such knowledge about interactions between muscle cells, T cells, DC, chemokine and cytokine systems could justify the evaluation of new anti-inflammatory therapies in inflammatory myopathies.

Competing interests: None.

REFERENCES

1. **Dalakas MC,** Hohlfeld R. Polymyositis and dermatomyositis. *Lancet* 2003;**362**:971–82.
2. **Bohan A,** Peter JB. Polymyositis and dermatomyositis. *N Engl J Med* 1975;**292**:344–7.
3. **Hengstman GJ,** van Engelen BG. Polymyositis, invasion of non-necrotic muscle fibres, and the art of repetition. *BMJ* 2004;**329**:1464–7.
4. **Page G,** Chevrel G, Miossec P. Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: interaction with chemokines and Th1 cytokine-producing cells. *Arthritis Rheum* 2004;**50**:199–208.
5. **Emslie-Smith AM,** Arahata K, Engel AG. Major histocompatibility complex class I antigen expression, immunolocalization of interferon subtypes, and T cell-mediated cytotoxicity in myopathies. *Hum Pathol* 1989;**20**:224–31.
6. **Hoogendijk JE,** Amato AA, Lecky BR, Choy EH, Lundberg IE, Rose MR, *et al*. 119th ENMC international workshop: Trial design in adult idiopathic inflammatory myopathies, with the exception of inclusion body myositis. *Neuromuscul Disord* 2004;**14**:337–45.
7. **Lundberg I,** Ulfgren A.K, Nyberg P, Andersson U, Klreskog L. Cytokine production in muscle tissue of patients with idiopathic inflammatory myopathies. *Arthritis Rheum* 1997;**40**:865–74.
8. **Bell D,** Young J.W, Banchereau J. Dendritic cells. *Adv Immunol* 1999;**72**:255–324.
9. **Steinman RM,** Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* 2003;**21**:685–711.
10. **Greenberg SA,** Pinkus JL, Pinkus GS, Burleson T, Sanoudou D, Tawil R, *et al*. Interferon-alpha/beta-mediated innate immune mechanisms in dermatomyositis. *Ann Neurol* 2005;**57**:664–78.
11. **López de Padilla CM,** Vallejo AN, McNallan KT, Vehe R, Smith SA, Dietz AB, *et al*. Plasmacytoid dendritic cells in inflamed muscle of patients with juvenile dermatomyositis. *Arthritis Rheum* 2007;**56**:1658–68.
12. **Greenberg SA,** Pinkus GS, Amato AA, Pinkus JL. Myeloid dendritic cells in inclusion-body myositis and polymyositis. *Muscle Nerve* 2007;**35**:17–23.
13. **Eloranta ML,** Barbasso Helmers S, Ulfgren AK, Rönnblom L, Alm GV, Lundberg IE. A possible mechanism for endogenous activation of the type I interferon system in myositis patients with anti-Jo-1 or anti-Ro 52/anti-Ro 60 autoantibodies. *Arthritis Rheum* 2007;**56**:3112–24.
14. **Walsh RJ,** Kong SW, Yao Y, Jallal B, Kiener PA, Pinkus JL, *et al*. Type I interferon-inducible gene expression in blood is present and reflects disease activity in dermatomyositis and polymyositis. *Arthritis Rheum* 2007;**56**:3784–92.
15. **Halloran PF,** Urmson J, Van der Meide PH, Autenried P. Regulation of MHC expression in vivo. II. IFN-alpha/beta inducers and recombinant IFN-alpha modulate MHC antigen expression in mouse tissues. *J Immunol* 1989;**142**:4241–7.
16. **Nagaraju K,** Raben N, Loeffler L, Parker T, Rochon PJ, Lee E, *et al*. Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies. *Proc Natl Acad Sci U S A* 2000;**97**:9209–14.
17. **Olson TS,** Ley K. Chemokines and chemokine receptors in leukocyte trafficking. *Am J Physiol Regul Integr Comp Physiol* 2002;**283**:R7–28.
18. **Civatte M,** Bartoli C, Schleinitz N, Chetaille B, Pellissier JF, Figarella-Branger D. Expression of the beta chemokines CCL3, CCL4, CCL5 and their receptors in idiopathic inflammatory myopathies. *Neuropathol Appl Neurobiol* 2005;**31**:70–9.
19. **Dieu MC,** Vanbervliet B, Vicari A, Bridon JM, Oldham E, Ait-Yahia S, *et al*. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* 1998;**188**:373–86.
20. **Page G,** Lebecque S, Miossec P. Anatomic localization of immature and mature dendritic cells in an ectopic lymphoid organ: correlation with selective chemokine expression in rheumatoid synovium. *J Immunol* 2002;**168**:5333–41.
21. **Ngo VN,** Tang HL, Cyster JG. Epstein–Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J Exp Med* 1998;**188**:181–91.
22. **Minetti C,** Gattorno M, Repetto S, Gregorio A, Pedemonte M, Assereto S, *et al*. Chemokine receptor CCR7 is expressed in muscle fibers in juvenile dermatomyositis. *Biochem Biophys Res Commun* 2005;**333**:540–3.
23. **Tateyama M,** Fujihara K, Misu T, Feng J, Onodera Y, Itoyama Y. Expression of CCR7 and its ligands CCL19/CCL21 in muscles of polymyositis. *J Neurol Sci* 2006 15;**249**:158–65.
24. **Lepidi H,** Frances V, Figarella-Branger D, Bartoli C, Machado-Baeta A, Pellissier JF. Local expression of cytokines in idiopathic inflammatory myopathies. *Neuropathol Appl Neurobiol* 1998;**24**:73–9.
25. **Tews DS,** Goebel HH. Cytokine expression profile in idiopathic inflammatory myopathies. *J Neuropathol Exp Neurol* 1996;**55**:342–7.
26. **De Bleecker JL,** Meire VI, Declercq W, Van Aken EH. Immunolocalization of tumor necrosis factor-alpha and its receptors in inflammatory myopathies. *Neuromuscul Disord* 1999;**9**:239–46.
27. **Grundtman C,** Salomonsson S, Dorph C, Bruton J, Andersson U, Lundberg IE. Immunolocalization of interleukin-1 receptors in the sarcolemma and nuclei of skeletal muscle in patients with idiopathic inflammatory myopathies. *Arthritis Rheum* 2007;**56**:674–87.

28. **De Bleecker JL,** Engel AG. Expression of cell adhesion molecules in inflammatory myopathies and Duchenne dystrophy. *J Neuropathol Exp Neurol* 1994;**53**:369–76.
29. **Lundberg I,** Brengman JM, Engel AG. Analysis of cytokine expression in muscle in inflammatory myopathies, Duchenne dystrophy, and non-weak controls. *J Neuroimmunol* 1995;**63**:9–16.
30. **Tucci M,** Quatraro C, Dammacco F, Silvestris F. Interleukin-18 overexpression as a hallmark of the activity of autoimmune inflammatory myopathies. *Clin Exp Immunol* 2006;**146**:21–31.
31. **Kaser A,** Kaser S, Kaneider NC, Enrich B, Wiedermann CJ, Tilg H. Interleukin-18 attracts plasmacytoid dendritic cells (DC2s) and promotes Th1 induction by DC2s through IL-18 receptor expression. *Blood* 2004;**103**:648–55.
32. **Harrington LE,** Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, *et al*. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005;**6**:1123–32.
33. **Park H,** Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, *et al*. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005;**6**:1133–41.
34. **Chabaud M,** Fossiez F, Taupin JL, Miossec P. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J Immunol* 1998;**161**:409–14.
35. **Chevrel G,** Page G, Granet C, Streichenberger N, Varennes A, Miossec P. Interleukin-17 increases the effects of IL-1 beta on muscle cells: arguments for the role of T cells in the pathogenesis of myositis. *J Neuroimmunol* 2003;**137**:125–33.
36. **De Rossi M,** Bernasconi P, Baggi F, de Waal Malefyt R, Mantegazza R. Cytokines and chemokines are both expressed by human myoblasts: possible relevance for the immune pathogenesis of muscle inflammation. *Int Immunol* 2000;**12**:1329–35.
37. **Bartocionni E,** Gallucci S, Scuderi F, Ricci E, Servidei S, Broccolini A, *et al*. MHC class I, MHC class II and intercellular adhesion molecule-1 expression in inflammatory myopathies. *Clin Exp Immunol* 1994;**95**:166–72.
38. **Gallucci S,** Provenzano C, Mazzarelli P, Scuderi F, Bartoccioni E. Myoblasts produce IL-6 in response to inflammatory stimuli. *Int Immunol* 1998;**10**:267–73.
39. **Nagaraju K,** Raben N, Merritt G, Loeffler L, Kirk K, Plotz P. A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli. *Clin Exp Immunol* 1998;**113**:407–14.
40. **Jo C,** Kim H, Jo I, Choi I, Jung SC, Kim J, *et al*. Leukemia inhibitory factor blocks early differentiation of skeletal muscle cells by activating ERK. *Biochim Biophys Acta* 2005;**1743**:187–97.
41. **Ebisui C,** Tsujinaka T, Morimoto T, Kan K, Iijima S, Yano M, *et al*. Interleukin-6 induces proteolysis by activating intracellular proteases (cathepsins B and L, proteasome) in C2C12 myotubes. *Clin Sci (Lond)* 1995;**89**:431–9.
42. **Austin L,** Burgess AW. Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines. *J Neurol Sci* 1991;**101**:193–7.
43. **Casciola-Rosen L,** Nagaraju K, Plotz P, Wang K, Levine S, Gabrielson E, *et al*. Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy.*J Exp Med* 2005;**201**:591–601.
44. **Howard OM,** Dong HF, Yang D, Raben N, Nagaraju K, Rosen A, *et al*. Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, autoantigens in myositis, activate chemokine receptors on T lymphocytes and immature dendritic cells. *J Exp Med* 2002;**196**:781–91.
45. **Levine SM,** Rosen A, Casciola-Rosen LA. Anti-aminoacyl tRNA synthetase immune responses: insights into the pathogenesis of the idiopathic inflammatory myopathies. *Curr Opin Rheumatol* 2003;**15**:708–13.
46. **Suber TL,** Casciola-Rosen L, Rosen A. Mechanisms of disease: autoantigens as clues to the pathogenesis of myositis. *Nat Clin Pract Rheumatol* 2008;**4**:201–9.
47. **Goebels N,** Michaelis D, Wekerle H, Hohlfeld R. Human myoblasts as antigen-presenting cells. *J Immunol* 1992;**49**:661–7.
48. **Michaelis D,** Goebels N, Hohlfeld R. Constitutive and cytokine-induced expression of human leukocyte antigens and cell adhesion molecules by human myotubes. *Am J Pathol* 1993;**143**:1142–9.
49. **Marino M,** Scuderi F, Mazzarelli P, Mannella F, Provenzano C, Bartoccioni E. Constituve and cytokine-induced expression of MHC and intercellular adhesion molecule-1 on human myoblasts. *J Neuroimmunol* 2001;**116**:94–101.
50. **Lundberg I,** Kratz A.K, Alexanderson H, Patarroyo M. Decreased expression of interleukine-1α, interleukine-1β, and cell adhesion molecules in muscle tissue following corticosteroid treatment in patients with polymyositis and dermatomyositis. *Arthritis Rheum* 2000;**43**:336–48.
51. **Bernasconi P,** Confalonieri P, Andreetta F, Baggi F, Cornelio F, Mantegazza R. The expression of co-stimulatory and accessory molecules on cultured human muscle cells is not dependent on stimulus by proinflammatory cytokines: relevance for the pathogenesis of inflammatory myopathy. *J Neuroimmunol* 1998;**85**:52–8.
52. **Nagaraju K,** Raben N, Villalba ML. Costimulatory markers in muscle of patients with idiopathic inflammatory myopathies and in cultured muscle cells. *Clin Immunol* 1999;**92**:161–9.
53. **Murata K,** Dalakas MC. Expression of the costimulatory molecule BB-1, the ligands CTLA-4 and CD28, and their mRNA in inflammatory myopathies. *Am J Pathol* 1999;**155**:453–60.
54. **Wiendl H,** Mitsdoerffer M, Schneider D, Chen L, Lochmüller H, Melms A, *et al*. Human muscle cells express a B7-related molecule, B7-H1, with strong negative immune regulatory potential: a novel mechanism of counterbalancing the immune attack in idiopathic inflammatory myopathies. *FASEB J* 2003;**17**:1892–4.
55. **Chevrel G,** Granet C, Miossec P. Contribution of tumour necrosis factor alpha and interleukin (IL) 1beta to IL6 production, NF-kappaB nuclear translocation, and class I MHC expression in muscle cells: in vitro regulation with specific cytokine inhibitors. *Ann Rheum Dis* 2005;**64**:1257–62.
56. **Iannone F,** Scioscia C, Falappone PC, Covelli M, Lapadula G. Use of etanercept in the treatment of dermatomyositis: a case series. *J Rheumatol* 2006;**33**:1802–4.
57. **Efthimiou P,** Schwartzman S, Kagen LJ. Possible role for tumour necrosis factor inhibitors in the treatment of resistant dermatomyositis and polymyositis: a retrospective study of eight patients. *Ann Rheum Dis* 2006;**65**:1233–6.
58. **Hengstman GJ,** De Bleecker JL, Feist E, Vissing J, Denton CP, Manoussakis MN, *et al*. Open-label trial of anti-TNF-alpha in dermato- and polymyositis treated concomitantly with methotrexate. *Eur Neurol* 2008;**59**:159–63.
59. **Dastmalchi M,** Grundtman C, Alexanderson H, Mavragani CP, Einarsdottir H, Barbasso Helmers S, *et al*. A high incidence of disease flares in an open pilot study of infliximab in patients with refractory inflammatory myopathies. *Ann Rheum Dis* 2008;**67**:1670–7.
60. **Chabaud M,** Page G, Miossec P. Enhancing effect of IL-1, IL-17, and TNF-alpha on macrophage inflammatory protein-3alpha production in rheumatoid arthritis: regulation by soluble receptors and Th2 cytokines. *J Immunol* 2001;**167**:6015–20.
61. **Levine TD.** Rituximab in the treatment of dermatomyositis: an open-label pilot study. *Arthritis Rheum* 2005;**52**:601–7.
62. **Mok CC,** Ho LY, To CH. Rituximab for refractory polymyositis: an open-label prospective study.*J Rheumatol* 2007;**34**:1864–8.
63. **Kremer JM,** Dougados M, Emery P, Durez P, Sibilia J, Shergy W, *et al*. Treatment of rheumatoid arthritis with the selective costimulation modulator abatacept: twelve-month results of a phase iib, double-blind, randomized, placebo-controlled trial. *Arthritis Rheum* 2005;**52**:2263–71.
64. **Nagaraju K,** Casciola-Rosen L, Lundberg I, Rawat R, Cutting S, Thapliyal R, *et al*. Activation of the endoplasmic reticulum stress response in autoimmune myositis: potential role in muscle fiber damage and dysfunction. *Arthritis Rheum* 2005;**52**:1824–35.

IV-c Profil cytokinique des myopathies inflammatoires

IV-c-1. Cytokines proinflammatoires IL1, TNFα, IL6.

L'expression dans le tissu musculaire des PM et DM des cytokines pro inflammatoires, IL1 et TNFα, est retrouvée dans de nombreuses études alors qu'elle est absente dans le tissu musculaire normal [72]. Les techniques immunohistochimiques permettent de localiser cette expression aux cellules mononuclées et aux cellules endothéliales mais pas aux fibres musculaires, qui néanmoins expriment à proximité des cellules productrices d'IL1 le récepteur associé [73]. Localement, l'IL1 et le TNFα peuvent participer au recrutement des cellules mononuclées en induisant la production de diverses chémokines et en augmentant l'expression à la surface des cellules endothéliales des molécules d'adhésion qui permettent la migration des cellules inflammatoires dans le périmysium et l'endomysium. D'autre part, la forte expression d'IL1α dans les cellules endothéliales des biopsies musculaires de PM et DM s'associe à des changements morphologiques des capillaires, veinules et artérioles, similaires à ceux notés dans les tissus lymphoïdes, et à un appauvrissement du nombre de capillaires [29, 37]. Ces modifications morphologiques des capillaires peuvent être comparées aux « High Endothelial Venules (HEV) » des ganglions lymphatiques, site majeur de la diapédèse des lymphocytes. Outre sa participation à la migration des cellules de la réponse immunitaire, l'atteinte de la microvascularisation pourrait favoriser l'hypoxie, elle-même responsable d'une augmentation de la production d'IL1 [74], ainsi qu'un dysfonctionnement métabolique du tissu musculaire. L'IL1 et le TNFα contribuent également à la maturation des cellules dendritiques.

Sous l'effet synergique de l'IL1, du TNFα et de l'IL17, les cellules mononuclées et les cellules mésenchymateuses, dont les cellules musculaires, produisent de l'IL6 [13, 66, 72, 75]. L'IL6 est une cytokine proinflammatoire responsable de la réaction inflammatoire aigüe via la production des protéines de l'inflammation et de la différentiation des lymphocytes B et T. En particulier, l'IL6 est avec le TGFβ, l'IL1, et l'IL23 produite par les monocytes et les cellules dendritiques, une cytokine clé dans la différentiation des lymphocytes Th17 [76]. Lorsqu'elle est associée au TGFβ, elle a un effet inhibiteur sur le développement des lymphocytes T régulateurs Foxp3 positifs induits par le TGFβ [77].

Sur la cellule musculaire elle-même, l'effet catabolique ou anabolique du TNFα et de l'IL6 n'est pas clairement établi, et pourrait dépendre du stade de différentiation de la cellule musculaire. Ainsi, le TNFα semble nécessaire au développement et à la différentiation des myoblastes mais son action sur les cellules plus différentiées telles que les myotubes est de type catabolique avec une activation de la voie NF-κB [78]. L'IL6 semble aussi jouer un rôle important dans les stades précoces de la différentiation musculaire favorisant pour certains la prolifération des myoblastes [79, 80]. A l'inverse, plus tardivement au cours de la différentiation, l'IL6 favorise le processus de protéolyse au sein des myotubes en augmentant l'activité du protéasome et des cathepsines [81]. L'IL1 et le TNFα augmentent également *in vitro* la surexpression des antigènes HLA de classe I à la surface des cellules musculaires, caractéristique des myopathies inflammatoires [35].

Tournadre A, Miossec P. Cytokine response in inflammatory myopathies. *Curr Rheumatology Rep.*2007;9:286-290

Contribution spécifique de la candidate:

- Revue de la littérature et écriture de l'article.

.

Cytokine Response in Inflammatory Myopathies

Anne Tournadre, MD, and Pierre Miossec, MD, PhD

Corresponding author
Pierre Miossec, MD, PhD
Clinical Immunology Unit, Department of Immunology and Rheumatology, Hospital Edouard Herriot, 69437 Lyon Cedex 03, France.
E-mail: pierre.miossec@univ-lyon1.fr

Current Rheumatology Reports 2007, **9:**286–90
Current Medicine Group LLC ISSN 1523-3774

After background information about pathologic findings, this review focuses on the cytokine response in the pathogenesis of polymyositis and dermatomyositis. Cytokines are important mediators of the immune response and play a key role in these diseases by acting on inflammatory immune cells, muscle cells, and vessel cells. Various cytokines are found in myositis samples, in particular interleukin-1 and tumor necrosis factor–α, which are associated with the migration, differentiation, and maturation of inflammatory cells. Recent advances indicate that the muscle cell itself could participate in the inflammatory process. Cytokines promote changes in muscle metabolism resulting in a self-sustaining inflammatory response. Accordingly, cytokines may represent new targets for therapies.

Introduction

Inflammatory myopathies (IM) are a group of chronic muscle disorders of unknown origin, which have in common clinical symptoms such as muscle weakness, an inflammatory infiltrate in muscle tissue, and autoantibodies against self-antigens. Based on clinical and histologic features, these diseases can be separated into polymyositis (PM), dermatomyositis (DM), and inclusion body myositis (IBM). IBM is a rare subset characterized by vacuolar formations with amyloid deposits that coexist with immunologic features rather similar to PM. This report focuses primarily on PM and DM. Even if DM and PM are both considered muscle autoimmune diseases, their pathologic mechanism differs. DM is a microangiopathy affecting skin and muscles, whereas in PM, muscle is the primary target of the immune attack via T cell–mediated muscle cell cytotoxicity. However, if DM and PM are two different muscle autoimmune diseases, they have in common the immunologic basis involving muscle tissue, immune cells, and microvasculature. Cytokines allow a network of interactions between these three components, and the understanding of such cytokine response is essential because it can lead to therapeutic applications. This review describes first the common pathologic background and then the role of the cytokines in PM and DM.

Basis of Immunopathology in DM and PM

Muscle fiber and tissue

To form striated muscle, myoblasts defined as myogenic cells proliferate and fuse into primary multinucleated cells called myotubes, which then differentiate into mature muscle fibers. Muscle regeneration depends on satellite cells, which are mononuclear myogenic cells in close contact to muscle fibers. Satellite cells proliferate following muscle injuries and behave as myoblasts to form new mature myofibers. Therefore, three developmental stages can be

described: myoblasts or satellite cells, myotubes, and myofibers as the last step of maturation. Interestingly, major histocompatibility complex (MHC) antigen expression on muscle cells is regulated during these different stages of differentiation. Expression of MHC class I antigens is upregulated on myoblasts or satellite cells and then downregulated during fusion and differentiation into normal mature muscle fiber. Under normal physiologic condition, normal mature muscle fibers express neither MHC class I nor MHC class II antigens [1,2].

In IM, histologic characteristics show evidence of tissue destruction with degenerating and regenerating fibers, variation in fiber size, and centralized nuclei. In contrast to normal muscle tissue, increased MHC class I expression on the surface of muscle fibers is a common feature [1]. This abnormal feature is the first major marker of IM.

Immune cells

Normal muscle is poorly infiltrated by immune cells. Criteria for the diagnosis of IM include inflammatory infiltrate in affected muscle tissue [3]. In PM, endomysial CD8+ T cells and macrophages surround and invade non-necrotic muscle fibers expressing MHC class I antigens. In DM, mononuclear cells consisting of perimysial CD4+ T cells and B cells predominate in perivascular regions, and their association with a reduction of capillary density contributes to perifascicular atrophy. MHC class I expression predominates on perifascicular fibers. Both mature dendritic cells (DCs), defined as CD1a-positive cells, and immature DCs, defined as DC-LAMP-positive cells, have been localized in the lymphocytic infiltrates of DM and PM [4•]. The presence of DCs alone is abnormal and reflects an ongoing immune-mediated reaction.

Microvasculature

DM is distinguishable from PM by the presence of perifascicular atrophy, which probably reflects hypoperfusion due to focal depletion of capillaries. Vascular changes result from the deposition of complement membrane attack complexes on capillaries [5]. Involvement of microvasculature in IM is also suggested by phenotypic changes of the capillaries seen in both in DM and PM with a strong expression of proinflammatory cytokines by endothelial cells [6].

Cytokine Response in PM and DM

Cytokines and inflammatory cells

Proinflammatory cytokines (interleukin [IL]-1α, IL-1β, and tumor necrosis factor [TNF]-α) are mainly produced by macrophages, monocytes, and DCs. In IM, most studies show a strong expression of proinflammatory cytokine messenger RNA (mRNA) not found in normal muscles [7]. By immunohistochemistry, the expression is mainly localized to mononuclear cell infiltrate and to endothelial cells but not to muscle fibers [6,8–10]. Conversely, IL-1 receptor expression is widely localized in muscle fibers, inflammatory cells, and endothelial cells and is increased in IM. This is most pronounced in muscle fibers close to cells expressing IL-1α and IL-1β [10]. TNF-α interacts with IL-1 to stimulate IL-6 production and expression of adhesion molecules by monocytes and endothelial cells. These cytokines can also act on muscle fibers by promoting IL-6 production and MHC class I expression.

Although debated, most studies suggest a main T helper (Th) response in inflammatory myopathies. In PM and DM, interferon (IFN)-γ is frequently detected by reverse transcriptase polymerase chain reaction [7,11]. It is detected by immunohistochemistry in mononuclear cells but not in muscle fibers [6,7]. Less often, IL-2 and IL-4 mRNA is found in PM and DM [7,12]. In a study by Tews and Goebel [8], IL-4 was strongly expressed in inflammatory. All these cytokines are implicated in T-cell differentiation and function. Th1 cells stimulated by IL-12 and IL-18 secrete IFN-γ and IL-2 and mediate "delayed-type" hypersensitivity and cell-mediated immunity, whereas IL-4 induces a Th2 immune response characterized by humoral immune responses and allergic responses. On muscle fibers, IFN-γ is a strong inducer of MHC class I antigens.

However, an update must take into account the recently described Th17 cells [13•,14•]. IL-17 is a new cytokine produced by T cells, first described in 1995 and 1996 for its ability to induce the production of IL-6 and IL-8 by fibroblasts [15]. A synergistic effect on IL-6 production between IL-1, TNF-α, and IL-17 has been demonstrated, and IL-17 increases the production of IL-1 and TNF-α, suggesting a regulatory role for IL-17 [16]. Immunostaining of DM and PM muscle biopsies show a moderate expression of IL-17 in the T lymphocyte–rich area, whereas normal muscle is negative [17]. IL-17 is not expressed in muscle cells. In functional studies, IL-17 in combination with IL-1β strongly increases IL-6 production by human myoblasts and has an additive effect at lower concentrations. IL-17 increases in a synergistic fashion the inducing effect of IL-1β on chemokine production (CCL-20) by myoblasts, which may induce the recruitment of mature DCs and T cells. In combination with IL-1β, it can increase HLA class I expression. Because IL-17 is associated with matrix destruction, it may contribute to IM pathogenesis.

Transforming growth factor (TGF)-β has inhibiting effects on lymphocytic growth and stimulatory effects on synthesis of extracellular matrix, promoting healing and fibrosis. It is strongly expressed in muscle biopsies of IM compared with controls [6]. For TGF-β1 and TGF-β3, immunostaining is mainly located in mononuclear cells and muscle fibers. Expression of TGF-β2 is predominantly vascular, in endothelial cells of capillaries and larger vessels.

TGF-β1 protein and mRNA is downregulated in muscle biopsies of patients with DM successfully treated with intravenous immunoglobulin [18]. In addition TGF-β stimulates the IL-17 pathway when combined with IL-17.

Taken together, these data suggest that inflammatory cells (macrophages, monocytes, DCs, T cells, and B cells) promote their own differentiation and proliferation by the production of proinflammatory cytokines (IL-1α, IL-1β and TNF-α) and T-cell cytokines (IFN-γ, IL-17) and also act on muscle fiber metabolism through IL-6 and MHC class I upregulation.

Cytokines and muscle cells

Human myoblasts constitutively produce low levels of IL-6 and TGF-β, which are increased in a dose-dependent manner after stimulation with proinflammatory cytokines (eg, IL-1α, IL-1β, TNF-α) and with IFN-γ [2,19,20]. On intact muscle, the effect of TNF-α is more potent than that of IL-1β in inducing IL-6 production [21]. IL-6 can act on T-cell and plasma-cell differentiation and could play a major role in the early stage of muscle cell differentiation [22]. Its exact effect remains unclear with a catabolic response seen by some investigators [23] and an anabolic response seen by others [24]. TNF-α inhibits muscle regeneration and may contribute to muscle wasting [25]. Myoblasts may also be implicated in the recruitment of leukocytes and DCs by a local production of chemokines (CCL-20) induced by IL-1β and IL-17 [17,26].

In contrast to the culture condition, normal mature muscle fibers express neither MHC class I nor MHC class II molecules [1,2]. In inflammatory myopathies, increased MHC class I expression on the surface membrane of muscle fibers is a common feature. In vitro, MHC class I expression can be induced on muscle cells by proinflammatory cytokines (IL-1α, IL-1β, TNF-α, IFN-γ) and chemokine (macrophage inflammatory protein–1α), whereas TGF-β reduces its expression [20]. Results from a mouse model of myositis induced by muscle MHC class I overexpression have suggested that MHC class I upregulation alone could affect muscle function and could play a role in the pathogenesis of IM [27]. In this hypothesis, the microenvironment is essential, in particular the presence of cytokines such as IL-1, TNF-α, and IFN-γ, which can upregulate MHC expression on the surface of muscle fibers.

These data suggest that muscle fiber itself under proinflammatory conditions could contribute to the inflammatory process through the production of cytokines and chemokines and the overexpression of MHC class I molecules.

Cytokines and vessels

Involvement of microvessels, as supported by morphologic changes, could cause a disturbed circulation or metabolism in muscle tissue leading to increased IL-1α expression. In several cases, IL-1α expression in endothelial cells is associated with morphologic changes in capillaries, venules, and arterioles, with an appearance similar to the high endothelial venules seen in lymph nodes and other inflammatory tissues [28,29]. Moreover, it has been reported that hypoxia can upregulate IL-1 [30].

Endothelial cells are implicated in the inflammatory process through the migration of inflammatory cells to the target tissue. Cell adhesion molecules on both leukocytes and endothelial cells are responsible for this step. IL-1 and TNF-α are mainly produced by activated macrophages, but also by endothelial cells, upregulated adhesion molecules on endothelial cells, and production of chemokines. Chemokines produced by leukocytes, DCs, and endothelial cells induce cell migration to target tissues. In particular, chemokines play a major role in monocytes and T-cell migration. In myositis tissue, endothelial cells support the migration of mononuclear inflammatory cells to the perimysial and endomysial spaces through the expression of adhesion molecules (intercellular adhesion molecule [ICAM]-1, vascular cell adhesion molecule-1), which bind to T-cell and macrophage integrins (LFA-1, VLA-4, Mac-1) [31]. In normal muscle tissues, capillaries are weakly stained for endothelial cell marker (CD146) and neoangiogenesis marker (αVβ3), whereas an intense staining is observed in DM biopsies associated with up-regulation of the proteins implicated in leukocyte recruitment (CX3CL1, CCR1, CD47, vascular cell adhesion molecule-1, ICAM-1, platelet/endothelial cell adhesion molecule-1, ICAM-2) [32]. The chemokines CCL2 (monocyte chemoattractant protein-1), CCL3, CCL4 and their receptors CCR2, CCR1, CCR5 are upregulated in IM [33], and they may contribute to the inflammatory response in muscle tissue.

DCs, as the most efficient professional antigen-presenting cells, support the generation and the proliferation of CD4+ helper T cells and CD8+ cytotoxic T lymphocytes. In IM, immature DC expression is always associated with expression of the chemokine CCL20/macrophage inflammatory protein–3α and its associated receptor CCR9, suggesting that immature DCs are attracted to muscle tissue in response to local production of chemokines. Proinflammatory cytokines such as IL-1 and TNF-α upregulated in IM may contribute to local DC maturation [4•].

Myositis autoantigens (Mi-2, histidyl transfer RNA [tRNA] synthetase) may play a role in the migration of immune cells to muscle tissue. They are found at high levels in myositis muscle, particularly in regenerating muscle cells. However, they are expressed at low levels in normal muscle [34•]. Furthermore, autoantigenic aminoacyl-tRNA synthetases (histidyl and asparaginyl tRNA synthetases) induce migration of CD4+ and CD8+ lymphocytes and immature DCs [35]. These data suggest that myositis autoantigens liberated from damaged muscle cells during the cell death process possess chemoattractant properties and may be responsible for an amplification of a specific immune response [36].

Physiopathologic Overview and Hypothesis

During the regeneration process occurring after muscle damage, MHC class I expression is upregulated on muscle fibers. In pathologic situations or in susceptible individuals, the accumulation of class I MHC and the activation of the endoplasmic reticulum stress response pathway may induce the liberation of myositis autoantigens from apoptotic cells [34,37•]. Autoantigens and muscle cells induce migration of T cells, B cells, and DCs by a local production of chemokines. Inflammatory cells produce proinflammatory cytokines (ie, IL-1, TNF-α), IFN-γ, and IL-17, which in turn promote differentiation, proliferation, and maturation of T cells, B cells, and DCs and their migration by upregulation of adhesion molecules on endothelial cells. These cytokines then act on muscle fibers by inducing IL-6 production with metabolic and inflammatory effects and by upregulation of MHC class I. In addition, muscle cells can participate in immune reactions, by acting as antigen-presenting cells with expression of costimulatory molecules that allow a functional immunologic synapse with T cells [38•]. All these conditions create a self-sustaining autoimmune loop.

Cytokines and Therapeutic Applications

TNF-α and IL-1 participate to the pathogenesis of inflammatory myopathies. As seen in rheumatoid arthritis, cytokine inhibitors could be used to control the disease when traditional immunosuppressive agents have failed. In vitro, their soluble receptors decrease IL-6 production by muscle samples, downregulate nuclear factor-κB translocation induced by TNF-α and IL-1β and inhibits MHC class I expression [21]. Limited uncontrolled and off-label studies on cytokine inhibitors in IM are conflicting [39,40], and larger controlled studies are needed. In one study, all five DM patients treated with etanercept experienced exacerbation of the disease [39], whereas in another study, six of eight patients with DM or PM treated with etanercept or infliximab improved [40]. As TNF-α, IL-1, and IL-17 have synergistic effects in vitro, IL-17 may represent a new target for the treatment of PM and DM. In ex vivo models of synovial inflammation and bone destruction, combination therapy by soluble receptors of TNF-α, IL-1, and IL-17 increase inhibitory effects on IL-6 production and collagen degradation [41]. These experimental results suggest that IL-17 inhibitors could be used to control synovial inflammation and could be extended for IM. B cell–targeted therapy using a CD20 monoclonal antibody (rituximab) appears as a promising therapeutic approach in IM with beneficial effects in open-label studies and case reports [42,43]. Other biologic therapies influence the production of immunomodulatory cytokines by targeting T-cell activation. Following antigen recognition, T cells require costimulation to become fully activated. Abatacept (CTLA4Ig), a soluble human CTLA4, selectively modulates the costimulatory pathway of T-cell activation by preventing the engagement of CD80/CD86 on antigen-presenting cells with CD28 on T cells. In patients with rheumatoid arthritis, selective modulation of costimulation with abatacept was effective in reducing chronic inflammation [44]. In IM, low levels of CTLA4 and CD28 are observed on muscle fibers but not in controls [45], and costimulatory pathway may be a new therapeutic target.

Conclusions

Although recent concept suggests that muscle cell itself may participate in immune reactions, the cytokine network plays a key role in PM and DM pathogenesis. Cytokines allow a self-sustaining inflammatory response by acting on the three elements of IM pathogenesis (ie, inflammatory cells, muscle cells, vessels). Proinflammatory cytokines promote migration, differentiation, and maturation of inflammatory cells in target tissue. Inflammatory conditions sustained by inflammatory cells and muscle cells themselves induce changes in muscle cell metabolism that can lead to damage. Such knowledge about the cytokine response could justify the development of new anti-inflammatory therapies.

Acknowledgments

DISCLOSURE

References and Recommended Reading

Papers of particular interest, published recently,
have been highlighted as:

- • Of importance
- •• Of major importance

1. Emslie-Smith AM, Arahata K, Engel AG: **Major histocompatibility complex class I antigen expression, immunolocalization of interferon subtypes, and T cell-mediated cytotoxicity in myopathies.** *Hum Pathol* 1989, **20:**224–231.
2. Bartocionni E, Gallucci S, Scuderi F, et al.: **MHC class I, MHC class II and intercellular adhesion molecule-1 expression in inflammatory myopathies.** *Clin Exp Immunol* 1994, **95:**166–172.
3. Hoogendijk JE, Amato AA, Lecky BR, et al.: **119th ENMC international workshop: Trial design in adult idiopathic inflammatory myopathies, with the exception of inclusion body myositis.** *Neuromuscular Disorders* 2004, **14:**337–345.

4.• Page G, Chevrel G, Miossec P: **Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: Interaction with chemokines and Th1 cytokine-producing cells.** *Arthritis Rheum* 2004, **50:**199–208.

This study reports the presence of both immature and mature DCs, the expression of IL-17, IFN-γ, and chemokines and associated receptors in inflammatory myopathy biopsies, supporting interactions among DCs, T cells, and chemokines.

5. Dalakas MC, Hohlfeld R: **Polymyositis and dermatomyositis.** *Lancet* 2003, **362:**971–982.

6. Lundberg I, Ulfgren AK, Nyberg P, et al.: **Cytokine production in muscle tissue of patients with idiopathic inflammatory myopathies.** *Arthritis Rheum* 1997, **40:**865–874.

7. Lepidi H, Frances V, Figarella-Branger D, et al.: **Local expression of cytokines in idiopathic inflammatory myopathies.** *Neuropathol Appl Neurobiol* 1998, **24:**73–79.

8. Tews DS, Goebel HH: **Cytokine expression profile in idiopathic inflammatory myopathies.** *J Neuropathol Exp Neurol* 1996, **55:**342–347.

9. De Bleecker JL, Meire VI, Declercq W, Van Aken EH. **Immunolocalization of tumor necrosis factor-alpha and its receptors in inflammatory myopathies.** *Neuromuscul Disord* 1999, **9:**239–246.

10. Grundtman C, Salomonsson S, Dorph C, et al.: **Immunolocalization of interleukin-1 receptors in the sarcolemma and nuclei of skeletal muscle in patients with idiopathic inflammatory myopathies.** *Arthritis Rheum* 2007, **56:**674–687.

11. Lundberg I, Brengman JM, Engel AG: **Analysis of cytokine expression in muscle in inflammatory myopathies, Duchenne dystrophy, and non-weak controls.** *J Neuroimmunol* 1995, **63:**9–16.

12. Adams EM, Kirkley J, Eidelman G, et al.: **The predominance of beta (CC) chemokine transcripts in idiopathic inflammatory muscle diseases.** *Proc Assoc Am Physicians* 1997, **109:**275–285.

13.• Harrington LE, Hatton RD, Mangan PR, et al.: **Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.** *Nat Immunol* 2005, **6:**1123–1132.

Along with the study by Park et al. [14•], this study favors the idea of a distinct effector lineage called Th17.

14.• Park H, Li Z, Yang XO, et al.: **A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.** *Nat Immunol* 2005, **6:**1133–1141.

In addition to the above study by Harrington et al. [13•], this study favors the idea of a distinct effector lineage called Th17.

15. Miossec P: **Interleukin-17 in rheumatoid arthritis: if T cells were to contribute to inflammation and destruction through synergy.** *Arthritis Rheum* 2003, **48:**594–601.

16. Chabaud M, Fossiez F, Taupin JL, Miossec P: **Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines.** *J Immunol* 1998, **161:**409–414.

17. Chevrel G, Page G, Granet C, et al.: **Interleukin-17 increases the effects of IL-1 beta on muscle cells: arguments for the role of T cells in the pathogenesis of myositis.** *J Neuroimmunol* 2003, **137:**125–133.

18. Amemiya K, Semino-Mora C, Granger RP, Dalakas MC: **Downregulation of TGF-beta1 mRNA and protein in the muscles of patients with inflammatory myopathies after treatment with high-dose intravenous immunoglobulin.** *Clin Immunol* 2000, **94:**99–104.

19. Gallucci S, Provenzano C, Mazzarelli P, et al.: **Myoblasts produce IL-6 in response to inflammatory stimuli.** *Int Immunol.* 1998, **10:**267–273.

20. Nagaraju K, Raben N, Merritt G, et al.: **A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli.** *Clin Exp Immunol* 1998, **113:**407–414.

21. Chevrel G, Granet C, Miossec P: **Contribution of tumour necrosis factor alpha and interleukin (IL) 1beta to IL6 production, NF-kappaB nuclear translocation, and class I MHC expression in muscle cells: in vitro regulation with specific cytokine inhibitors.** *Ann Rheum Dis* 2005, **64:**1257–1262.

22. Jo C, Kim H, Jo I, et al.: **Leukemia inhibitory factor blocks early differentiation of skeletal muscle cells by activating ERK.** *Biochim Biophys Acta* 2005, **1743:**187–197.

23. Ebisui C, Tsujinaka T, Morimoto T, et al.: **Interleukin-6 induces proteolysis by activating intracellular proteases (cathepsins B and L, proteasome) in C2C12 myotubes.** *Clin Sci (Lond)* 1995, **89:**431–439.

24. Austin L, Burgess AW: **Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines.** *J Neurol Sci* 1991, **101:**193–197.

25. Langen RC, van der Velden JL, Schols AM, et al.: **Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization.** *FASEB J* 2004, **18:**227–237.

26. De Rossi M, Bernasconi P, Baggi F, et al.: **Cytokines and chemokines are both expressed by human myoblasts: possible relevance for the immune pathogenesis of muscle inflammation.** *Int Immunol* 2000, **12:**1329–1335.

27. Nagaraju K, Raben N, Loeffler L, et al.: **Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies.** *Proc Natl Acad Sci U S A* 2000, **97:**9209–9214.

28. Lundberg I, Ulfgren AK, Nyberg P, et al.: **Cytokine production in muscle tissue of patients with idiopathic inflammatory myopathies.** *Arthritis Rheum* 1997, **40:**865–874.

29. Nyberg P, Wikman AL, Nennesmo I, Lundberg I: **Increased expression of interleukin 1alpha and MHC class I in muscle tissue of patients with chronic, inactive polymyositis and dermatomyositis.** *J Rheumatol* 2000, **27:**940–948.

30. Shreeniwas R, Koga S, Karakurum M: **Hypoxia-mediated induction of endothelial cell interleukine-1 alpha. An autocrine mechanism promoting expression of leukocyte adhesion molecules on the vessel surface.** *J Clin Investigation* 1992, **90:**2333–2339.

31. De Bleecker JL, Engel AG: **Expression of cell adhesion molecules in inflammatory myopathies and Duchenne dystrophy.** *J Neuropathol Exp Neurol* 1994, **53:**369–376.

32. Nagaraju K, Rider LG, Fan C, et al.: **Endothelial cell activation and neovascularization are prominent in dermatomyositis.** *J Autoimmune Dis* 2006, **3:**2.

33. Civatte M, Bartoli C, Schleinitz N, et al.: **Expression of the beta chemokines CCL3, CCL4, CCL5 and their receptors in idiopathic inflammatory myopathies.** *Neuropathol Appl Neurobiol* 2005, **31:**70–79.

34.• Casciola-Rosen L, Nagaraju K, Plotz P, et al.: **Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy.** *J Exp Med* 2005, **201:**591–601.

This article reports the preferential expression of myositis autoantigens in regenerating muscle cells implicating regenerating muscle as potential source of autoantigens.

35. Howard OM, Dong HF, Yang D, et al.: **Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, autoantigens in myositis, activate chemokine receptors on T lymphocytes and immature dendritic cells.** *J Exp Med* 2002, **196:**781–791.

36. Levine SM, Rosen A, Casciola-Rosen LA: **Anti-aminoacyl tRNA synthetase immune responses: insights into the pathogenesis of the idiopathic inflammatory myopathies.** *Curr Opin Rheumatol* 2003, **15:**708–713.

37. • Nagaraju K, Casciola-Rosen L, Lundberg I, et al.: **Activation of the endoplasmic reticulum stress response in autoimmune myositis: potential role in muscle fiber damage and dysfunction.** *Arthritis Rheum* 2005, **52:**1824–1835.

This article addresses the role of MHC class I overexpression and endoplasmic reticulum stress response in myositis pathogenesis.

38. • Wiendl H, Hohlfeld R, Kieseier BC: **Immunobiology of muscle: advances in understanding an immunological microenvironment.** *Trends Immunol* 2005, **26:**373–380.

A detailed and informative review on the immunopathogenesis of inflammatory myopathies.

39. Iannone F, Scioscia C, Falappone PC, et al.: **Use of etanercept in the treatment of dermatomyositis: a case series.** *J Rheumatol* 2006, **33:**1802–1804.

40. Efthimiou P, Schwartzman S, Kagen LJ: **Possible role for tumour necrosis factor inhibitors in the treatment of resistant dermatomyositis and polymyositis: a retrospective study of eight patients.** *Ann Rheum Dis* 2006, **65:**1233–1236.

41. Chabaud M, Page G, Miossec P: **Enhancing effect of IL-1, IL-17, and TNF-alpha on macrophage inflammatory protein-3alpha production in rheumatoid arthritis: regulation by soluble receptors and Th2 cytokines.** *J Immunol* 2001, **167:**6015–6020.

42. Noss EH, Hausner-Sypek DL, Weinblatt ME: **Rituximab as therapy for refractory polymyositis and dermatomyositis.** *J Rheumatol* 2006, **33:**1021–1026.

43. Levine TD: **Rituximab in the treatment of dermatomyositis: an open-label pilot study.** *Arthritis Rheum* 2005, **52:**601–607.

44. Kremer JM, Dougados M, Emery P, et al.: **Treatment of rheumatoid arthritis with the selective costimulation modulator abatacept: twelve-month results of a phase iib, double-blind, randomized, placebo-controlled trial.** *Arthritis Rheum* 2005, **52:**2263–2271.

45. Nagaraju K, Raben N, Villalba ML, et al.: **Costimulatory markers in muscle of patients with idiopathic inflammatory myopathies and in cultured muscle cells.** *Clin Immunol* 1999, **92:**161–169.

Inflammatory Muscle Disease
Cytokine Response in Inflammatory Myopathies
Tournadre and Miossec

IV-c-2. Cytokines Th1 et Th17.

Bien que débattu, la plupart des études suggèrent un profil cytokinique Th1 dans les myopathies inflammatoires [23, 66, 72]. Au sein des biopsies musculaires de PM ou DM, la production de la cytokine Th1, l'IFNγ, est largement détectée que ce soit en biologie moléculaire (RT-PCR) ou en immunohistochimie [23, 82, 83]. De plus, l'IL18, cytokine produite par les macrophages et les cellules dendritiques et qui avec l'IL12 oriente la réponse immunitaire vers la voie Th1, est surexprimée dans les régions périvasculaires et endomysiales des biopsies musculaires des PM et DM comparées au tissu musculaire normal [84]. Dans le sérum des PM et DM, les taux d'IL18 et d'IFNγ sont significativement plus élevés que dans le sérum des sujets contrôles, et sont corrélés à l'activité de la maladie alors que les taux sériques d'IL4, cytokine Th2, sont comparables aux sujets contrôles [84].

La classique dichotomie des lymphocytes Th1 et Th2 s'est récemment enrichie des lymphocytes Th17, producteurs de la cytokine proinflammatoire IL17 décrite pour la première fois en 1995 pour ses capacités à induire la production d'IL6 et d'IL8 par les fibroblastes et par les synoviocytes de patients atteints de PR [76]. Le rôle pathogénique de la voie Th17 dans les pathologies inflammatoires chroniques est particuliérement bien démontré dans la PR. Ainsi, nous avons pu démontrer une surexpression des 2 récepteurs spécifiques de l'IL17, IL-17RA et IL-17RC, dans le sang périphérique de patients atteints de PR ainsi que leur expression diffuse dans le tissu synovial rhumatoïde [85]. En immunohistochimie, l'expression d'IL17 est détectée dans le tissu synovial de PR alors qu'elle est absente dans le tissu synovial arthrosique [86]. L'expression d'IL17A et d'IL17F, autre membre de la famille IL17 possédant 50 % d'homologie avec l'IL17A, est principalement localisée au sein de l'infiltrat lymphocytaire, dans des cellules de morphologie semblable aux plasmocytes, caractérisées par un large noyau et un cytoplasme réduit [86]. *In vitro*, l'IL17A et l'IL17F ont un effet régulateur sur la production par les synoviocytes de cytokines proinflammatoires, de chémokines et de métallo protéases [85, 86]. L'interaction de l'IL17A avec ses récepteurs spécifiques, IL17-RA et IL17-RC, induit la production par les synoviocytes de PR d'IL6, et des chémokines CCL20 et IL8, avec un effet synergique lorsque l'IL17 est associée au TNFα [85].

Avec l'identification récente de l'IL17 et de l'IL23 comme acteurs majeurs des pathologies inflammatoires chroniques et de l'autoimmunité [76], le rôle potentiel de la voie

Th17 dans la physiopathologie des myopathies inflammatoires peut être envisagé. *In vitro*, un effet synergique de l'IL17 et de l'IL1 sur la production par les myoblastes humains d'IL6 et de la chémokine CCL20, elle-même facteur chémotactique des lymphocytes Th17, est démontré [13]. Contrairement au tissu musculaire normal, plusieurs études en immunohistochimie démontrent la présence d'IL17 au sein de l'infiltrat lymphocytaire musculaire des PM et DM [2, 13, 23].

Nous avons analysé le profil cytokinique de biopsies musculaires de 13 patients atteints de PM et DM, ainsi que l'association aux différents types de cellules dendritiques et le lien avec la réponse au traitement par IVIG [23]. L'expression des cellules productrices d'IL17 et d'IFNγ, des lymphocytes T CD4+ et CD8+, des cellules dendritiques immatures CD1a+ et matures DC-LAMP+, a été quantifiée par immunohistochimie en utilisant la méthode des «hot spots» avant traitement par IVIG. Si l'expression d'IL17 est observée dans la majorité des prélèvements musculaires (11/13 soit 85 %), le nombre de cellules productrices d'IL17 est faible (0.095±0.166 par mm^2) et le nombre de cellules productrices d'IFNγ est significativement plus élevé (1.310±1.085 par mm^2, p< 0;05), suggérant un profil Th1 dans les myopathies inflammatoires. Le faible nombre de DM (3 patients) ne permet pas de distinguer le profil cytokinique selon le type de myopathie inflammatoire. D'autre part, nous n'avons pas noté de lien entre l'expression des cytokines Th1 (IFNγ), ou Th17 (IL17), et la présence de cellules dendritiques immatures ou matures. Les IVIG sont un traitement efficace des DM et PM réfractaires aux traitements conventionnels [22]. Leur action immunomodulatrice, qui s'explique probablement par une interaction avec les autoanticorps et immuns complexes, le complément, les cytokines et chémokines, reste mal expliquée. Nous nous sommes donc demandé s'il existait un lien entre la réponse ultérieure au traitement par IVIG et le profil cytokinique musculaire initial estimé par le ratio de cellules productrices IFNγ/IL17. Le nombre de lymphocytes T CD4+ ou CD8+ ainsi que le nombre de cellules dendritiques matures ou immatures, n'est pas significativement différent entre patients répondeurs et patients non-répondeurs aux IVIG. Par contre, le nombre moyen de cellules productrices d'IFNγ est significativement plus élevé chez les patients non-répondeurs comparé aux répondeurs aux IVIG (2.09±1.06 par mm^2 vs 0.64±0.54 par mm^2, p<0.05) et le ratio de cellules productrices IFNγ/IL17 est également plus élevé chez les patients non-répondeurs (64.2±44.6 versus 23.9±27.6 par mm^2, p=0.05). Ces résultats suggèrent donc un

profil Th1 prépondérant dans les PM et DM, mais un rôle de la voie Th17 dans la réponse thérapeutique aux IVIG.

Zrioual S, Toh ML, **Tournadre A** , Zhou Y, Cazalis MA, Pachot A, Miossec V, Miossec P.IL-17RA and IL-17RC Receptors Are Essential for IL-17A-Induced ELR+ CXC Chemokine Expression in Synoviocytes and Are Overexpressed in Rheumatoid Blood. *J Immunol.* 2008;180:655-63.

Contribution spécifique de la candidate:

- Réalisation des techniques d'immunomarquages en im munohistochimie pour IL17RA et IL17RC dans la synoviale rhumatoïde et arthrosique, avec mise au point des anticorps spécifiques.
- Analyse des immunomarquages, réalisation des photographies
- Conception et réalisation de la figure 2 de l'article.

IL-17RA and IL-17RC Receptors Are Essential for IL-17A-Induced ELR$^+$ CXC Chemokine Expression in Synoviocytes and Are Overexpressed in Rheumatoid Blood[1]

Saloua Zrioual, Myew-Ling Toh, Anne Tournadre, Yuan Zhou, Marie-Angélique Cazalis, Alexandre Pachot, Vincent Miossec, and Pierre Miossec[2]

IL-17A is a cytokine secreted by the newly described Th17 cells implicated in rheumatoid arthritis (RA). Less is known about its receptors in synoviocytes. IL-17RA and IL-17RC were found to be overexpressed in RA peripheral whole blood and their expression was detected locally in RA synovium. In vitro, IL-17A synergized with TNF-α to induce IL-6, IL-8, CCL-20, and matrix metalloproteinase-3. Using microarrays, a specific up-regulation of Glu-Leu-Arg$^+$ CXC chemokines was observed in IL-17A-treated synoviocytes. Using both posttranslational inhibitions by silencing interfering RNA and extracellular blockade by specific inhibitors, we showed that both IL-17RA and IL-17RC are implicated in IL-17A-induced IL-6 secretion, whereas in the presence of TNF-α, the inhibition of both receptors was needed to down-regulate IL-17A-induced IL-6 and CCL-20 secretion. Thus, IL-17A-induced IL-6, IL-8, and CCL20 secretion was dependent on both IL-17RA and IL-17RC, which are overexpressed in RA patients. IL-17A-induced pathogenic effects may be modulated by IL-17RA and/or IL-17RC antagonism. ***The Journal of Immunology,*** **2008, 180: 655–663.**

The newly characterized IL-17-secreting T cells (1, 2), named Th17, play a pathogenic role in rheumatoid arthritis (RA).[3] Th17 can now be placed alongside the classically known Th1 and Th2 cells, these three cell types developing by way of distinct lineages. In mouse models, TGF-β and IL-6 are implicated in the differentiation of Th17 cells from naive T cells (3, 4), and IL-23 contributes to their survival and proliferation. The crucial role of Th17 in chronic inflammatory disorders comes also from studies in mice showing that Th17 and regulatory T cells result from reciprocal differentiation pathways (3). The important role of IL-17/IL-23 axis in diseases such as RA is supported by substantial anti-inflammatory and joint protective effects of IL-17 antagonism (soluble IL-17RFc fusion protein and neutralizing anti-IL-17 Abs) in murine and human models of arthritis. In addition, IL-17 deficient mice show markedly diminished collagen-induced arthritis (5).

In RA patients, high local levels of IL-17A are present in both synovium and synovial fluid (6, 7). IL-17A induces proinflammatory cytokines (IL-1β, TNF-α, IL-6) (8, 9) and CXC chemokines which recruit neutrophils into the joint (10, 11), stimulate angiogenesis (12, 13), and are implicated in joint degradation (14). The relevance of this cytokine has been further underlined by in vitro studies performed in the presence of TNF-α and IL-1β, two key pathogenic proinflammatory cytokines overexpressed in RA joints (8, 15, 16). IL-17A often acts synergistically with these two cytokines to induce inflammatory mediators in synoviocytes, human osteoblasts, myoblasts, and chondrocytes (8, 17–19). Furthermore, a recent clinical study demonstrated that synovial IL-17A expression is associated with joint destruction (20).

Chemokines are small cytokines which have been classified into four groups according to their structure. C, CC, CXC, or CX3C chemokines differ by the presence of conserved cysteines. Moreover, CXC chemokines are grouped into Glu-Leu-Arg or ELR$^-$ chemokines depending on the presence or absence of a sequence of three amino acids (Glu-Leu-Arg) in their N terminus. This ELR motif is implicated in CXC chemokine-associated regulatory effects on angiogenesis, with ELR$^+$ chemokines having angiogenic properties and non-ELR chemokines acting mainly as angiostatic factors (21, 22). The neutrophil attractant characteristics of CXC chemokines have also been linked to the presence of the ELR motif (23). To study the contribution of IL-17A in RA pathogenesis, we analyzed its regulatory effect on chemokine expression by synoviocytes using microarrays and focusing on CCL20/MIP-3α, which is implicated in the recruitment of activated lymphocytes and immature dendritic cells (18, 24).

Although IL-17A has affinities with IL-17RA, binding assays have suggested the involvement of additional receptor components (25). To date, four additional receptors have been identified in the IL-17R family based on sequence homology to IL-17R (IL-17Rh1, IL-17RC, IL-17RD, and IL-17RE) and among them, IL-17RC has been shown recently to physically associate with IL-17RA, suggesting that it may be a functional component in the IL-17R complex (26). Nevertheless, the final structure of the functional IL-17 receptor and the mechanisms of interactions with IL-17A remain unknown.

Department of Immunology and Rheumatology, Mixed Unit Civil Hospital of Lyon-Biomerieux, Edouard Herriot Hospital, Lyon, France

Received for publication April 24, 2007. Accepted for publication October 23, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by grants from the Hospices Civils of Lyon and the Region Rhône-Alpes. S.Z. was supported by a scholarship from the Region Rhône-Alpes. M.-L.T. and Y.Z. were supported by a fellowship from the Region Rhône-Alpes.

[2] Address correspondence and reprint requests to Prof. Pierre Miossec, Clinical Immunology Unit, Department of Immunology and Rheumatology, Hospital Edouard Herriot, 69437 Lyon Cedex 03, France. E-mail address: pierre.miossec@univ-lyon1.fr

[3] Abbreviations used in this paper: RA, rheumatoid arthritis; siRNA, silencing interfering RNA; OA, osteoarthritis; HV, healthy volunteer; DAS, disease activity score; MMP, matrix metalloproteinase; HPRT1, hypoxanthine phosphoribosyltransferase 1; AU, arbitrary unit; ELR, Glu-Leu-Arg; PPIB, peptidylpropyl isomerase B.

We analyzed the expression of IL-17RA and IL-17RC in peripheral whole blood and demonstrated their overexpression in RA patients. In RA synoviocytes, we observed that both IL-17RA and IL-17RC are required to transduce IL-17A signals leading to increased expression of critical genes for RA pathogenesis. Our data suggest that IL-17RA and IL-17RC overexpression may play an important role in RA pathogenesis as shown by their modulation using silencing interfering RNA (siRNA) delivery or extracellular inhibitors.

Materials and Methods

Patients and healthy volunteers

Forty RA patients fulfilling the American College of Rheumatology 1987 revised criteria for RA (27) and 19 healthy volunteers (HV) were enrolled for determination of gene expression profiles in whole peripheral blood using U133A microarrays (Affymetrix). Clinical indices and biological markers in this study included age, sex, disease duration, right Larsen wrist x-ray index, rheumatoid factor, C-reactive protein, and number of Disease Modifying Anti-Rheumatic Drugs. Patients were divided into two groups according to the right Larsen wrist x-ray index: destructive RA (Larsen wrist score ≥ 2) and nondestructive arthritis (Larsen wrist score <2). On the basis of the modified disease activity score 28 (DAS 28) joint index, 31 were evaluated as severe RA (DAS 28 $>$ 3.2) and 9 as moderate RA (DAS 28 $\leq$ 3.2). All the participants gave their written informed consent. The protocol was approved by the committee for protection of persons participating in biomedical research.

Immunohistochemistry

Synovium pieces kept for immunohistochemistry analysis were directly fixed in 10% phosphate-buffered formaldehyde. After paraffin embedding, samples were cut into 4-μm serial sections, mounted on glass slides, and treated for removal of paraffin (OTTIX Plus; DiaPath). To detect Ag expression in paraffin-embedded sections, Ag retrieval procedure was performed by incubation in citrate buffer (pH 6) for 40 min at 99°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min before the application of primary Ab. The sections were then incubated for 1 h with primary Abs (R&D Systems): 10 μg/ml mouse monoclonal anti-IL-17A (IgG2b), 10 μg/ml mouse monoclonal anti-IL-17RA (IgG1), or 10 μg/ml goat polyclonal anti-IL-17RC Abs. In negative control sections, irrelevant Ab (mouse IgG2b, mouse IgG1, or goat serum, respectively) was applied at the same concentration as the primary Ab. After washing, the sections were incubated with biotinylated anti-mouse and anti-goat Igs for 15 min, followed by streptavidin-peroxidase complex for 15 min and 3,3′ diaminobenzidine chromogen solution (DakoCytomation). The sections were then counterstained with Mayer's hematoxylin.

Cell culture

RA synoviocytes were obtained from synovium tissue of RA patients undergoing joint surgery who fulfilled the American College of Rheumatology criteria for RA as previously described (27). In brief, synovium tissue was minced into small pieces and then incubated for 2 h at 37°C with proteolytic enzymes (1 mg/ml collagenase and hyaluronidase from Sigma-Aldrich). Synoviocytes were cultured in DMEM (Invitrogen Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, streptomycin at 37°C in a humidified 5% CO_2 incubator as previously described (28). Experiments were performed in synoviocytes at passages 3–7, which were $>$99% negative for CD45, CD1, CD3, CD19, CD14, and HLA-DR and were positive for the expression of CD44 (Abs obtained from BD Pharmingen).

Cell separation

PBMCs cells were isolated from RA patients by Ficoll-Paque plus density gradient centrifugation of heparinized blood (Amersham Biosciences). $CD14^+$ monocytes, $CD3^+$ lymphocytes, or $CD19^+$ lymphocytes were then isolated from PBMC by positive selection using MACS isolation kits (Miltenyi Biotec). The purity of the $CD14^+$ monocyte subset (90–95%), $CD3^+$ lymphocyte subset ($>$95%), or $CD19^+$ lymphocyte subset ($>$90%) were determined by flow cytometry analysis (FACS scan; BD Biosciences) using specific mAbs against human PE-conjugated CD14, human PE-conjugated CD3, human PE-conjugated CD19 (BD Pharmingen), or isotype control Abs (BD Pharmingen) as per the manufacturer's instructions as described (28).

Cytokines, Abs, and ELISAs

Human recombinant TNF-α was purchased from Sigma-Aldrich. Human rIL-17A, the mAb against human IL-17RA and the polyclonal goat Ab against IL-17RC, were purchased from R&D Systems. RA synoviocytes seeded in 96-well plates (1×10^4 cells/well), were stimulated with IL-17A or IL-17F 50 ng/ml alone or in combination with 0.5 ng/ml TNF-α for 36 h. IL-6, IL-8, and CCL20 levels were quantified in removed supernatants by ELISA (eBioscience; Diaclone; R&D Systems, respectively).

RNA extraction and purification

Whole blood samples were collected in PAXgene Blood RNA tubes (PreAnalytiX) to minimize postsampling activation of mRNA (29) and total RNA extracted using the PAXgene Blood RNA kit (PreAnalytix). Synoviocytes seeded in 6-well plates (5×10^5 cells/well) were serum deprived for 2 h and then stimulated for 12 h with IL-17A 50 ng/ml, alone or in combination with 0.5 ng/ml TNF-α in DMEM/10% FCS. RNA from synoviocytes and blood cell subsets ($CD3^+$, $CD14^+$, $CD19^+$) were extracted using TRIzol reagents (Invitrogen Life Technologies), according to the manufacturer's instructions. RNA was purified using RNeasy kits (Qiagen). The concentration of RNA was quantified by spectrophotometry at 260 nm (SmartSpecTM3000; Bio-Rad).

Real-time quantitative RT-PCR analysis

One microgram of total RNA was reverse transcribed using ThermoScript RT-PCR System (Invitrogen Life Technologies). Briefly, total RNA was denatured by incubating for 5 min at 65°C with 4 μM oligo(dT) primer and then reverse transcribed by using a final concentration of 0.5 mM dNTP, 40 U/μl RNase OUT, 0.01 M DTT, and 10 U/μ of ThermoScript reverse transcriptase. Reverse transcription was performed by incubation at 50°C for 60 min followed by 85°C for 5 min. The obtained cDNA was diluted 1/10 with distilled water and 10 μl were used for amplification.

Specific primer sets for IL-6, CXCL8/IL-8, matrix metalloproteinase (MMP)-3, RANTES/CCL5, GAPDH, and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were optimized for the LightCycler instrument (Roche Molecular Biochemicals), and purchased from Search-LC. Primer sets for IL-17RA (GenBank accession no. NM_014339) and IL-17RC (GenBank accession no. NM_153461) were synthesized by Eurogentec: IL-17RA forward: 5′-AGACACTCCAGAACCAATTCC-3′, IL-17RA reverse: 5′-TCTTAGAGTTGCTCTCCACCA-3′, IL-17RC forward: 5′-AC CAGAACCTCTGGCAAGC-3′, IL-17RC reverse: 5′-GAGCTGTTCAC CTGAACACA-3. The PCR was performed using the LightCycler FastStart DNA SYBR Green I kit (Roche Molecular Biochemicals) according to the protocol provided with the parameter-specific kits (45 amplification cycles, denaturation at 96°C, primer annealing at 68°C with touchdown to 58°C, amplicon extension at 72°C), except for IL-17RC amplification (45 amplification cycles, denaturation at 99°C, primer annealing at 68°C with touchdown to 58°C, amplicon extension at 72°C). Because of the large number of isoforms ($>$90) generated from the *IL-17RC* gene (30), IL-17RC amplification products have been sequenced (Genome Express). The designed primers led to the amplification of a 191-bp sequence which corresponds to exons 11 through 13. No amplification products corresponding to deletion of exon 12 were detected. The copy number of target mRNA was normalized by the housekeeping gene *GAPDH* or *HPRT1*.

mRNA microarray hybridization and analysis

Five micrograms of RNA from peripheral whole blood (31 RA patients and 19 healthy volunteers) and 2 μg of RNA from in vitro experiment done in RA synoviocytes were analyzed using HG-U133A arrays (Affymetrix), according to the manufacturer's instructions. RNA integrity number was assessed using RNA 6000 nanochips and the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was used to prepare double-stranded cDNA containing the T7 promoter sequence. cRNA was synthesized and labeled with biotinylated ribonucleotide (GeneChip IVT Labeling kit; Affymetrix). The fragmented cRNA was hybridized onto HG-U133A oligonucleotide arrays (22,283 probe sets). The arrays were washed and stained using the fluidic station FS450 (Affymetrix) (protocol EukGE-WS2v4). The array was scanned with the Agilent G2500A GeneArray Scanner. The NETAFFX web site (www.affymetrix.com) was used to select candidate genes.

Silencing interfering RNA

A mixture of four siRNA duplexes (siGENOME SMARTPool siRNA) specific for IL-17RA (NM_014339) and IL-17RC (NM_032732) were purchased from Dharmacon. RA synoviocytes were seeded at a density of 5×10^5 cells/60-mm plate before transfection and used at 70–80% confluence. Cells were transfected with control siRNAs (siCONTROL siRNA as a

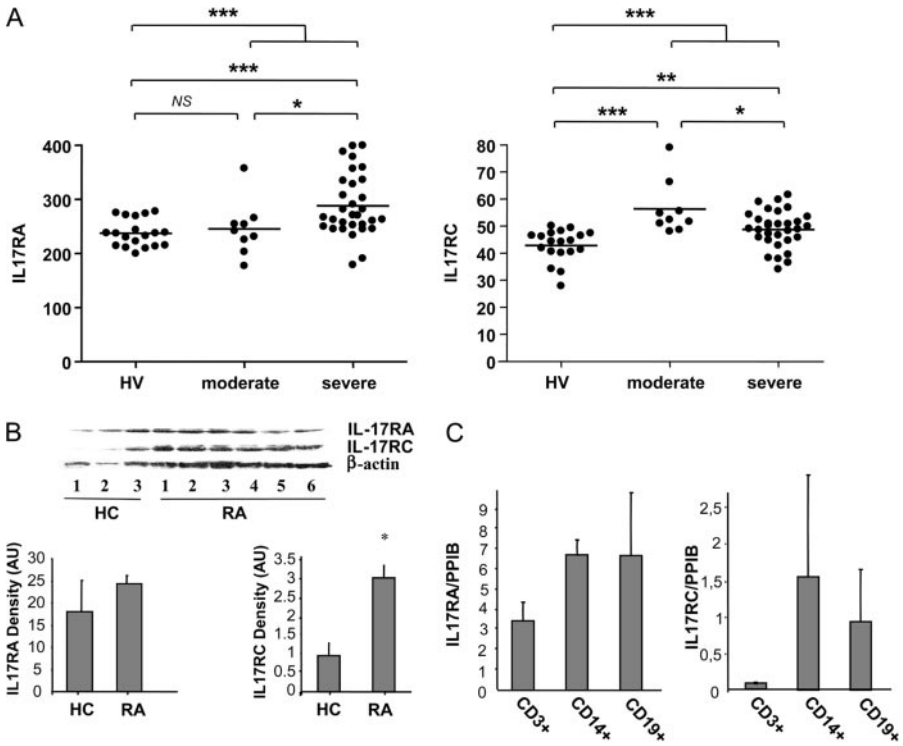


FIGURE 1. IL-17RA and IL-17RC mRNA expression in blood. *A*, IL-17RA and IL-17RC mRNA expression in peripheral whole blood from 40 RA patients (31 patients with severe RA and 9 with moderate RA) and from 19 healthy volunteers (HV), as determined by microarray analysis. Results are presented as fluorescent intensity (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ by Mann-Whitney U test). *B*, IL-17RA or IL-17RC were detected in whole blood from HV ($n = 3$) or RA patients ($n = 6$) by Western blotting using actin as a loading control. Density ratios of IL-17RA or IL-17RC were normalized to actin and expressed as AU (*, $p < 0.05$ by Mann-Whitney test). *C*, IL-17RA and IL-17RC mRNA expression in peripheral blood subsets from three RA patients. $CD14^+$ monocytes, $CD3^+$ lymphocytes, and $CD19^+$ lymphocytes were isolated from PBMC by positive selection. IL-17RA and IL-17RC mRNA expression were assessed by real-time RT-PCR. PPIB was used to normalize gene expression. Values represent the mean ± SEM expression from three RA patients (*, $p < 0.05$ by Mann-Whitney test).

negative control and siGLO peptidylpropyl isomerase B (PPIB) (cyclophilin B) as a positive control) or target siRNAs (siGENOME SMARTPool IL-17RA siRNA and IL-17RC siRNA) by nucleofection (Amaxa) according to the manufacturer's instructions (program U23; Human Dermal Fibroblast Nucleofector kit). Dose- and time-response experiments were performed to determine the best time point and the lowest suitable concentration of siRNA duplexes needed for efficacious RNA silencing. Thus, cells were nucleofected with 0.5 or 0.05 μg of either IL-17RA or IL-17RC siRNA duplexes, respectively, per 5×10^5 cells. Forty-eight hours posttransfection, the cells were stimulated for 12 or 36 h with IL-17A, IL-17F, or IL-17A plus TNF-α. Some of the results performed with siGENOME SMARTPool reagents were confirmed with the new ON-TARGET plus SMARTPool reagents used to reduce off-target effects (Dharmacon).

Western blotting

IL-17RA and IL-17RC expression was measured by Western blot using mouse anti-human mAb against IL-17RA or goat anti-human polyclonal Ab against IL-17RC (R&D Systems) as described previously (31). Protein concentration was measured using the BCA Protein assay reagent kit (Pierce). Eighty micrograms of protein were separated on 10% SDS-polyacrylamide electrophoresis gels and transferred to Hybond-C extra nitrocellulose membranes (Millipore). Membranes were stripped and serially reprobed with Abs against actin (Chemicon International). Western blots were scanned and densitometry ratios of IL-17RA or IL-17RC were normalized to actin content and expressed as arbitrary units (AU) using Image Gauge software (version 3.46).

Statistical analysis

Protein levels are expressed as mean ± SEM. mRNA expression of target genes was normalized with GAPDH or HPRT1 mRNA expression in synoviocytes and PPIB mRNA expression in whole blood. Data from functional analysis are expressed as the fold induction compared with untreated controls. For functional experiments in RA synoviocytes, statistical analysis was done using one-way ANOVA and post-hoc comparisons were conducted using Dunnett's t test. In ex vivo studies, Mann-Whitney's statistical test was used to compare two groups, whereas correlations were done using Spearman's statistical test. Differences resulting in p values <0.05 were considered to be statistically significant.

Results

IL-17RA and IL-17RC are overexpressed in peripheral whole blood from RA patients

Although the expression of IL-17A has already been shown in peripheral blood from RA patients (32), data concerning its receptors is missing. Thus, to determine whether IL-17RA and IL-17RC expression could be differentially expressed in RA patients when compared with healthy volunteers, we examined their expression at the mRNA level in whole blood using microarrays (Fig. 1*A*). A significant up-regulation of both IL-17RA and IL-17RC mRNA expression was observed in RA patients ($n = 40$) when compared with healthy volunteers ($n = 19$) (IL-17RA median: 262.5 vs 237.2; $p = 0.003$ and IL-17RC median: 50.46 vs 44.29; $p = 0.0002$). For IL-17RA mRNA expression, such difference was in fact the consequence of a specific overexpression in patients with severe RA (IL-17RA median in HV vs moderate or severe RA patients: 237.2 vs 241.9 or 270.2, respectively; $p = 0.8$ or $p = 0.0004$). This was not the case for IL-17RC mRNA expression which was higher in moderate vs severe RA patients (IL-17RC median in HV vs moderate or severe RA patients: 44.3 vs 52.4 or 49.1, respectively; $p < 0.0001$ or $p = 0.003$).

Because it was recently suggested that IL-17RA and IL-17RC act as an heterodimer to transduce IL-17A signals (26), we analyzed whether the expression of these two receptors was correlated in whole blood cells ($n = 59$). We failed to demonstrate a correlation between the mRNA expression of IL-17RA and IL-17RC ($R = -0.098$, $p = 0.5$). Moreover, it should be noted that IL-17RA mRNA levels were 5-fold higher than those of IL-17RC.

To determine whether IL-17RA and IL-17RC overexpression in RA patients could have a physiopathological significance, we analyzed their association with clinical activity or severity markers of RA. No correlation was found between IL-17RA or IL-17RC mRNA and DAS 28 index, C-reactive protein, rheumatoid factor, shared

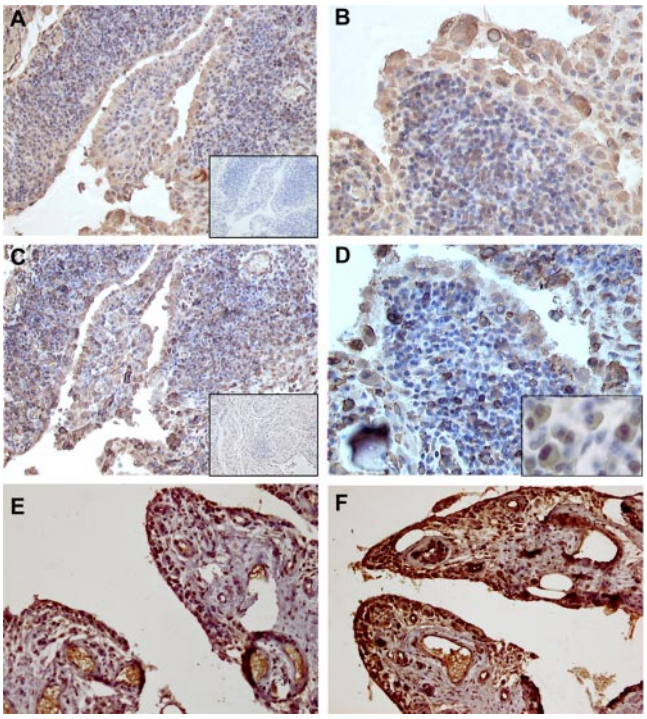


FIGURE 2. Detection of IL-17RA and IL-17RC in RA synovium. Immunostaining of paraffin-embedded serial sections using mouse anti-IL-17RA mAb (*A* and *B*, respectively, ×200 and ×400) and goat anti-IL-17RC polyclonal Ab (*C* and *D*, respectively, ×200 and ×400) shows a diffuse positive staining (brown) in both the lining and sublining area. Control staining was performed using mouse IgG1 and goat serum (×200) (*insets* in *A* and *C*). Immunostaining of IL-17RA and IL-17RC were also performed in OA synovium (*E* and *F*, respectively; ×200). Some of the IL-17A-producing cells have a plasmacytoid-like appearance as suggested by the remote nucleus in a large cytoplasm (*inset* in *D*, ×600).

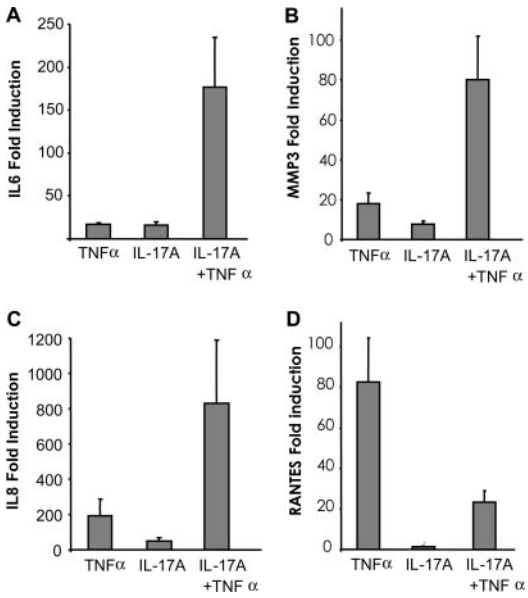


FIGURE 3. Effect of IL-17A, alone or in combination with TNF-α, on the mRNA expression of proinflammatory mediators. After serum deprivation, RA synoviocytes were stimulated for 12 h with IL-17A (50 ng/ml), alone or in combination with TNF-α (0.5 ng/ml). Total RNA was extracted and reverse transcribed. IL-6 (*A*), MMP-3 (*B*), IL-8 (*C*), and RANTES (*D*) mRNA levels were quantified by real-time RT-PCR. The values were normalized with GAPDH mRNA expression (mean ± SEM in untreated cells: 0.20 ± 0.12, 0.08 ± 0.02, 0.05 ± 0.02, 0.0004 ± 0.0001, respectively) and are expressed as fold induction compared with the untreated condition. Values represent the mean ± SEM of four independent experiments (*, $p < 0.05$ by Dunnett's test).

epitope (33), Larsen wrist score, or patient age, sex, or disease duration (data not shown).

IL-17RA and IL-17RC expression was also analyzed at the protein level by Western blotting (Fig. 1*B*). As for the mRNA level, IL-17RA levels were found to be significantly higher than those of IL-17RC (IL-17RA vs IL-17RC median at the mRNA ($n = 59$) or protein level ($n = 9$): 253.7 vs 48.2; $p < 0.0001$ or 22.8 vs 2.5; $p < 0.0001$, respectively). Moreover, a significant up-regulation of IL-17RC but not of IL-17RA protein expression was observed in whole blood from RA patients when compared with healthy volunteers (IL-17RC median: 0.9 vs 2.9; $p = 0.02$ and IL-17RA median: 13.0 vs 23.1; $p = 0.5$, respectively).

Members of the IL-17R family are known to be widely expressed (14, 34). Nevertheless, we wondered which cell type in RA peripheral blood could express IL-17RA and/or IL-17RC. Thus, we investigated IL-17RA and IL-17RC mRNA expression in T cells, B cells, and monocytes from RA patients by RT-PCR. Both IL-17RA and IL-17RC were expressed in $CD14^+$ monocytes and $CD19^+$ lymphocytes. In contrast, lower levels of IL-17RA and IL-17RC were measured in $CD3^+$ cells (Fig. 1*C*).

IL-17RA and IL-17RC are broadly expressed in whole synovium tissue from RA patients

Although IL-17RA and IL-17RC expression has been demonstrated in vitro in RA synoviocytes (35, 36), their expression in situ in the joint remains unclear. We performed immunohistochemical analysis to investigate whether IL-17Rs were expressed in synovium tissues from RA patients. Both IL-17RA and IL-17RC receptors were found to be broadly expressed within the synovium of RA patients (Fig. 2, *A–D*). Moreover, both receptors displayed overlapping staining within the synovium, as demonstrated using serial sections. Such diffuse staining was also observed in osteoarthritis (OA) synovium tissues (Fig. 2, *E* and *F*), where IL-17RA and IL-17RC expression was detected in the lining and sublining area. This diffuse staining confirmed the ubiquitous expression of IL-17RA and IL-17RC, both by stromal cells and infiltrating immune cells. We next examined expression of IL-17A in the same samples. IL-17A-positive cells were detected in lymphocytic infiltrates. When studied at high magnification, these IL-17-producing cells had a plasma cell-like morphology with a remote nucleus in a large cytoplasm (*inset* of Fig. 2*D*), as we have previously described (37).

IL-17A regulates key inflammatory mediators

The regulatory effect of IL-17A on RA synoviocytes was examined using microarrays, and confirmed by RT-PCR for four major genes involved in RA pathogenesis: IL-6, MMP-3, IL-8, and RANTES. After 12 h of stimulation, IL-17A stimulation up-regulated TNF-α-induced IL-6, MMP-3, and IL-8/CXCL8 mRNA expression and inhibited TNF-α-induced RANTES/CCL5 mRNA expression. For confirmation, we analyzed target gene expression from independent experiments done with synoviocytes obtained from four different RA patients using RT-PCR (Fig. 3). We confirmed that IL-17A or TNF-α alone significantly induced IL-6 and MMP-3 mRNA expression (15.8 ± 4.1- and 17 ± 2.8-fold induction, respectively, for IL-6, $p < 0.05$ and 7.6 ± 1.9- and 18.0 ± 4.8-fold induction, respectively, for MMP-3, $p < 0.05$). In the presence of TNF-α, IL-17A synergistically induced IL-6 and MMP-3 mRNA expression (175.9 ± 57.7- and 80.1 ± 22.0-fold

Table I. *Effect of IL-17A and/or TNF-α on chemokine mRNA expression in RA synoviocytes, as determined by microarrays*[a]

ID	Symbol	Fold Change			*p* Value		
		IL-17A	TNF-α	IL-17A + TNF-α	IL-17A	TNF-α	IL-17A + TNF-α
CXC chemokines							
204470_at	CXCL1	++	+	+++	0.00002	0.000027	0.00002
209774_x_at	CXCL2	+++	++	++++	0.00002	0.00002	0.00002
207850_at	CXCL3	+++	++	++++	0.00002	0.00003	0.00002
215101_s_at	CXCL5	+	++	+++	0.094279	0.000774	0.00002
206336_at	CXCL6	‖	‖	‖	0.00002	0.00002	0.00002
205592_at	CXCL8/IL8	‖	‖	‖‖	0.5	0.030967	0.5
211506_s_at	CXCL8/IL8	0			0.00002	0.00002	0.00002
203915_at	CXCL9	0	++	+	0.532344	0.078937	0.5
204533_at	CXCL10	0	+	+	0.838962	0.00006	0.000241
210163_at	CXCL11	0	0	0	0.5	0.00002	0.018128
209687_at	CXCL12	0	0	0	0.980376	0.999899	0.999932
205242_at	CXCL13	0	0	0	0.998664	0.958799	0.999886
218002_s_at	CXCL14	0	0	0	0.454766	0.441923	0.284967
CC chemokines							
207533_at	CCL1	0	0	0	0.161038	0.5	0.284967
216598_s_at	CCL2	0	+	+	0.00002	0.00002	0.00002
204103_at	CCL4	0	0	0	0.645558	0.39129	0.065566
204655_at	CCL5	0	+	0	0.998923	0.000101	0.013078
208075_s_at	CCL7	+	+	+	0.00002	0.00002	0.00002
214038_at	CCL8	0	+	+	0.000692	0.000114	0.00002
210133_at	CCL11	0	0	0	0.5	0.185981	0.274048
216714_at	CCL13	0	0	0	0.657563	0.118009	0.633407
205392_s_at	CCL14///CCL15	0	0	0	0.757415	0.5	0.5
207354_at	CCL16	0	0	0	0.153232	0.868657	0.596188
207900_at	CCL17	0	0	0	0.5	0.596188	0.846768
32128_at	CCL18	0	0	0	0.326797	0.5	0.799953
210072 at	CCL19	0	0	0	0.532344	0.5	0.786812
205476_at	CCL20	+++	+++	+++++	0.00002	0.00002	0.00002
204606 at	CCL21	0	0	0	0.342437	0.065566	0.138386
207861_at	CCL22	0	0	0	0.5	0.747149	0.5
210548 at	CCL23	0	0	0	0.041201	0.021224	0.213188
221463_at	CCL24	0	0	0	0.138386	0.467656	0.37888
206988_at	CCL25	0	0	0	0.805199	0.921063	0.958799
207955_at	CCL27	0	0	0	0.596188	0.830902	0.5
CX3C chemokines							
823_at	CX3CL1	0	+	+	0.5	0.000014	0.002991
XC chemokines							
206365 at	XCL1	0	0	0	0.725952	0.961585	0.846768
206366 x at	XCL2	0	0	0	0.366593	0.232549	0.145682

[a] The fold changes are normalized to untreated fibroblast-like synovial cells. RA synoviocytes were stimulated for 12 h with IL-17A (50 ng/ml), alone or in combination with TNF-α (0.5 ng/ml). The expression of chemokines, which were grouped according to the structural-based classification, and is represented as fold induction when compared to the untreated group (fold induction: 0, 0–2; +, 2–10; ++, 10–100; +++, 100–500; ++++, 500–1000) (HG-U133A microarrays are lacking the probe sets specific for: CXCL4, CXCL7, CCL3, CCL6, CCL9, CCL10, CCL12, CCL26, and CCL28).

induction, respectively). IL-17A alone up-regulated IL-8 mRNA levels by RA synoviocytes (47.1 ± 21.7-fold induction) and it synergized with TNF-α to induce a 800-fold induction (829.0 ± 358.1-fold induction for IL-17A plus TNF-α). In contrast, IL-17A inhibited TNF-α-induced RANTES mRNA expression (mean ± SEM by TNF-α compared with TNF-α plus IL-17A, 82.5 ± 21.9 compared with 23.4 ± 5.8).

IL-17A specifically induced ELR$^+$ CXC chemokines

To extend the results observed with IL-8, we next analyzed by microarrays the regulatory effect of IL-17A on chemokine expression (Table I). We observed a specific up-regulation of all ELR$^+$ CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8). In contrast, IL-17A had no effect on the expression of non-ELR CXC chemokines (CXCL9, CXCL10 and CXCL11). Moreover, IL-17A inhibited TNF-α-induced CXCL10 and CXCL11 mRNA expression. Among the CC chemokines, IL-17A had a positive effect on CCL7 and particularly on CCL20 mRNA levels whereas it inhibited CCL2 and CCL5 mRNA levels. Finally, IL-17A inhibited TNF-α-induced CX3CL1 mRNA expression, whereas it had no effect on C chemokine expression (XCL1 and XCL2).

Both IL-17RA and IL-17RC are needed for IL-17A-induced IL-6 secretion

Among the described IL-17R family members, IL-17RA and IL-17RC appear the most critical in mediating cellular responsiveness to IL-17A. Both receptors are expressed by synoviocytes, as demonstrated by baseline expression assessed by real-time RT-PCR. As observed in PBMCs, levels of IL-17RA were 5-fold higher than those of IL-17RC. We investigated the functional contribution of IL-17RA and IL-17RC in mediation of IL-17A biological effects by using siRNA. We first determined the lowest suitable dose of target siRNA to efficiently diminish IL-17RA and IL-17RC expression. A total of 80 and 62% specific knockdown of IL-17RA and IL-17RC mRNA expression were observed at 24 h posttransfection using 0.5 and 0.05 μg of siRNA, respectively (Fig. 4*A*). The knockdown efficiency was also confirmed at the protein level by Western blotting (Fig. 4*B*). Indeed, we observed a reduced immunostaining using anti-IL-17RA Ab in synoviocytes transfected

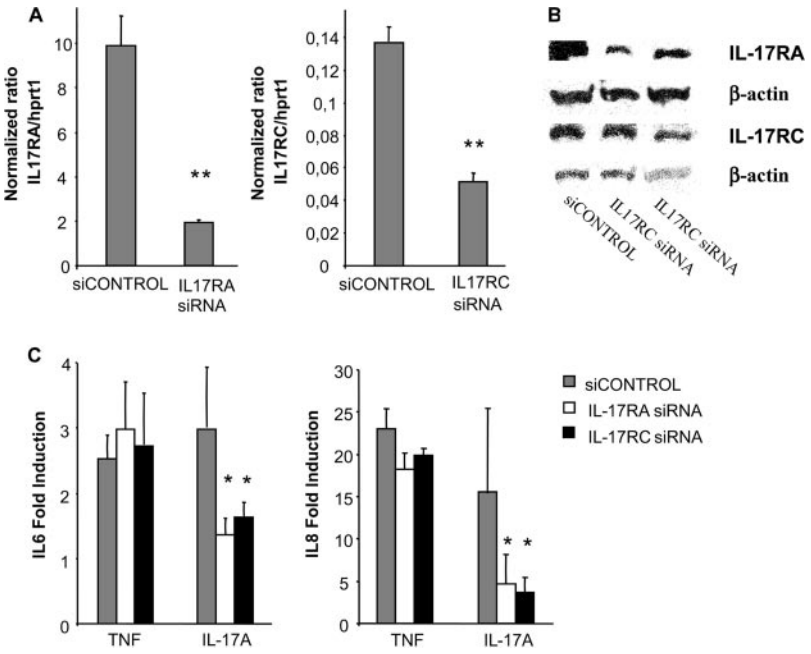


FIGURE 4. Effect of IL-17RA siRNA and IL-17RC siRNA on IL-17A- or IL-17F-induced IL-6 and IL-8 secretion by RA synoviocytes. RA synoviocytes were transfected with IL-17RA siRNA and IL-17RC siRNA at 0.5 and 0.05 μg, respectively. A scrambled nonsilence siRNA, siCONTROL, was used as a negative control. *A*, Knockdown efficiency of IL-17RA siRNA and IL-17RC siRNA was assessed by real-time RT-PCR. The values represent data obtained 24 h posttransfection and are expressed as mean ± SEM of three independent experiments (*, $p < 0.05$ by Dunnett's test). *B*, Knockdown efficiency of IL-17RA siRNA and IL-17RC siRNA was assessed by Western blotting 48 h posttransfection, using actin as a loading control. A representative western blot among three separate experiments is shown. *C*, Effect of IL-17RA siRNA and IL-17RC siRNA on IL-17A-induced IL-6 and IL-8 secretion by RA synoviocytes. Forty-eight hours after siRNA delivery (siCONTROL, IL-17RA siRNA, or IL-17RC siRNA), RA synoviocytes were stimulated for 12 h with IL-17A at 50 ng/ml or TNF-α at 0.5 ng/ml. IL-6 and IL-8 levels were quantified in removed supernatants by ELISA. The values are expressed as fold induction compared with the untreated condition (mean ± SEM in untreated cells transfected with siCONTROL siRNA: 2.4 ± 0.8 ng/ml and 367 ± 39.6 pg/ml, respectively) and are represented the mean ± SEM of three independent experiments (*, $p < 0.05$ by Dunnett's test).

with IL-17RA siRNA duplexes, and a reduced immunostaining using anti-IL-17RC Ab in synoviocytes transfected with IL-17RC siRNA duplexes. Forty-eight hours after transfection with IL-17RA siRNA, IL-17RC siRNA or the scrambled negative control (siCONTROL), RA synoviocytes were stimulated with IL-17A or IL-17F for 12 h and supernatants analyzed for IL-6 and IL-8 levels by ELISA. Stimulation with TNF-α alone was used as a control. As shown in Fig. 4*C*, TNF-α-induced IL-6 or IL-8 secretion by RA synoviocytes was not significantly affected by IL-17RA or IL-17RC knockdown (mean ± SEM by IL-17RA siRNA or IL-17RC siRNA compared with siCONTROL siRNA transfected cells: 2.9 ± 0.7 or 2.7 ± 0.7 compared with 2.5 ± 0.4 respectively, p was NS for IL-6 secretion and 12.8 ± 1.8 or 19.8 ± 1.2 compared with 23.0 ± 2.4 respectively, p was NS for IL-8 secretion), whereas IL-17A-induced IL-6 or IL-8 secretion was significantly reduced after both IL-17RA knockdown and IL-17RC knockdown (mean ± SEM by IL-17RA siRNA and IL-17RC siRNA compared with siCONTROL siRNA: 1.3 ± 0.2 and 1.6 ± 0.3 compared with 3 ± 0.9, respectively, $p < 0.05$ for IL-6 secretion and 4.7 ± 3.6 and 3.6 ± 1.8 compared with 15.6 ± 9.9, respectively, $p < 0.05$ for IL-8 secretion) (Fig. 4*B*). However, the contribution of both receptors to IL-17F response could not be estimated because of the low induction of proinflammatory cytokines by IL-17F alone (IL-6 and IL-8-fold induction compared with the untreated situation ± SEM, 1.52 ± 0.48 and 1.28 ± 0.28).

In the presence of TNF-α, the combined inhibition of IL-17RA and IL-17RC is needed to diminish IL-17A-induced IL-6 or CCL20 secretion by RA synoviocytes

We then investigated the efficiency of IL-17RA and IL-17RC inhibition on IL-17A-induced IL-6 and CCL20 secretion in the presence of TNF-α, this latest cytokine being critical to consider with clinical applications in mind. Thus, transfected RA synoviocytes were stimulated with IL-17A alone or in combination with TNF-α, and supernatants were analyzed for IL-6 levels by ELISA. As shown in Fig. 5*A*, after 36 h of stimulation, IL-17RA or IL-17RC knockdown alone had no effect on IL-17A plus TNF-induced IL-6 secretion (mean ± SEM by IL-17RA siRNA and IL-17RC siRNA compared with siCONTROL siRNA-transfected cells, 33.6 ± 5.1 and 36.1 ± 4.1 compared with 32.6 ± 6.6; $p > 0.9$), whereas the combination of IL-17RA siRNA and IL-17RC siRNA had an inhibitory effect (mean ± SEM by IL-17RA siRNA plus IL-17RC siRNA compared with siCONTROL siRNA transfected cells, 23.9 ± 8.8 compared with 32.6 ± 6.6; $p < 0.05$).

The effect of IL-17RA knockdown by siRNA was then compared with its extracellular blockade using neutralizing Abs against IL-17RA or IL-17RC. RA synoviocytes were preincubated with anti-IL-17RA and/or anti-IL-17RC Abs for 2 h and then stimulated for 36 h with IL-17A alone or in combination with TNF-α. IL-6 and CCL20 levels were analyzed by ELISA (Fig. 5, *B* and *C*). Results similar to those observed using siRNA were obtained: anti-IL-17RA and -IL-17RC Abs decreased IL-17A-induced IL-6 or CCL20 secretion by RA synoviocytes (mean ± SEM by anti-IL-17RA or -IL-17RC Ab compared with the situation without inhibitors, 2.0 ± 0.7 or 3.0 ± 1.1 compared with 5.2 ± 1.8; $p < 0.05$ for IL-6 secretion and 0.0 ± 0.2 and 1.9 ± 0.8 compared with 8.8 ± 4.6; $p < 0.05$ for CCL20 secretion), but had no suppressive effects in the presence of TNF-α (mean ± SEM by anti-IL-17RA Ab and -IL-17RC Ab compared with the situation without inhibitors, 34.7 ± 6.8 and 40.6 ± 9.1 compared with 37.6 ± 6.3; NS for IL-6 secretion and 20.5 ± 4.4 and 12.5 ± 1.2 compared with 20.6 ± 9.7; NS for CCL20 secretion). Moreover, the combination

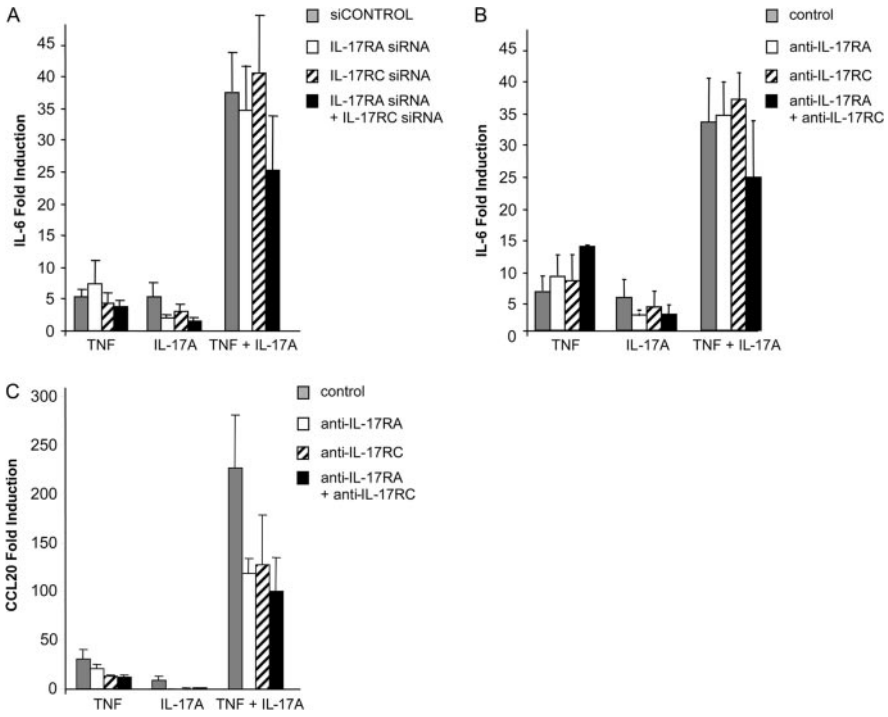


FIGURE 5. Effect of IL-17RA and/or IL-17RC inhibition by siRNA or extracellular inhibitors, on IL-17 plus TNF-α-induced IL-6 secretion. *A*, Efficiency of posttranscriptional inhibition of IL-17RA and/or IL-17RC using siRNA. RA synoviocytes from the three cell populations (transfected with siCONTROL, IL-17RA siRNA, and IL-17RC siRNA) were stimulation for 36 h with IL-17A (50 ng/ml), alone or in combination with TNF-α (0.5 ng/ml). IL-6 levels were quantified in removed supernatants by ELISA. The values represent the mean $\pm$ SEM of three independent experiments (*, $p < 0.05$ by Dunnett's test). *B* and *C*, Efficiency of extracellular inhibition using Abs. RA synoviocytes were preincubated for 2 h (37°C, 5% CO2) with anti-IL-17RA Ab (10 μg/ml) and/or anti-IL-17RC Ab (10 μg/ml). The cells were then stimulated with IL-17A (50 ng/ml), alone or in combination with TNF-α (0.5 ng/ml). IL-6 and CCL20 levels were quantified in removed supernatants by ELISA. The values represent the mean $\pm$ SEM of three independent experiments (mean $\pm$ SEM in untreated cells: 1.7 $\pm$ 0.6 ng/ml and 1.4 $\pm$ 1.6 pg/ml, respectively) (*, $p < 0.05$ by Dunnett's test).

of anti-IL-17RA and IL-17RC Abs induced a decrease of IL-17A plus TNF-α-induced IL-6 secretion (mean $\pm$ SEM by anti-IL-17RA Ab plus anti-IL-17RC Ab compared with the situation without inhibitors, 25.3 $\pm$ 8.6 compared with 37.6 $\pm$ 6.3 for IL-6 secretion and 226.7 $\pm$ 54.4 pg/ml compared with 100.6 $\pm$ 59.7; $p < 0.05$ for CCL20 secretion).

Discussion

RA is a systemic inflammatory disease characterized by chronic synovium inflammation leading to matrix destruction. RA synovium and synovial fluid are highly infiltrated by immune cells. Among them, activated $CD4^+$ T cells contribute to RA pathogenesis with a pivotal role of the newly characterized Th17 cells, which secrete IL-17A and IL-17F, IL-22, TNF-α, and IL-6 (38–41).

We showed that IL-17A synergized with TNF-α to induce IL-6 mRNA expression by RA synoviocytes. IL-6 is an important proinflammatory cytokine, regulating both local and systemic inflammation. Moreover, IL-6 was recently demonstrated to be a major mediator of Th17 differentiation. This differentiation pathway presents unique features because of its relationship with regulatory T cell developmental program. Indeed, in mouse models, the differentiation of $CD4^+$ naive T cells into regulatory T cells in the presence of TGF-β is completely inhibited by IL-6, whereas it promotes Th17 cells. The developmental program induced by IL-6 in the presence of TGF-β induces the expression of the nuclear receptor RORγt which controls Th17 differentiation leading to IL-17A and IL-17F secretion (42). Thus, IL-17A-induced IL-6 secretion by RA synoviocytes results in a positive feedback which could be involved in the promotion of local Th17 induction.

One key property of IL-17A is its orchestral role in mediating the migration of inflammatory cells, which takes a central place in RA pathogenesis (43). The list of chemokines has been growing extensively, a condition where the use of extensive gene analysis is well-suited to compare levels. Chemokine secretion by activated RA synoviocytes regulates different phases in this multistep process: recruitment through the activation of endothelial cells, local retention and activation of immune cells, and transmigration to the synovial fluid. Data obtained using microarrays suggested that IL-17A promotes the selective expression of ELR^+ CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and, CXCL8) in RA synoviocytes, especially in the presence of TNF-α, whereas it has no effect or an inhibitory effect on the expression of ELR^- CXC chemokines (CXCL9, CXCL10, CXCL11, and PF4). This specific regulatory effect of IL-17A highlights its role in neutrophil recruitment into the joint, but also supports its specific promoting effect on angiogenesis. Indeed, the ELR motif has been implicated in the neutrophil attractant properties of CXC chemokines, and in their angiogenic effects. IL-17A regulatory effects on angiogenic (ELR^+) vs angiostatic (non-ELR) chemokines are just opposite to those of IFN-γ. Indeed, it was demonstrated that IFN-γ inhibits angiogenesis in part through the inhibition of ELR^+ CXC chemokines (44).

In the group of CC chemokines, we observed an inhibitory effect of IL-17A on TNF-α-induced CCL2, CCL5, and CX3CL1 mRNA expression in RA synoviocytes, which suggests a negative effect on the migration of mononuclear cell types including activated T cells, macrophages, NK cells, and regulatory T cells. In contrast, IL-17A up-regulated the expression of the CC chemokines, CCL20 and CCL7, which are both expressed in RA joint (45–47). CCL20 is implicated in the local recruitment of CCR6-positive cells, which include immature dendritic cells, naive B cells, and memory T cells (18, 24, 46, 47). Indeed, we have previously demonstrated the expression of CCR6 in subsets of IL-17- and IFN-γ-producing cells within RA synovium (45).

Among IL-17R family members, we confirmed the expression of IL-17RA and IL-17RC by RA synoviocytes. Using both siRNA delivery and extracellular inhibitors, we showed that the inhibition of IL-17RA or IL-17RC alone was sufficient to induce a significant reduction in IL-17A-induced IL-6, IL-8, and CCL20 secretion, demonstrating that both IL-17RA and IL-17RC are implicated in transducing IL-17A signals in RA synoviocytes. This result is consistent with the current concept of a functional receptor composed

of IL-17RA and IL-17RC subunits (26). Nevertheless, we observed that the inhibition of IL-17RA or IL-17RC when used alone failed to significantly reduce IL-17A and TNF-α-induced IL-6 or CCL20 secretion. In that case, the combined inhibition of IL-17RA and IL-17RC was needed for a significant decrease in IL-17A and TNF-α-induced IL-6 or CCL20 secretion. At the mRNA level, IL-17RA had a 5-fold higher expression than IL-17RC systemically and locally. However, although there was a trend to more potent inhibition of synoviocyte IL-17A-induced IL-6 by IL-17RA antagonism compared with IL-17RC inhibition, this was not statistically significant. This suggests that despite increased expression of IL-17RA compared with IL-17RC, their absolute expression levels may be less important compared with functional outcomes following antagonism.

In conclusion, we demonstrated that IL-17RA and IL-17RC are overexpressed systemically in RA patients. This overexpression could make synoviocytes and immune cells more sensitive to IL-17, a major regulatory cytokine implicated in RA pathogenesis. Finally, we showed that both IL-17RA and IL-17RC are implicated in IL-17A-induced inflammatory signals in RA synoviocytes. These receptors could be targets for therapeutic intervention, in combination with conventional anti-TNF treatment.

Disclosures

The authors have no financial conflict of interest.

References

1. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6: 1123–1132.
2. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6: 1133–1141.
3. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235–238.
4. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179–189.
5. Nakae, S., A. Nambu, K. Sudo, and Y. Iwakura. 2003. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J. Immunol.* 171: 6173–6177.
6. Chabaud, M., J. M. Durand, N. Buchs, F. Fossiez, G. Page, L. Frappart, and P. Miossec. 1999. Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum.* 42: 963–970.
7. Kotake, S., N. Udagawa, N. Takahashi, K. Matsuzaki, K. Itoh, S. Ishiyama, S. Saito, K. Inoue, N. Kamatani, M. T. Gillespie, et al. 1999. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J. Clin. Invest.* 103: 1345–1342.
8. Chabaud, M., F. Fossiez, J. L. Taupin, and P. Miossec. 1998. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J. Immunol.* 161: 409–414.
9. Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicoeur, Y. He, M. Zhang, F. Mineau, and J. P. Pelletier. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines: IL-β and TNF-α, by human macrophages. *J. Immunol.* 160: 3513–3521.
10. Katz, Y., O. Nadiv, and Y. Beer. 2001. Interleukin-17 enhances tumor necrosis factor α-induced synthesis of interleukins 1,6, and 8 in skin and synovial fibroblasts: a possible role as a "fine-tuning cytokine" in inflammation processes. *Arthritis Rheum.* 44: 2176–2184.
11. Kehlen, A., K. Thiele, D. Riemann, and J. Langner. 2002. Expression, modulation and signalling of IL-17 receptor in fibroblast-like synoviocytes of patients with rheumatoid arthritis. *Clin. Exp. Immunol.* 127: 539–546.
12. Numasaki, M., M. T. Lotze, and H. Sasaki. 2004. Interleukin-17 augments tumor necrosis factor-α-induced elaboration of proangiogenic factors from fibroblasts. *Immunol. Lett.* 93: 39–43.
13. Takahashi, H., M. Numasaki, M. T. Lotze, and H. Sasaki. 2005. Interleukin-17 enhances bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells. *Immunol. Lett.* 98: 189–193.
14. Miossec, P. 2003. Interleukin-17 in rheumatoid arthritis: if T cells were to contribute to inflammation and destruction through synergy. *Arthritis Rheum.* 48: 594–601.
15. Granet, C., W. Maslinski, and P. Miossec. Increased AP-1 and NF-κB activation and recruitment with the combination of the proinflammatory cytokines IL-1β, tumor necrosis factor α and IL-17 in rheumatoid synoviocytes. 2004. *Arthritis Res. Ther.* 6: R190–R198.
16. Chabaud, M., and P. Miossec. 2001. The combination of tumor necrosis factor α blockade with interleukin-1 and interleukin-17 blockade is more effective for controlling synovial inflammation and bone resorption in an ex vivo model. *Arthritis Rheum.* 44: 1293–1303.
17. Granet, C., and P. Miossec. 2004. Combination of the pro-inflammatory cytokines IL-1: TNF-α and IL-17 leads to enhanced expression and additional recruitment of AP-1 family members, Egr-1 and NF-κB in osteoblast-like cells. *Cytokine* 26: 169–177.
18. Chabaud, M., G. Page, and P. Miossec. 2001. Enhancing effect of IL-1: IL-17, and TNF-α on macrophage inflammatory protein-3α production in rheumatoid arthritis: regulation by soluble receptors and Th2 cytokines. *J. Immunol.* 167: 6015–6020.
19. Chevrel, G., G. Page, C. Granet, N. Streichenberger, A. Varennes, and P. Miossec. 2003. Interleukin-17 increases the effects of IL-1β on muscle cells: arguments for the role of T cells in the pathogenesis of myositis. *J. Neuroimmunol.* 137: 125–133.
20. Kirkham, B. W., M. N. Lassere, J. P. Edmonds, K. M. Juhasz, P. A. Bird, C. S. Lee, R. Shnier, and I. J. Portek. 2006. Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort). *Arthritis Rheum.* 54: 1122–1131.
21. Strieter, R. M., P. J. Polverini, S. L. Kunkel, D. A. Arenberg, M. D. Burdick, J. Kasper, J. Dzuiba, J. Van Damme, A. Walz, D. Marriott, et al. 1995. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J. Biol. Chem.* 270: 27348–27357.
22. Strieter, R. M., M. D. Burdick, B. N. Gomperts, J. A. Belperio, and M. P. Keane. 2005. CXC chemokines in angiogenesis. *Cytokine Growth Factor Rev.* 16: 593–609.
23. Clark-Lewis, I., B. Dewald, M. Loetscher, B. Moser, and M. Baggiolini. 1994. Structural requirements for interleukin-8 function identified by design of analogs and CXC chemokine hybrids. *J. Biol. Chem.* 269: 16075–16081.
24. Page, G., G. Chevrel, and P. Miossec. 2004. Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: interaction with chemokines and Th1 cytokine-producing cells. *Arthritis Rheum.* 50: 199–208.
25. Yao, Z., M. K. Spriggs, J. M. Derry, L. Strockbine, L. S. Park, T. VandenBos, J. D. Zappone, S. L. Painter, and R. J. Armitage. 1997. Molecular characterization of the human interleukin (IL)-17 receptor. *Cytokine* 9: 794–800.
26. Toy, D., D. Kugler, M. Wolfson, T. Vanden Bos, J. Gurgel, J. Derry, J. Tocker, and J. Peschon. 2006. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J. Immunol.* 177: 36–39.
27. Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. McShane, J. F. Fries, N. S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, H. S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31: 315–324.
28. Toh, M. L., H. Marotte, J. L. Blond, U. Jhumka, A. Eljaafari, B. Mougin, and P. Miossec. 2006. Overexpression of synoviolin in peripheral blood and synoviocytes from rheumatoid arthritis patients and continued elevation in nonresponders to infliximab treatment. *Arthritis Rheum.* 54: 2109–2118.
29. Pachot, A., J. L. Blond, B. Mougin, and P. Miossec. 2004. Peptidylpropyl isomerase B (PPIB): a suitable reference gene for mRNA quantification in peripheral whole blood. *J. Biotechnol.* 114: 121–124.
30. Haudenschild, D. R., S. B. Curtiss, T. A. Moseley, and A. H. Reddi. 2006. Generation of interleukin-17 receptor-like protein (IL-17RL) in prostate by alternative splicing of RNA. *Prostate* 66: 1268–1274.
31. Lacey, D., A. Sampey, R. Mitchell, R. Bucala, L. Santos, M. Leech, and E. Morand. 2003. Control of fibroblast-like synoviocyte proliferation by macrophage migration inhibitory factor. *Arthritis Rheum.* 48: 103–109.
32. Kageyama, Y., M. Takahashi, E. Torikai, M. Suzuki, T. Ichikawa, T. Nagafusa, Y. Koide, and A. Nagano. 2007. Treatment with anti-TNF-α antibody infliximab reduces serum IL-15 levels in patients with rheumatoid arthritis. *Clin. Rheumatol.* 26: 505–509.
33. Marotte, H., B. Pallot-Prades, L. Grange, J. Tebib, P. Gaudin, C. Alexandre, J. L. Blond, M. A. Cazalis, B. Mougin, and P. Miossec. 2006. The shared epitope is a marker of severity associated with selection for, but not with response to, infliximab in a large rheumatoid arthritis population. *Ann. Rheum. Dis.* 65: 342–347.
34. Moseley, T. A., D. R. Haudenschild, L. Rose, and A. H. Reddi. 2003. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev.* 14: 155–174.
35. Hwang, S. Y., J. Y. Kim, K. W. Kim, M. K. Park, Y. Moon, W. U. Kim, and H. Y. Kim. 2004. IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-κB- and PI3-kinase/Akt-dependent pathways. *Arthritis Res. Ther.* 6: R120–R128.
36. Hwang, S. Y., and H. Y. Kim. 2005. Expression of IL-17 homologs and their receptors in the synovial cells of rheumatoid arthritis patients. *Mol. Cell.* 19: 180–184.
37. Page, G., A. Sattler, S. Kersten, A. Thiel, A. Radbruch, and P. Miossec. 2004. Plasma cell-like morphology of Th1-cytokine-producing cells associated with the loss of CD3 expression. *Am. J. Pathol.* 164: 409–417.
38. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23

drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.
39. Hymowitz, S. G., E. H. Filvaroff, J. P. Yin, J. Lee, L. Cai, P. Risser, M. Maruoka, W. Mao, J. Foster, R. F. Kelley, et al. 2001. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* 20: 5332–5341.
40. Chung, Y., X. Yang, S. H. Chang, L. Ma, Q. Tian, and C. Dong. 2006. Expression and regulation of IL-22 in the IL-17-producing $CD4^+$ T lymphocytes. *Cell Res.* 16: 902–907.
41. Liang, S. C., X. Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L. A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* 203: 2271–2279.
42. Ivanov, I. I., B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory $IL\text{-}17^+$ T helper cells. *Cell* 126: 1121–1133.
43. Cross, A., D. Bakstad, J. C. Allen, L. Thomas, R. J. Moots, and S. W. Edwards. 2005. Neutrophil gene expression in rheumatoid arthritis. *Pathophysiology* 12: 191–202.
44. Belperio, J. A., M. P. Keane, D. A. Arenberg, C. L. Addison, J. E. Ehlert, M. D. Burdick, and R. M. Strieter. 2000. CXC chemokines in angiogenesis. *J. Leukocyte Biol.* 68: 1–8.
45. Page, G., and P. Miossec. 2004. Paired synovium and lymph nodes from rheumatoid arthritis patients differ in dendritic cell and chemokine expression. *J. Pathol.* 204: 28–38.
46. Matsui, T., T. Akahoshi, R. Namai, A. Hashimoto, Y. Kurihara, M. Rana, A. Nishimura, H. Endo, H. Kitasato, S. Kawai, et al. 2001. Selective recruitment of CCR6-expressing cells by increased production of MIP-3α in rheumatoid arthritis. *Clin. Exp. Immunol.* 125: 155–161.
47. Haringman, J. J., T. J. Smeets, P. Reinders-Blankert, and P. P. Tak. 2006. Chemokine and chemokine receptor expression in paired peripheral blood mononuclear cells and synovial tissue of patients with rheumatoid arthritis, osteoarthritis, and reactive arthritis. *Ann. Rheum. Dis.* 65: 294–300.

Zrioual S, Ecochard R, **Tournadre A** , Lenief V, Cazalis MA, Miossec P. Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. *J Immunol.* 2009;182:3112-20

Contribution spécifique de la candidate:

- Réalisation des techniques d' immunomarquages en immunohistochimie pour IL17A et IL17F dans la synoviale rhumatoïde et arthrosique, avec mise au point des anticorps spécifiques.
- Analyse des immunomarquages, réalisation des photographies
- Conception et réalisation de la figure 1 de l'article.

Genome-Wide Comparison between IL-17A- and IL-17F-Induced Effects in Human Rheumatoid Arthritis Synoviocytes[1]

Saloua Zrioual,* René Ecochard,† Anne Tournadre,† Vanina Lenief,* Marie-Angélique Cazalis,* and Pierre Miossec[2]*

IL-17A is implicated in rheumatoid arthritis (RA) pathogenesis; however, the contribution of IL-17F remains to be clarified. Using microarrays and gene-specific expression assays, we compared the regulatory effects of IL-17A and IL-17F alone or in combination with TNF-α on RA synoviocytes. IL-17A and IL-17F expression was studied in osteoarthritis and RA synovium by immunohistochemistry. The comparison between the IL-17A and IL-17F stimulatory effect on RA synoviocytes was assessed at the protein level by ELISA and at the mRNA level by microarrays and real-time RT-PCR. TNFRII expression was studied by real-time RT-PCR and immunofluorescence, and neutralizing Ab was used to analyze its contribution to CCL20 secretion. IL-17A and IL-17F were detected in plasma cell-like cells from RA but not osteoarthritis synovium. In microarrays, IL-17A and IL-17F alone had similar regulatory effects, IL-17F being quantitatively less active. Both cytokines induced a similar expression pattern in the presence of TNF-α. Based on a cooperation index, 130 and 203 genes were synergistically induced by IL-17A or IL-17F plus TNF-α, respectively. Among these, the new target genes CXCR4, LPL, and IL-32 were validated by real-time RT-PCR. IL-17A and IL-17F up-regulated TNFRII expression, but had no effects on TNFRI, IL-17RA or IL-17RC. TNFRII blockade inhibited the synergistic induction of CCL20 by IL-17A or IL-17F and TNF-α. IL-17A and IL-17F are both expressed in RA synovium. In the presence of TNF-α, they induced a similar expression pattern in RA synoviocytes. Accordingly, IL-17F appears as a target in Th17-mediated diseases such as RA. ***The Journal of Immunology,*** **2009, 182: 3112–3120.**

Rheumatoid arthritis (RA)[3] is a complex chronic disorder leading to joint destruction and characterized by synovium hyperplasia, neoangiogenesis, and local infiltration by immune cells. Among the inflammatory mediators expressed within RA synovium and synovial fluid, IL-17A is associated with disease severity (1). This proinflammatory cytokine is produced almost exclusively by Th17 cells. Mouse models have suggested a critical role of IL-17A in RA since IL-17A deficiency or antagonism has profound antiarthritic effects (2, 3). Data coming from human in vitro experiments demonstrated their effects on joint degradation through the induction of RANKL, metalloproteinases (4–6), and neutrophil survival, in part through GM-CSF (7).

Results on IL-17F, another member of the IL-17 family, have suggested that the genes encoding for IL-17A and IL-17F are coming from a mechanism of duplication since they are located adjacent. Moreover, both cytokines exhibit a similar expression pattern, driven by the nuclear receptor RORγt (8–10), and have 50% identity (11). They also exhibit a similar cysteine knot configuration (11), acting both as homodimer but also as IL-17A-IL-17F heterodimer (12–14), and they induce common transducing pathways through a complex composed of the IL-17RA and IL-17RC receptors (15). Thus, the common features shared by IL-17A and IL-17F suggest that they may have a similar regulatory effect. However, it is still unknown whether IL-17A and IL-17F induce similar, overlapping, or divergent gene expression profiles.

To investigate their possible implication in RA, the expression of IL-17A and IL-17F was studied in osteoarthritis (OA) and RA synovium and their regulatory effects were examined on cultured RA synoviocytes at the mRNA and protein levels. Since cooperative effects with other proinflammatory cytokines are common, the effects of IL-17A and IL-17F were compared when used alone or in the presence of TNF-α. High throughput cDNA microarrays were used to obtain an extensive evaluation of these interactions, focusing on the genes synergistically induced by IL-17A or IL-17F and TNF-α.

Materials and Methods

Cell culture and experimental design

RA synoviocytes were obtained from synovial tissue obtained from RA patients undergoing joint surgery who fulfilled the American College of Rheumatology's criteria for RA (16). In brief, synovial tissues were minced into small pieces and then incubated for 2h at 37°C with proteolytic enzymes (1 mg/ml collagenase and hyaluronidase; Sigma-Aldrich). Synoviocytes were cultured in DMEM (Invitrogen) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator and used at passages three to seven, which were >99% negative for CD45, CD1, CD3, CD19, CD14, and HLA-DR and were positive for the expression of CD44 (Abs obtained from BD Pharmingen). The effect of IL-17A and IL-17F alone or in combination with TNF-α was compared both at the protein and mRNA levels.

*Department of Immunology and Rheumatology and Hospices Civils de Lyon-bioMerieux Mixed Research Unit, Hospital Edouard Herriot, Lyon; and †Department of Biostatistics, Hospices Civils, Lyon, France

Received for publication June 17, 2008. Accepted for publication December 19, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work has been supported in part by grants from the Hospices Civils de Lyon and the Region Rhône-Alpes. S.Z. is supported by a scholarship from the Region Rhône-Alpes.

[2] Address correspondence and reprint requests to Prof. Pierre Miossec, Clinical Immunology Unit, Department of Immunology and Rheumatology, Hospital Edouard Herriot, 69437 Lyon Cedex 03, France. E-mail address: pierre.miossec@univ-lyon1.fr

[3] Abbreviations used in this paper: RA, rheumatoid arthritis, OA, osteoarthritis; LPL, lipoprotein lipase; ARE, AU-rich element.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0801967

Synoviocytes seeded in 96-well plates (1×10^4 cells/well) were stimulated for 48 h with IL-17A or IL-17F (0.1–100 ng/ml) alone or in combination with TNF-α (0.5 ng/ml) to examine protein secretion by ELISA. Synoviocytes seeded in 6-well plates (5×10^5 cells/well) were stimulated for 1–12 h with IL-17A or IL-17F (0.5 ng/ml) alone or in combination with TNF-α (0.5 ng/ml) to examine target gene mRNA expression by real-time RT-PCR. For microarray analysis, the cells were stimulated for 12 h.

Cytokines, Abs, and ELISA

Human recombinant TNF-α was purchased from Sigma-Aldrich. Human recombinants IL-17A and IL-17F were purchased from R&D Systems. CCL20/MIP-3α and IL-6 levels were quantified in removed supernatants by ELISA (R&D Systems and eBioscience, respectively). Human anti-IL-17A mAb (clone 41809) and anti-IL-17F mAb (clone 197315) were purchased from R&D Systems. Monoclonal anti-human TNFRII/TNFRSF1B Ab (clone 22210) used for neutralizing assays and PE-conjugated mouse anti-human TNFRII mAb (clone 22235) and PE-conjugated mouse IgG2A monoclonal Ig isotype control were purchased from R&D Systems.

Immunohistochemistry

OA and RA synovial tissues were fixed in 10% phosphate-buffered formaldehyde. After paraffin embedding, samples were cut into 4-μm sections, mounted on glass slides, and treated for deparaffination (OTTIX Plus; DiaPath). An Ag retrieval procedure was performed by incubation in citrate buffer (pH 6) for 40 min at 99°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min before the application of primary Ab. The sections were then incubated for 1 h with primary Abs: 10 μg/ml mouse monoclonal anti-IL-17A (IgG2b) or 10 μg/ml mouse monoclonal anti-IL-17F (IgG1). In negative control sections, irrelevant Ab (mouse IgG2b) was applied at the same concentration as the primary Ab. After washing, the sections were incubated with biotinylated anti-mouse Ab for 15 min, followed by streptavidin-peroxidase complex for 15 min and 3,3′-diaminobenzidine chromogen solution (DakoCytomation). The sections were then counterstained with Mayer's hematoxylin.

RNA extraction and purification

Synoviocytes seeded in 6-well plates (5×10^5 cells/well) were serum deprived for 2 h and then stimulated for 1, 3, 6, or 12 h with IL-17A or IL-17F (50 ng/ml) alone or in combination with TNF-α (0.5 ng/ml) in DMEM/10% FCS. RNA was extracted using TRIzol reagents (Life Technologies) and purified using RNeasy kits (Qiagen). The concentration of RNA was quantified by spectrophotometry at 260 nm (SmartSpec 3000; Bio-Rad).

Real-time RT-PCR

One microgram of total RNA was reverse transcribed using a ThermoScript RT-PCR System (Invitrogen Life Technologies). Briefly, total RNA was denatured by incubating for 5 min at 65°C with 4 μM oligo(dT) primer and then reverse transcribed by using a final concentration of 0.5 mM dNTP, 40 U/μl RNase OUT, 0.01 M DTT, and 10 U/μl of ThermoScript reverse transcriptase. Reverse transcription was performed by incubation at 50°C for 60 min followed by 85°C for 5 min. The obtained cDNA was diluted 1/10 with distilled water and 10 μl was used for amplification. Specific primer sets for IL-6, CXCL8/IL-8, CXCL5/ENA-78, RANTES/CCL5, CXCR4, and GAPDH were optimized for the LightCycler instrument (Roche Molecular Biochemicals) and purchased from Search-LC. Primer-specific nucleotide sequences for TNFR type I (GenBank accession no. NM_001065), TNFR type II (GenBank accession no. NM_001066), IL-32 (GenBank accession no. NM_001012631), lipoprotein lipase (LPL; GenBank accession no. NM_000237), and CDC42EP3 (GenBank accession no. NM_006449) were synthesized by Eurogentec: TNFRI forward: 5′-ctcctgtagtaactgtaagaa-3′ and TNFRI reverse: 5′-gtctaggctctgtggctt-3′; TNFRII forward: 5′-ccccaccagatctgtaacg-3′ and TNFRII reverse: 5′-tatcggcaggcaagtgagg-3′; IL-32 forward: 5′-ggagacagtggcggcttat-3′ and IL-32 reverse: 5′-ggcaccgtaatccatctctt-3′; LPL forward: 5′-ccatgacaagtctctgaataagaa-3′, LPL reverse: 5′-ccccaaacactgggtatgtt-3; and CDC42EP3 forward: 5′-agactcggctggatctgc-3′, CDC42EP3 reverse: 5′-gaccacaaccaggacaaacc-3′. The PCR was performed using the LightCycler FastStart DNA SYBR Green I kit (Roche Molecular Biochemicals) according to the protocol provided with the parameter-specific kits (45 amplification cycles, denaturation at 96°C, primer annealing at 68 °C with touchdown to 58°C, amplicon extension at 72°C). The copy number of target mRNA was normalized by the housekeeping gene *GAPDH*.

Microarray hybridization

Two micrograms of RNA from RA synoviocytes were analyzed using HG-U133A arrays (Affymetrix) according to the manufacturer's instructions (in vitro transcribed labeling protocol). RNA integrity number was assessed using RNA 6000 nano chips (mean ± SD: 8.3 ± 0.1) and the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was used to prepare double-stranded cDNA containing the T7 promoter sequence. cRNA was synthesized and labeled with biotinylated ribonucleotide (GeneChip IVT Labeling Kit; Affymetrix). The fragmented cRNA was hybridized onto HG-U133A oligonucleotide arrays (22,283 probe sets). The arrays were washed and stained using the fluidic station FS450 (Affymetrix; protocol EukGE-WS2v4). The array was scanned with the Agilent G2500A GeneArray Scanner. Array hybridization quality controls were analyzed (mean ± SD of scaling factor: 1.7 ± 0.8; GAPDH ratio: 1.0 ± 0.1; and β-actin ratio: 2.3 ± 0.1).

Microarray data preprocessing and validation

Statistical analysis was performed using the Affymetrix Data Mining Tool Software (version MAS 5.0). There were 12,074, 12,912, 12,238, 12,391, and 12,779 probe set signals detected (present call) after IL-17A, IL-17F, TNF-α, IL-17A plus TNF-α, and IL-17A plus TNF-α, respectively. Gene products that showed a signal intensity of 30 or greater were considered as significantly expressed and genes that showed a change of 2-fold or greater compared with the control situation were considered as significantly regulated. A global comparison between the regulatory effects of IL-17A and IL-17F was first assessed using the comparison call from the MAS 5.0 algorithm. The signal intensity of 705 and 438 probe sets showed a change of 2-fold or greater when compared with the untreated condition upon IL-17A and IL-17F stimulation, respectively. From these subsets, a list of 680 distinct probe sets (601 genes) induced by IL-17A and/or IL-17F showed a signal intensity of 30 or greater. A hierarchical clustering was then applied to compare the effects of IL-17A or IL-17F alone or in combination with TNF-α. The signal intensity of 705, 438, 1032, 1493, and 1288 probe set signals showed a change of 2-fold or greater when compared with the untreated condition upon the stimulation with IL-17A, IL-17F, TNF-α, IL-17A plus TNF-α, and IL-17F plus TNF-α, respectively. From these subsets, a common list of 2408 probe sets (1979 genes) showed a signal intensity of 30 or greater and was analyzed by hierarchical clustering using Spotfire (Spotfire DecisionSite 8.2) on Z-transformed row data. The NETAFFX web site (www.affymetrix.com) was used to select candidate genes. Two ways were used for independent confirmation of the results: in silico analysis using published results and experimental validation by real-time RT-PCR. Three levels of analysis were investigated for in silico validation: cell type specificity, constitutive mRNA expression, and inducible effect upon IL-17A or TNF-α stimulation. The validation by real-time RT-PCR was tested on four target genes (IL-6, IL-8, MMP-3, and CXCR4). Due to the allometric relation between PCR- and microarray-derived data, the correlation was calculated after log transformation.

Analysis of the cooperative effect between IL-17A or IL-17F and TNF-α

The following approach was used to quantify the cooperation between IL-17A or IL-17F and TNF-α from large-scale expression data sets. For each gene, H defined the value of gene induction expressed as fold change when compared with the untreated situation and I defined a variable which depends both on IL-17A and TNF-α pathways: $H_{\text{TNF + IL-17A}} = H_{\text{TNF}} + H_{\text{IL-17A}} + I_{\text{(TNF,IL-17A)}}$.

To avoid a bias leading to an underestimation of poorly expressed genes, data were normalized. H' defined the normalized values of induction and I' defined the associated value of cooperation as follows: $H'_{\text{TNF + IL-17A}} = H_{\text{TNF + IL-17A}}/(H_{\text{TNF}} + H_{\text{IL-17A}})$; $H'_{\text{TNF}} = H_{\text{TNF}}/(H_{\text{TNF}} + H_{\text{IL-17A}})$; and $H'_{\text{IL-17}} = H_{\text{IL-17A}}/(H_{\text{TNF}} + H_{\text{IL-17A}})$.

Thus, a cooperation index (I') was defined as follows: $I'_{\text{(IL-17A, TNF)}} = H'_{\text{TNF + IL-17A}} - (H'_{\text{TNF}} + H'_{\text{IL-17A}})$. I' was not calculable for 11 genes of 1979. A cutoff of 2 was arbitrarily taken to represent significant agreement beyond chance for genes to be classified in the group of inhibition ($I' < -2$), additivity ($-2 \leq I' < 2$), or synergy ($I' \geq 2$) depending on the type of cooperation between IL-17A or IL-17F and TNF-α. A part of the apparent cooperation effect might be artifactual due to random variations. To verify the statistical significance of I', a linear mixed model was used in which the variance of the cooperation index among genes was tested. The dependent covariate in this model was the signal and independent covariates were the experimental conditions (control, IL-17A, TNF-α, and IL-17A plus TNF-α). The four different signals of

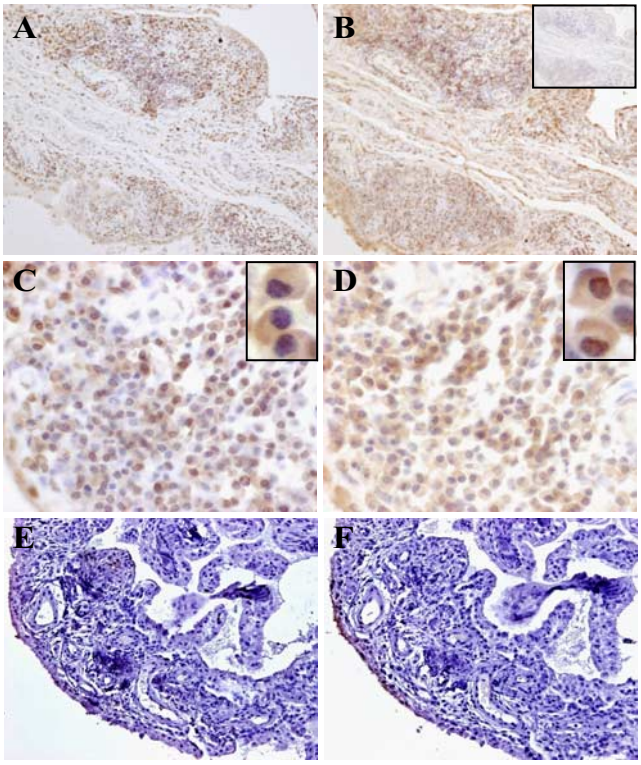


FIGURE 1. IL-17A- and IL-17F-producing cells are detected in the lymphocytic infiltrates of RA synovium. Immunostaining of paraffin-embedded RA serial sections using mouse anti-IL-17A mAb (original magnification for *A* and *C*, respectively, ×100 and ×600) and mouse anti-IL-17F mAb (original magnification for *B* and *D*, respectively, ×100 and ×600) shows a positive staining (brown) in the sublining area. IL-17A- and IL-17F-positive cells are mainly detected in lymphocytic infiltrates (*A* and *B*). IL-17A- and IL-17F-producing cells have a plasmacytoid-like appearance and a remote nucleus in a large cytoplasm (*inset*s in *C* and *D*). Sections of RA synovium with an irrelevant mouse IgG2a Ab were used as control (*inset* in *B*, original magnification, ×100). IL-17A and IL-17F immunostaining were analyzed in OA serial sections (original magnification for *E* and *F*, respectively, ×100).

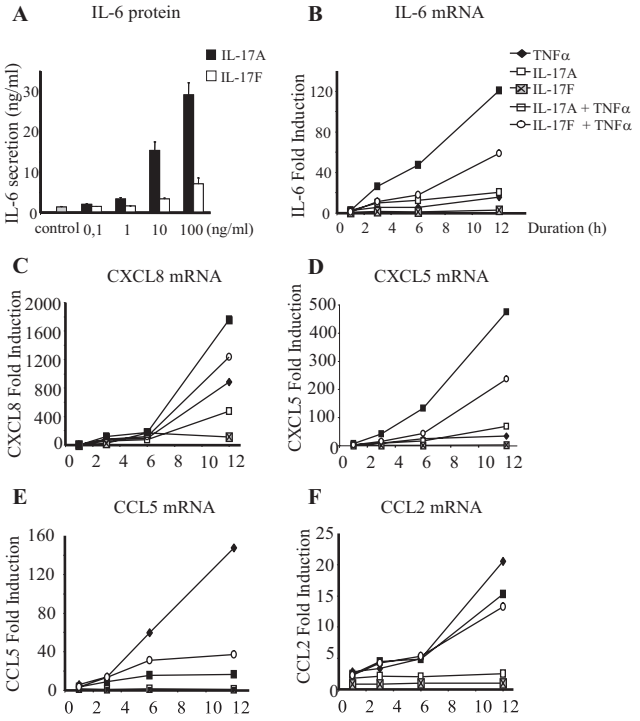


FIGURE 2. Effect of IL-17A and IL-17F, alone or in combination with TNF-α, on the expression of proinflammatory mediators. *A*, RA synoviocytes were stimulated for 48 h with of IL-17A or IL-17F (0.1–100 ng/ml) and IL-6 levels were quantified by ELISA. *B*, RA synoviocytes were stimulated for 48 h with IL-17A or IL-17F (50 ng/ml) alone or in combination with TNF-α (0.5 ng/ml) and IL-6 levels were quantified by ELISA. *C–F*, RA synoviocytes were stimulated for 1, 3, 6, and 12 h with IL-17A or IL-17F (50 ng/ml) alone or in combination with TNF-α (0.5 ng/ml). IL-6 (*C*), CXCL8/IL-8 (*D*), CXCL5/ENA-78 (*E*), and CCL5/RANTES (*F*) mRNA levels were quantified by real-time RT-PCR. The values, normalized with GAPDH mRNA expression, are expressed as the fold induction compared with the untreated condition. One representative experiment of three is shown.

each gene were considered as repeated measurements and I' was introduced as a random covariate. A non-null variance of I' was considered as evidence of heterogeneity of I' among genes. The three gene subsets (inhibition, addition, synergy) were compared for the presence of AU-rich elements (ARE) using the ARE-mRNA database (http://brp.kfshrc.edu.sa/ARED/) (17).

Immunofluorescence of TNFRII surface expression

Synoviocytes (10^5 cells/condition) were seeded in 12-mm cover slides and stimulated with cytokines. FcRs were blocked using PBS/3% BSA After 1 h of incubation with PE-conjugated anti-TNFRII, cells were fixed and permeabilized using BD Biosciences Cytofix/Cytoperm. Nuclear staining was performed by incubation with Hoechst for 1 h. Fluorescent images were captured by using a Nikon Eclipse 50i microscope connected to a Nikon digital sight DS-U1 camera and merged with the NIS-Elements F 2.20 software.

Statistical analysis

Protein levels were expressed as mean ± SEM. mRNA expression of target genes was normalized with GAPDH mRNA expression and data are expressed as the fold induction compared with untreated controls. Results are expressed as the mean ± SEM. Statistical inference was performed using Mann-Whitney U test, Pearson χ^2 tests, and mixed linear models. Values of $p < 0.05$ were considered statistically significant. All data are the result of at least three separate experiments.

Results

IL-17A and IL-17F are expressed in synovial tissue from RA patients

We have previously demonstrated the selective expression of IL-17A protein in RA synovial tissue when compared with OA samples (18). To extend this study to IL-17F, we performed immunohistochemical analysis of both IL-17A and IL-17F on serial sections from OA and RA synovium (Fig. 1). Both cytokines were expressed in RA but not OA synovial tissues and a more sustained staining for IL-17F compared with IL-17A was observed in the synovium from three different RA patients. IL-17A and IL-17F were expressed in the same areas, particularly in lymphocytic aggregates and hyperplasic lining cells (Fig. 1, *A* and *B*). Moreover, IL-17F-secreting cells had a plasma cell-like morphology similar to that we had described for IL-17A-secreting cells (Fig. 1, *inset* in *C* and *D*) (18).

Effect of IL-17A or IL-17F, alone or in combination with TNF-α, on RA synoviocytes

Although the effect of IL-17A, alone or in combination with TNF-α, has been studied in RA synoviocytes, the effect of IL-17F still remained to be clarified. First, we compared the effect of IL-17A and IL-17F (0.1–100 ng/ml) on IL-6 secretion by synoviocytes. IL-17F dose-dependently induced IL-6 secretion, but to a lesser extent compared with IL-17A (Fig. 2*A*). At 100 ng/ml,

Table I. *In silico validation of microarray data sets*

Identification	Microarray Data			Literature	
	Gene	Signal	Detection	Notes	Ref.
Celltypecharacterization					
215313_x_at	HLA-A	6710.9	p	Nucleated human cells	19
203507_at	CD68	110.9	p	Macrophages	19
205456_at	CD3E	7.9	A	T cells	19
202112_at	VWF	4.7	A	Endothelial cells	19
206398_s_at	CD 19	6.6	A	B cells	19
208850_s_at	CD90/THY1	1371.5	p	Fibroblasts	20, 21
212063_at	CD44	5433.5	p	Fibroblasts	22
222125_s_at	PH-4	319.3	p	Fibroblasts	23
201925_s_at	CD 55	584.8	p	Type B FLS	24
Constitutive expression					
212077_at	CALD1	3224.8	p	Constitutive[a]	25
201262_s_at	BGN	816.3	p	Constitutive[a]	25
205679_x_at	AGC1	792.5	p	Constitutive[a]	25
203868_s_at	VCAM-1	813.2	p	Constitutive[a]	26, 27
211959_at	IGFBP5	2022.6	p	Constitutive[a]	26, 28
209687_at	CXCL12	371.3	p	Constitutive[a]	26, 28
208677_s_at	BSG/CD147	769.9	p	Constitutive[a]	29
201069_at	MMP2	4965.4	p	Constitutive[a]	29
207433_at	IL-10	1.7	A	Not expressed	19
207844_at	IL-13	5.8	A	Not expressed	19
207113_s_at	TNF	3.2	A	Not expressed	19
217371_s_at	IL-15	12.7	A	Not expressed	19
206295_at	IL-18	8	A	Not expressed	19
205207_at	IL-6	703.4	p	Constitutive	19, 25
209774_x_at	CXCL2	4.9	A	Low in RA[b]	25
206336_at	CXCL6	11.6	p	Low in RA[b]	25

Identification	Gene	Microarray data				Literature	
		IL-17A		TNF-α			
		Fold change	*p*	Fold change	*p*	Note	Ref.
Inducible expression							
205476_at	CCL20	548.7	0.00002	238.9	0.00002	↑ by IL-17A ↑ by TNF-α	16
207386_at	CYP7B1	2.6	0.2	7.0	0.00003	↑ by IL-17A ↑ by TNF-α	30, 31
220054_at	IL-23A	22.6	0.00002	16.0	0.00008	↑ by IL-17A ↑ by TNF-α	34
207160_at	IL-12A	1.0	0.5	1.0	0.8	∅ by IL-17A ∅ by TNF-α	34
203868_s_at	VCAM-1	−1.9	0.9	3.7	0.00002	∅ by IL-17A ↑ by TNF-α	18, 35
205207_at	IL-6	14.9	0.00002	9.2	0.00002	↑ by IL-17A ↑ by TNF-α	6, 32
205266_at	LIF	8.6	0.00002	4.6	0.00003	↑ by IL-17A ↑ by TNF-α	32, 33
211506_s_at	IL-8	207.9	0.00002	208.0	0.00002	↑ by IL-17A ↑ by TNF-α	6
207442_at	CSF3	9.2	0.009	1.1	0.5	↑ by IL-17A ? by TNF-α	6

[a] More specifically expressed in RA syn compared to OA.
[b] More specifically expressed in OA syn compared to RA.

IL-17A induced a 4-fold higher induction of IL-6 secretion compared with IL-17F (29.2 ± 3 ng/ml vs 7.1 ± 1.4 ng/ml, respectively, $p < 0.05$). We compared the effect of IL-17A and IL-17F in the presence of TNF-α and demonstrated that although IL-17F alone had a limited effect alone, it potently induced IL-6 mRNA expression in the presence of TNF-α (18-fold increase compared with the basal level; Fig. 2*B*).

We then examined by real-time RT-PCR the time course expression of target genes known to be induced (IL-6, IL-8/CXCL8, and CXCL5) (19–21) or repressed (RANTES/CCL5) by IL-17A. IL-17A, IL-17F, or TNF-α induced IL-6 mRNA expression as early as 1 h, which increased over 12 h, leading to a 20-, 3-, and 16-fold induction of IL-6 mRNA levels, respectively. After 12 h of exposure, the combination of IL-17A and TNF-α induced a 2-fold induction of IL-6 mRNA expression compared with the combination of IL-17F plus TNF-α (120- and 60-fold increase compared with the basal level; Fig. 2*C*). IL-17F had a limited effect on IL-8 mRNA levels compared with IL-17A. At 12 h of stimulation, IL-17A induced a 30-fold higher in IL-8 mRNA expression compared with IL-17F alone. In the presence of TNF-α, both IL-17A and IL-17F further induced IL-8 mRNA expression (Fig. 2*D*). We observed a similar trend in ENA-78/CXCL5 mRNA expression: at 12 h of stimulation, IL-17A, IL-17F, or TNF-α alone induced a 69-, 2.5-, and 35-fold induction, respectively, which was further enhanced upon combined stimulation with IL-17A or IL-17F plus TNF-α (Fig. 2*E*). IL-17A or IL-17F had no significant effect on RANTES mRNA expression between 1 and 12 h of stimulation, whereas TNF-α was effective, with a 147-fold increase over control at 12 h. We observed that not only IL-17A, but also IL-17F induced a massive inhibition of TNF-α-induced RANTES expression. At 12 h, IL-17A or IL-17F inhibited 89 or 75% of TNF-α-induced RANTES mRNA expression, respectively (Fig. 2*F*).

Transcript profiling data analysis and validation

To further extend these studies to a large number of genes, mRNA profiles from IL-17A- and IL-17F-stimulated cells were studied using microarrays. The expression of several cell-specific markers

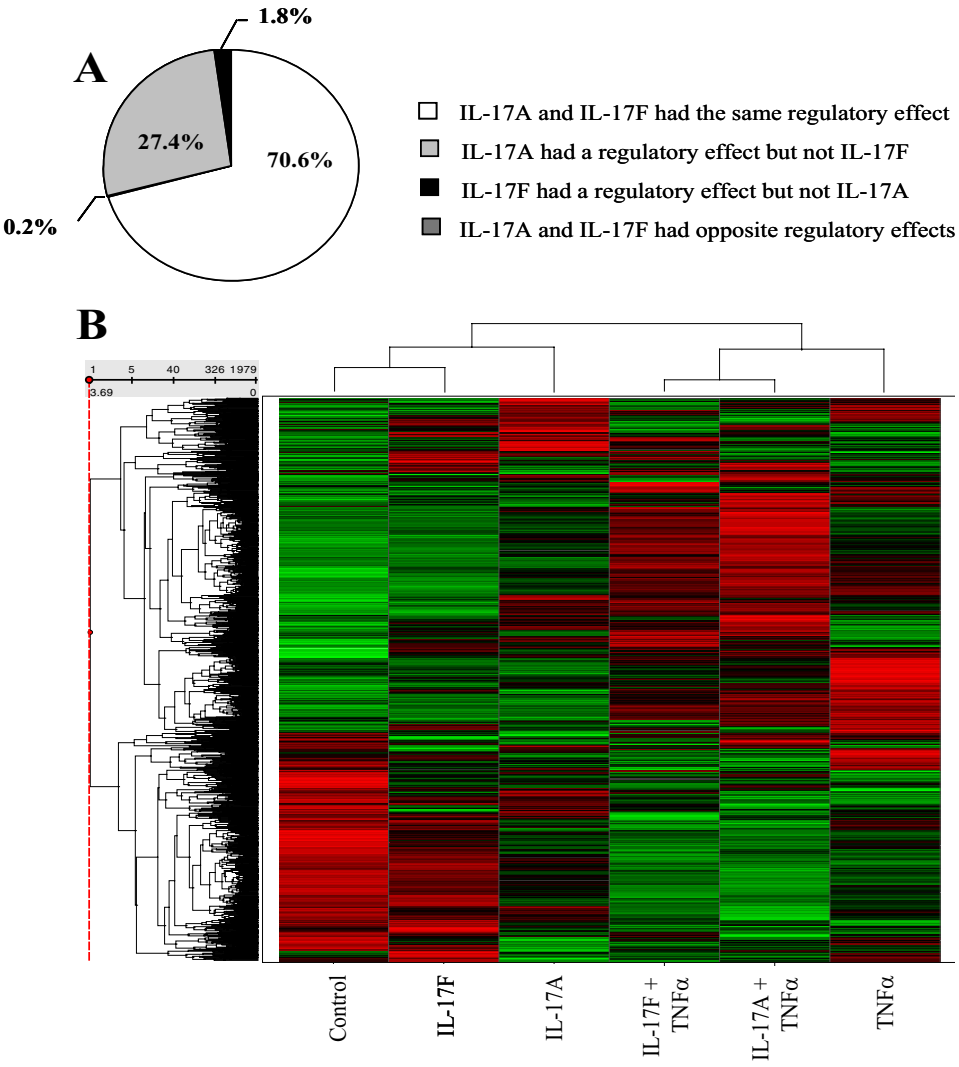


FIGURE 3. Microarray-based comparison of IL-17A- and IL-17F-induced effects alone or in combination with TNF-α. RA synoviocytes were stimulated with IL-17A or IL-17F (50 ng/ml) alone or in combination with TNF-α (0.5 ng/ml) for 12 h. Gene products that showed a signal intensity of 30 or greater were considered as expressed and genes with a 2-fold or greater change were considered as regulated (see *Materials and Methods*). *A*, The regulatory effects of IL-17A and IL-17F were compared using the comparison call from the MAS 5.0 algorithm. Among the 601 genes selected for this analysis, 424 were regulated in the same way by IL-17A and IL-17F (70.6%), 165 were exclusively regulated by IL-17A (27.4%), 11 were exclusively regulated by IL-17F (1.8%), and only 1 gene was regulated in the opposite way by IL-17A and IL-17F (0.2%). *B*, The analysis of IL-17A- and IL-17F-induced expression profile, alone or in combination with TNF-α, was performed on Z-transformed row data through hierarchical clustering (Spotfire DecisionSite 8.2).

was analyzed to characterize the population used in the experiment. As described in Table I, the synovium-cell derived population was negative for CD19, CD3E, von Willebrand factor, markers which are specifically expressed by B cells, T cells, and endothelial cells, respectively (22). CD68, a marker of monocytes/macrophages, was expressed at very low levels, whereas high levels of fibroblastic markers CD90 (23, 24), CD44 (25), prolyl 4-hydroxylase (26), and CD55 (27) were detected. These results indicated that the cell population was mainly composed of type B synoviocytes, with a residual proportion of type A synoviocytes. These results extend the observations made at the protein level by flow cytometry with cells >99% negative for CD3, CD19, and CD14.

As a way of confirmation, we also analyzed the mRNA expression of genes based on published knowledge from synoviocyte studies. Table I shows genes known to be constitutively overexpressed in RA synoviocytes compared with OA synoviocytes (caldesmon 1 (28), biglycan (28), aggrecan 1 (28), VCAM-1 (29, 30), IGFBP-5 (29, 31), CXCL12 (29, 31), CD147/basigin (32), MMP-2 (32), and IL-6 (22, 28)), genes known to be expressed at low levels compared with OA synoviocytes (CXCL2 (28) and CXCL6 (28)) and genes known not to be expressed in RA synoviocytes (IL-10, IL-13, TNF-α, IL-15, and IL-18 (22)). The accuracy of our microarray datasets was further tested through the analysis of expression data upon stimulation (Table I). The known regulatory effects of IL-17A or TNF-α on RA synoviocytes were confirmed in our microarray data sets. This included the positive regulatory effect of IL-17A and TNF-α on CCL20 (20), CYP7B1 (33, 34), IL-6 (12, 35), LIF (35, 36), IL-8 (12), and IL-23p19 (37) expression, along with the specific regulatory effect of TNF-α on VCAM-1 mRNA expression (38, 39). Moreover, no regulatory effect on IL-12p35 mRNA expression was observed upon IL-17A or TNF-α stimulation, as previously described (37). Finally, real-time RT-PCR experiments performed with cells from four different RA donors confirmed the regulatory effects of IL-17A and TNF-α on IL-6 and IL-8 mRNA expression.

Comparison between IL-17A- and IL-17F-induced regulatory effect

Among the 601 genes considered as significantly expressed and regulated upon IL-17A and/or IL-17F stimulation, we observed that IL-17A and IL-17F had similar regulatory effects on 424 genes (70.6%; Fig. 3*A*). Only one gene, CDC42EP3, was classified as regulated in the opposite way by IL-17A and IL-17F. However, such regulatory effect was not significant (−1.9 and +2.0-fold induction after IL-17A and IL-17F stimulation, respectively; NS). The lack of significant effect was confirmed by real-time RT-PCR (+1.5 and +2.0-fold induction after IL-17A and IL-17F stimulation respectively; NS). Interestingly, 165 genes were specifically regulated by IL-17A (27.4%) but only 11 were specifically regulated by IL-17F (1.8%). Again, the level of induction and/or expression in the latter group of genes was poorly significant. Based

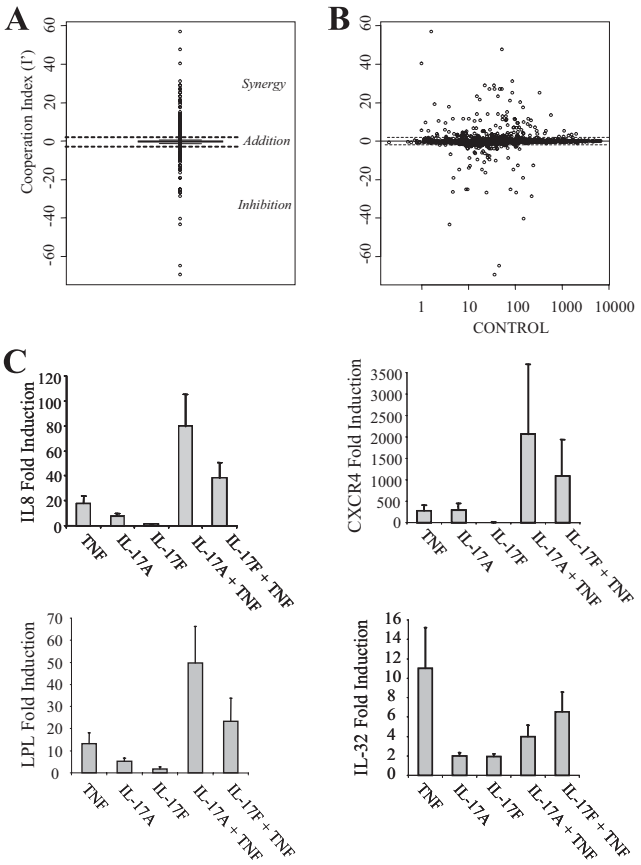


FIGURE 4. Cooperative effect between IL-17A and TNF-α. The cooperation index was used to analyze gene mRNA expression upon IL-17A and/or TNF-α stimulation (see *Materials and Methods*). Accordingly, gene expression patterns were classified as inhibition, additivity, or synergy (*A* and *B*). *A*, Distribution of genes according to the cooperation index. *B*, Distribution of the cooperation index according to control signal values. *C*, Validation of target gene mRNA expression by real-time RT-PCR. Four genes were validated by real-time RT-PCR: IL-8, CXCR4, LPL, and IL-32. The values were normalized with GAPDH mRNA expression and expressed as the fold induction compared with the untreated condition. Values represent the mean ± SEM of four independent experiments.

on this analysis, IL-17A and IL-17F had a very similar regulatory effect on RA synoviocytes (McNemar's χ^2: 101; df = 1; $p < 0.00001$), but at the same time, IL-17A regulated the expression of more genes than IL-17F (McNemar's χ^2: 133; df = 1; $p < 0.00001$).

We then compared IL-17A- and IL-17F-induced signals in the presence of TNF-α. A subset of 1979 genes considered as significantly expressed and regulated upon stimulation with at least one stimulus was analyzed by hierarchical clustering (Fig. 3*B*). IL-17A induced a distinct expression profile when compared with TNF-α, as demonstrated by the metric distance in the hierarchical clustering. The profile induced by IL-17F was closer to the control than to IL-17A-stimulated cells. In the presence of TNF-α, the expression patterns of IL-17A and IL-17F were rather similar.

Cooperative effect between IL-17A or IL-17F and TNF-α

To study the cooperative effect between IL-17A or IL-17F and TNF-α, we used the cooperation index (I') resulting in inhibitory, additive, or synergistic interactions (see *Materials and Methods* for details). Fig. 4*A* represents the distribution of genes according to I' and showed the presence of outlier genes. These outliers were either positive ($I'_{(IL17A,\ TNF)} > 2$; synergy) or negative ($I'_{(IL17A,\ TNF)} < 2$; inhibition). Fig. 4*B* shows that these cooperation effects were observed for genes independently of signal intensity. The linear mixed model showed that the observed variability of the cooperation index (I') was statistically significant (likelihood ratio test: 34.17; 2 df; $p < 0.0001$). Among the 1968 genes, 80 genes showed a pattern of inhibition ($I'_{(IL17A,\ TNF)} < 2$), 1758 a pattern of additivity ($-2 < I'_{(IL17A,\ TNF)} < 2$), and 130 a pattern of synergy ($I'_{(IL17A,\ TNF)} > 2$) according to the cooperation index. Among the genes synergistically induced by IL-17A or IL-17F and TNF-α, we found again IL-6, IL-8, CXCL5, but also IL-23p19, E-selectin, Egr-1, and G-CSF. We validated by real-time RT-PCR the synergistic induction of IL-8, a chemoattractant for neutrophils, of CXCR4, a chemokine receptor implicated in the migration of hematopoietic and metastatic cells, and of LPL, which catalyzes the hydrolysis of chylomicrons and very low-density lipoproteins (Fig. 4*C*). Furthermore, we validated the inhibitory effect of IL-17A and IL-17F on TNF-α-induced IL-32 expression, a proinflammatory cytokine

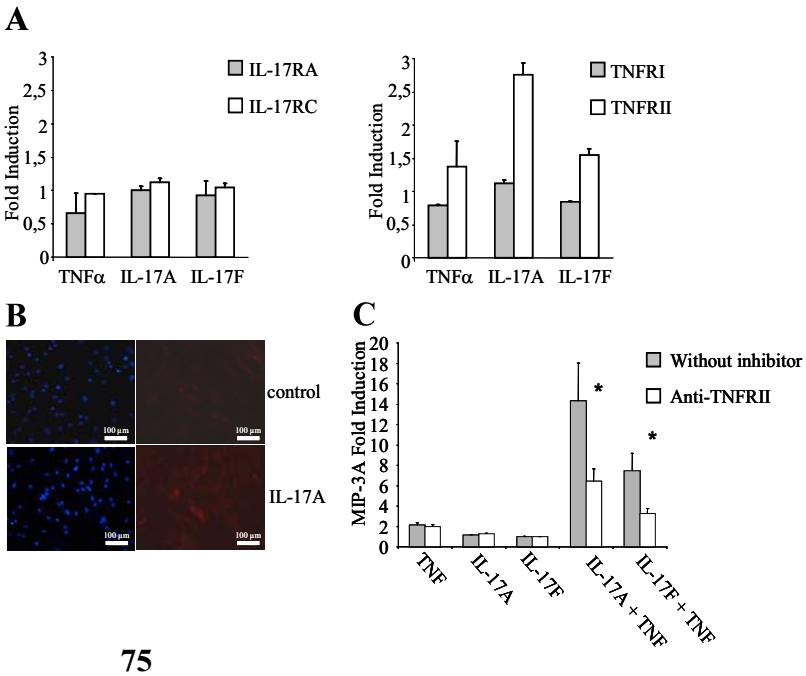


FIGURE 5. IL-17- and TNF-associated receptor expression and contribution to IL-17A or IL-17F and TNF-α synergistic effect. *A*, RA synoviocytes were stimulated with IL-17A or IL-17F (50 ng/ml) alone or in combination with TNF-α (0.5 ng/ml) for 3 h. IL-17RA, IL-17RC (*A*), TNFR type I, and type II mRNA levels were quantified by real-time RT-PCR. Levels were normalized with GAPDH mRNA expression and expressed as fold induction compared with the untreated condition. Values represent the mean ± SEM of four independent experiments. *B*, RA synoviocytes were stimulated for 12 h with or without IL-17A (50 ng/ml) and TNFRII was detected by immunofluorescence. One representative result among three is shown. *C*, RA synoviocytes were preincubated for 4 h with anti-TNFRII (10 μg/ml) and then stimulated for 12 h with IL-17A or IL-17F (50 ng/ml) alone or in combination with TNF-α (0.5 ng/ml). CCL20/MIP-3α protein levels were quantified by ELISA. Values represent the mean ± SEM of three independent experiments.

overexpressed in RA synoviocytes when compared with OA synoviocytes (40). Additional examples of such regulation can be found in a supplemental file[4] added to this article.

Mechanisms implicated in the regulatory effects of IL-17A or IL-17F alone or in combination with TNF-α

Multiple mechanisms are likely to drive cooperation between IL-17A or IL-17F and TNF-α in RA synoviocytes. Among them, posttranscriptional regulations, mainly through ARE, have been previously described (41). Thus, we looked for the presence of ARE in our gene subsets using the ARE-mRNA database (http://brp.kfshrc.edu.sa/ARED/). No ARE enrichment was found in synergistically induced genes ($n = 130$ genes) when compared with the subsets of additivity and inhibition ($n = 1818$ genes) (χ^2: 0.7091; df = 1; $p = 0.4$). Moreover, the mRNA expression of the well-known ARE-BP (AUF1, TTP, HuR, TIAR, TIA-1, BRF1; (42)) was not regulated by IL-17A or IL-17F alone or in combination with TNF-α (data not shown). We then tested whether a feedback mechanism involving IL-17A/F or TNF receptors could be associated with the cooperative effect between IL-17A/F and TNF-α. The time course mRNA expression of IL-17RA, IL-17RC, TNFR type 1 (p55), and TNFR type 2 (p75) was studied upon IL-17A or IL-17F stimulation alone or in combination with TNF-α (data not shown). The stimulation with TNF-α had no effect on IL-17RA, IL-17RC, TNFR type 1, or TNFR type 2 in RA synoviocytes. Conversely, IL-17A or IL-17F alone or in combination with TNF-α up-regulated TNFRII mRNA expression, which peaked after 3–6 h of stimulation, whereas the expression of TNFRI, IL-17RA and IL-17RC remained stable (Fig. 5*A*). We showed by immunofluorescence an up-regulation of TNFRII surface expression upon IL-17A stimulation (Fig. 5*B*). Finally, we showed that TNFRII neutralization using a mAb inhibited the synergistic effects of IL-17A or IL-17F and TNF-α on CCL20/MIP-3α (Fig. 5*C*). Thus, part of the cooperation effects between IL-17A/F and TNF-α is possibly mediated at the level of receptor expression.

Discussion

The recent discovery of Th17 cells as additional effector T cells distinct from Th1 and Th2 cells challenged the traditional concept of a Th1-driven RA disease (43). These cells are characterized by the production of a distinct profile of effector cytokines, including IL-17A, IL-17F, IL-21, and IL-22. In RA patients, IL-17A is present in both synovium and synovial fluid (4, 44). We showed here that both IL-17A and IL-17F are expressed within RA synovium, whereas no expression was detected in OA samples. IL-17F-expressing cells are characterized by a plasma cell-like morphology, with a large nucleus and a reduced cytoplasm, as we have previously described for IL-17A (18). Moreover, IL-17F expression was strongest than IL-17A in RA synovial tissues. These observations are consistent with previous studies reporting higher levels of IL-17F secretion compared with IL-17A upon stimulation of $CD4^+$ T cells (10, 14, 45).

Using high throughput microarrays, we showed that IL-17A and IL-17F have a significant similar regulatory effect on RA synoviocytes, IL-17F being less active quantitatively. Moreover, IL-17A and IL-17F induced similar expression profiles in the presence of TNF-α, as demonstrated by the hierarchical clustering analysis. These expression data, taken together with the specific expression of IL-17A and IL-17F in RA synovium, are in line with a role for both cytokines in joint degradation, neutrophil attraction, and angiogenesis in the context of chronic inflammation.

The cooperative effect between IL-17A and TNF-α was analyzed using the cooperation index. The majority of regulated genes were additively induced by IL-17A and TNF-α (89%), whereas the patterns of inhibition and synergy only represented 11% of the relevant genes. Among the synergistically induced genes, we validated by real-time RT-PCR the regulation of LPL and CXCR4. CXCR4 is a chemokine receptor mainly expressed on immune cells, except for stem cells and tumor cells in which CXCR4 play a key role for tissue homing. Its expression on synoviocytes is still controversial (46–48). Our report demonstrates for the first time the inducible expression of CXCR4 on RA synoviocytes. Indeed, IL-17A and IL-17F synergistically induced CXCR4 in the presence of TNF-α, while no regulatory effect was seen for all other chemokine receptors (data not shown). The cognate ligand of CXCR4 is CXCL12/stromal-derived factor-1, which is overexpressed in synovial tissue and synovial fluid from RA patients compared with OA patients (46, 49, 50). In RA joints, its expression is detected in synovial lining, synovial high endothelial venules, and synovial fluid (46, 49, 51). Thus, these data suggest a role for IL-17A and IL-17F in synoviocyte migration toward the CXCL12 gradient.

We showed that both IL-17A and IL-17F inhibited TNF-α-induced IL-32 mRNA expression. IL-32 is overexpressed in RA synovium when compared with OA, and its expression is mainly detected in lymphocytic infiltrates (52, 53). Furthermore, IL-32 was associated with disease severity (52). Interestingly, it was shown in epithelial cells that IFN-γ and IL-18 were potent inducers of IL-32 expression (54). Its induction by Th1-associated cytokines remains to be established in synoviocytes to clarify whether IL-17-induced IL-32 inhibition could represent a negative regulatory mechanism of the Th1 pathway.

We showed that the differences in expression patterns (inhibition, addition, synergy) were not linked to a preferential distribution of ARE in the promoter of target genes nor to a specific regulation of ARE-BP. Although ARE, which are key players in the mRNA life time, were found in the 3′ untranslated region of many inflammatory mediators (55), they may not be crucially involved in synergistic cooperation between IL-17A or IL-17F and TNF-α. Conversely, both IL-17A and IL-17F up-regulated TNFR type 2 receptor mRNA and protein expression. Such a positive regulatory loop may in part participate in the potentiating effect of IL-17A or IL-17F on TNF-α-induced target gene mRNA expression, as demonstrated by the effect of TNFRII neutralization on CCL20/MIP-3α secretion. In human bronchial epithelial cells, it has been demonstrated that blockade of both TNFRI or TNFRII down-regulated IL-17A or IL-17F plus TNF-α induced G-CSF secretion (56). Finally, the distribution of the cooperation index highlighted the complexity of the networks implicated in gene regulation. Previous studies supported the existence of multiple mechanisms implicated in the cross-talk between IL-17- and TNF-associated pathways. These could lead to either activation or inhibition of gene expression: activation of signaling pathways (MAPKs, Akt/protein kinase B) (57, 58), recruitment of specific transcription factors (C/EBP, NF-κB, AP-1, IFN regulatory factor 1) (59–61), and mRNA stabilization (41).

In conclusion, we demonstrated that IL-17A and IL-17F are specifically expressed in RA synovium when compared with OA. In RA synoviocytes, both cytokines induced a similar expression pattern in the presence of TNF-α. Accordingly, IL-17F appears as a target in Th17-mediated diseases such as RA.

Disclosures

The authors have no financial conflict of interest.

[4] The online version of this article contains supplemental material.

References

1. Kirkham, B. W., M. N. Lassere, J. P. Edmonds, K. M. Juhasz, P. A. Bird, C. S. Lee, R. Shnier, and I. J. Portek. 2006. Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort). *Arthritis Rheum.* 54: 1122–1131.
2. Lubberts, E., M. I. Koenders, B. Oppers-Walgreen, L. van den Bersselaar, C. J. Coenen-de Roo, L. A. Joosten, and W. B. van den Berg. 2004. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. *Arthritis Rheum.* 50: 650–659.
3. Nakae, S., A. Nambu, K. Sudo, and Y. Iwakura. 2003. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J. Immunol.* 171: 6173–6177.
4. Kotake, S., N. Udagawa, N. Takahashi, K. Matsuzaki, K. Itoh, S. Ishiyama, S. Saito, K. Inoue, N. Kamatani, M. T. Gillespie, et al. 1999. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J. Clin. Invest.* 103: 1345–1352.
5. Van Bezooijen, R. L., L. Van Der Wee-Pals, S. E. Papapoulos, and C. W. Lowik. 2002. Interleukin 17 synergises with tumour necrosis factor α to induce cartilage destruction in vitro. *Ann. Rheum Dis.* 61: 870–876.
6. Koshy, P. J., N. Henderson, C. Logan, P. F. Life, T. E. Cawston, and A. D. Rowan. 2002. Interleukin 17 induces cartilage collagen breakdown: novel synergistic effects in combination with proinflammatory cytokines. *Ann. Rheum Dis.* 61: 704–713.
7. Parsonage, G., A. Filer, M. Bik, D. Hardie, S. Lax, K. Howlett, L. D. Church, K. Raza, S. H. Wong, E. Trebilcock, et al. 2008. Prolonged, granulocyte-macrophage colony-stimulating factor-dependent, neutrophil survival following rheumatoid synovial fibroblast activation by IL-17 and TNFα. *Arthritis Res. Ther.* 10: R47.
8. Ivanov, I. I., B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17$^+$ T helper cells. *Cell* 126: 1121–1133.
9. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.
10. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4$^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6: 1123–1132.
11. Hymowitz, S. G., E. H. Filvaroff, J. P. Yin, J. Lee, L. Cai, P. Risser, M. Maruoka, W. Mao, J. Foster, R. F. Kelley, et al. 2001. IL-17s adopt a cysteine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* 20: 5332–5341.
12. Fossiez, F., O. Djossou, P. Chomarat, L. Flores-Romo, S. Ait-Yahia, C. Maat, J. J. Pin, P. Garrone, E. Garcia, S. Saeland, et al. 1996. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J. Exp. Med.* 183: 2593–2603.
13. Liang, S. C., A. J. Long, F. Bennett, M. J. Whitters, R. Karim, M. Collins, S. J. Goldman, K. Dunussi-Joannopoulos, C. M. Williams, J. F. Wright, and L. A. Fouser. 2007. An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. *J. Immunol.* 179: 7791–7799.
14. Chang, S. H., and C. Dong. 2007. A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. *Cell Res.* 17: 435–440.
15. Toy, D., D. Kugler, M. Wolfson, T. Vanden Bos, J. Gurgel, J. Derry, J. Tocker, and J. Peschon. 2006. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J. Immunol.* 177: 36–39.
16. Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. McShane, J. F. Fries, N. S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, H. S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31: 315–324.
17. Bakheet, T., M. Frevel, B. R. Williams, W. Greer, and K. S. Khabar. 2001. ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Res.* 29: 246–254.
18. Page, G., A. Sattler, S. Kersten, A. Thiel, A. Radbruch, and P. Miossec. 2004. Plasma cell-like morphology of Th1-cytokine-producing cells associated with the loss of CD3 expression. *Am. J. Pathol.* 164: 409–417.
19. Ruddy, M. J., F. Shen, J. B. Smith, A. Sharma, and S. L. Gaffen. 2004. Interleukin-17 regulates expression of the CXC chemokine LIX/CXCL5 in osteoblasts: implications for inflammation and neutrophil recruitment. *J. Leukocyte Biol.* 76: 135–144.
20. Chabaud, M., G. Page, and P. Miossec. 2001. Enhancing effect of IL-1, IL-17, and TNF-α on macrophage inflammatory protein-3α production in rheumatoid arthritis: regulation by soluble receptors and Th2 cytokines. *J. Immunol.* 167: 6015–6020.
21. Zrioual, S., M. L. Toh, A. Tournadre, Y. Zhou, M. A. Cazalis, A. Pachot, V. Miossec, and P. Miossec. 2008. IL-17RA and IL-17RC receptors are essential for IL-17A-induced ELR$^+$ CXC chemokine expression in synoviocytes and are overexpressed in rheumatoid blood. *J. Immunol.* 180: 655–663.
22. Hirth, A., A. Skapenko, R. W. Kinne, F. Emmrich, H. Schulze-Koops, and U. Sack. 2002. Cytokine mRNA and protein expression in primary-culture and repeated-passage synovial fibroblasts from patients with rheumatoid arthritis. *Arthritis Res.* 4: 117–125.
23. Palmer, D. G., Y. Selvendran, C. Allen, P. A. Revell, and N. Hogg. 1985. Features of synovial membrane identified with monoclonal antibodies. *Clin. Exp. Immunol.* 59: 529–538.
24. Saalbach, A., R. Kraft, K. Herrmann, U. F. Haustein, and U. Anderegg. 1998. The monoclonal antibody AS02 recognizes a protein on human fibroblasts being highly homologous to Thy-1. *Arch. Dermatol. Res.* 290: 360–366.
25. Henderson, K. J., J. C. Edwards, and J. G. Worrall. 1994. Expression of CD44 in normal and rheumatoid synovium and cultured synovial fibroblasts. *Ann. Rheum Dis.* 53: 729–734.
26. Schwachula, A., D. Riemann, A. Kehlen, and J. Langner. 1994. Characterization of the immunophenotype and functional properties of fibroblast-like synoviocytes in comparison to skin fibroblasts and umbilical vein endothelial cells. *Immunobiology* 190: 67–92.
27. Hamann, J., J. O. Wishaupt, R. A. van Lier, T. J. Smeets, F. C. Breedveld, and P. P. Tak. 1999. Expression of the activation antigen CD97 and its ligand CD55 in rheumatoid synovial tissue. *Arthritis Rheum.* 42: 650–658.
28. Scaife, S., R. Brown, S. Kellie, A. Filer, S. Martin, A. M. Thomas, P. F. Bradfield, N. Amft, M. Salmon, and C. D. Buckley. 2004. Detection of differentially expressed genes in synovial fibroblasts by restriction fragment differential display. *Rheumatology* 43: 1346–1352.
29. Seki, T., J. Selby, T. Haupl, and R. Winchester. 1998. Use of differential subtraction method to identify genes that characterize the phenotype of cultured rheumatoid arthritis synoviocytes. *Arthritis Rheum.* 41: 1356–1364.
30. Schwarting, A., J. Schlaak, J. Lotz, I. Pfers, K. H. Meyer zum Buschenfelde, and W. J. Mayet. 1996. Endothelin-1 modulates the expression of adhesion molecules on fibroblast-like synovial cells (FLS). *Scand. J. Rheumatol.* 25: 246–256.
31. Kasperkovitz, P. V., T. C. Timmer, T. J. Smeets, N. L. Verbeet, P. P. Tak, L. G. van Baarsen, B. Baltus, T. W. Huizinga, E. Pieterman, M. Fero, et al. 2005. Fibroblast-like synoviocytes derived from patients with rheumatoid arthritis show the imprint of synovial tissue heterogeneity: evidence of a link between an increased myofibroblast-like phenotype and high-inflammation synovitis. *Arthritis Rheum.* 52: 430–441.
32. Zhu, P., N. Lu, Z. G. Shi, J. Zhou, Z. B. Wu, Y. Yang, J. Ding, and Z. N. Chen. 2006. CD147 overexpression on synoviocytes in rheumatoid arthritis enhances matrix metalloproteinase production and invasiveness of synoviocytes. *Arthritis Res. Ther.* 8: R44.
33. Dulos, J., A. Kaptein, A. Kavelaars, C. Heijnen, and A. Boots. 2005. Tumour necrosis factor-α stimulates dehydroepiandrosterone metabolism in human fibroblast-like synoviocytes: a role for nuclear factor-κB and activator protein-1 in the regulation of expression of cytochrome p450 enzyme 7b. *Arthritis Res. Ther.* 7: R1271–R1280.
34. Dulos, J., M. A. van der Vleuten, A. Kavelaars, C. J. Heijnen, and A. M. Boots. 2005. CYP7B expression and activity in fibroblast-like synoviocytes from patients with rheumatoid arthritis: regulation by proinflammatory cytokines. *Arthritis Rheum.* 52: 770–778.
35. Chabaud, M., F. Fossiez, J. L. Taupin, and P. Miossec. 1998. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J. Immunol.* 161: 409–414.
36. Dechanet, J., J. L. Taupin, P. Chomarat, M. C. Rissoan, J. F. Moreau, J. Banchereau, and P. Miossec. 1994. Interleukin-4 but not interleukin-10 inhibits the production of leukemia inhibitory factor by rheumatoid synovium and synoviocytes. *Eur. J. Immunol.* 24: 3222–3228.
37. Kim, H. R., M. L. Cho, K. W. Kim, J. Y. Juhn, S. Y. Hwang, C. H. Yoon, S. H. Park, S. H. Lee, and H. Y. Kim. 2007. Up-regulation of IL-23p19 expression in rheumatoid arthritis synovial fibroblasts by IL-17 through PI3-kinase-, NF-κB- and p38 MAPK-dependent signalling pathways. *Rheumatology* 46: 57–64.
38. Marlor, C. W., D. L. Webb, M. P. Bombara, J. M. Greve, and M. L. Blue. 1992. Expression of vascular cell adhesion molecule-1 in fibroblastlike synoviocytes after stimulation with tumor necrosis factor. *Am. J. Pathol.* 140: 1055–1060.
39. Schnyder, B., S. Schnyder-Candrian, A. Pansky, M. L. Schmitz, M. Heim, B. Ryffel, and R. Moser. 2005. IL-17 reduces TNF-induced RANTES and VCAM-1 expression. *Cytokine* 31: 191–202.
40. Cagnard, N., F. Letourneur, A. Essabbani, V. Devauchelle, S. Mistou, A. Rapinat, C. Decraene, C. Fournier, and G. Chiocchia. 2005. Interleukin-32, CCL2, PF4F1 and GFD10 are the only cytokine/chemokine genes differentially expressed by in vitro cultured rheumatoid and osteoarthritis fibroblast-like synoviocytes. *Eur. Cytokine Network* 16: 289–292.
41. Hartupee, J., C. Liu, M. Novotny, X. Li, and T. Hamilton. 2007. IL-17 enhances chemokine gene expression through mRNA stabilization. *J. Immunol.* 179: 4135–4141.
42. Wilusz, C. J., M. Wormington, and S. W. Peltz. 2001. The cap-to-tail guide to mRNA turnover. *Nat. Rev. Mol. Cell Biol.* 2: 237–246.
43. Toh, M. L., and P. Miossec. 2007. The role of T cells in rheumatoid arthritis: new subsets and new targets. *Curr. Opin. Rheumatol.* 19: 284–288.
44. Chabaud, M., J. M. Durand, N. Buchs, F. Fossiez, G. Page, L. Frappart, and P. Miossec. 1999. Human interleukin-17: a T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum.* 42: 963–970.
45. Wright, J. F., Y. Guo, A. Quazi, D. P. Luxenberg, F. Bennett, J. F. Ross, Y. Qiu, M. J. Whitters, K. N. Tomkinson, K. Dunussi-Joannopoulos, et al. 2007. Identification of an interleukin 17F/17A heterodimer in activated human CD4$^+$ T cells. *J. Biol. Chem.* 282: 13447–13455.
46. Kanbe, K., K. Takagishi, and Q. Chen. 2002. Stimulation of matrix metalloprotease 3 release from human chondrocytes by the interaction of stromal cell-derived factor 1 and CXC chemokine receptor 4. *Arthritis Rheum.* 46: 130–137.

47. Nanki, T., K. Nagasaka, K. Hayashida, Y. Saita, and N. Miyasaka. 2001. Chemokines regulate IL-6 and IL-8 production by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *J. Immunol.* 167: 5381–5385.
48. Garcia-Vicuna, R., M. V. Gomez-Gaviro, M. J. Dominguez-Luis, M. K. Pec, I. Gonzalez-Alvaro, J. M. Alvaro-Gracia, and F. Diaz-Gonzalez. 2004. CC and CXC chemokine receptors mediate migration, proliferation, and matrix metalloproteinase production by fibroblast-like synoviocytes from rheumatoid arthritis patients. *Arthritis Rheum.* 50: 3866–3877.
49. Santiago, B., F. Baleux, G. Palao, I. Gutierrez-Canas, J. C. Ramirez, F. Arenzana-Seisdedos, and J. L. Pablos. CXCL12 is displayed by rheumatoid endothelial cells through its basic amino-terminal motif on heparan sulfate proteoglycans. *Arthritis Res. Ther.* 8: R43, 2006.
50. Grassi, F., A. Piacentini, S. Cristino, S. Toneguzzi, C. Cavallo, A. Facchini, and G. Lisignoli. 2003. Human osteoclasts express different CXC chemokines depending on cell culture substrate: molecular and immunocytochemical evidence of high levels of CXCL10 and CXCL12. *Histochem. Cell Biol.* 120: 391–400.
51. Kanbe, K., T. Takemura, K. Takeuchi, Q. Chen, K. Takagishi, and K. Inoue. 2004. Synovectomy reduces stromal-cell-derived factor-1 (SDF-1) which is involved in the destruction of cartilage in osteoarthritis and rheumatoid arthritis. *J. Bone Joint Surg. Br.* 86: 296–300.
52. Joosten, L. A., M. G. Netea, S. H. Kim, D. Y. Yoon, B. Oppers-Walgreen, T. R. Radstake, P. Barrera, F. A. van de Loo, C. A. Dinarello, and W. B. van den Berg. 2006. IL-32, a proinflammatory cytokine in rheumatoid arthritis. *Proc. Natl. Acad. Sci. USA* 103: 3298–3303.
53. Shoda, H., K. Fujio, Y. Yamaguchi, A. Okamoto, T. Sawada, Y. Kochi, and K. Yamamoto. 2006. Interactions between IL-32 and tumor necrosis factor α contribute to the exacerbation of immune-inflammatory diseases. *Arthritis Res. Ther.* 8: R166.
54. Kim, S. H., S. Y. Han, T. Azam, D. Y. Yoon, and C. A. Dinarello. 2005. Interleukin-32: a cytokine and inducer of TNFα. *Immunity* 22: 131–142.
55. Clark, A. 2000. Post-transcriptional regulation of pro-inflammatory gene expression. *Arthritis Res.* 2: 172–174.
56. McAllister, F., A. Henry, J. L. Kreindler, P. J. Dubin, L. Ulrich, C. Steele, J. D. Finder, J. M. Pilewski, B. M. Carreno, S. J. Goldman, et al. 2005. Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-α and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. *J. Immunol.* 175: 404–412.
57. Kehlen, A., K. Thiele, D. Riemann, and J. Langner. 2002. Expression, modulation and signalling of IL-17 receptor in fibroblast-like synoviocytes of patients with rheumatoid arthritis. *Clin. Exp. Immunol.* 127: 539–546.
58. Hwang, S. Y., J. Y. Kim, K. W. Kim, M. K. Park, Y. Moon, W. U. Kim, and H. Y. Kim. 2004. IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-κB- and PI3-kinase/Akt-dependent pathways. *Arthritis Res. Ther.* 6: R120–R128.
59. Andoh, A., S. Fujino, S. Bamba, Y. Araki, T. Okuno, T. Bamba, and Y. Fujiyama. 2002. IL-17 selectively down-regulates TNF-α-induced RANTES gene expression in human colonic subepithelial myofibroblasts. *J. Immunol.* 169: 1683–1687.
60. Granet, C., W. Maslinski, and P. Miossec. 2004. Increased AP-1 and NF-κB activation and recruitment with the combination of the proinflammatory cytokines IL-1β, tumor necrosis factor α and IL-17 in rheumatoid synoviocytes. *Arthritis Res. Ther.* 6: R190–R198.
61. Ruddy, M. J., G. C. Wong, X. K. Liu, H. Yamamoto, S. Kasayama, K. L. Kirkwood, and S. L. Gaffen. 2004. Functional cooperation between interleukin-17 and tumor necrosis factor-α is mediated by CCAAT/enhancer-binding protein family members. *J. Biol. Chem.* 279: 2559–2567.

Tournadre A, Porcherot M, Chérin P, Marie I, Hachulla E, Miossec P. Th1 and Th17 balance in inflammatory myopathies: Interaction with dendritic cells and possible link with response to high-dose immunoglobulins.
Cytokine. 2009;46:297-301.

Contribution spécifique de la candidate:

- Recueil des données cliniques et des données quantitatives immunohistochimiques des biopsies musculaires.
- Exploitation et analyse des données cliniques et immunohistochimiques.
- Ecriture de l'article, réalisation des tableaux et figures.
- Réponses aux commentaires des rapporteurs.

Cytokine 46 (2009) 297–301

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

Short Communication

Th1 and Th17 balance in inflammatory myopathies: Interaction with dendritic cells and possible link with response to high-dose immunoglobulins

Anne Tournadre [a], Marine Porcherot [a], Patrick Chérin [b], Isabelle Marie [c], Eric Hachulla [d], Pierre Miossec [a,*]

[a] Department of Immunology and Rheumatology, Clinical Immunology Unit, Hospital Edouard Herriot, University of Lyon, Hospices Civils de Lyon, 69437 Lyon Cedex 03, France
[b] Department of Internal Medicine, Hôpital Pitié-Salpêtrière, Paris, France
[c] Department of Internal Medicine, Rouen University Hospital, Rouen, France
[d] Department of Internal Medicine, Claude Huriez Hospital, Lille, France

ARTICLE INFO

Article history:
Received 19 December 2008
Received in revised form 6 February 2009
Accepted 14 February 2009

Keywords:
Th1
Th17
Dendritic cells
Myositis
Intravenous immunoglobulins

ABSTRACT

To clarify the interactions between dendritic cells (DCs) and Th1 and Th17 T cell subsets and the mode of action of IVIG in inflammatory myopathies, Expression of CD4(+) and CD8(+) T cells, immature (CD1a) and mature (DC-LAMP) DCs, interleukin-17 (IL-17) and interferon-γ (IFN-γ), was quantified by immunohistochemistry in muscle biopsies from 13 patients (11 with polymyositis (PM) and 2 dermatomyositis (DM)) obtained before treatment with IVIG. The Th1/Th17 cytokine and the immature/mature DC ratio were studied according to the response to IVIG. Immature DCs were rarely detected compared to mature DCs, observed in all samples except one PM. IFN-γ-producing cell count was higher than IL-17 count. Neither the expression of IFN-γ nor IL-17 was correlated with that of DC subsets. Seven of the 13 patients (6 PM and 1 DM) responded to IVIG. T cells and DC subsets were not differentially expressed between responders and non-responders. The frequency of IFN-γ-producing cells was significantly higher in non-responders with an increased IFN-γ/IL-17-producing-cell ratio. In conclusion, mature rather than immature DC and IFN-γ-rather than IL-17-producing cells accumulate in inflamed muscle. Increased IFN-γ-producing cell count and IFN-γ/IL-17-ratio were found in IVIG non-responders, suggesting a role for the Th17 mediated pathway in the response to IVIG.

This work has been supported by a grant from the laboratoire français de fractionnement et des biotechnologies (LFB). The authors thank Raphaelle Beaudet and Dr Sophie Puget for their help in getting some of the clinical data.

1. Introduction

Polymyositis (PM) and dermatomyositis (DM) are inflammatory myopathies (IM) which have in common muscle weakness and an inflammatory infiltrate in muscle [1]. DM is considered as a CD4-driven disease with microangiopathy affecting skin and muscle whereas PM is considered as a CD8-driven disease where muscle is the primary target of the immune attack [1]. Although debated, most studies have suggested an association with a main Th1 response. However, today, this has to be updated with the recently described Th17 cells [2]. In both PM and DM, the two key Th1 and Th17 cytokines, IFN-γ and IL-17, and both mature and immature dendritic cells (DCs) have been detected in the lymphocytic infiltrates [3,4].

Administration of IVIG has been shown to be effective both in PM and DM [5]. Its exact mechanism of action that could interfere with the production/action of antibodies, cytokines, chemokines, adhesion molecules and complement remains unclear.

To clarify the interaction between DCs and T cell subsets (Th1 and Th17) and to provide data on the mode of action of IVIG in IM, we have extended our previous studies [3,4] using immunohistochemistry to study the expression of immature CD1a+, mature DC-LAMP+ DC subsets and of T cell-derived cytokines IL-17 and IFN-γ according to patient response to IVIG.

2. Patients and methods

2.1. Patients

Thirteen patients with refractory IM (11 PM and 2 DM) were included (Table 1). All patients fulfilled the diagnostic criteria of Bohan and Peter for definite PM/DM [6], with inflammatory lesions on muscle biopsy and persistent muscle weakness and disability. These patients received IVIG, 2 g/kg, once a month for 3–24 months. All patients had previously been treated with high-dose steroids (daily dose of 1 mg/kg for at least 3 weeks) with no or limited improvement. Muscle strength was assessed using a

* Corresponding author. Fax: +33 472 11 74 29.
E-mail address: pierre.miossec@univ-lyon1.fr (P. Miossec).

1043-4666/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cyto.2009.02.013

Table 1
Clinical data on the 13 patients with refractory inflammatory myopathy.

Patients/ diagnosis	Age (years)	Sex	Duration before IVIG	BMRC score before/ after IVIG	MDS score before/ after IVIG	CK prior to/after IVIG (normal values UI/L)	Therapy prior to IVIG	No of IVIG courses	Dose of steroids before/after IVIG (mg/day)	Outcome
1/PM	61	F	4 Months (Mo)	Quadriplegia/67.5	47/17	955/62 (<200)	STER	25	110/45	RESP
2/PM	47	M	2 Mo	65/79.5	17/1	3348/90 (<200)	STER	16	50/27.5	RESP
3/PM	33	F	2 Mo	69/75	12/9	76/396 (<200)	STER	18	60/30	Non-RESP
4/PM	25	F	1 Mo	75/78	10.5/6	14666/777 (<170)	STER	4	60/60	Non-RESP
5/PM	27	F	4 Mo	48/84	13/1	2220/210 (<170)	STER	7	85/15	RESP
6/PM	22	F	24 Mo	57/69	21/1	1400/377 (<120)	STER	10	65/65	RESP
7/PM	53	M	3 Mo	54/62	34/6	3237/45 (<195)	STER	10	50/30	RESP
8/PM	53	M	18 Mo	70/78	19/3	2509/245 (<200)	STER, MTX	11	20/15	RESP
9/DM	16	M	1 Mo	63/81	NR	5340/40 (<200)	STER	9	50/15	RESP
10/DM	58	M	30 Mo	73/NR	NR	275/125 (<110)	STER, MTX	3	80/80	Non-RESP dead
11/PM	53	F	30 Mo	58/58.5	15/11	144/1330 (<160)	STER, MTX, AZA	12	70/35	Non-RESP
12/PM	51	F	8 Mo	73/74	8/12	15/50 (<200)	STER	3	20/15	Non-RESP
13/PM	23	F	18 Mo	51/61.5	31/27	18351/12390 (<160)	STER	7	80/50	Non-RESP

PM, polymyositis; DM, dermatomyositis; CK, creatine kinase; STER, steroids; AZA, azathioprine; MTX, methotrexate; RESP, responders.

modification of the British Medical Research Council (BMRC) grading for an increased precision: each value from 0 to 5 assigned to muscle power was subdivided into an intermediary score from 0 to 11 resulting in a maximal score of 88 obtained by the quotation of eight selected muscles (neck flexors, trapezius, deltoid, biceps, psoas, maximus and medius gluteus and quadriceps) [7]. The muscle disability scale (MDS) score, for PM only, was constructed to explore both proximal strength (proximal upper limbs, proximal lower limbs), axial and pharyngeal muscle disability. Total scores range from 75 (maximum disability) to 0 (no disability) [7]. Resistance to treatment was defined by a muscle strength score under 76 and a MDS score upper 8. Response to IVIG was obtained if the muscle strength improved by 6 points or greater, the MDS decreased of 8 points or greater from initial score and serum creatine kinase (CK) level decreased by >50% from initial values. The outcome measure was performed at a maximum of 6 months after the first infusion. The steroid dose before and after IVIG therapy was recorded. All patients gave their informed consent and the local ethics committee approved the study.

2.2. Muscle biopsy data

Muscle tissue used for this study was obtained at the time of the diagnosis, fixed in paraformaldehyde and embedded in paraffin. Antigen retrieval procedures were performed, including incubation in either citrate buffer (10 mM, pH 6) or EDTA buffer (1 mM, pH 8).

2.2.1. Detection of IL-17- and IFN-γ-producing cells

After dewaxing and dehydratation, endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Sections were incubated with 5 μg/ml goat polyclonal anti-IL-17 (R&D Systems Europe, London, UK) or rabbit polyclonal anti-IFN-γ (Genzyme, Cambridge, USA). In negative control sections, the primary antibodies were omitted. After overnight incubation at 4 °C and washing, sections were incubated with biotinylated mouse anti-goat IgG or mouse anti-rabbit IgG antibodies for 30 min, followed by streptavidin-peroxidase (Dako, Glostrup, Denmark) for 15 min, 3,3′ diaminobenzidine tetrahydrochloride (DAB, Dako) for 20 min, and counterstained with Mayer's hematoxylin (Dako).

2.2.2. Detection of T cell and DC subsets

After blocking endogenous peroxidase activity with 3% hydrogen peroxide, sections were incubated overnight at 4 °C with mouse monoclonal antibodies against: CD4 (IgG2a Novocastra), CD8 (IgG1 Dako), CD1a (IgG2b; Becton Dickinson, Pont de Claix, France), and DC-LAMP (IgG1; Immunotech). Sections were incubated with biotinylated anti-mouse IgG, followed by Streptavidin-peroxidase (Dako). Peroxidase was developed by DAB and Mayer's hematoxylin used for counterstaining. In negative control sections, the primary antibodies were omitted. Examples of these staining pictures can be seen in our previous publication [3]. and in Figs. 1 and 2.

2.2.3. Quantification of positive cells

Because of high sample heterogeneity and the low frequency of DC subsets and Th1- and Th17-producing cells, quantification was performed with the hot spot method used for the estimation of new blood vessel formation in breast tumor [8] and later applied to myositis [3]. This method allowed the quantification of cells in hot spots defined as muscle areas containing the highest density of infiltrating cells. Accordingly, two hot spots per muscle section were selected. Of note, these hot spots were not selected on the basis of the presence of a particular cell subset. In each spot, positive cells were counted in 10 consecutive high power fields (HPF) (500×). One field corresponded to 0.3 mm^2 and thus one spot to 3 mm^2. The number of positive cells per two hot spots was aver-

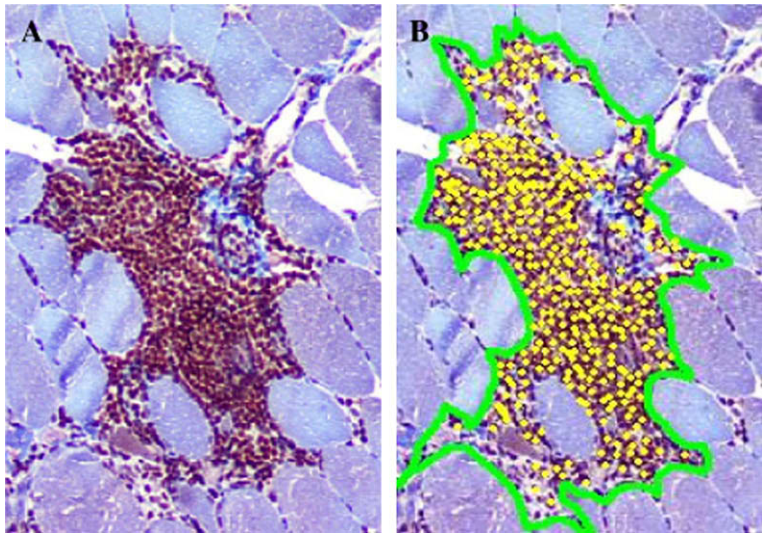


Fig. 1. Quantification of IFN-γ-producing cells in muscle section from myositis patient. A hot spot was defined as a muscle area containing the highest density of infiltrating cells (A). In each selected hot spot, positive cells for IFN-γ were counted (B). (400×).

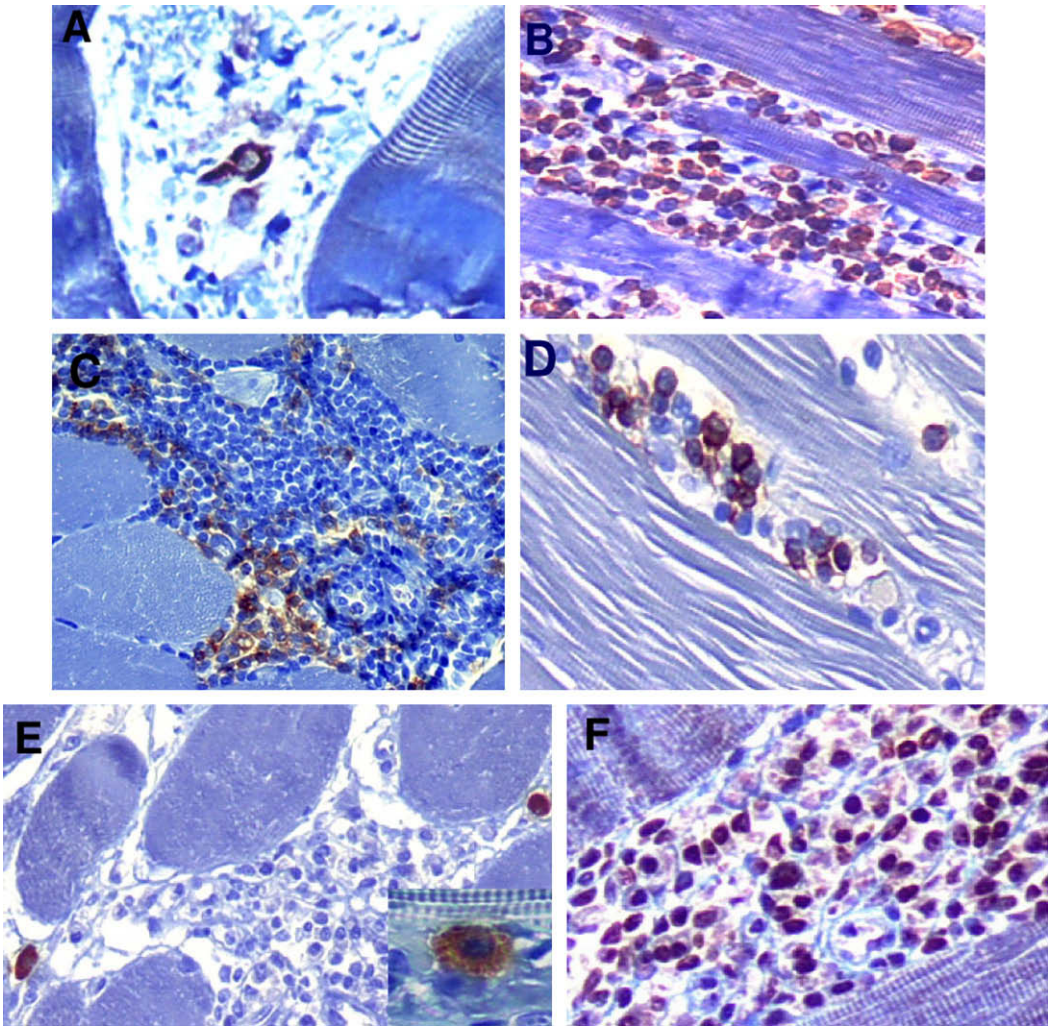


Fig. 2. Muscle staining for DCs and cytokine-producing cells. Immature CD1a+ DCs (A), mature DC-LAMP DCs (B), CD4+ (C) and CD8+ (D) T cells, IL-17 (E) and IFN-γ (F)-producing cells were detected in muscle sections from PM and DM (brown staining). 400× (C, inset E), 200× (A–F). Immature DCs (A) and IL-17-producing cells (E) were rarely detected as compared with mature DCs (B) and IFN-γ-producing cells (F).

aged and expressed as number of cells per mm^2. Pictures were analyzed with a Lucia® image analyzer (Nikon, Champigny-sur-Marne, France) to quantify the total number of ho0t spots in hot spots per mm^2 as well as the total tissue area of respective sections from each biopsy. Fig. 1 shows an example of quantification of IFN-γ positive cells using the hot spot method.

2.3. Statistical analysis

Results were expressed as mean ± SD. Levels of cell markers and cytokine expression were compared using the nonparametric Mann–Whitney test. Correlations were analysed using the Spearman's statistical test. A p value <0.05 was considered to be significant.

3. Results

3.1. DC and T cell subset expression in IM muscle tissue samples

We investigated DC and T cell subsets in sections from 13 IM samples (Table 2). Fig. 2 shows representative sections of muscle samples immunostained for the expression of immature and mature DCs, and T cell subsets. CD4+ and CD8+ T cells were detected in 6 samples (2 DM and 4 PM) and 10 samples (2 DM and 8 PM), respectively. CD4+ and CD8+ cells per mm^2 ranged from 0.178 to 0.908 (mean ± SD 0.2475 ± 0.3456 per mm^2) and from 0.071 to 1.321 (mean ± SD 0.2792 ± 0.3563 per mm^2), respectively.

Immature DCs, defined by CD1a expression, were detected in 5 samples (1 DM and 4 PM). CD1a+ cells per mm^2 ranged from 0.01 to 0.118 (mean ± SD 0.015 ± 0.034 per mm^2). DC-LAMP expression was observed in all samples except in one PM (Patient 1). In contrast to the low numbers of immature DCs, mature DCs defined by the expression of DC-LAMP, were detected in PM and DM in larger numbers (mean ± SD 0.81 ± 0.96 per mm^2, $p < 0.001$). No significant correlation was found between mature DC-LAMP DCs and CD4+ or CD8+ T cells.

3.2. Detection of IFN-γ- and IL-17-producing cells

We then investigated the presence of IFN-γ and IL-17, two key Th1 and Th17 cytokines (Fig. 2). Results are listed in Table 2. IFN-γ- and IL-17-producing cells were each one detected in 11 of 13 samples. IFN-γ-producing cell count was higher than IL-17 count (1.310 ± 1.085 and 0.095 ± 0.166 per mm^2, respectively, $p < 0.05$). No correlation was found between the number of IFN-γ- or IL-17-producing cells and that of DC subsets.

3.3. DC, T cell subsets, Th1 and Th17 expression and response to IVIG

Seven of the 13 patients were classified as responders as defined by a clinical improvement in muscle strength, disability and a decrease in muscle enzymes (Table 1). There were no significant differences in the age, disease duration, muscle testing score and the MDS score before IVIG treatment and the initial steroid doses between the responders and non-responders. Steroid doses could be significantly reduced after IVIG treatment in responders (mean initial steroid doses before IVIG therapy 61.43 mg/day [SD 28.97], mean steroid doses after IVIG 30.36 mg/day [SD 18.84], $p < 0.05$) whereas the change was not significant in non-responders (mean initial steroid doses before IVIG therapy 61.67 mg/day [SD 22.29], mean steroid doses after IVIG 45 mg/day [SD 23.24].

We compared DC and T cell subsets, IFN-γ and IL-17 expression, according to the IVIG response (Table 2). There were similar numbers of CD4+ or CD8+ cells in responders (mean ± SD 0.18 ± 0.28 and 0.15 ± 0.13 per mm^2, respectively) and non-responders (mean ± SD 0.32 ± 0.43 and 0.43 ± 0.48 per mm^2, respectively). The CD1a/DC-LAMP ratio, which defines the ratio of immature to mature cells, was similar in responders and non-responders (0.047 ± 0.09 per mm^2 and 0.021 ± 0.05 per mm^2, respectively). The mean number of IFN-γ-producing cells was significantly higher in samples from non-responders (2.09 ± 1.06 per mm^2) compared to non-responders (mean ± SD 0.64 ± 0.54 per mm^2), $p < 0.05$. IL-17-producing cells were not differentially expressed in samples from responders compared to non-responders. The IFN-γ/IL-17-producing cell ratio was found higher in non-responders (mean ± SD 64.2 ± 44.6 versus 23.9 ± 27.6 per mm^2, $p = 0.05$).

4. Discussion

Immature DCs were rarely detected in IM samples compared to mature DCs which were observed in all samples except one PM. This appears to be in line with previous data in IM and in contrast with the situation in rheumatoid arthritis (RA) synovium, where we have observed a relative accumulation of immature DCs has been observed [3,9].

Although debated, most studies suggest a main Th1 response in IM characterized by the overexpression of IFN-γ in PM and DM muscle tissues [10]. Today, the Th1/Th2 classification has to be revisited to take into account the Th17 cells [11]. In PM and DM inflammatory infiltrates, Th1 and Th17 cytokine expression has been reported and their contribution to DC homing and function has been suggested through chemokine up-regulation [3,4]. When considering the contribution of cytokine environment in DC

Table 2
Quantification of immature (CD1a) and mature (DC-LAMP) DCs and T cell-derived cytokines (IFNγ and IL-17) and link with response to IVIG.

	Patients	CD1a	DC-LAMP	CD1a/DC-LAMP	IFN-γ	IL-17	IFN-γ/IL-7
Responders	1		0.00		1.347	0.165	8.164
	2	0.011	3.223	0.003	1.215	0.018	67.50
	5	0.00	0.121	0.00	0.372	0.00	
	6	0.00	0.171	0.00	0.00	0.00	
	7	0.00	0.510	0.00	0.805	0.087	9.253
	8	0.033	0.147	0.224	0.00	0.618	0.00
	9	0.010	0.181	0.055	0.764	0.022	34.727
	Mean ± SD	0.009 ± 0.013	0.62 ± 1.2	0.047 ± 0.09	0.64 ± 0.54	0.13 ± 0.22	23.9 ± 27.6
Non-responders	3	0.011	2.131	0.005	3.912	0.041	95.415
	4	0.00	0.780	0.00	0.715	0.055	13.00
	10	0.00	1.525	0.00	2.129	0.043	49.512
	11	0.00	0.689	0.00	1.658	0.017	97.529
	12	0.00	0.108	0.00	2.388	0.16	14.925
	13	0.118	0.956	0.123	1.726	0.015	115.067
	Mean ± SD	0.022 ± 0.047	1 ± 0.71	0.021 ± 0.05	2.09 ± 1.06	0.055 ± 0.054	64.2 ± 44.6
	P	NS	NS	NS	<0.05	NS	NS

Results are expressed as mean of positive cells per mm^2 based on the hot spot analysis (see Section 2). The p value compares responders to non-responders.

recruitment and maturation, we did not find a correlation between the number of IFN-γ- or IL-17-producing cells and that of DC subsets. The proinflammatory properties of IL-17 were demonstrated by the synergistic interaction with TNF-α and IL-1 on IL-6 production by synoviocytes and myoblasts [12]. Conversely, IFN-γ has been shown to have an inhibitory effect on the production of IL-17 [11].

Modalities and position of IVIG in the treatment of IM have not been fully established, in part because their exact mechanism of action remains to be defined. As shown previously [13], T cells and DC subsets were not differentially expressed in IVIG-responder and IVIG-non-responder patients. When considering the Th1 and Th17 profiles according to the response to IVIG, we found an increased number of IFN-γ positive cells and an increased IFN-γ/IL-17-producing cell ratio in IVIG-non-responders. This could suggest a Th17 mediated inflammatory profile in IVIG-responders, sensitive to the immunomodulatory effect of IVIG on the Th1/Th17 balance. Response to IVIG may then result from the inhibition of an inflammatory Th17 mediated pathway.

References

[1] Dalakas MC, Hohlfeld R. Polymyositis and dermatomyositis. Lancet 2003;362(20):971–82.

[2] Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123–32.

[3] Page G, Chevrel G, Miossec P. Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: interaction with chemokines and Th1 cytokine-producing cells. Arthritis Rheum 2004;50:199–208.

[4] Chevrel G, Page G, Granet C, Streichenberger N, Varennes A, Miossec P. Interleukin-17 increases the effects of IL-1 beta on muscle cells: arguments for the role of T cells in the pathogenesis of myositis. J Neuroimmunol 2003;137:125–33.

[5] Dalakas MC. Intravenous immunoglobulin in autoimmune neuromuscular diseases. JAMA 2004;291:2367–75.

[6] Bohan A, Peter JB. Polymyositis and dermatomyositis. N Engl J Med 1975;292:344–7.

[7] Cherin P, Pelletier S, Teixeira A, Laforet P, Genereau T, Simon A, et al. Results and long-term follow-up of intravenous immunoglobulin infusions in chronic refractory polymyositis: an open study with 35 adult patients. Arthritis Rheum 2002;46:467–74.

[8] Bertin N, Clezardin P, Kubiak R, Frappart L. Thrombospondin-1 and -2 messenger RNA expression in normal, benign, and neoplastic human breast tissues: correlation with prognostic factors, tumor angiogenesis, and fibroblastic desmoplasia. Cancer Res 1997;57:396–9.

[9] Page G, Lebecque S, Miossec P. Anatomic localization of immature and mature dendritic cells in an ectopic lymphoid organ: correlation with selective chemokine expression in rheumatoid synovium. J Immunol 2002;168:5333–41.

[10] Lundberg I, Ulfgren AK, Nyberg P, Andersson U, Klreskog L. Cytokine production in muscle tissue of patients with idiopathic inflammatory myopathies. Arthritis Rheum 1997;40:865–74.

[11] Miossec P. Interleukin-17 in fashion at last: ten years after its description, its cellular source has been identified. Arthritis Rheum 2007;56:2111–5.

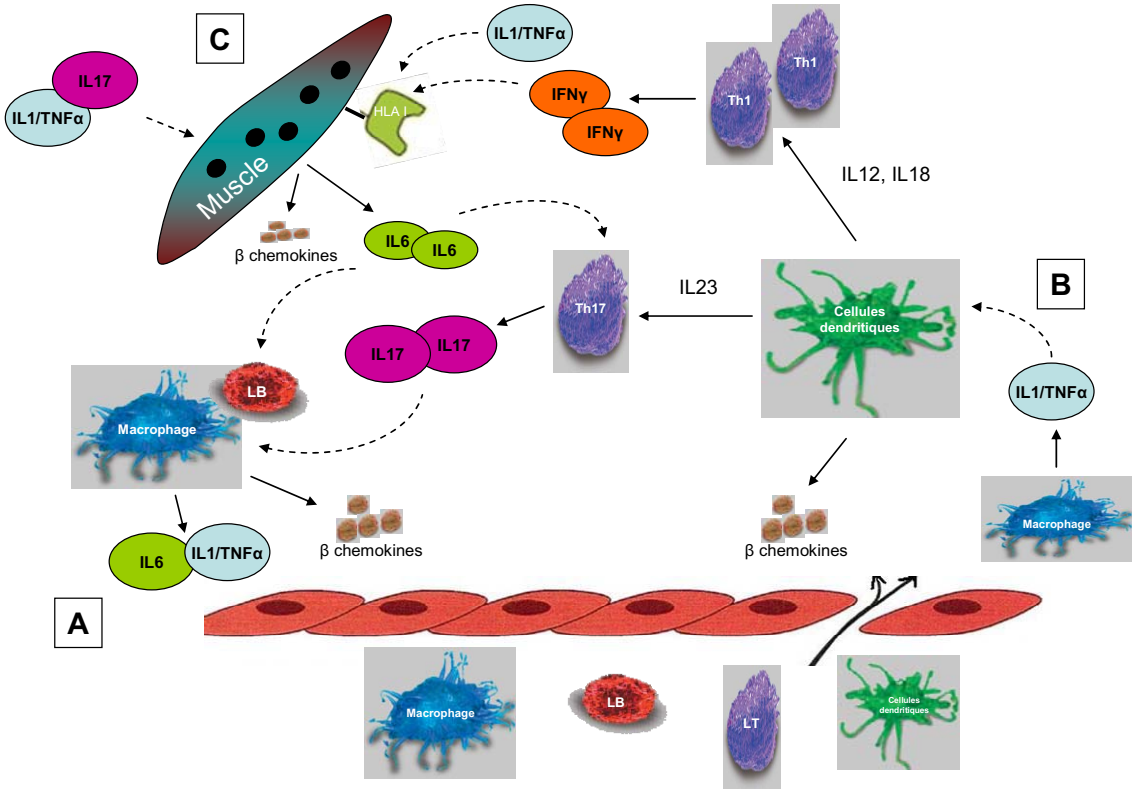
[12] Chabaud M, Fossiez F, Taupin JL, Miossec P. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. J Immunol 1998;161:409–14.

[13] Helmers SB, dastmalchi M, Alexanderson H, Nennesmo I, Esbjornsson M, Lindvall B, et al. Limited effects of high-dose intravenous immunoglobulin (IVIG) treatment on molecular expression in muscle tissue of patients with inflammatory myopathies. Ann Rheum dis 2007;66:1276–83.

IV-c-3. Conclusion.

Ainsi, les cytokines proinflammatoires (IL1, TNFα, IL6) produites par les cellules mononuclées de l'infiltrat inflammatoire musculaire mais aussi par la cellule musculaire elle-même, créent un microenvironnement inflammatoire au sein du tissu musculaire des PM et DM, propice au recrutement, à la prolifération et à la différentiation des lymphocytes T activés et cellules dendritiques. Elles sont également responsables de modifications métaboliques et morphologiques du tissu musculaire lui-même. Les interactions entre cellules dendritiques, cytokines, chémokines et la cellule musculaire sont schématisées par la figure 4.

Figure 4. Interactions entre cellules dendritiques, cytokines et chémokines dans le tissu musculaire inflammatoire.



A. Les cytokines proinflammatoires (IL1 et TNFα) et les β chémokines produites par les cellules mononuclées de l'infiltrat inflammatoire, et par la cellule musculaire elle-même, permettent la migration et le recrutement des cellules immunitaires dont les cellules dendritiques.

B. L'IL1 et le TNFα contribuent à la maturation des cellules dendritiques, dont la production de cytokines IL12/IL18 et IL23 oriente la différentiation des lymphocytes T en Th1 et Th17 respectivement. L'IL17 induit la production d'IL1/TNFα, d'IL6 et de β chémokines par les cellules mononuclées inflammatoires. En retour, l'IL6 et la β chémokine CCL20 sont des facteurs de différentiation et de recrutement des lymphocytes Th17.

C. La cellule musculaire elle-même participe à l'amplification du processus inflammatoire en produisant de l'IL6 et la β chémokine CCL20 sous l'effet synergique de l'IL17 et de l'IL1. Les lymphocytes Th1, présents en nombre important dans l'infiltrat inflammatoire musculaire, en produisant de l'IFNγ, favorisent avec l'IL1 et le TNFα la surexpression des antigènes HLA de classe I à la surface des fibres musculaires.

Chapitre V

Récepteurs de l'immunité innée et myopathies inflammatoires.

Sommaire

V-a Les Toll-like récepteurs.

V-a-1. Introduction.

L'immunité innée est la première ligne de défense de l'organisme via la détection et l'élimination des éléments pathogènes. Pour cela, des récepteurs spécifiques, pattern recognition receptors (PRRs), comprenant 3 familles de molécules exprimées à la surface ou dans le cytoplasme des cellules de l'immunité (les Toll-like récepteurs, les C-type lectin récepteurs et les NOD-like récepteurs), reconnaissent des motifs microbiens conservés au cours de l'évolution, et appelés pathogen-associated molecular patterns (PAMPs). Parmi ces récepteurs, les Toll-like récepteurs (TLRs) ont été observés pour la première fois en 1997 chez la *Drosophile* [87]. Depuis, treize variétés de TLRs, reconnaissant une grande diversité de composants bactériens, viraux ou fungiques, ont été identifiées chez les mammifères. Dans l'espèce humaine, dix TLRs sont répertoriés et le rôle de 9 d'entre eux (TLR1 à TLR9) est plus précisément connu [87]. Les TLR1, TLR2, TLR4, TLR5, TLR6 reconnaissent des motifs lipidiques ou glycopeptidiques (lipoprotéines, lipopolysaccharide, flagelline, peptidoglycane…) et sont présents à la surface cellulaire (Tableau 2). Les TLR3, TLR7, TLR8, TLR9, sont activés par des motifs dérivés des acides nucléiques (ADN ou ARN) et leur expression est restreinte au compartiment intracellulaire (endosome, lysosome) afinn d'éviter une activation aberrante par les acides nucléiques endogènes (Tableau 2). Les TLRs sont exprimés par les cellules de l'immunité dont les lymphocytes B, les macrophages, les monocytes, les cellules dendritiques et mais aussi plus largement par les cellules épithéliales et endothéliales. L'activation des lymphocytes B via leurs TLRs entraîne leur prolifération et la production d'anticorps. Les IgM ainsi produites vont participer à l'élimination des débris microbiens ainsi que des débris apoptotiques et nécrotiques. Les monocytes, macrophages et cellules dendritiques répondent à l'activation des TLRs par la production de cytokines proinflammatoires et par l'expression des molécules de costimulation permettant leur maturation [88]. Il apparaît donc que si le rôle premier de l'immunité innée est de contenir le processus infectieux, elle est secondairement responsable de l'activation de l'immunité adaptative.

Le lien étroit entre immunité innée et la réponse antigénique de l'immunité adaptative implique un rôle potentiel de l'immunité innée dans la réponse auto antigénique et donc dans le développement des maladies auto-immunes. L'association entre infection et auto-immunité

est depuis longtemps admise et deux théories sont proposées pour tenter d'expliquer le lien entre un processus infectieux et le début ou l'exacerbation d'une maladie auto-immune. La première théorie concerne le mimétisme moléculaire entre un agent microbien et un antigène du soi qui partagent une même structure moléculaire expliquant ainsi une perte de tolérance vis-à-vis de cet auto antigène. Le second concept repose sur une activation aberrante du système immunitaire non spécifique d'un antigène, soit par la libération anormale de protéines endogènes conséquence de la mort cellulaire induite par l'infection, soit par des cellules présentatrices d'antigènes stimulées par l'agent microbien et qui présentent secondairement de manière trop effective un auto antigène. Ainsi, des molécules endogènes, dont les protéines de choc thermique ou HSP (pour Heat Shock Proteins), la fibronectine, l'acide urique ou les protéines HMGB1 (high mobility group box 1 protein) composant structural de la chromatine libéré des cellules nécrotiques, peuvent induire la maturation des cellules dendritiques et la production d'IL6 et de TNFα par les macrophages via leur liaison à de multiples TLRs parmi lesquels TLR2 et TLR4 [65, 88] (Tableau 2). L'engagement d'un TLR par son ligand dérivé de composants microbiens, ou par un composant inhabituel modifié ou dérivé du soi, pourrait ainsi représenter une voie d'activation qui dans certaines circonstances pourrait conduire à une réponse auto-immune.

Plusieurs études récentes suggèrent que les récepteurs de l'immunité innée peuvent détecter les auto antigènes modifiés ou non [9-12]. Ainsi, l'activation de TLR9 par de l'ADN bactérien peut être reproduite par des nucléotides synthétiques contenant des motifs CpG et par de la chromatine endogène contenant des immuns complexes [88]. Des clones B auto réactifs peuvent être activés par des immuns complexes liés à la chromatine via le double engagement d'un BCR à la surface cellulaire puis secondairement d'un ou plusieurs TLRs intracellulaires dont TLR9 [9]. De plus, la signature IFN type I observée dans le lupus systémique pourrait être liée à l'activation des cellules dendritiques plasmacytoïdes par la chromatine endogène contenant des immuns complexes via l'engagement de TLR9 [65, 88]. Une autre source majeure d'auto antigènes est représentée par les protéines associées à l'ARN. L'ARN simple brin et double brin sont les ligands respectifs de TLR7/8 et TLR3. Tout comme pour le double engagement BCR/TLR9 par des autoantigènes associés à l'ADN, l'activation de clones B auto réactifs peut être obtenue par le co-engagement de BCR puis de TLR7 par l'ARN et les autoantigènes associés [10]. Dans la PR, plusieurs études suggèrent un rôle physiopathologique de l'immunité innée et des TLRs. Une surexpression de TLR3, TLR4, TLR7 est notée dans la synoviale rhumatoïde comparée à la synoviale arthrosique [11,

89, 90]. De nombreux ligands endogènes des TLRs (HSPs, fibronectine, protéines HMGB1, et dérivés d'acide hyaluronique) sont identifiés dans le sérum et les articulations de patients atteints de PR et sont corrélés à l'activité de la maladie [91]. De plus, la production de cytokines proinflammatoires et de métalloprotéases par les synoviocytes de PR en culture est diminuée après blocage de la voie de signalisation de TLR2 et TLR4 [92]. Il est également démontré que l'ARN dérivé des cellules nécrotiques contenues dans le liquide synovial de patients atteints de PR est un ligand endogène de TLR3 et que l'activation de la voie TLR3 induit la production par les synoviocytes d'IL6, d'IFNβ et de chémokines associées au Th1 [11].

Au cours des myopathies inflammatoires, la signature IFN type I, la présence d'autoantigènes spécifiques liés à l'ARN ou aux mécanismes impliqués dans sa traduction, et la présence de cellules dendritiques plasmacytoïdes activées et matures, sont autant d'arguments suggérant un rôle possible des TLRs dans la physiopathologie, en particulier des TLRs associés à l'ARN, TLR3 et TLR7. Nous nous sommes donc intéressés à l'expression musculaire *in vivo* de TLR3 et TLR7 ainsi qu'à leur régulation et fonction *in vitro* [93].

Tableau 2 [88]. Ligands exogènes et endogènes des TLRs.

Table 3. Toll-like receptor (TLR) endogenous ligands

		Potential endogenous ligands
Toll-like receptor	Exogenous ligands*	Ligand
TLR1 (with TLR2)	Tri-acyl lipopeptides (bacteria)	
TLR2	Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid (Gram-positive bacteria) Lipoarabinomannan (mycobacteria) Glycoinositolphospholipids (Trypanosomes) Glycolipids (*Treponema*) Porins (*Neisseria*) Zymogen (fungi) Lipopeptides	Heat shock proteins HMGB1
TLR3	Double-stranded RNA (virus)	mRNA
TLR4	Lipopolysaccharides, lipid A (Gram-negative bacteria) Taxol (plant) Protein F (respiratory syncytial virus) Hyphae (*Aspergillus*) HSP60 (*Chlamydia*) Envelope proteins (MMTV)	Heat shock proteins HMGB1 Fibronectin extra domain A Fibrinogen Lung surfactant protein A Minimally modified-low-density lipoprotein Heparan sulfate Hyaluronan fragments
TLR5	Flagellin (bacteria)	
TLR6 (with TLR2)	Di-acyl lipopeptides	
TLR7	Single-stranded RNA (viral)	
TLR8	Single-stranded RNA (viral)	
TLR9	DNA (bacteria and Herpes simplex virus)	DNA
TLR11	Uropathogenic *Escherichia coli*	

V-a-2 Expression de TLR3 et TLR7 *in vivo* dans le tissu musculaire.

Nous avons analysé par immunohistochimie, dans des biopsies musculaires de myopathies inflammatoires, l'expression tissulaire de TLR3 et TLR7, et nous l'avons corrélée à l'expression musculaire de CD56, un marqueur de précurseur musculaire immature et de régénération, ainsi qu'à l'expression des antigènes HLA de classe I surexprimés dans les myopathies inflammatoires [93]. Les biopsies musculaires de 11 patients atteints de PM (n=8) et de DM (n=3), remplissant les critères diagnostic de *Bohan et Peter* [14], ont été comparées à 5 biopsies contrôles dont 4 biopsies musculaires normales obtenues lors d'une arthroplastie de hanche et 1 biopsie musculaire obtenue chez un patient porteur d'une myopathie non-inflammatoire (maladie de Mac Ardle ou glycogénose type V).

L'expression de TLR3 et de TLR7 est retrouvée dans le tissu musculaire inflammatoire des patients atteints de PM et DM alors qu'elle est absente dans le tissu musculaire sain ou dans le tissu musculaire myopathique non-inflammatoire. Dans les myopathies inflammatoires, TLR3 et TLR7 sont largement détectés au sein de l'infiltrat inflammatoire mononucléé mais également au sein de quelques fibres musculaires dont l'irrégularité de taille et la présence d'un noyau centralisé évoquent un phénotype immature. Ces fibres musculaires exprimant TLR3 et TLR7 ont été mieux caractérisées en utilisant un marqueur de cellules musculaires immatures et de régénération, CD56, ainsi qu'un marquage dirigé contre les antigènes HLA de classe I régulés au cours de la différentiation musculaire [34] et surexprimés dans les myopathies inflammatoires. L'immunomarquage pour TLR3, TLR7 et CD56 sur des coupes musculaires successives de PM et DM permet d'identifier les fibres musculaires exprimant TLR3 et TLR7 comme étant des fibres musculaires immatures en cours de régénération, co-exprimant CD56. De plus, les précurseurs musculaires immatures, CD56 positifs, expriment également fortement les antigènes HLA de classe I suggérant donc que l'expression de TLR3 et TLR7 par les fibres musculaires est restreinte aux cellules de phénotype immature et s'accompagne d'une surexpression des antigènes HLA de classe I.

V-a-3 Régulation et fonction de TLR3 *in vitro*.

Après avoir étudié *in vivo* l'expression musculaire de TLR3 et TLR7, nous avons analysé *in vitro*, sur des cultures de myoblastes et de cellules musculaires plus différentiées, les myotubes, l'expression de TLR3 et TLR7 en immunofluorescence et en quantifiant l'ARN messager (ARNm) par RT-PCR quantitative (qRT-PCR). Une étude fonctionnelle de la voie TLR3 a été réalisée après stimulation des cellules musculaires par le ligand synthétique de TLR3 (Poly(I:C)) ou de TLR7 (CL097), les cytokines Th1 et Th17 et par des cellules musculaires nécrotiques en présence ou non de l'anticorps neutralisant anti-TLR3. La production par les cellules musculaires de la cytokine proinflammatoire IL6 et des chémokines CCL20 et IL8 a été mesurée dans le surnageant de culture par ELISA.

L'expression protéique musculaire de TLR3 et TLR7 est confirmée *in vitro* sur des cultures de myoblastes et myotubes. Le double marquage en immunofluorescence permet de détecter la co-expression intracytoplasmique de TLR3 et TLR7, à la fois dans les myoblastes et, après différentiation, dans les myotubes. Lorsque le niveau d'expression d'ARNm est quantifié, la stimulation durant 48h des myoblastes par Poly(I:C) et par la cytokine Th1, IFNγ, augmente de façon significative l'expression de TLR3 par rapport aux myoblastes non stimulés (moyenne ± SEM 18.25 ± 3.54 *vs* 4.253 ± 0.62, $p<0.005$). A l'inverse, l'IL17A diminue de façon significative l'expression de TLR3 par rapport aux myoblastes non stimulés (moyenne ± SEM 0.49 ± 0.07, $p<0.0001$) et inhibe l'augmentation induite par l'IFNγ (moyenne ± SEM 2.61 ± 0.68, $p<0.05$ comparé aux myoblastes stimulés par IFNγ). Concernant TLR7, le niveau d'expression d'ARNm est augmenté en présence de son ligand synthétique CL097 mais nous n'avons pas pu démontrer une régulation par les cytokines Th1 ou Th17.

Après avoir démontré un effet régulateur et opposé des cytokines Th1 et Th17 sur l'expression de TLR3 par les myoblastes, nous nous sommes intéressés à l'effet de l'activation de la voie TLR3 sur les cellules musculaires en culture. Après stimulation par l'agoniste de TLR3, Poly(I:C), les myoblastes produisent de façon importante la cytokine pro inflammatoire IL6. L'IL17A, mais pas l'IFNγ, en combinaison avec Poly(I:C), augmente de façon synergique la production musculaire d'IL6. Nous avons également analysé la production de la βchémokine CCL20 en raison de son implication dans la migration des cellules dendritiques et des lymphocytes T Th17, notamment dans le tissu musculaire

inflammatoire. L'association d'IL17A et de Poly(I:C) induit la production de CCL20 alors que cet effet synergique n'est pas observé pour l'IFNγ. D'autre part, aucun effet de Poly(I:C) ou des cytokines IL17 et IFNγ, sur la production de la chémokine CXC IL8, impliquée dans la migration des neutrophiles, n'est détecté.

Les myopathies inflammatoires étant caractérisées par un processus de destruction et de nécrose musculaire, nous avons examiné l'effet des cellules musculaires nécrotiques sur les cultures de myoblastes normaux, en particulier sur la voie TLR3. La présence dans le milieu de culture des cellules musculaires nécrotiques induit la production d'IL6 par les myoblastes. Comme pour le ligand synthétique de TLR3, Poly(I:C), la combinaison des cellules nécrotiques à l'IL17 est fortement synergique alors que l'IFNγ n'a aucun effet. Afin de déterminer le rôle de la voie TLR3 dans l'effet inducteur des cellules nécrotiques, la production d'IL6 par les myoblastes a été analysée après blocage de la voie TLR3 par un anticorps neutralisant anti-TLR3. Le blocage de la voie TLR3 entraine une diminution de la production d'IL6 induite par les cellules nécrotiques combinées à l'IL17 de 21 % (moyenne ± SEM 8.609±1.994 vs 6.829±1.521, $p<0.05$), ce qui est comparable à la diminution observée pour la production d'IL6 induite par Poly(I:C) (moyenne ± SEM 6.815±1.611 vs. 5.379±1.451, $p<0.05$). Malgré l'impossibilité d'obtenir un blocage complet de la voie TLR3 avec l'anticorps neutralisant, ces résultats suggèrent donc que la présence de cellules nécrotiques musculaires peut induire l'activation des myoblastes et que cette activation dépend au moins en partie de la voie TLR3.

Les études immunohistochimiques *in vivo* ayant permis de localiser l'expression de TLR3 au sein des précurseurs musculaires immatures, nous avons analysé l'expression de TLR3 *in vitro* au cours de la différentiation des myoblastes en myotubes. En immunofluorescence, l'expression de TLR3 et de TLR7 est détectée dans les myotubes, et ce de façon comparable aux myoblastes. Les résultats de qRT-PCR sont cohérents avec ceux notés en immunofluorescence et montrent une expression persistante de TLR3 par les myotubes. En effet, nous avons retrouvé un niveau d'expression d'ARNm de TLR3 plus important dans les myotubes que dans les myoblastes, avec également une augmentation du niveau d'expression plus importante dans les myotubes après stimulation par Poly(I:C) et IFNγ. Comme pour les myoblastes, l'IL17 inhibe l'effet inducteur de l'IFNγ. A l'inverse, les productions d'IL6 et de CCL20 sont plus importantes pour les myoblastes que les myotubes, excepté après stimulation par Poly(I:C).

V-a-4. Discussion et conclusion.

En accord avec les données rapportées dans la PR [11] ou la myosite à inclusion [94], ces travaux démontrent l'expression spécifique dans le tissu musculaire inflammatoire des PM et DM de TLR3 et TLR7, impliqués dans la reconnaissance de motifs dérivés des acides nucléiques et de ligands endogénes,. La confrontation de l'expression tissulaire de TLR3, TLR7, des antigènes HLA de classe I caractéristiques des myopathies inflammatoires, et de CD56, sur coupes musculaires successives de PM et DM, révèle que les fibres musculaires exprimant TLR3 ou TLR7 expriment également fortement les antigènes HLA de classe I et CD56. *In vivo*, l'expression de TLR3 et TLR7 semble donc d'une part spécifique du tissu musculaire inflammatoire, et d'autre part localisée aux cellules inflammatoires mononuclées et aux précurseurs musculaires immatures CD56 positifs. La présence dans le tissu musculaire des PM et DM de nombreuses fibres musculaires CD56 positives (Figure 2, p.19) témoigne d'un processus de régénération en cours, mais non fonctionnel à terme puisque suivi d'une destruction et d'une perte musculaire. *In vitro*, nous n'avons pas pu confirmer une régulation de l'expression de TLR3 en fonction de la différentiation musculaire, probablement en raison d'un stade de différentiation insuffisant par rapport au tissu musculaire. D'autre part, l'identification de ces fibres musculaires immatures en cours de régénération comme source possible d'auto antigènes myositiques [5] renforce le rôle des TLRs dans l'initiation et la propagation du processus inflammatoire musculaire (Figure 5). Si le processus de régénération rend compte de la surexpression musculaire de TLR3 et TLR7, il s'y associe également une surexpression musculaire des antigènes HLA de classe I qui pourrait alors contribuer au processus de destruction musculaire via soit une cytotoxicité directe médiée par les lymphocytes T, soit une accumulation intracellulaire des molécules HLA et la génération d'un stress du réticulum endoplasmique [43]. Ainsi, de possibles autoantigènes et ligands endogénes des TLRs peuvent être libérés des cellules nécrotiques et peuvent participer à l'activation de la voie TLR3 (Figure 5). Nous avons montré que la présence dans le milieu de culture de cellules nécrotiques musculaires est responsable d'une activation musculaire se traduisant par la production de cytokine et de chémokine proinflammatoires (IL6, CCL20). Malgré l'absence d'anticorps neutralisant anti-TLR3 suffisamment performant pour obtenir un blocage complet, que ce soit après activation de la voie TLR3 par le ligand synthétique Poly(I :C) ou les cellules nécrotiques, l'activation musculaire induite par les cellules nécrotiques est en partie dépendante de la voie TLR3.

Afin de reproduire le microenvironnement inflammatoire des PM et DM, les myoblastes en culture ont été stimulés par les cytokines Th1 (IFNγ) et Th17 (IL17A), démontrant un effet synergique de l'IL17 sur l'activation des cellules musculaires via la voie TLR3, alors que l'IFNγ n'a aucun effet. Cette balance cytokinique Th1/Th17 est également observée en ce qui concerne la régulation de l'expression de TLR3 par les cellules musculaires. En effet, l'expression de TLR3 est régulée de façon différentielle par l'IFNγ, qui augmente le niveau d'expression d'ARNm, et par l'IL17 qui à l'inverse le diminue et inhibe l'augmentation induite par l'IFNγ. Ainsi, un profil Th1 prédominant, suggéré par l'accumulation musculaire de cellules productrices d'IFNγ [23], pourrait favoriser la surexpression musculaire de TLR3 observée dans le tissu des PM et DM. L'IL17, impliquée dans la régulation de l'expression de TLR3 via la balance cytokinique Th1/Th17, induit l'activation de la voie TLR3 et la production d'IL6 et de CCL20 en synergie avec les cellules nécrotiques ou le ligand synthétique Poly(I :C). Le rôle régulateur de la balance Th1/Th17 ainsi que l'interaction entre lymphocytes T régulateurs et lymphocytes Th17 est connu [76]. Il est ainsi démontré que l'IFNγ a un effet inhibiteur sur la production d'IL17 et d'IL6, et que l'IL6 a un effet inhibiteur sur le développement des lymphocytes T régulateurs Foxp3+. En accord avec les données histopathologiques des PM et DM démontrant une accumulation de cellules inflammatoires mononuclées, nous n'avons pas retrouvé de production musculaire de la chémokine CXC, IL8, impliquée dans le recrutement des polynucléaires neutrophiles, mais une induction de la β chémokine CCL20 qui contribue au recrutement des cellules dendritiques et des lymphocytes T dont les Th17. L'IL6, produite par les cellules mononucléées de l'infiltrat inflammatoire et par la cellule musculaire elle-même, participe à la différentiation des cellules de l'immunité adaptative et peut orienter la réponse immunitaire vers la voie Th17 en association avec l'IL23 [76]. L'IL6 joue également un rôle qui reste à déterminer dans la différentiation musculaire [78, 79, 80].

En conclusion, nos résultats indiquent un possible rôle de l'immunité innée dans les myopathies autoimmunes impliquant l'activation de la voie TLR3 par des ligands endogènes libérés des cellules nécrotiques. L'expression de TLR3 et de TLR7, caractéristique des myopathies inflammatoires, se localise au sein du tissu musculaire, dans les fibres musculaires immatures en cours de régénération, source possible d'autoantigènes et de ligands endogénes, contribuant ainsi à l'initiation et la propagation de la boucle inflammatoire qui fait intervenir lymphocytes Th1 et Th17 (Figure 5).

Tournadre A, Lenief V, Miossec P. Expression of TLR3 and TLR7 in muscle is characteristic of inflammatory myopathy and is differentially regulated by Th1 and Th17 cytokines. *Arthritis Rheum.* 2010;62:2144-51.

Contribution spécifique de la candidate:

- Idée originale et proposition du travail de recherche.
- Réalisation des techniques d'immunomarquages en immunohistochimie pour TLR3, TLR7, CD56, HLA classe I, sur les biopsies musculaires. Sélection des patients selon les critéres d'inclusion. Mise au point des anticorps spécifiques, analyse des immunomarquages et réalisation des photographies.
- Mise au point des techniques d'isolement des myoblastes en culture à partir de tissu musculaire et des techniques de différentiation en myotubes.
- Analyse et interprétation des données immunohistochimiques *in vivo* et des données *in vitro* sur cultures de myoblastes et myotubes après stimulation (RT-PCR, ELISA).
- Ecriture de l'article, réalisation des figures.
- Réponses aux commentaires des rapporteurs.

ARTHRITIS & RHEUMATISM
Vol. 62, No. 7, July 2010, pp 2144–2151
DOI 10.1002/art.27465

Expression of Toll-like Receptor 3 and Toll-like Receptor 7 in Muscle Is Characteristic of Inflammatory Myopathy and Is Differentially Regulated by Th1 and Th17 Cytokines

A. Tournadre,[1] V. Lenief,[2] and P. Miossec[2]

Objective. **To assess the expression of Toll-like receptor 3 (TLR-3) and TLR-7 in muscle tissue from patients with polymyositis (PM) and dermatomyositis (DM) and to investigate the function and regulation of TLR-3 in cultured muscle cells.**

Methods. **The expression of TLR-3, TLR-7, HLA class I, and CD56, a marker of immature myoblast precursors, was analyzed using immunohistochemistry. TLR-3 regulation and signaling were assessed in myoblasts and in differentiated myotubes with the TLR-3 agonist poly(I-C), necrotic myoblasts, and Th1 and Th17 cytokines, in the presence or absence of neutralizing anti–TLR-3 antibody. Levels of TLR-3 messenger RNA (mRNA) were quantified by reverse transcription–polymerase chain reaction. Levels of interleukin-6 (IL-6), CCL20, and IL-8 were determined by enzyme-linked immunosorbent assay.**

Results. **TLR-3 and TLR-7 were expressed in PM/DM tissues, but not in noninflammatory muscle tissues, and were primarily detected in inflammatory infiltrates, although a few muscle cells were also positive. These TLR-3– and TLR-7–positive fibers expressed high levels of CD56 and HLA class I antigens. A synergy between poly(I-C) and IL-17 was observed for the production of IL-6 and CCL20. Similarly, stimulation with necrotic myoblasts increased IL-6 production, and stimulation with necrotic myoblasts in combination with IL-17 further increased the induction of IL-6. TLR-3 blockade decreased the inducing effect of necrotic myoblasts and IL-17 on IL-6 production. Stimulation with interferon-γ (IFNγ) increased TLR-3 mRNA levels, but IL-17 down-regulated the inducing effect of IFNγ.**

Conclusion. **Our findings indicate that TLR-3 and TLR-7 are expressed in inflammatory myopathic tissues, particularly in immature myoblast precursors. Necrotic muscle cells activate cytokine production, in part, through the TLR-3 pathway, with a differential regulatory effect of Th1 and Th17 cytokines.**

Polymyositis (PM) and dermatomyositis (DM) are chronic muscle disorders of unknown origin that lead to muscle destruction. They have in common clinical symptoms such as muscle weakness and inflammatory infiltrates in muscle tissue. Although the mechanisms of PM/DM remain unclear, evidence of an autoimmune origin includes an association with autoantibodies, class I major histocompatibility complex overexpression in muscle cells, and evidence of a T cell–mediated cytotoxicity (1). In the lymphocytic infiltrates in PM/DM muscle tissue, detection of the 2 key Th1 and Th17 cytokines, interferon-γ (IFNγ) and interleukin-17 (IL-17), respectively, suggests the involvement of activated T cells in the pathophysiology of the disease, although their respective contributions remain unclear (2,3). Furthermore, the Th1 and Th17 proinflammatory cytokines present in myositis tissues are associated with the migration, differentiation, and maturation of inflammatory cells, including dendritic cells (DCs) (2).

Toll-like receptors (TLRs) are key sentinels that link innate and adaptive immunity. TLRs mediate inflammatory stimuli from pathogens and endogenous danger signals, including bacterial cell components, bacterial DNA, or viral RNA. TLR-1, TLR-2, TLR-4, and TLR-6 recognize lipid-based pathogen-associated mo-

Supported by funding from the interregional Programme Hospitalier de Recherche Clinique to the Centre Hospitalier Universitaire Clermont-Ferrand, the Region Rhône-Alpes, and the Hospices Civils de Lyon.

[1]A. Tournadre, MD: University of Lyon, Edouard Herriot Hospital, and Centre Hospitalier Universitaire Clermont-Ferrand, Lyon, France; [2]V. Lenief, P. Miossec, MD, PhD: University of Lyon, and Edouard Herriot Hospital, Lyon, France.

Address correspondence and reprint requests to P. Miossec, MD, PhD, Clinical Immunology Unit, Department of Immunology and Rheumatology, Edouard Herriot Hospital, 69437 Lyon Cedex 03, France. E-mail: pierre.miossec@univ-lyon1.fr.

Submitted for publication November 13, 2009; accepted in revised form March 12, 2010.

lecular patterns (PAMPs), whereas TLR-3, TLR-7, TLR-8, and TLR-9 recognize nucleic acid–based PAMPs. TLR activation leads to the generation of cytokines and chemokines and to the maturation of antigen-presenting cells (APCs) by up-regulating costimulatory molecules that promote efficient interactions between APCs and T cells. The recent identification of endogenous ligands for TLR-3 and TLR-7, such as RNA, suggests that TLRs may be involved in autoimmune disease pathogenesis (4–8).

In the present study, we investigated the expression, regulation, and function of TLR-3 and TLR-7 in PM and DM muscle tissues and isolated cultured human myoblasts. Our results indicate that TLR-3 and TLR-7 are overexpressed in myositis muscle tissue, as compared with normal or noninflammatory myopathic muscle tissue. Stimulation of the TLR-3 pathway in human myoblasts by the TLR-3 ligand poly(I-C), as well as by necrotic myoblasts, induced the production of IL-6 and of the β chemokine CCL20, which is involved in the differentiation and the migration of Th17 cells. The inducing effect of necrotic myoblasts in combination with IL-17 on IL-6 production was decreased after TLR-3 blockade. The expression of TLR-3 was differentially regulated by Th1 and Th17 cytokines.

MATERIALS AND METHODS

Patients and muscle biopsies. Muscle biopsy specimens were obtained from 11 patients with myositis (8 with PM and 3 with DM). Muscle tissue samples from 4 healthy patients undergoing joint replacement surgery and from 1 patient with a metabolic myopathy (McArdle disease/glycogenosis disease type V) were used as controls. Patients with PM/DM all had disease that met the Bohan and Peter criteria (9), with characteristic inflammatory lesions observed on muscle biopsy. For immunohistochemical analysis, tissues were snap frozen in liquid nitrogen and stored at −80°C until further use.

Immunohistochemistry and immunofluorescence. Frozen sections of muscle tissues (10 μm) were thawed, fixed in acetone for 10 minutes, air dried, and then rehydrated in phosphate buffered saline (PBS). Immunohistochemical staining was performed for the detection of TLR-3, TLR-7, CD56, and HLA class I. The serial sections were incubated overnight at 4°C with the following primary antibodies: 10 μg/ml of mouse monoclonal anti–TLR-3.7 (IgG1; Santa Cruz Biotechnology), 10 μg/ml of goat polyclonal anti–TLR-7 (Santa Cruz Biotechnology), 1 μg/ml of mouse monoclonal anti-CD56 (IgG1; Novocastra), and 4 μg/ml of mouse monoclonal anti–HLA class I (IgG2a; Immunotech). In control experiments, goat IgG or matched mouse IgG isotype was applied at the same concentration as the primary antibodies. After washing, the sections were incubated with biotinylated anti-mouse and anti-goat immunoglobulins for 15 minutes, followed by streptavidin–peroxidase complex for 15 minutes and 3,3′-diaminobenzidine chromogen solution (Dako). The sections were then counterstained with Mayer's hematoxylin.

Double immunofluorescence staining was performed on unstimulated and stimulated myoblasts and myotubes. After removal of culture medium, muscle cells were incubated for 1 hour with 20 μg/ml of fluorescein isothiocyanate–conjugated mouse monoclonal anti–TLR-3.7 (IgG1; Santa Cruz Biotechnology) and 20 μg/ml of goat polyclonal anti–TLR-7 (Santa Cruz Biotechnology), followed by incubation with phycoerythrin-conjugated porcine anti-goat antibody (IgG; R&D Systems).

Isolation and culture of human myoblasts. Normal muscle samples were obtained from patients with osteoarthritis who were undergoing hip joint replacement. Informed consent was obtained according to the policies of the local ethics committee. After surgery, muscle samples were immediately placed in sterile PBS with antibiotics (penicillin and streptomycin) and washed, and the fat and fibrous tissue was removed. Muscle samples were cut into fragments (1–2 mm^3) and incubated at 37°C for 30 minutes with proteolytic enzymes (1 mg/liter of collagenase; Sigma-Aldrich) and 0.05% trypsin (Invitrogen Life Technologies). After washing and filtration, a first selection was done to remove fibroblasts by incubating the supernatants in petri dishes at 37°C for 1 hour. Unattached myoblasts were then transferred and cultured in Ham's F-10 nutrient mixture (supplemented with 20% fetal calf serum [FCS], 2 m*M* L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin; Lonza) at 37°C in a humidified 5% CO_2 incubator. Medium was changed every 4 days. After 10 days, adherent cells were detached with trypsin (Invitrogen Life Technologies), and myoblasts were purified by positive selection with CD56 microbeads, according to the instructions of the manufacturer (Miltenyi Biotec). Cultured myoblasts were used between passages 2 and 8.

Differentiated myotubes were obtained from myoblasts cultured initially in growth medium and later in Dulbecco's modified Eagle's medium (supplemented with 2% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin; Lonza) for at least 14 days before the addition of cytokines. Differentiation into myotubes was confirmed by the low expression level of CD56 and the high expression level of desmin, which were quantified using real-time polymerase chain reaction (PCR) (10).

Stimulation assays. Cultured muscle cells were grown in 6-well plates (3.5×10^5 cells/well) and stimulated for 48 hours with the following agents: IFNγ (50 ng/ml or 10^3 units/ml; R&D Systems), IL-17A (50 ng/ml; R&D Systems), poly(I-C) (25 μg/ml; InvivoGen), and CL097 (5 μg/ml; InvivoGen). For the stimulation assay with necrotic cells, necrotic myoblasts were obtained by heating normal myoblasts at 60°C for 30 minutes and were then added to myoblast cultures at a 5:1 ratio for 24 hours. Cultured myoblasts were incubated with the neutralizing purified anti-human TLR-3 antibody (eBioscience) at a concentration of 10 μg/ml for 4 hours prior to stimulation, when indicated. Then, supernatants were collected, and IL-6, CCL20, and IL-8 protein levels were determined using the DuoSet enzyme-linked immunosorbent assay development system, according to the instructions of the manufacturer (R&D Systems).

Real-time PCR. RNA was extracted using TRIzol reagent, according to the instructions of the manufacturer

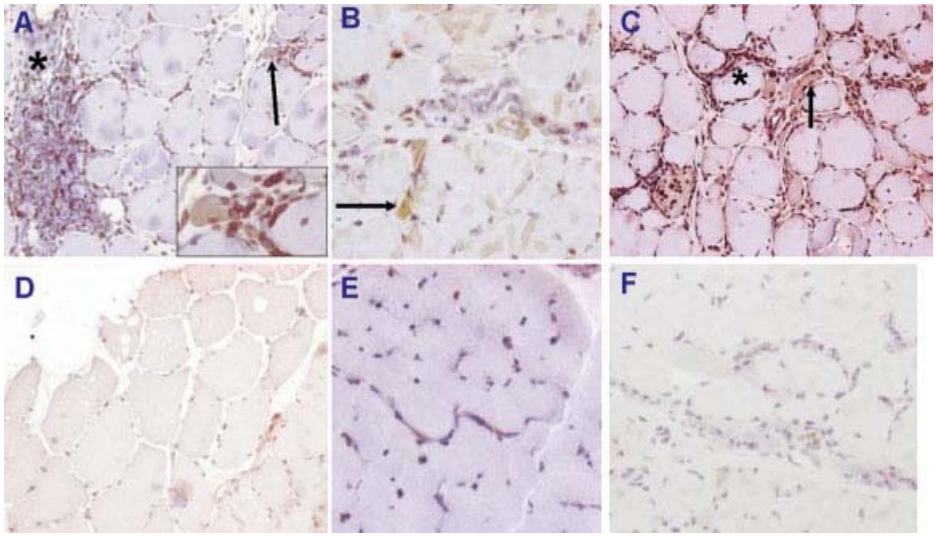


Figure 1. Overexpression of Toll-like receptor 3 (TLR-3) and TLR-7 in polymyositis (PM) and dermatomyositis (DM) muscle tissue. **A–C**, Expression of TLR-3 (**A** and **B**) and TLR-7 (**C**) was detected in muscle tissue from patients with PM (**A** and **C**) and DM (**B**). **D** and **E**, No expression of TLR-3 was observed in muscle tissue from a patient with metabolic myopathy (**D**) or in normal muscle tissue (**E**). In PM/DM tissue, TLR-3 and TLR-7 expression was observed mainly in cells from inflammatory infiltrates (**asterisks**), although a few muscle cells were also positive (**arrows**). **F**, No expression was observed in a section stained with isotype control. (Original magnification × 200; × 400 in **inset** in **A.**)

(Gibco BRL). The concentration of RNA was quantified using a SmartSpec 3000 spectrophotometer at 260 nm (Bio-Rad). Total RNA (1 μg) was reverse transcribed using the ThermoScript reverse transcription–PCR (RT-PCR) system, according to the instructions of the manufacturer (Invitrogen Life Technologies). Specific messenger RNA (mRNA) was quantified using real-time PCR. Briefly, PCR was performed in a LightCycler 2.0 using the Fast-Start DNA Master SYBR Green I real-time PCR kit, according to the instructions of the manufacturer (Roche Molecular Biochemicals). Thermocycling was performed in a final volume of 20 μl, with 3 m*M* $MgCl_2$ and 0.5 μM of each of the required primers. PCR was performed with an initial denaturation step (10 minutes at 95°C) followed by 40 touchdown cycles (10 seconds at 95°C, annealing for 10 seconds at 58–68°C, and extension for 16 seconds at 72°C). Primer-specific nucleotide sequences for TLR-3 (GenBank accession no. NM_003265) (forward 5′-GAGCTATCTCAACTTTCTG-3′ and reverse 5′-AGCTGAACCTGAGTTCCTA-3′) were synthesized by Eurogentec. Specific primer sets for TLR-7 and GAPDH were purchased from Search-LC and optimized for the LightCycler. The copy number of target mRNA was normalized to the housekeeping gene GAPDH. The relative gene expression of TLR-3 was calculated in relation to expression in unstimulated myoblasts.

Statistical analysis. Data are presented as the mean ± SEM. The nonparametric Mann-Whitney U test or Wilcoxon's matched pairs test, when appropriate, was used to compare groups. *P* values less than 0.05 were considered significant.

RESULTS

Increased expression of TLR-3 and TLR-7 in myositis tissue. Immunohistochemistry was used to investigate TLR-3 and TLR-7 protein expression in muscle tissue from patients with PM/DM, and the expression pattern was compared with that found in normal and noninflammatory metabolic myopathic muscle tissue. TLR-3 and TLR-7 proteins were broadly expressed in inflammatory infiltrates from PM/DM muscle tissue (Figures 1A–C). Small muscle fibers expressing TLR-3 or TLR-7 could be identified in muscle samples from PM/DM patients (Figures 1A–C). In contrast, neither TLR-3 nor TLR-7 protein expression was detected in normal or noninflammatory myopathic muscle tissue (Figures 1D and E).

Muscle cells expressing TLR-3 or TLR-7 also express high levels of CD56 and HLA class I. The muscle fibers expressing TLR-3 and TLR-7 were further characterized using antibodies to CD56, a marker of immature myoblasts, and antibodies to HLA class I antigens, which are known to be overexpressed in myositis (1,11). Staining of serial PM/DM muscle sections with antibodies to CD56, TLR-3, or TLR-7 exhibited

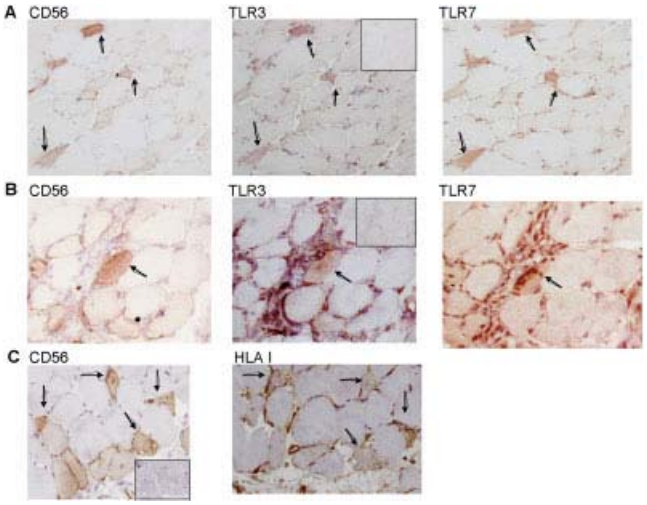


Figure 2. Expression of TLR-3, TLR-7, and HLA class I in muscle cells expressing CD56. **A** and **B,** Serial DM (**A**) and PM (**B**) muscle biopsy sections were stained with antibodies against CD56, TLR-3, or TLR-7. These antibodies stained an overlapping subset of cells (**arrows**) expressing high levels of CD56, a marker of muscle cell regeneration, and either TLR-3 or TLR-7. **C,** Serial PM muscle biopsy sections were stained with antibodies against CD56 and HLA class I antigens. Antibodies stained an overlapping subset of cells (**arrows**) expressing high levels of CD56 and HLA class I antigens. **Insets** show isotype control staining. (Original magnification × 200.) See Figure 1 for definitions.

strong staining for TLR-3 and TLR-7 in immature myoblast precursors (Figures 2A and B). Interestingly, cells expressing CD56, a marker of muscle cell regeneration, also expressed high levels of HLA class I (Figure 2C). Together, these data demonstrate that TLR expression was particularly enhanced in less differentiated muscle cells expressing CD56 and high levels of HLA class I antigens.

Expression of TLR-3 mRNA in human myoblasts is differentially regulated by the Th1 and Th17 cytokines IFNγ and IL-17. Analysis using double immunofluorescence staining confirmed that TLR-3 and TLR-7 proteins were expressed by myoblasts (Figure 3A). Coexpression of TLR-3 with TLR-7 was identified by overlay. TLR-3 mRNA expression was quantified using RT-PCR in cultured myoblasts and after stimulation for 48 hours with the TLR-3 ligand poly(I-C), the TLR-7 ligand CL097, the Th1 cytokine IFNγ, and the Th17 cytokine IL-17A (Figure 4A). Human myoblasts constitutively expressed TLR-3 mRNA. Stimulation with poly(I-C) and IFNγ induced strong expression of TLR-3 mRNA, as compared with unstimulated myoblasts (mean ± SEM 18.25 ± 3.54–fold and 4.253 ± 0.62–fold, respectively; $P < 0.005$) (Figure 4A). In contrast, stimulation with IL-17A significantly down-regulated TLR-3 mRNA expression (mean ± SEM 0.49 ± 0.07–fold; $P < 0.0001$) and inhibited the inducing effect of IFNγ on TLR-3 expression (mean ± SEM 2.61 ± 0.68–fold; $P < 0.05$ versus stimulation with IFNγ alone) (Figure 4A). TLR-7 mRNA expression levels were increased after stimulation with CL097 but were not modulated by stimulation with either IFNγ or IL-17 (data not shown). These

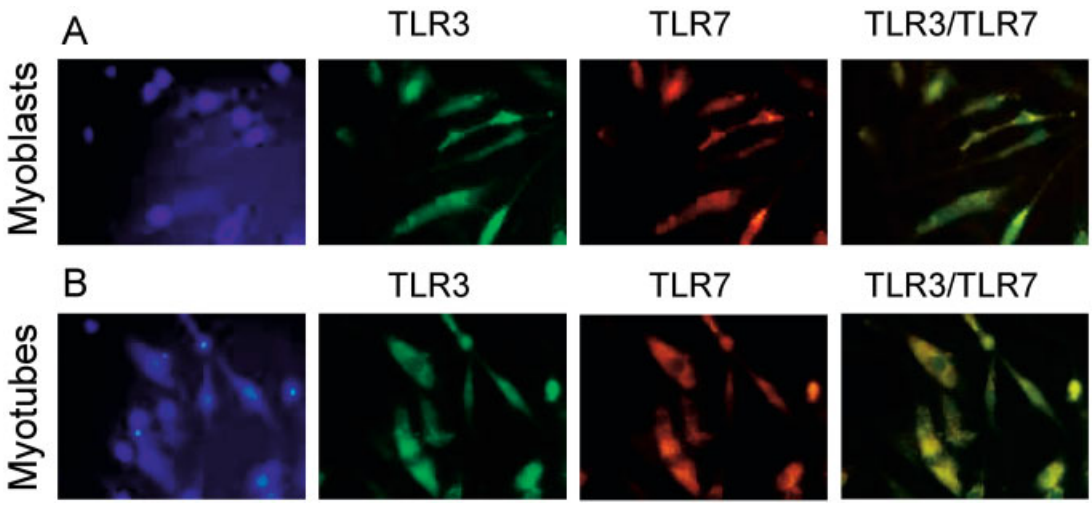


Figure 3. Detection of Toll-like receptor 3 (TLR-3) and TLR-7 protein expression in myoblasts and myotubes. Double immunofluorescence staining with anti–TLR-3 antibody (green) and anti–TLR-7 (red) was performed in cultured myoblasts (**A**) and myotubes (**B**). An overlay of cells that were positive for TLR-3 and TLR-7 is also shown (yellow). Blue fluorescent Hoechst dye was used for nuclear staining. (Original magnification × 400.)

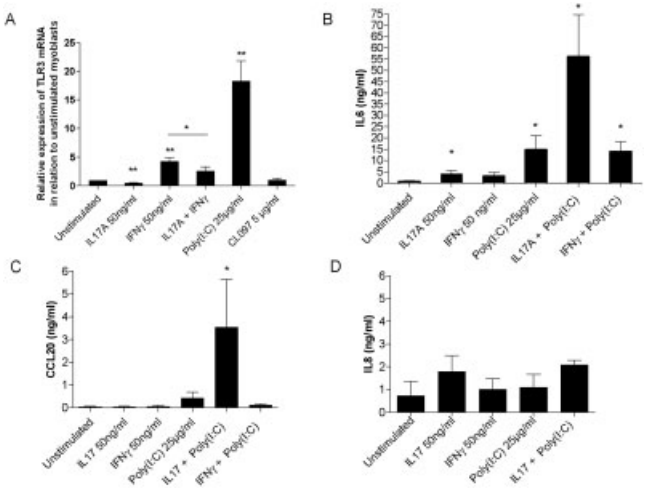


Figure 4. Expression of Toll-like receptor 3 (TLR-3) mRNA and production of interleukin-6 (IL-6), CCL20, and IL-8 by cultured myoblasts following stimulation with poly(I-C), IL-17, and interferon-γ (IFNγ). Cultured myoblasts were stimulated for 48 hours with poly(I-C) or the TLR-7 agonist CL097, IL-17 (50 ng/ml), and IFNγ (50 ng/ml) in various combinations. TLR-3 mRNA induction (**A**) was analyzed by real-time reverse transcription–polymerase chain reaction, and IL-6 (**B**), CCL20 (**C**), and IL-8 (**D**) protein production in supernatants was determined using enzyme-linked immunosorbent assay. Bars show the mean and SEM of at least 5 different myoblast cultures. $* = P < 0.05$; $** = P < 0.005$, versus unstimulated myoblasts. $*$ over horizontal line $= P < 0.05$ for the indicated comparison.

results demonstrated a differential effect of Th1 and Th17 cytokines on TLR-3 expression.

Induction of myoblast activation following stimulation with necrotic muscle cells and IL-17. To further investigate the opposite effects of IFNγ and IL-17 on TLR-3 expression, we analyzed the function of TLR-3 following stimulation with poly(I-C), IFNγ, and IL-17A. In response to poly(I-C), myoblasts produced significant amounts of the proinflammatory cytokine IL-6 (Figure 4B). Moreover, when added to poly(I-C), IL-17A, but not IFNγ, increased in a synergistic manner the production of IL-6 by myoblasts (Figure 4B). For the β chemokine CCL20, which is involved in DC and Th17 cell recruitment, and the CXC chemokine IL-8, which is involved in the recruitment of neutrophils, IL-17A, but not IFNγ, added to poly(I-C) induced a strong production of CCL20, whereas no effect was detected with IL-17A, poly(I-C), or their combination on the production of IL-8 (Figures 4C and D).

Since myositis is characterized by high levels of local muscle death, the effects of necrotic muscle cells were examined on cultured myoblasts. Similarly to poly(I-C), necrotic myoblasts induced a strong production of IL-6 (Figure 5). IL-17 increased in a synergistic manner the inducing effect of necrotic muscle cells on IL-6 production, whereas IFNγ had no effect (Figure 5). The production of IL-6 induced by the combination of necrotic cells and IL-17 was significantly decreased by 21% after TLR-3 blockade (mean ± SEM 8.609 ± 1.994 ng/ml versus 6.829 ± 1.521 ng/ml; $P < 0.05$) (Figure 5). A similar 21% decrease in IL-6 was also observed for poly(I-C)–induced IL-6 production (mean ± SEM 6.815 ± 1.611 ng/ml versus 5.379 ± 1.451 ng/ml; $P < 0.05$) (Figure 5). These results indicated that muscle cell activation induced by necrotic myoblasts was, in part, dependent of the TLR-3 pathway.

Effect of myoblast differentiation on TLR-3 expression and cytokine and chemokine production. As for myoblasts, double immunofluorescence staining confirmed the coexpression of TLR-3 and TLR-7 in differentiated myotubes obtained by switching growth medium to fusion medium, as described previously (12) (Figure 3B). In order to evaluate the possible link with muscle cell differentiation, we analyzed whether the in vitro differentiation and fusion of myoblasts into myotubes had an effect on the expression levels of TLR-3 mRNA and on IL-6 and CCL20 production. TLR-3 mRNA levels were higher in unstimulated myotubes

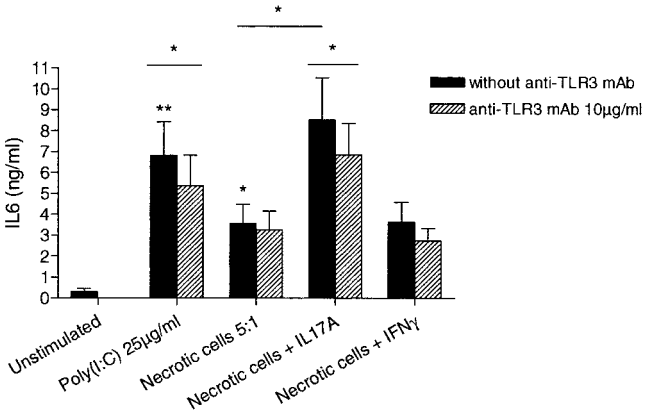


Figure 5. Production of interleukin-6 (IL-6) by normal cultured myoblasts following stimulation with necrotic muscle cells. Cultured myoblasts were stimulated for 24 hours with poly(I-C), necrotic myoblasts, or a combination of necrotic myoblasts and either IL-17 (50 ng/ml) or interferon-γ (IFNγ) (50 ng/ml). Myoblasts were incubated with neutralizing anti-human Toll-like receptor 3 (anti–TLR-3) monoclonal antibody (mAb) for 4 hours prior to stimulation. Bars show the mean and SEM of at least 5 different myoblast cultures. $* = P < 0.05$; $** = P < 0.005$, versus unstimulated myoblasts. $*$ over horizontal bars $= P < 0.05$ for the indicated comparisons.

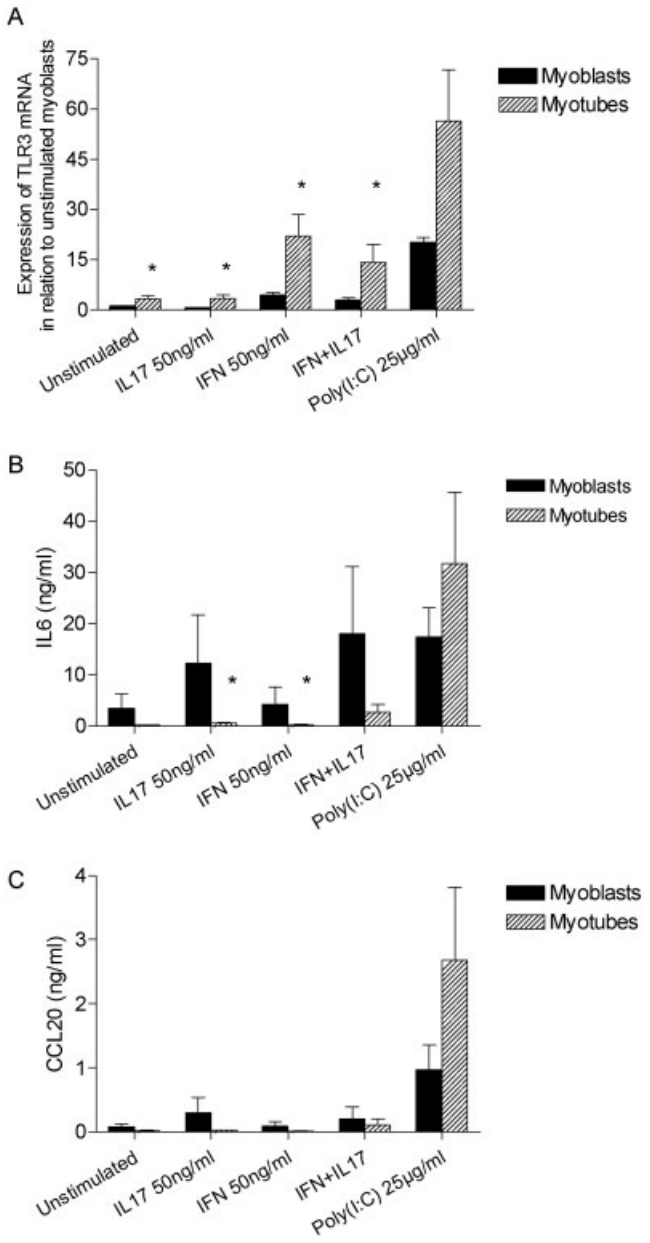


Figure 6. Effect of myoblast differentiation into myotubes on Toll-like receptor 3 (TLR-3) mRNA expression and on interleukin-6 (IL-6) and CCL20 production. Cultured myotubes were obtained from myoblasts cultured first in Ham's F-10 growth medium (supplemented with 20% fetal calf serum) and then in Dulbecco's modified Eagle's medium (supplemented with 2% horse serum) for at least 14 days before the addition of the TLR-3 agonist poly(I-C), IL-17 (50 ng/ml), and interferon-γ (IFNγ) (50 ng/ml) for 48 hours. TLR-3 mRNA induction (**A**) was analyzed by real-time reverse transcription–polymerase chain reaction, and IL-6 (**B**) and CCL20 (**C**) protein production in supernatants was determined using enzyme-linked immunosorbent assay. Bars show the mean and SEM of 5 different myoblast cultures. $* = P < 0.05$ versus myoblasts.

than in unstimulated myoblasts (mean ± SEM 3.194 ± 1.031-fold; $P < 0.05$) (Figure 6A). Stimulation with poly(I-C) and IFNγ induced higher levels of TLR-3 mRNA in myotubes than in myoblasts. In myotubes, IL-17 stimulation did not decrease TLR-3 mRNA expression, as compared with unstimulated myotubes (mean ± SEM 3.394 ± 0.99-fold versus 3.194 ± 1.03-fold). However, as in myoblasts, IL-17 stimulation inhibited the inducing effect of IFNγ on TLR-3 expression, but the difference was not significant (mean ± SEM 14.24 ± 5.35-fold versus 22.01 ± 6.54-fold). The secretion of IL-6 and CCL20 was higher in myoblasts, except after stimulation with poly(I-C) (Figures 6B and C).

DISCUSSION

The aim of this study was to investigate the role of the innate immune system via TLRs in the development of autoimmune muscle. As in other autoimmune diseases, such as rheumatoid arthritis, the expression of TLR-3 and TLR-7, the two TLRs involved in the recognition of nucleic acid-based PAMPs and endogenous ligands, was found to be specific to inflammatory tissues. TLR-3 and TLR-7 expression was detected in both PM and DM muscle samples, whereas no expression was detectable in normal or noninflammatory myopathic muscle. This is consistent with the findings of one study that showed muscle expression of TLR-3 in patients with sporadic inclusion body myositis but not in controls (13).

When we examined HLA class I expression in myositis muscle, which is characteristic of inflammatory muscle tissue (11), cells expressing high levels of TLR-3 also expressed high levels of HLA class I, as well as CD56, a marker of immature myoblast. The identification of muscle fibers expressing TLR-3 and TLR-7 as less differentiated muscle cells was confirmed by the coexpression of TLR-3, TLR-7, and CD56 in serial sections of PM and DM muscle biopsies. CD56 is constitutively expressed by proliferating immature myoblasts but is absent in mature cells (10,14). Quiescent myoblast precursors, also known as satellite cells, are present in normal muscle tissue. They can become proliferating cells after muscle damage and then can participate in the regeneration process. Thus, the presence of CD56+ cells in muscle tissue could represent a signal of damage and/or of possible regeneration. It is unclear if such active regeneration is effective in the context of inflammation.

In contrast to the in vivo results, we did not

observe a down-regulation of TLR-3 mRNA expression in cultured differentiated myotubes, as compared with proliferating myoblasts. This could be explained by a state of in vitro differentiation not similar enough to that observed in vivo in muscle tissue. These results, strengthened by the previous observations of an increased expression of myositis autoantigens and HLA class I antigens in regenerating muscle cells (15), are consistent with a potential role for TLR-3 and TLR-7 in disease initiation or propagation. Muscle tissues, in the context of nonspecific muscle damage (i.e., trauma, exposure to a toxin, viral infection, etc.) that increases the number of immature myoblast precursors, also termed regenerating cells (15), may be exposed to many PAMPs that could activate TLRs, leading to the maturation of DCs and the secretion of proinflammatory cytokines and chemokines.

Therefore, we investigated the function and regulation of TLR-3 in human myoblast cultures. Stimulation with the TLR-3 ligand poly(I-C), a synthetic analog of double-stranded RNA and necrotic muscle cells, induced a strong production of IL-6 by myoblasts. The inducing effect of both poly(I-C) and necrotic muscle cells was further increased in a synergistic manner when combined with IL-17, but not with IFNγ. Blockade of the TLR-3 pathway by a monoclonal antibody resulted in a significant reduction in the stimulatory effect of necrotic muscle cells in combination with IL-17, suggesting that necrotic cells play a role in myoblast activation through TLR-3 pathway. It should be noted, however, that the inhibitory effect of this antibody was modest, even on the poly(I-C)-induced effect. We were not able to identify better antibodies among those currently available. However, the degree of inhibition observed with necrotic cells was similar to that observed for poly(I-C), which was used here as a positive control for a TLR-3–mediated effect.

IL-6, a proinflammatory cytokine involved in T and B cell differentiation, can drive Th17 differentiation through the induction of IL-23 (16). IL-6 could also play a major role in the early stages of muscle cell differentiation (17). Its exact effect remains unclear, with some investigators reporting a catabolic response (18) and others reporting an anabolic response (19). In sharp contrast, activation of TLR-7 with CL097 and loxoribine had no effect (data not shown), suggesting a specific TLR-3 activation.

To reproduce the microenvironment found in PM/DM, we stimulated myoblasts with the Th1 and Th17 cytokines IFNγ and IL-17, which have been observed in PM/DM muscle samples (2,3). The proinflammatory properties of IL-17 have been demonstrated previously by synergistic interactions with tumor necrosis factor α and IL-1 on the production of IL-6 and CCL20 (20). In the present study, IL-17, but not IFNγ, synergized with poly(I-C) for the myoblast production of CCL20, which is in turn, a chemotactic factor for Th17 cells. Then, muscle fiber itself may be implicated in the recruitment of leukocytes and DCs by local production of β chemokines induced by IL-17 in combination with IL-1 and by TLR-3 activation. Consistent with the pathologic findings, IL-8 was not induced, and indeed, neutrophils are not found in myositis samples. Finally, we demonstrated a differential regulation of TLR-3 expression in myoblasts by Th1 and Th17 cytokines. This is consistent with the balance between Th1 and Th17 pathways and between Th17 and Treg cells (16). IFNγ has been shown to have an inhibitory effect on the production of IL-17 and IL-6. In addition, IL-6, which favors the Th17 pathway, has an inhibitory effect on the Treg cell pathway (21). In myoblasts, we observed an inhibitory effect of IL-17 on the inducing effect of IFNγ on TLR-3 expression.

In conclusion, our results indicate that the innate immune system may be involved in autoimmune muscle disorders through the activation of the TLR pathway, possibly by exogenous and/or endogenous activators released from necrotic cells. Overexpression of TLR-3 and TLR-7 was characteristic of inflammatory myopathy. IFNγ and IL-17 had an opposite effect on TLR-3 expression, indicating a differential contribution of Th1 and Th17 cells.

ACKNOWLEDGMENTS

We thank Professor P. Dechelotte and Dr. A. M. Beaufrere, Laboratory of Pathology, Centre Hospitalier Universitaire Clermont-Ferrand, for access to muscle biopsy tissues and for technical assistance.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Miossec had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Tournadre, Miossec.

Acquisition of data. Tournadre, Lenief.

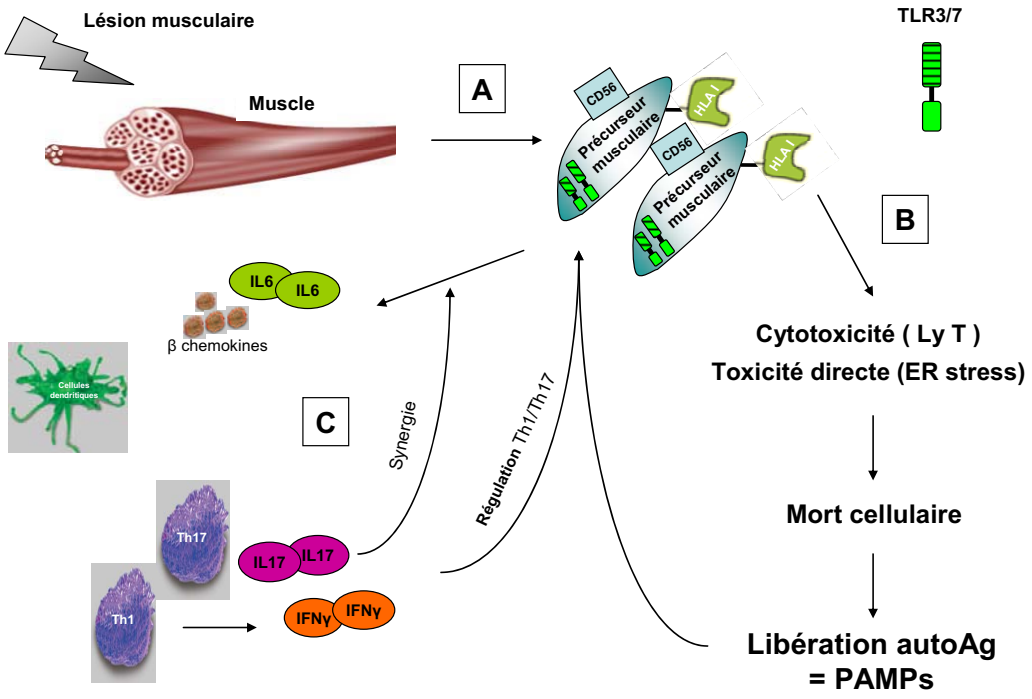
Analysis and interpretation of data. Tournadre, Lenief, Miossec.

REFERENCES

1. Dalakas MC, Hohlfeld R. Polymyositis and dermatomyositis. Lancet 2003;362:971–82.

2. Page G, Chevrel G, Miossec P. Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: interaction with chemokines and Th1 cytokine-producing cells. Arthritis Rheum 2004;50:199–208.
3. Chevrel G, Page G, Granet C, Streichenberger N, Varennes A, Miossec P. Interleukin-17 increases the effects of IL-1β on muscle cells: arguments for the role of T cells in the pathogenesis of myositis. J Neuroimmunol 2003;137:125–33.
4. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 2002;416:603–7.
5. Lau CM, Broughton C, Tabor AS, Akira S, Flavell RA, Mamula MJ, et al. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. J Exp Med 2005;202:1171–7.
6. Brentano F, Schorr O, Gay RE, Gay S, Kyburz D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via TOLL-like receptor 3. Arthritis Rheum 2005;52:2656–65.
7. Patole PS, Grone HJ, Segerer S, Ciubar R, Belemezova E, Henger A, et al. Viral double-stranded RNA aggravates lupus nephritis through Toll-like receptor 3 on glomerular mesangial cells and antigen-presenting cells. J Am Soc Nephrol 2005;16:1326–38.
8. Doughty LA, Carlton S, Galen B, Cooma-Ramberan I, Chung CS, Ayala A. Activation of common antiviral pathways can potentiate inflammatory responses to septic shock. Shock 2006;26:187–94.
9. Bohan A, Peter JB. Polymyositis and dermatomyositis. N Engl J Med 1975;292:344–7.
10. Stewart JD, Masi TL, Cumming AE, Molnar GM, Wentwoth BM, Sampath K, et al. Characterization of proliferating human skeletal muscle-derived cells in vitro: differential modulation of myoblast markers by TGF-β2. J Cell Physiol 2003;196:70–8.
11. Nagaraju K, Raben N, Loeffler L, Parker T, Rochon PJ, Lee E, et al. Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies. Proc Natl Acad Sci U S A 2000;97:9209–14.
12. Nagaraju K, Raben N, Merritt G, Loeffler L, Kirk K, Plotz P. A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli. Clin Exp Immunol 1998;113:407–14.
13. Schreiner B, Voss J, Wischhusen J, Dombrowski Y, Steinle A, Lochmuller H, et al. Expression of toll-like receptors by human muscle cells in vitro and in vivo: TLR3 is highly expressed in inflammatory and HIV myopathies, mediates IL-8 release and up-regulation of NKG2D-ligands. FASEB J 2006;20:118–20.
14. Illa I, Leon-Monzon M, Dalakas MC. Regenerating and denervated human muscle fibers and satellite cells express neural cell adhesion molecule recognized by monoclonal antibodies to natural killer cells. Ann Neurol 1992;31:46–52.
15. Casciola-Rosen L, Nagaraju K, Plotz P, Wang K, Levine S, Gabrielson E, et al. Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy. J Exp Med 2005;201:591–601.
16. Miossec P. Interleukin-17 in fashion, at last: ten years after its description, its cellular source has been identified. Arthritis Rheum 2007;56:2111–5.
17. Jo C, Kim H, Jo I, Choi I, Jung SC, Kim J, et al. Leukemia inhibitory factor blocks early differentiation of skeletal muscle cells by activating ERK. Biochim Biophys Acta 2005;1743:187–97.
18. Ebisui C, Tsujinaka T, Morimoto T, Kan K, Iijima S, Yano M, et al. Interleukin-6 induces proteolysis by activating intracellular proteases (cathepsins B and L, proteasome) in C2C12 myotubes. Clin Sci (Lond) 1995;89:431–9.
19. Austin L, Burgess AW. Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines. J Neurol Sci 1991;101:193–7.
20. Chabaud M, Fossiez F, Taupin JL, Miossec P. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. J Immunol 1998;161:409–14.
21. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235–8.

Figure 5. Hypothèse physiopathologique du rôle et de la fonction de TLR3 et TLR7 dans le tissu musculaire inflammatoire.



A. Suite à une lésion musculaire (virus, traumatisme, toxique…), initiation d'un processus de régénération avec augmentation dans le tissu musculaire du nombre de précurseurs musculaires CD56 positifs, exprimant également fortement les antigènes HLA de classe I et les TLR3 et TLR7.

B. La surexpression des antigènes de HLA de classe I est responsable d'une toxicité musculaire, via soit une cytotoxicité médiée par les lymphocytes T, soit un stress du réticulum endoplasmique. Il s'ensuit une destruction musculaire avec libération d'autoantigènes et de ligands endogènes à partir des cellules nécrotiques.

C. Ces ligands endogènes (PAMPs) vont pouvoir du fait d'une surexpression musculaire de TLR3, activer la voie TLR3 et induire la production par les cellules musculaires immatures de la cytokine proinflammatoire IL6 et de la β chémokine CCL20. En retour, la β chémokine CCL20 et l'IL6 participent au recrutement des cellules de l'immunité adaptative (cellules dendritiques, lymphocytes T) et à la différentiation des lymphocytes T. Les lymphocytes Th1 et Th17 ainsi présents régulent l'expression musculaire de TLR3, induisent la production de cytokines et chémokines proinflammatoires (IL1, TNFα, IL6, CCL20) par les cellules mononuclées et les cellules musculaires, et la surexpression des antigènes HLA de classe I, qui amplifient le processus inflammatoire.

V-b Les C-type lectin récepteurs (CLRs).

V-b-1. Introduction.

Les CLRs constituent le second type de récepteur de l'immunité innée après les TLRs. Ils sont exprimés à la surface des cellules dendritiques, monocytes et macrophages, et sont activés par la partie carbohydrate des glycoprotéines. Comme les TLRs, ils permettent donc le lien entre immunité innée et immunité adaptative et peuvent orienter la réponse immunitaire par l'activation de différents types de cellules dendritiques [65]. Ainsi, BDCA2 (Blood DC antigen 2) est spécifique des cellules dendritiques plasmacytoïdes, Langerin/CD207 des cellules de Langerhans, DC-SIGN/CD209 (DC-specific intercellular adhesion molecule-3-grabbing non-integrin) des cellules dendritiques interstitielles. D'autres CLRs ont une expression moins spécifique. C'est le cas d'ASGPR/CD301, (asialoglycoprotein receptor) exprimé par les cellules hépatiques, les cellules dendritiques interstitielles immatures et les polynucléaires [95], et de MMR/CD206 (macrophage mannose receptor) exprimé par les macrophages, les cellules dendritiques myéloïdes dérivées des monocytes, mais aussi par des cellules épithéliales hépatiques ou rétiniennes [65, 96]. La liaison des récepteurs DC-SIGN, ASGPR et MMR par le ligand spécifique induit un signal d'activation des cellules dendritiques conduisant à un processus d'endocytose et de présentation antigénique [95]. Outre leur fonction de reconnaissance des pathogènes, les CLRs peuvent remplir la fonction de molécule d'adhésion entre les cellules dendritiques et d'autres types cellulaires. Par exemple, DC-SIGN présent à la surface des cellules dendritiques peut se lier à CD11b présent sur les neutrophiles, ou à ICAM-2 présent à la surface des cellules endothéliales en contribuant alors à la diapédèse, ou encore à ICAM-3 molécule de costimulation présente à la surface des lymphocyte T [65].

Nous nous sommes intéressés à l'expression des CLRs associés aux cellules dendritiques et macrophages, DC-SIGN, ASGPR, MMR, dans le tissu musculaire des myopathies inflammatoires.

V-b-2. Expression *in vivo* dans le tissu musculaire inflammatoire.

Nous avons analysé par immunohistochimie, dans des biopsies musculaires de PM et DM, l'expression tissulaire de DC-SIGN, ASGPR et MMR, et leur association aux

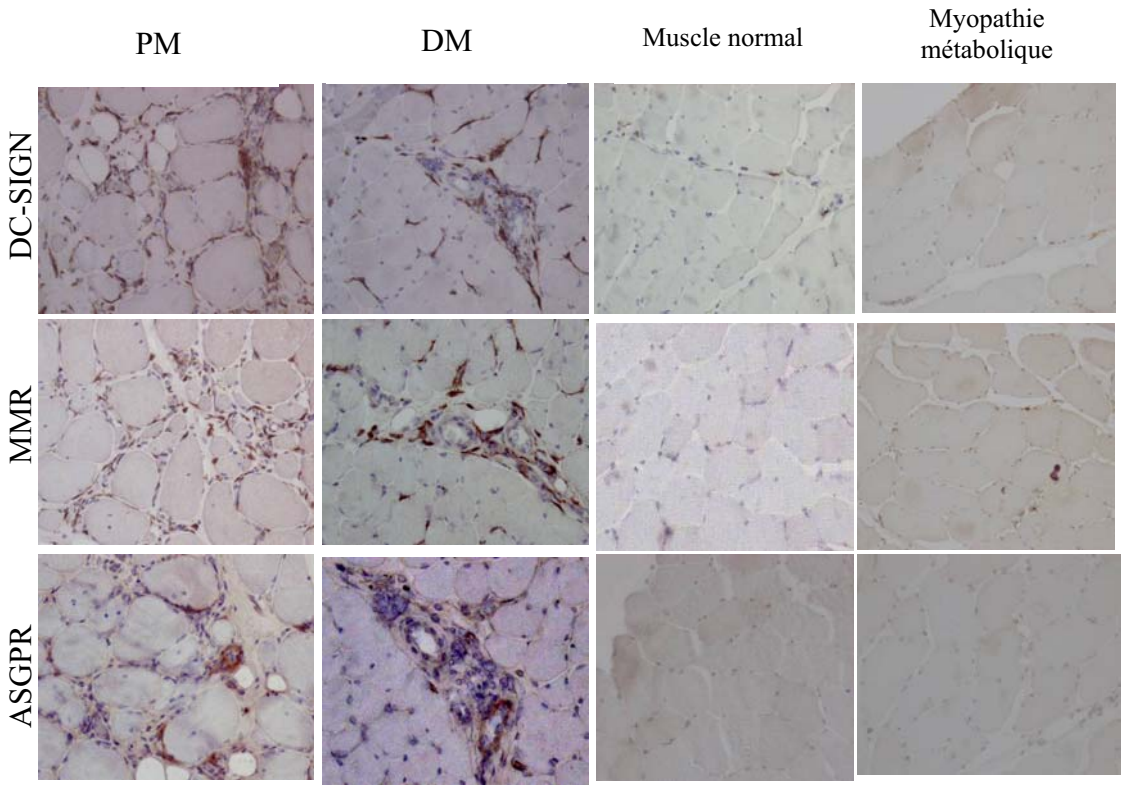
macrophages CD68+, aux cellules dendritiques myéloïdes CD11c +, et aux cytokines IL12 et IL23 produites par les cellules dendritiques activées et impliquées dans la différentiation des lymphocytes Th1 et Th17. Ainsi, les biopsies musculaires de 8 patients atteints de PM (n=5) et de DM (n=3), remplissant les critères diagnostic de *Bohan et Peter* [14], ont été comparées à 5 biopsies contrôles dont 4 biopsies musculaires normales obtenues lors d'une arthroplastie de hanche et 1 biopsie musculaire obtenue chez un patient porteur d'une myopathie non-inflammatoire (maladie de Mac Ardle ou glycogénose type V).

Les prélèvements musculaires ont été congelés et conservés dans l'azote liquide jusqu'à leur utilisation en coupes successives de 10 µm. Les sections musculaires ont ensuite été fixées dans de l'acétone, réhydratées dans un tampon PBS, puis incubées durant 45 mn à température ambiante avec les anticorps primaires suivants : 5 µg/ml d'anticorps monoclonal de souris anti-DC-SIGN (IgG2b, Dendritics, Lyon, France), 5 µg/ml d'anticorps monoclonal de souris anti-ASGPR (IgG1, Dendritics, Lyon, France), anticorps monoclonal de souris anti-MMR (IgG1, Dendritics, France) à la dilution 1:100, 25 µg/ml d'anticorps monoclonal de souris anti-IL12 (IgG1, Abcam, Cambridge, UK), 5 µg/ml of d'anticorps polyclonal de lapin anti-IL23 (IgG, Abcam, Cambridge, UK), anticorps monoclonal de souris anti-CD11c (IgG1, BD Biosciences, CA) à la dilution 1:50, anticorps monoclonal de souris anti-CD68 (IgG1, DAKO, Glostrup, Denmark) à la dilution 1:500. Les isotypes correspondants, IgG de souris ou de lapin, ont été utilisés à la même concentration et durant la même période d'incubation, comme contrôles négatifs. Les sections musculaires ont ensuite été incubées durant 15 mn avec l'anticorps secondaire biotinylé anti-souris et anti-lapin puis le marquage a été révélé par l'application du complexe enzymatique streptavidin-peroxidase suivi du chromogéne DAB (DAKO, Glostrup, Denmark). La solution d'hématoxyline de Mayer a été utilisée comme contre-coloration nucléaire.

L'expression des CLRs, DC-SIGN, ASGPR et MMR, est largement détectée dans l'infiltrat inflammatoire des biopsies musculaires de PM et DM, avec une répartition prédominante dans l'endomysium pour les PM et dans les régions périvasculaires pour les DM (Figure 6). A l'inverse, aucun marquage positif n'est détecté dans les biopsies contrôles saines ou de myopathie non inflammatoire (Figure 6). Afin de préciser le type de cellules exprimant ces CLRs, un immunomarquage pour CD11c, CD68, IL12 et IL23 a été réalisé sur des coupes successives de muscle de PM et DM. L'expression de DC-SIGN, ASGPR et MMR est associée à la présence de macrophages CD68 positifs et de cellules dendritiques

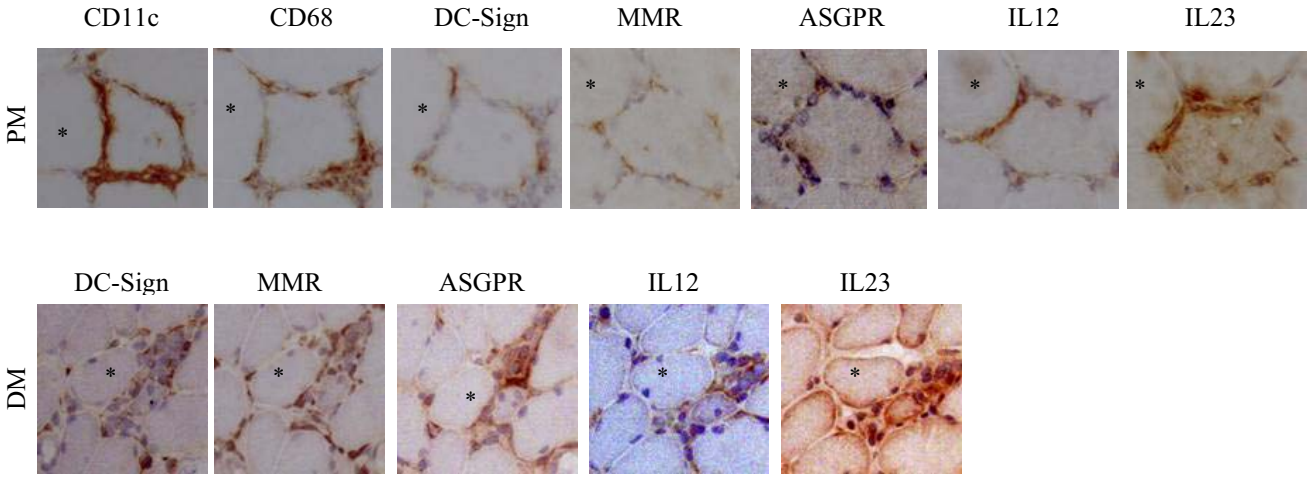
myéloïdes CD11c positives, ainsi qu'à la détection des cytokines IL12 et IL23 (Figure 7). La présence dans l'infiltrat inflammatoire des PM et DM des CLRs exprimés par les cellules dendritiques et les macrophages, en association avec les cytokines IL12 et IL23 produites par les cellules dendritiques activées, et impliquées dans la différentiation des lymphocytes T, est donc fortement évocatrice d'une réaction immunitaire en cours impliquant les voies Th1 et Th17.

Figure 6. L'expression des CLRs (DC-SIGN, ASGPR et MMR) est spécifique du tissu musculaire inflammatoire.



L'expression de DC-SIGN, MMR et ASGPR est détectée dans les biopsies musculaires de PM et DM alors qu'elle est absente dans le tissu musculaire sain ou de patient atteint de myopathie non inflammatoire. Chez les patients atteints de PM et DM, les CLRs sont exprimés au sein de l'infiltrat inflammatoire qui prédomine dans les régions endomysiales pour les PM, et dans les régions périvasculaires pour les DM. (x200)

Figure 7. Expression des CLRs (DC-SIGN, ASGPR et MMR) en association avec les cellules dendritiques, macrophages et cytokines IL12 et IL23, dans le tissu musculaire de PM et DM.



Des coupes musculaires successives de PM et DM ont été marquées avec des anticorps anti-CD11c, anti-CD68, anti-DC-SIGN, anti-MMR, anti-ASGPR, anti-IL12 et anti-IL23. Les astérisques (*) permettent de repérer une même fibre musculaire sur les différentes coupes successives.

Sur les biopsies musculaires de PM et DM, la présence des CLRs (DC-SIGN, MMR, ASGPR) est associée à la présence de cellules dendritiques myéloïdes CD11c positives et de macrophages CD68 positifs, ainsi qu'à la détection des cytokines IL12 et IL23. (x200)

Chapitre VI

Rôle des précurseurs musculaires dans la physiopathologie des myopathies inflammatoires.

Sommaire

VI-a Introduction.

La présence dans le tissu musculaire inflammatoire d'un processus de régénération est une des caractéristiques des PM et DM. Les précurseurs musculaires CD56 positifs, qui sont les cellules à potentiel de régénération, sont rarement observés dans le tissu musculaire non inflammatoire alors qu'ils très fréquents dans les biopsies musculaires de PM et DM (Figure 2, p.19). Néanmoins, cette activité de régénération n'est pas fonctionnelle et n'aboutit pas à un processus de réparation, comme en témoignent les signes de destruction associés, tels que la nécrose ou l'atrophie musculaire. L'expression spécifiquement dans le tissu inflammatoire des PM et DM, par les fibres musculaires immatures, de TLR3 et TLR7, des antigènes HLA de classe I, et des auto antigènes spécifiques des myosites, Mi2 et histidyl-tRNA synthétase, [5, 93], plaident pour un rôle central des précurseurs musculaires dans la physiopathologie des myopathies auto-immunes comme cibles potentielles de la réponse immunitaire.

Nous avons donc analysé la présence des précurseurs musculaires CD56 positifs au sein de la réaction inflammatoire musculaire précédemment caractérisée par la coexistence de cellules dendritiques et de macrophages activés, exprimant des CLRs et produisant les cytokines IL12 et IL23 qui orientent vers une réponse immunitaire Th1 et Th17. De plus, les PM et DM étant caractérisées par une signature IFN type I, nous nous sommes demandés si les cellules musculaires elles-mêmes, et en particulier les cellules musculaires immatures, pouvaient avoir un rôle dans la production locale d'IFNβ, et ainsi contribuer à la surexpression musculaire des antigènes HLA de classe I.

VI-b Les précurseurs musculaires au centre de la réaction inflammatoire.

Afin de déterminer le rôle des cellules musculaires immatures CD56 positives dans la réponse immune musculaire, nous avons réalisé un immunomarquage dirigé contre les antigènes CD56 et HLA de classe I en complément des immunomarquages dirigés contre les cellules dendritiques (CD11c), les macrophages (CD68), et les CLRs (DC-SIGN, ASGPR, MMR), sur des coupes successives de biopsies musculaires de 4 patients avec PM et 1 patient avec DM.

A la fois dans les biopsies musculaires de PM et de DM, la présence d'infiltrats inflammatoires constitués de cellules dendritiques CD11c positives et de macrophages CD68 positifs, exprimant DC-SIGN, ASGPR et MMR, est observée préférentiellement autour des précurseurs musculaires immatures, caractérisés par une forte expression de l'antigène CD56 et des antigènes HLA de classe I. Ces données immunohistochimiques sont en faveur d'une réaction immunitaire ciblée sur les fibres musculaires immatures de phénotype CD56 positif, surexprimant les antigènes HLA de classe I et les TLR3 et TLR7.

VI-c Les précurseurs musculaires comme source d'IFN de type I.

VI-c-1 Expression *in vivo* d'IFNβ dans le tissu musculaire.

Des données récentes suggérant un rôle du système IFN de type I dans la physiopathologie des myopathies inflammatoires [7, 53-58], nous avons analysé *in vivo* l'expression d'IFNβ au sein de biopsies musculaires de PM et de DM, de biopsies musculaires normales et de myopathie métabolique. Afin d'identifier les cellules productrices d'IFNβ dans le tissu musculaire inflammatoire des PM et DM, un immunomarquage dirigé contre l'antigène BDCA2, CLR spécifique des cellules dendritiques plasmacytoïdes, et contre les antigènes CD56 et HLA de classe I, marqueurs des précurseurs musculaires immatures, a été réalisé sur des coupes musculaires successives en complément de l'immunomarquage contre l'IFNβ.

L'expression d'IFNβ est observée de façon spécifique dans le tissu musculaire des PM et DM, alors qu'elle est absente du tissu musculaire normal ou myopathique non inflammatoire. L'IFNβ est largement détecté au sein des fibres musculaires à la fois des PM et DM, notamment les fibres caractérisées par leur petite taille et par leur localisation endomysiale pour les PM et périfasciculaire pour les DM. Les fibres musculaires exprimant l'IFNβ sont également caractérisées par une forte expression de l'antigène CD56 et des antigènes HLA de classe I, témoignant de leur phénotype immature. A l'inverse, la production d'IFNβ est plus rarement observée au sein des cellules de l'infiltrat inflammatoire. De façon cohérente, les cellules dendritiques plasmacytoïdes BDCA2 positives, productrices d'IFN de type I, sont également rarement détectées dans l'infiltrat inflammatoire à la fois des PM et DM, suggérant donc que la cellule musculaire elle-même est la principale source d'IFNβ.

VI-c-2 Production *in vitro* d'IFNβ par les cellules musculaire.

Après avoir identifié *in vivo* l'expression d'IFNβ principalement dans les précurseurs musculaires immatures, nous avons analysé *in vitro*, la production d'IFNβ par les myoblastes humains normaux en culture, après stimulation durant 48 heures par le ligand synthétique de TLR3, Poly(I:C), et par les cytokines Th1 (IFNγ) et Th17 (IL17A). Les myoblastes normaux produisent de façon constitutionnelle de faibles quantités d'IFNβ. L'activation de la voie TLR3 par son ligand synthétique spécifique, Poly(I:C), augmente de façon significative la production d'IFNβ ($p<0.05$). Par contre, l'IL17 ou l'IFNγ, seuls ou combinés à Poly(I:C), n'ont aucun effet sur la production musculaire d'IFNβ.

L'expression de TLR3 par les précurseurs musculaires pourrait donc contribuer à la production locale d'IFNβ par ces mêmes cellules, après activation de la voie TLR3. Les résultats précédents suggérant une activation de la voie TLR3 par les cellules nécrotiques musculaires, nous avons analysé la production d'IFNβ par les myoblastes normaux en présence de cellules musculaires nécrotiques. Les cellules nécrotiques augmentent la production d'IFNβ par les myoblastes, mais la différence par rapport aux myoblastes non stimulés reste non significative (moyenne ± SEM 10.06±5.611 vs. 5.05±2.02, $p=0.68$). L'association avec l'IL17 ou l'IFNγ n'augmente pas de façon synergique la production d'IFNβ.

VI-c-3 Rôle de l'IFNβ et de la voie TLR3 dans la surexpression musculaire des antigènes HLA de classe I.

Les IFN de type I, IFNα/β, induisant l'expression des molécules du CMH de classe I [58, 97], nous avons analysé le rôle de la voie TLR3 dans la surexpression musculaire des antigènes HLA de classe I via la production d'IFNβ. Pour cela, des myoblastes ont été stimulés durant 48 heures par le ligand spécifique de TLR3, Poly(I:C) 25 µg/ml ou par l'IFNγ 50 ng/ml, puissant inducteur de l'expression des antigènes HLA de classe I, en présence ou non d'un anticorps purifié neutralisant anti-IFNβ humain, utilisé aux concentrations de 10 µg/ml, 50 µg/ml et 100 µg/ml. L'expression de HLA de classe I induite après stimulation par IFNγ est utilisée comme contrôle positif et l'incubation avec un anticorps neutralisant anti-IFNγ humain 50 µg/ml comme contrôle négatif. L'expression des antigènes HLA de classe I à la surface des myoblastes a été déterminée par cytométrie de flux.

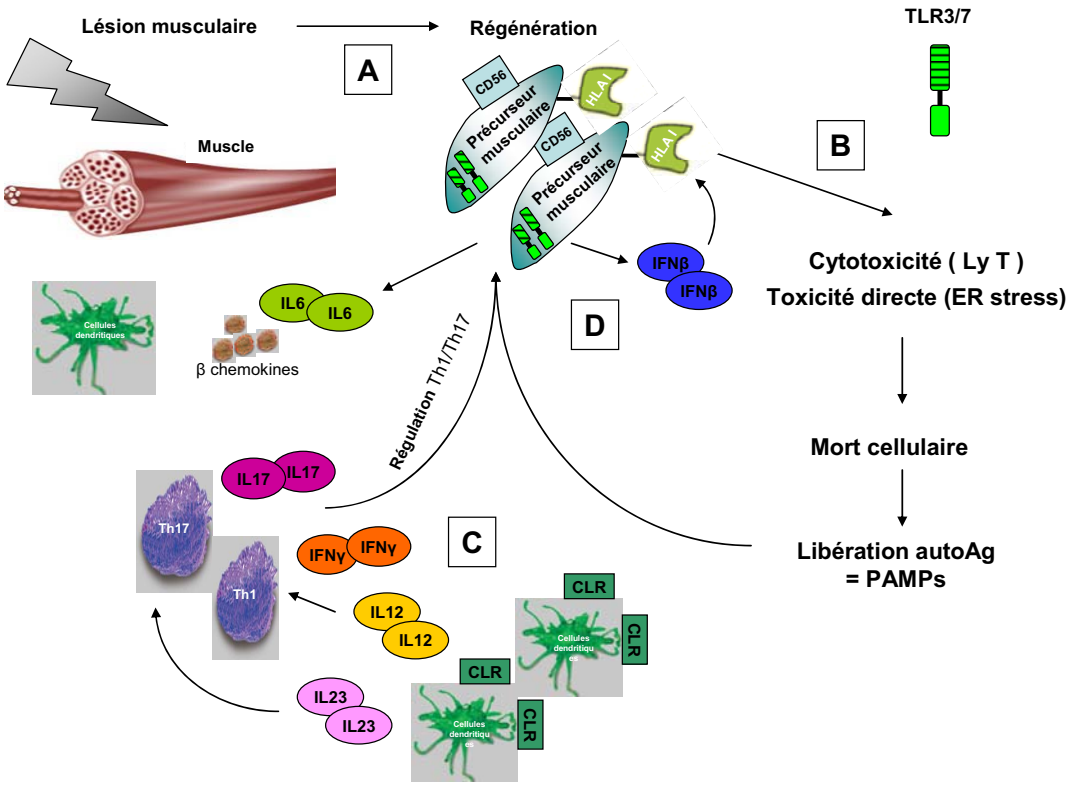
Les myoblastes normaux en culture expriment faiblement les antigènes HLA de classe I. L'IFNγ et l'agoniste de TLR3, Poly(I :C), augmentent de façon significative l'expression de HLA de classe I à la surface cellulaire. Lorsque la production d'IFNβ induite par Poly(I :C) est neutralisée par un anticorps monoclonal anti-IFNβ utilisé à dose croissante, il existe une diminution dose-dépendante de l'expression de HLA de classe I. Aucun effet inhibiteur avec l'anticorps non spécifique anti-IFNγ n'est observé. Le blocage de la production d'IFNβ induite par Poly(I :C) entraine une diminution de l'expression de HLA de classe I de 22.72 % par rapport aux myoblastes stimulés par Poly(I:C) seul ($p=0.08$), et de 38.6 % par rapport aux myoblastes stimulés par Poly(I :C) associé à un anticorps neutralisant non spécifique ($p<0.05$).

Ces données suggèrent donc que l'activation de la voie TLR3 par son ligand spécifique induit l'expression musculaire des antigènes HLA de classe I et que cette surexpression de HLA de classe I est dépendante en partie de la production induite d'IFNβ.

VI-d Discussion et conclusion.

Le rôle de la fibre musculaire elle-même dans la physiopathologie des myopathies inflammatoires est suggéré par plusieurs études, impliquant les fibres musculaires immatures en cours de régénération, la surexpression des antigènes HLA de classe I et l'activation de la voie TLR3 [3, 5, 43, 93]. En analysant l'expression musculaire de l'antigène CD56, marqueur de régénération musculaire, et des antigènes HLA de classe I, nous avons observé une réponse immunitaire faisant intervenir les cellules dendritiques et macrophages exprimant les CLRs et produisant les cytokines IL12 et IL23, ciblée sur les précurseurs musculaires immatures. Bien que plusieurs études suggérent un rôle de la voie IFN de type I dans la physiopathologie des myopathies inflammatoires [7, 8, 53-57], la source de la production d'IFN de type I n'est pas clairement établie, et les cellules dendritiques plasmacytoïdes, principale source suspectée, sont rarement détectée dans les biopsies musculaires de PM et DM [7]. Nous avons identifié les précurseurs musculaires immatures de phénotype CD56 positifs, et surexprimant les antigènes HLA de classe I comme source possible d'IFNβ. Les études i*n vitro* confirment la production musculaire d'IFNβ, qui est de plus fortement augmentée après activation de la voie TLR3. En retour, la production musculaire d'IFNβ induite par l'activation de la voie TLR3 contribue à la surexpression musculaire des antigènes HLA de classe I. Ainsi, les fibres musculaires immatures ont un rôle central dans la réponse immunitaire musculaire. Caractérisées par une surexpression du HLA de classe I, des TLR3 et TLR7, des autoantigènes myositiques, et par une production locale d'IFNβ, elles contribuent à amplifier le processus inflammatoire et de destruction tissulaire (Figure 8).

Figure 8. Rôle central des précurseurs musculaires dans la réaction inflammatoire chronique musculaire.



A. Suite à une lésion musculaire (virus, traumatisme, toxique…), initiation d'un processus de régénération avec augmentation dans le tissu musculaire du nombre de précurseurs musculaires CD56 positifs (cellules satellites), exprimant également fortement les antigènes HLA de classe I et les TLR3 et TLR7.

B. La surexpression des antigènes de HLA de classe I est responsable d'une toxicité musculaire, via soit une cytotoxicité médiée par les lymphocytes T, soit un stress du réticulum endoplasmique. Il s'ensuit une destruction musculaire avec libération d'autoantigènes et de ligands endogénes à partir des cellules nécrotiques.

C. Ces ligands endogénes (PAMPs) vont, du fait d'une surexpression musculaire de TLR3, activer la voie TLR3 et induire la production, par les cellules musculaires immatures, de la cytokine proinflammatoire IL6 et de la β chémokine CCL20. En retour, CCL20 et l'IL6 participent au recrutement et à la différentiation des cellules dendritiques et lymphocytes T. Les cytokines IL12 et IL23, produites par les cellules dendritiques, permettent la différentiation des lymphocytes Th1 et Th17 qui régulent l'expression musculaire de TLR3, et induisent la production de cytokines et chémokines proinflammatoires (IL1, TNFα, IL6, CCL20) par les macrophages et monocytes.

D. L'activation de la voie TLR3 induit la production musculaire d'IFNβ et contribue ainsi à la surexpression musculaire du HLA de classe I qui amplifie le processus inflammatoire et de destruction au sein du tissu musculaire.

Tournadre A, Lenief V, Eljaafari-Dany A, Miossec P. Contribution of immature muscle precursors to the adaptive and innate immune responses in inflammatory myopathy: A possible target of the immune attack and a source of type I interferon.
Submitted.

Contribution spécifique de la candidate:

- Idée originale et proposition du travail de recherche.
- Réalisation des techniques d'immunomarquages en immunohistochimie pour les CLRs (DC-SIGN, ASGPR, MMR), CD11c, CD68, BDCA2, IL12, IL23, IFNβ, CD56, HLA classe I, sur les biopsies musculaires. Sélection des patients selon les critéres d'inclusion. Mise au point des anticorps spécifiques, analyse des immunomarquages et réalisation des photographies.
- Mise au point des techniques d'isolement des myoblastes en culture à partir de tissu musculaire.
- Analyse et interprétation des données immunohistochimiques *in vivo* et des données *in vitro* sur cultures de myoblastes après stimulation (ELISA) et en cytométrie de flux.
- Ecriture de l'article, réalisation des figures.
- Réponses aux commentaires des rapporteurs.

CONTRIBUTION OF IMMATURE MUSCLE PRECURSORS TO THE ADAPTIVE AND INNATE IMMUNE RESPONSES IN INFLAMMATORY MYOSITIS: A POSSIBLE TARGET OF THE IMMUNE ATTACK AND A SOURCE OF TYPE I INTERFERON.

Anne Tournadre [1,2], Vanina Lenief [1], Assia Eljaafari-Dany [1], Pierre Miossec [1]

[1] Department of Immunology and Rheumatology and Immunogenomics and inflammation research Unit EA 4130, University of Lyon, Edouard Herriot Hospital, Lyon, France.

[2] CHU Clermont-Ferrand, Department of Rheumatology and Pathology

Address and correspondence to Professor Pierre Miossec, Clinical Immunology Unit, Department of Immunology and Rheumatology, Hospital Edouard Herriot, 69437 Lyon Cedex 03, France. Phone: +33-472-11-74-87. Fax: +33-472-11-74-29. E-mail: pierre.miossec@univ-lyon1.fr

Number of text pages: 18

Number of figures: 6

Running head (40 characters or less): Contribution of muscle precursors in myositis

The study was supported by funding from the PHRC interregional to the CHU of Clermont-Ferrand, the Region Rhône-Alpes and the Hospices Civils de Lyon.

ABSTRACT

We investigated the contribution of immature muscle precursors to the adaptive and innate immune responses in inflammatory myositis. Muscle biopsies from polymyositis and dermatomyositis were investigated for the expression of CD11c-positive myeloid dendritic cells (DCs), CD68-positive macrophages, IL12-, IL23- and IFNβ-producing cells, and for the C-type lectin receptors (CLRs) DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), asialoglycoprotein receptor (ASGPR), and macrophage mannose receptor (MMR). Immature muscle precursors were further characterized using immunostaining for CD56. In cultured myoblasts, the production of IFNβ was assessed after stimulation with the TLR3 agonist poly(I:C), Th1 and Th17 cytokines, and necrotic myoblasts. The expression of HLA class I by myoblasts was analyzed by FACS after activation of TLR3 and IFNβ neutralization. CLR expression by DC and macrophages was specific of myositis muscle tissue and was localized in association with IL12 and IL23 cytokines, around immature muscle fibers expressing CD56, HLA class I antigens and IFNβ. *In vitro* stimulation of TLR3 induced the production of IFNβ and up-regulated HLA class I expression which was decreased after IFNβ blockade. In conclusion, immature muscle precursors may represent the target of an immune response involving innate and adaptive immune system, leading to the production of IFNβ. In turn, such local production of IFNβ after TLR3 activation may explain HLA class I overexpression in myositis.

INTRODUCTION

Polymyositis (PM) and dermatomyositis (DM) are autoimmune muscle disorders of unknown origin that lead to muscle destruction. They share clinical symptoms such as muscle weakness and histopathologic features characterized by the presence of inflammation in muscle and that of regenerating and degenerating muscle fibres [1]. In PM, endomysial CD8+ T cells and macrophages surround and invade non necrotic muscle fibers expressing HLA class I antigens, suggesting a cytotoxic T cell response against an autoantigen expressed by muscle fibers. In DM, considered as a complement-mediated microangiopathy, mononuclear cells made of perimysial CD4+ T cells and B cells predominate in perivascular regions. However, this classification is limited by a clinical and histopathological overlap, and by a common inflammatory profile.

The detection of the Th1 and Th17 cytokines in the lymphocytic infiltrates of PM and DM, and the association with the migration, differentiation and maturation of dendritic cells (DCs) [2, 3], reflect an ongoing immune response involving activated T cells and DCs. Besides the role of the adaptive immunity, the detection of plasmacytoïd DCs in muscle tissue, the up-regulation of type I interferon (IFN) system and the overexpression of Toll-like receptors (TLRs), suggest a role for the innate immune system [4-8]. The adaptive immune response is dependent on the nature of the activating stimuli that DCs receive from the innate immune system in part by the activation of pattern recognition receptors, of which TLRs and C-type lectin receptors (CLRs), that make the link between innate and adaptive immunity [9].

In addition to the immune effectors, many studies reported a possible role for muscle cell itself, in particular for immature muscle precursors, in disease pathogenesis, through the up-regulation of MHC class I antigens [10, 11], the activation of TLR pathway [8], and as a source of autoantigens [12].

In the present study, we investigated the role of immature muscle precursors in inflamed muscle of PM and DM. We report the presence of the CLRs, DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN/CD209), asialoglycoprotein receptor (ASGPR/CD301), and macrophage mannose receptor (MMR/CD206), expressed by DCs and macrophages, specifically in myositis muscle, in association with IL12 and IL23 cytokines promoting respectively Th1 and Th17 differentiation. This immune response was focused on immature muscle precursors, characterized by high level expression of CD56 and HLA class I antigens, which were identified as a local source of type I IFN. Furthermore, *in vitro* stimulation of TLR3 pathway in human myoblasts induced IFNβ production and up-regulation of HLA class I antigens.

MATERIALS AND METHODS

Patients and muscle biopsies.

Muscle biopsy specimens were obtained from 8 patients with myositis (5 PM and 3 DM). Muscle tissues from 4 healthy patients undergoing joint replacement surgery and from 1 patient with a metabolic myopathy (Mac Ardle Disease or glycogenosis type V) were used as controls. Patients with PM and DM all had disease that met the Bohan and Peter criteria [13] with characteristic inflammatory lesions observed on muscle biopsy. For immuno-histochemistry studies, tissues were snap-frozen in liquid nitrogen and stored at -80°C until further use.

Immunohistochemistry.

Frozen sections of muscle tissues (10 µm) were thawed, fixed in acetone for 10 mn, air-dried and then rehydrated in phosphate buffered saline (PBS). Immunohistochemical staining was performed for the detection of CLRs (DC-SIGN, ASGPR, MMR), C11c+ myeloid DCs, BDCA2+ plasmacytoid DC, CD68+ macrophages, IL12-, IL23-, IFNβ-producing cells, CD56 and HLA class I antigens. The use of serial sections was required to investigate all the markers. Then, the serial muscle sections were incubated for 45 minutes with the following primary antibodies: 5 µg/ml of mouse monoclonal anti-DC-SIGN (IgG2b, Dendritics, Lyon, France), 5 µg/ml of mouse monoclonal anti-ASGPR (IgG1, Dendritics, Lyon, France), 1:100 dilution of mouse monoclonal anti-MMR (IgG1, Dendritics, France), 25 µg/ml of mouse monoclonal anti-IL12 (IgG1, Abcam, Cambridge, UK), 5 µg/ml of rabbit polyclonal anti-IL23 (IgG, Abcam, Cambridge, UK), 1:50 dilution of mouse monoclonal anti-CD11c (IgG1, BD Biosciences, CA), 1:500 dilution of mouse monoclonal anti-CD68 (IgG1, DAKO, Glostrup, Denmark), 5 µg/ml of mouse monoclonal anti-IFNβ (IgG1, R&D Systems, London, UK), 1 µg/ml of mouse monoclonal anti-CD56 (IgG1, Novocastra, Newcastle, UK), 4 µg/ml of mouse monoclonal anti-HLA class I (IgG2a, Immunotech, Marseille, France), 10 µg/ml of mouse monoclonal anti-BDCA2 (IgG1, Dendritics, France). In control experiments, rabbit IgG or matched mouse IgG isotype was applied at the same concentration as the primary antibodies. After washing, the sections were incubated with biotinylated anti-mouse and anti-rabbit immunoglobulins for 15 minutes, followed by streptavidin-peroxidase complex for 15 minutes and 3,3' diaminobenzidine chromogen solution (DAB) (DAKO, Glostrup, Denmark). The sections were then counterstained with Mayer's hematoxylin.

Isolation and culture of human myoblasts.

Normal muscle samples were obtained from patients with osteoarthritis who were undergoing hip joint replacement. Informed consent was obtained according to the policies of the local ethics committee. After surgery, muscles samples were immediately placed in sterile PBS with antibiotics (penicillin, streptomycin) and washed, and the fat and fibrous tissue were removed. Muscle samples were cut into fragments (1-2 mm^3) and incubated at 37°C for 30 minutes with proteolytic enzymes [1 mg/l Collagenase (Sigma-Aldrich, St Louis, MO) and trypsin 0.05% (Invitrogen Life Technologies, CA)]. After washing and filtration, a first selection was done to remove fibroblasts by incubating the supernatants in petri dishes at 37°C for 1 hour. Unattached myoblasts were then transferred and cultured in F10 growth medium (Ham's F10 nutrient mixture (Lonza, France) supplemented with 20 % fetal calf serum (FCS), 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin) at 37°C in a humidified 5% CO2 incubator. Medium was changed every 4 days. After 10 days, adherent cells were detached with trypsin (Invitrogen Life Technologies, CA) and myoblasts were purified by positive selection with CD56 microbeads (MACS, Miltenyi Biotec, Germany) according to the instructions of manufacturer. Cultured myoblasts were used between passages 2 and 8.

Stimulation assays.

Cultured muscle cells were grown in 6-well plates ($3.5x10^5$ cells/well) and stimulated for 48 h with the following agents: IFNγ 50 ng/ml (R&D Systems, London UK), IL17A 50 ng/ml (R&D Systems, London UK), Poly(I:C) 25 µg/ml (Invivogen, CA). For the stimulation assay with necrotic cells, necrotic myoblasts were obtained by heating normal myoblasts at 60°C for 30 minutes and were then added to myoblast culture at a 5:1 ratio for 24 hours. Then, supernatants were collected and IFNβ protein levels were determined by ELISA using Verikine Human IFNβ ELISA Kit according to the instructions of manufacturer (PBL InterferonSource, NJ).

Flow cytometry.

Cultured muscle cells were grown in 24-well plates (10^5 cells/well) and stimulated for 48 h with the following agents: Poly(I:C) 25 µg/ml (Invivogen, CA), IFNγ 50 ng/ml (R&D Systems, London UK). During stimulation, cultured myoblasts were incubated with increasing concentrations from 10 to 100 µg/ml of the neutralizing purified mouse anti-human

IFNβ antibody (eBioscience, CA) or with 50 µg/ml of mouse anti-human IFNγ antibody (R&D Systems, London UK).

Cells were harvested using 0.05 % trypsin (Invitrogen Life Technologies, CA), washed with PBS containing 5 % FCS, resuspended in 50 µl PBS with 5 % FCS and incubated at 4°C for 20 min with monoclonal antibody against HLA (ABC) class I-FITC (BD Biosciences). Then, the cells were washed 2 times, resuspended in 250 µl 1 % paraformaldehyde, and analysed using FACScan. The results were expressed as average fluorescence intensity of the entire population of cells.

Statistical analysis.

Data are presented as mean ± SEM. The non-parametric Mann Whitney *U* test was used to compare groups. P values less than 0.05 were considered significant.

RESULTS

CLRs are expressed in association with IL12 and IL23 cytokines in myositis tissue.

Immunohistochemistry was performed to investigate DC-SIGN, MMR and ASGPR protein expression in muscle tissues from patients with PM and DM. Expression pattern was compared with normal and noninflammatory metabolic myopathic muscle tissues. DC-SIGN, MMR and ASGPR proteins were broadly expressed in inflammatory infiltrates from PM and DM muscle tissues whereas no expression was detected in normal and non inflammatory myopathic muscle tissues (Figure 1). CLR-expressing cells showed a perivascular pattern in DM and an endomysial pattern in PM (Figure 1).

To investigate the expression of CLRs, CD11c, CD68, IL12 and IL23, single step immunostaining for all these markers was performed in serial sections of myositis tissue. Expression of DC-SIGN, MMR and ASGPR in inflammatory infiltrates was associated with the presence of CD11+ myeloid DCs and CD68+ macrophages, and with the expression of IL12 and IL23 cytokines (Figure 2). The presence in inflamed muscle of CLRs expressed by DCs and macrophages, in association with IL12 and IL23 cytokines involved in the differentiation of Th1 and Th17 cells, strongly suggests an ongoing immune process involving activated T cells and DCs.

Inflammatory infiltrates with CLR-expressing cells are localized around immature muscle fiber precursors.

To further investigate whether muscle cell itself could contribute to the immune response in myositis tissue, immunohistochemistry for CD56, a marker of immature myoblasts [14], and for HLA class I antigens known to be overexpressed in myositis [10], was performed in serial muscle biopsy sections in addition to CLRs, CD11c and CD68 immunostaining. Inflammatory cells, with DCs positive for CD11c and macrophages positive for CD68, expressing DC-SIGN, MMR and ASGPR, were observed around immature myoblast precursors, expressing CD56, a marker of muscle cell regeneration, and also high levels of HLA class I (Figure 3). These data suggest local immune response focused on immature muscle precursors expressing CD56 and HLA class I antigens.

Immature muscle precursors are identified as a source of IFNβ.

Recent studies have suggested a role for the type I IFN pathway in the pathogenesis of PM and DM [4-7]. To define the mechanism production and the cell source, we examined IFNβ expression in control and inflamed muscle by immunohistochemistry. IFNβ expression was observed specifically in PM and DM (Figure 4A). While a weak expression was detected in inflammatory infiltrates, strong staining was seen in some muscle fibers characterized by a small size, with a perifascicular pattern in DM and an intrafascicular pattern in PM (Figure 4A). This pattern of rare expression of IFNβ in inflammatory cells is consistent with the rare detection of BDCA2 positive plasmacytoid DCs, the major type I IFN producer (Figure 4A). Interestingly, staining of serial PM or DM muscle sections with antibodies to IFNβ, CD56 and HLA class I, identified IFNβ-positive muscle fibers as immature myoblast precursors, expressing CD56 and HLA class I (Figure 4B). These data strongly implicate that immature muscle precursors are the local source of type I IFN production rather than plasmacytoid DCs in myositis. We were not able to perform a double staining of muscle fibers with CD56 and IFNβ as primary antibodies of different species or directly labelled primary antibodies should be used to exclude the possibility that secondary antibody bound to the same primary antibody. Unfortunately, directly labelled first antibody against IFNβ was not available and we were unable to obtain a positive staining using goat anti-CD56 antibody as primary antibody and biotinylated donkey anti-goat antibody as secondary antibody.

We next analyzed i*n vitro* the production of IFNβ by normal cultured myoblasts, and after stimulation for 48 hours with the TLR3 ligand Poly(I:C), the Th1 cytokine IFNγ and the Th17 cytokine IL17A. Human myoblasts constitutively produced low levels of IFNβ. Stimulation with the TLR3 ligand, Poly(I:C), strongly induced IFNβ production ($p<0.05$) (Figure 5A). In contrast, IL17A or IFNγ, alone or in combination with Poly(I:C), had no effect on IFNβ

production (Figure 5A). Then, a possible mechanism for the IFNβ-producing capacity of immature myoblast precursors could be the activation of TLR3 pathway. Because necrotic muscle cells can activate cytokine production, in part through the TLR3 pathway [8], we examined the effect of necrotic muscle cells on the production of IFNβ by cultured myoblasts. Necrotic myoblasts increased the production of IFNβ but the difference was not significant compared to unstimulated myoblasts (mean ± SEM 10.06±5.611 vs. 5.05±2.02, p=0.68) (Figure 5B). The combination of necrotic myoblasts with IL17 or IFNγ had no effect on the production of IFNβ (Figure 5B).

We next analyzed i*n vitro* the production of IFNβ by normal cultured myoblasts, and after stimulation for 48 hours with the TLR3 ligand Poly(I:C), the Th1 cytokine IFNγ and the Th17 cytokine IL17A. Human myoblasts constitutively produced low levels of IFNβ. Stimulation with the TLR3 ligand, Poly(I:C), strongly induced IFNβ production ($p<0.05$) (Figure 5A). In contrast, IL17A or IFNγ, alone or in combination with Poly(I:C), had no effect on IFNβ production (Figure 5A). Then, a possible mechanism for the IFNβ-producing capacity of immature myoblast precursors could be the activation of TLR3 pathway. Because necrotic muscle cells can activate cytokine production, in part through the TLR3 pathway [8], we examined the effect of necrotic muscle cells on the production of IFNβ by cultured myoblasts. Necrotic myoblasts increased the production of IFNβ but the difference was not significant compared to unstimulated myoblasts (mean ± SEM 10.06±5.611 vs. 5.05±2.02, p=0.68) (Figure 5B). The combination of necrotic myoblasts with IL17 or IFNγ had no effect on the production of IFNβ (Figure 5B).

Stimulation of TLR3 pathway increases HLA class I expression in part through IFNβ induction.

The explanation for HLA class I overexpression in myositis could be type I IFN [15, 16]. Then, we investigated in cultured myoblasts the effect of TLR3 activation on HLA class I expression through the IFNβ induction. HLA class I expression was assessed by flow cytometry in cultured myoblasts after stimulation for 48 hours with the TLR3 ligand, Poly(I:C), or with IFNγ known to be a strong inducer of HLA class I [17], alone or in combination with neutralizing anti-human IFNβ monoclonal antibody. Stimulation with poly(I:C) and IFNγ induced strong expression of HLA class I as compared with unstimulated myoblasts (Figure 6). IFNβ blockade with increasing concentrations of neutralizing anti-IFNβ monoclonal antibody decreased TLR3-induced-HLA class I expression in a dose dependent manner (Figure 6A). The expression of HLA class I in cultured myoblasts was decreased by

38.6 % after IFNβ neutralization ($p<0.05$) (Figure 6B). These results indicate that HLA class I up-regulation induced by TLR3 stimulation was, in part, dependent of the IFNβ production.

DISCUSSION

An important development in the field of innate immunity was the identification of pattern recognition receptors (PRRs), of which TLRs and CLRs, that link the innate and adaptive immunity. DCs, equipped with a range of PRRs such as CLRs, play a central role in the formation of the immune response upon activation through PRRs. We have previously shown the involvement of the innate immune system in PM and DM through the activation of the TLR pathway [8]. In addition to the overexpression of TLR3 and TLR7 in PM and DM [8], the present study reported the expression of DC-SIGN, MMR and ASGPR, the main CLRs expressed by DC/macrophages lineage cells, in muscle tissue, specifically in inflammatory myopathic tissues. In PM and DM, their expression, predominantly localized in inflammatory infiltrates, was associated with the presence of the two cytokines, IL12 and IL23, mainly produced by macrophages and DCs, and promoting Th1 and Th17 differentiation respectively [18]. This is in accordance with the detection of both Th1 and Th17 cells in lymphocytic infiltrates of PM and DM muscle tissues [2, 3]. Together, these data suggest the presence in myositis muscle tissue of an ongoing immune response involving activated T cells and DCs, the components of adaptive immunity, as well as the receptors of the innate immune system.

We further analyzed whether muscle cell itself may contribute to or even induce the immune process. The role of muscle fiber in the pathogenesis of inflammatory myopathies was suggested by many studies and may implicate immature regenerating muscle cells, up-regulation of HLA class I antigens, and activation of TLR pathway [8, 10, 11, 12]. When we examined CD56, a marker of immature myoblast, as well as HLA class I expression which is a key characteristic of myositis, we found that the immune response involving CLR-expressing cells, and IL12- and IL23-producing cells, was focused on muscle cells expressing high levels of CD56 and HLA class I antigens. This suggests that immature muscle precursors may represent the target of an immune response involving innate and adaptive immune system. Thus by focusing damage onto those immature muscle cells in charge of repair, it could explain muscle wasting and why the regeneration is not effective in the context of such inflammation.

There are many evidences for a role of the type I IFN system in the pathogenesis of inflammatory myopathies [4-7]. However, the cause and origin of the type I IFN production are unclear. Plasmacytoid DCs, known as the major source of type I IFN, were rare and

scattered in muscle tissues from PM and DM [5]. In contrast, we identified CD56 positive immature muscle cells up-regulating HLA class I antigens as a major source of IFNβ. *In vitro* study confirmed the constitutional production of IFNβ by cultured myoblasts, which was strongly induced after activation of the TLR3 pathway. In turn, the local production of type I IFN by muscle cells may contribute in part to the up-regulation of HLA class I antigens on muscle cells, which may be one of the mechanisms of muscle fiber damage [11]. Furthermore, activation of TLR3 pathway possibly by exogenous and/or endogenous activators released from damaged cells [8] amplifies the induction of IFNβ, and contributes to the formation of a self-sustained inflammatory loop. This is consistent with previous reports of an increased expression of myositis autoantigens in such regenerating muscle cells [12]. In addition, the induction of type I IFN could be further increased by immune complexes composed of myositis autoantibodies [5].

Finally, these results strengthen the critical role of immature muscle fibers in the pathogenesis of PM and DM. It appears that inflamed muscle of PM and DM is characterized by the overexpression of HLA class I antigens, myositis autoantigens, TLR3 and TLR7, and type I IFN, all localized to immature muscle precursors [8, 12].

In conclusion, our results indicate an immune response involving innate and adaptive immune system focused on immature muscle precursors. These muscle cells usually responsible for the regenerating process may be the target of the immune attack as well as a major source of type I IFN.

Acknowledgments

We thank the Pr. P. Dechelotte and Dr. A.M. Beaufrere from the laboratory of pathology of CHU Clermont-Ferrand for access to muscle biopsies and technical assistance.

References

1. Hoogendijk JE, Amato AA, Lecky BR, Choy EH, Lundberg IE, Rose MR, Vencovsky J, de Visser M, Hughes RA.119th ENMC international workshop: Trial design in adult idiopathic inflammatory myopathies, with the exception of inclusion body myositis. Neuromuscular Disorders 2004;14:337–345.

2. Page G, Chevrel G, Miossec P. Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: Interaction with chemokines and Th1 cytokine-producing cells. Arthritis Rheum. 2004 ;50:199-208.

3. Chevrel G, Page G, Granet C, Streichenberger N, Varennes A, Miossec P. Interleukin-17 increases the effects of IL-1 beta on muscle cells: arguments for the role of T cells in the pathogenesis of myositis. J Neuroimmunol. 2003;137:125-33.

4. Greenberg SA, Pinkus JL, Pinkus GS, Burleson T, Sanoudou D, Tawil R et al. Interferon-alpha/beta-mediated innate immune mechanisms in dermatomyositis. Ann Neurol. 2005;57:664-78.

5. Eloranta ML, Barbasso Helmers S, Ulfgren AK, Rönnblom L, Alm GV, Lundberg IE. A possible mechanism for endogenous activation of the type I interferon system in myositis patients with anti-Jo-1 or anti-Ro 52/anti-Ro 60 autoantibodies.Arthritis Rheum. 2007;56:3112-24.

6. Walsh RJ, Kong SW, Yao Y, Jallal B, Kiener PA, Pinkus JL, Beggs AH, Amato AA, Greenberg SA. Type I interferon-inducible gene expression in blood is present and reflects disease activity in dermatomyositis and polymyositis. Arthritis Rheum. 2007;56:3784-92.

7. López de Padilla CM, Vallejo AN, McNallan KT, Vehe R, Smith SA, Dietz AB et al. Plasmacytoid dendritic cells in inflamed muscle of patients with juvenile dermatomyositis. Arthritis Rheum. 2007;56:1658-68.

8. Tournadre A, Lenief V, Miossec P. Expression of TLR3 and TLR7 in muscle is characteristic of inflammatory myopathy and is differentially regulated by Th1 and Th17 cytokines. Arthritis Rheum. 2010 Mar 22.

9. Nagaraju K, Raben N, Loeffler L, Parker T, Rochon PJ, Lee E, Danning C, Wada R, Thompson C, Bahtiyar G, Craft J, Hooft Van Huijsduijnen R, Plotz P. Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies. Proc Natl Acad Sci U S A. 2000;97:9209-14.

10. Nagaraju K, Casciola-Rosen L, Lundberg I, Rawat R, Cutting S, Thapliyal R, Chang J, Dwivedi S, Mitsak M, Chen YW, Plotz P, Rosen A, Hoffman E, Raben N. Activation of the

endoplasmic reticulum stress response in autoimmune myositis: potential role in muscle fiber damage and dysfunction. Arthritis Rheum. 2005;52:1824-35.

11. Casciola-Rosen L, Nagaraju K, Plotz P, Wang K, Levine S, Gabrielson E, Corse A, Rosen A. Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy.J Exp Med. 2005;201:591-601.

12. Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, Di Pucchio T, Connolly J, Fay JW, Pascual V, Palucka AK, Banchereau J. Dendritic cell subsets in health and disease. Immunol Rev. 2007;219:118-42.

13. Bohan A, Peter JB. Polymyositis and dermatomyositis. N Engl J Med 1975;292:344-347.

14. Illa I, Leon-Monzon M, Dalakas MC. Regenerating and denervated human muscle fibers and satellite cells express neural cell adhesion molecule recognized by monoclonal antibodies to natural killer cells. Ann Neurol. 1992;31:46-52.

15. Halloran PF, Urmson J, Van der Meide PH, Autenried P. Regulation of MHC expression in vivo. II. IFN-alpha/beta inducers and recombinant IFN-alpha modulate MHC antigen expression in mouse tissues. J Immunol. 1989;142:4241-7.

16. Garofalo R, Mei F, Espejo R, Ye G, Haeberle H, Baron S, Ogra PL, Reyes VE. Respiratory syncytial virus infection of human respiratory epithelial cells up-regulates class I MHC expression through the induction of IFN-beta and IL-1 alpha. J Immunol. 1996 ;157:2506-13.

17. Nagaraju K, Raben N, Merritt G, Loeffler L, Kirk K, Plotz P. A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli. Clin. Exp. Immunol. 1998 ; 113 : 407-414.

18. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med. 2009;361:888-98.

Figure 1. Expression of the CLRs, DC-SIGN, MMR and ASGPR, was specific of inflammatory muscle tissues.

The expression of DC-SIGN, MMR and ASGPR was detected both in PM and DM. No expression of CLRs was observed in normal muscle section or in muscle from metabolic myopathy. CLR expression was observed mainly in cells from inflammatory infiltrates with a predominantly endomysial localization in PM and perivascular localization in DM (Original magnification x200)

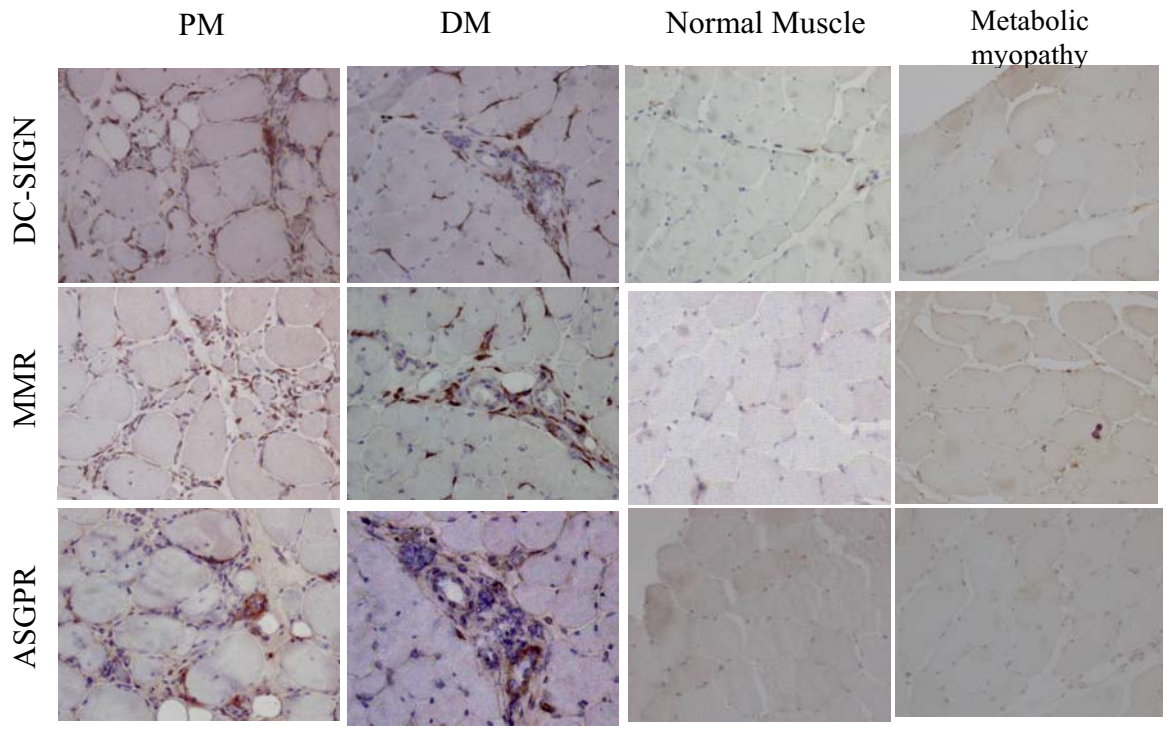


Figure 2. Detection of CLRs (DC-SIGN, MMR, ASGPR) in association with CD11+ myeloid DCs, CD68+ macrophages, and with the IL12 and IL23 cytokines, in PM and DM muscle tissue samples.

Serial PM and DM muscle biopsy sections were stained with antibodies against CD11c, CD68, DC-SIGN, MMR, ASGPR, IL12 and IL23. For both DM and PM, the presence of CLRs was associated with the presence of cells of DC/macrophages lineage, positive for CD11c and CD68, and with the detection of IL12 and IL23 cytokines. Stars (*) serve as markers for same muscle fibers on serial muscle sections (Original magnification x200)

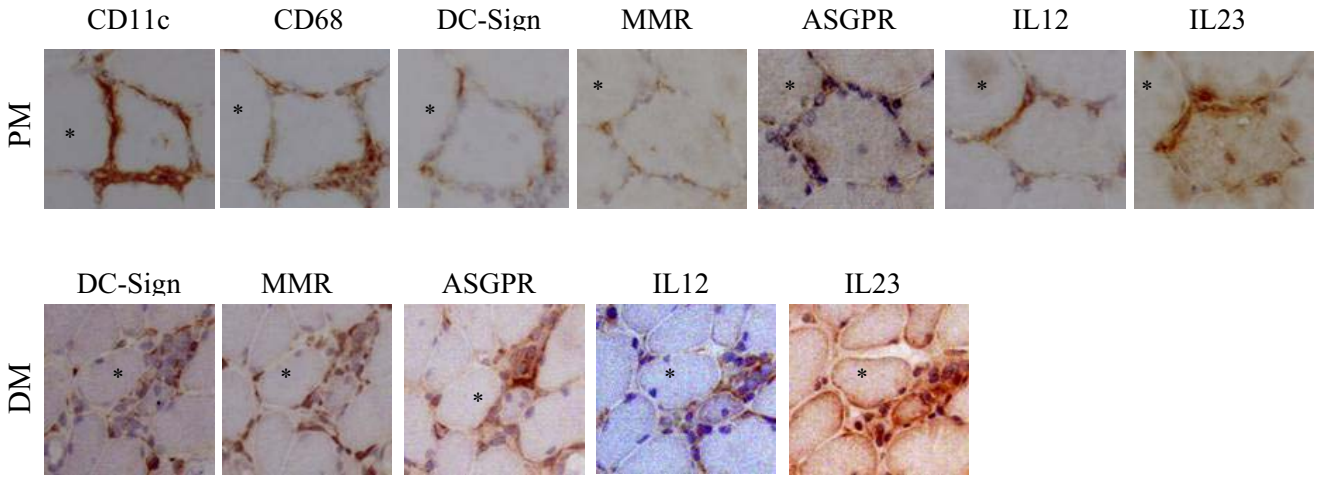


Figure 3. High levels of cells expressing CLRs (DC-SIGN, MMR, ASGPR) were found around immature myoblast precursors expressing marker of muscle cell regeneration CD56, and HLA class I.

Serial PM (A) or DM (B) muscle biopsy sections were stained with antibodies against CD11c, CD68, DC-SIGN, MMR, ASGPR, CD56 and HLA class I. Inflammatory infiltrates consisting in DCs and macrophages expressing CLRs were preferentially detected around muscle fibers expressing high levels of CD56, a marker of immature myoblasts and of muscle cell regeneration. Cells expressing CD56 antigens expressed also high levels of HLA class I antigens (arrows). Stars (*) serve as markers for same muscle fibers on serial muscle sections (Original magnification x200)

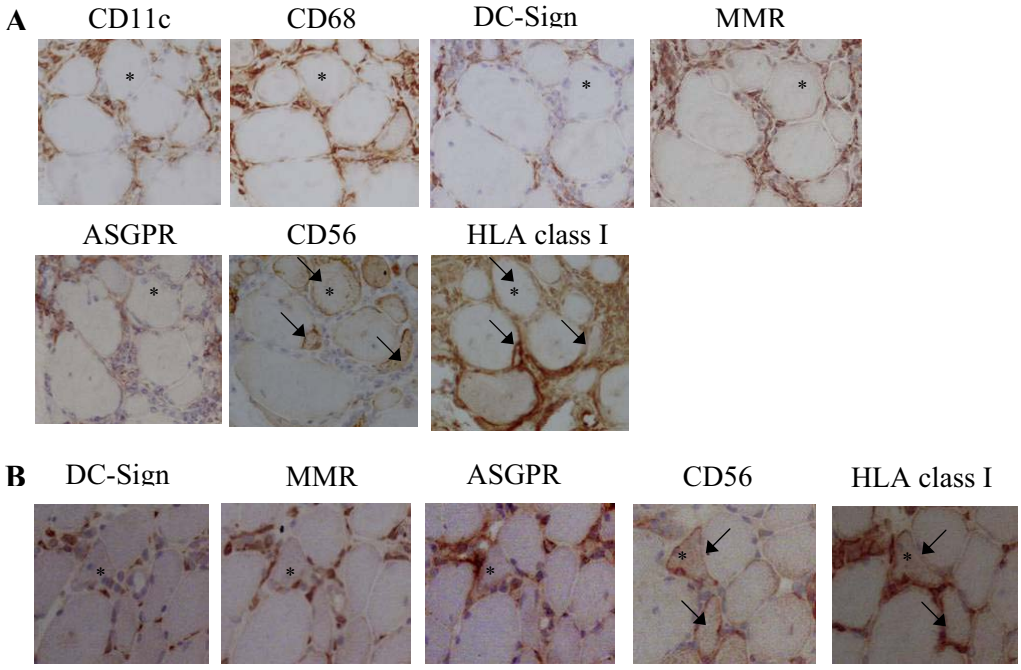


Figure 4. Expression of IFNβ in PM and DM muscle biopsies.

A. In DM and PM muscle biopsies, immunostaining with anti-IFNβ antibody (brown staining) showed many muscle fibers expressing IFNβ, with endomysial localization in PM and perivascular localization in DM. Rare IFNβ-producing cells were detected in inflammatory infiltrates (inset). Serial PM and DM muscle biopsy sections were stained with antibodies against IFNβ and BDCA2 (Stars (*) serve as markers for same muscle fibers on serial muscle sections). Consistent with the rare expression of IFNβ in mononuclear cells, scarce BDCA2 positive plasmacytoid DCs were detected in mononuclear inflammatory cells. In normal muscle and non-inflammatory myopathy, no positive muscle fibers for IFNβ were detected. (Original magnification x200)

B. Serial PM muscle biopsy sections were stained with antibodies against IFNβ, CD56, and HLA class I. IFNβ positive muscle fibers also expressed high levels of CD56 and HLA class I antigens (arrows). (Original magnification x200)

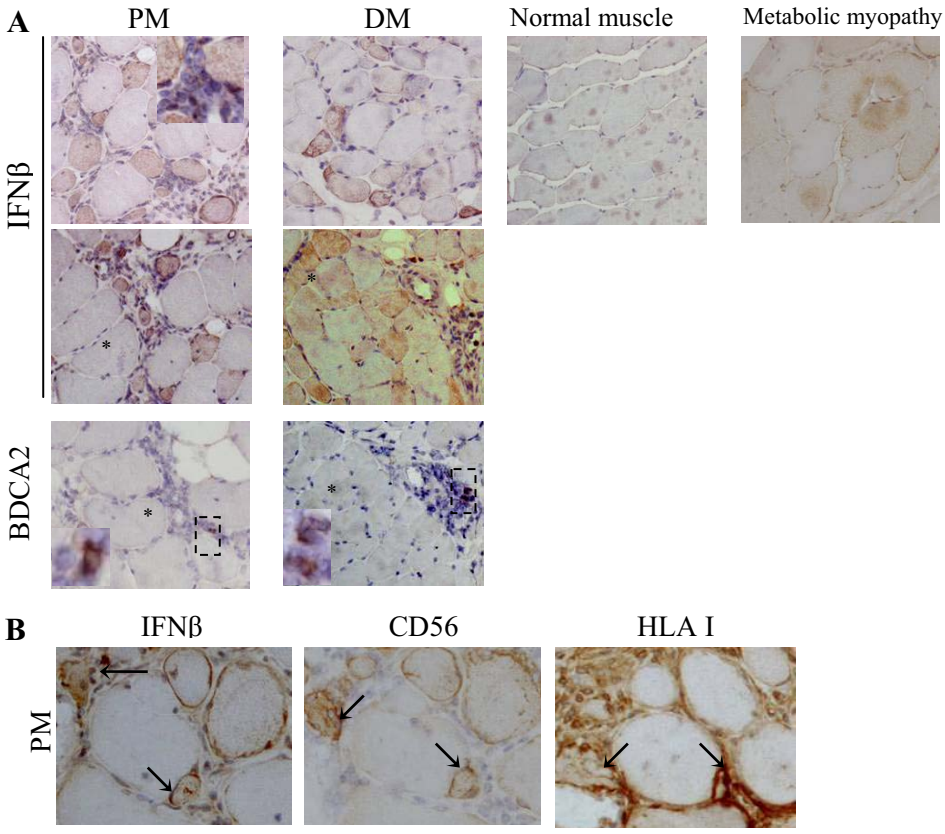


Figure 5. IFNβ production in normal cultured myoblasts, stimulated by the TLR3 agonist Poly(I:C), Th1 and Th17 cytokines, and by necrotic muscle cells.

A. Cultured myoblasts were stimulated for 48 hours with TLR3 agonist Poly(I:C) 25 µg/ml, IL17 50 ng/ml and IFNγ 50 ng/ml. IFNβ protein production was determined in supernatants by ELISA. Values are the mean and SEM of 5 different myoblast cultures. *=p<0.05 versus unstimulated myoblasts.

B. Cultured myoblasts were stimulated for 24 hours with TLR3 agonist Poly(I:C) 25 µg/ml, necrotic muscle cells, alone or combined with IL17 50 ng/ml and IFNγ 50 ng/ml. IFNβ protein production was determined in supernatants by ELISA. Values are the mean and SEM of 4 different myoblast cultures. *=p<0.05 versus unstimulated myoblasts.

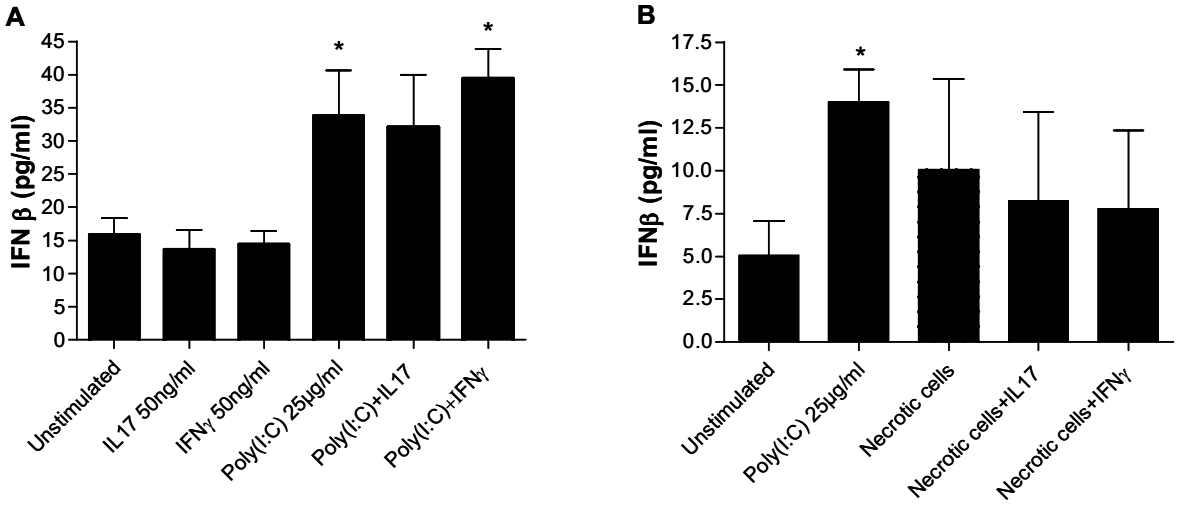
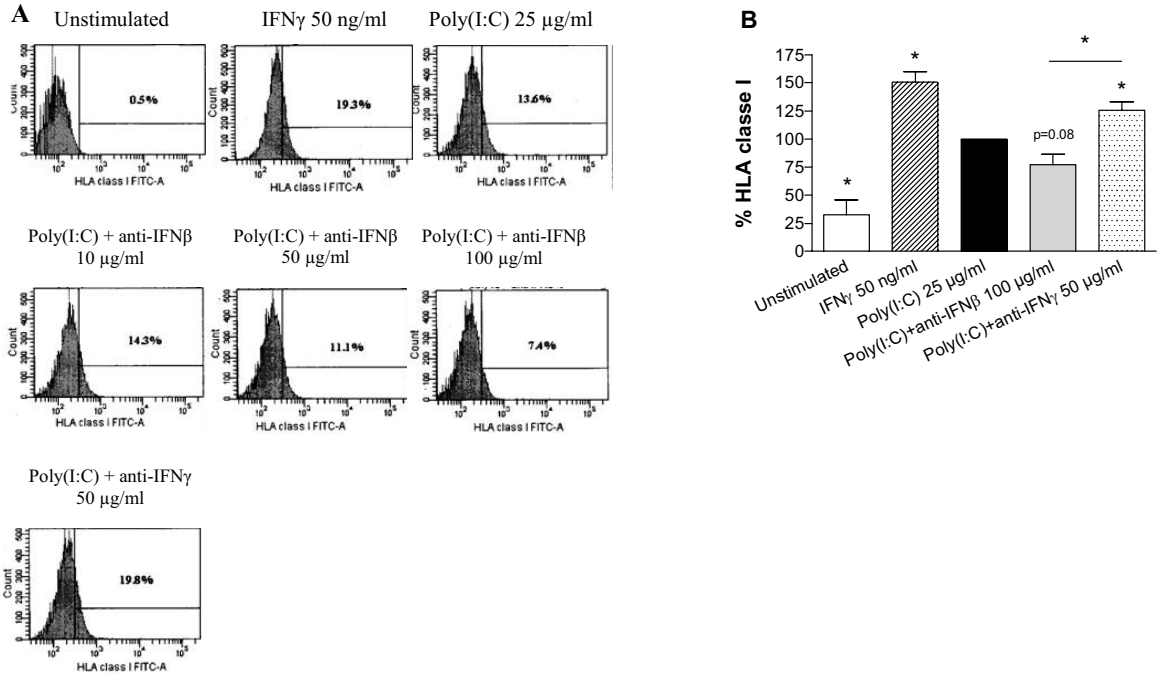


Figure 6. Effect of IFNβ neutralization on Poly(I:C)-induced-HLA class I expression by normal skeletal myoblasts.

Cultured myoblasts were analyzed by flow cytometry for the expression of HLA class I after stimulation for 48 hours with Poly(I:C) 25 µg/ml used alone or in combination with increasing concentrations from 10 to 100 µg/ml of neutralizing anti-human IFNβ monoclonal antibody. The expression of HLA class I induced after stimulation with IFNγ (50 ng/ml) is shown as a positive control. Incubation with neutralizing anti-human IFNγ monoclonal antibody (50 µg/ml) was used as a negative control.

A, Dose response curve of IFNβ neutralization on Poly(I:C)-induced-HLA class I expression by normal skeletal myoblasts. Percent represent the mean number of cells over defined fluorescence intensity.

B, Expression of HLA class I by unstimulated myoblasts or myoblasts stimulated with IFNγ, Poly(I:C) in combination with neutralizing anti-IFNβ or anti-IFNγ antibodies, compared to HLA class I expression of myoblasts stimulated with Poly(I:C) alone taken as the reference (100 %). Bars show the mean of 4 different myoblast cultures. *=p<0.05 versus myoblasts stimulated with Poly(I:C) alone. * over horizontal line = p<0.05 for the indicated comparison.



Chapitre VII

Conclusion et perspectives.

Les myopathies inflammatoires, au premier rang desquelles les PM et DM, sont des maladies auto-immunes rares mais qui représentent un modèle intéressant pour différentes raisons : a) il s'agit d'une maladie chronique aboutissant à un processus de destruction d'un tissu cible bien défini, le muscle squelettique, facilement identifiable et accessible à un prélèvement local ; b) le tissu musculaire cible est le siège d'une réaction inflammatoire et immunitaire impliquant à la fois des éléments de l'immunité innée (récepteurs spécifiques, voie de l'IFN type I), adaptative (lymphocytes T, cellules dendritiques) et humorale (autoantigènes et autoanticorps spécifiques); c) à ces manifestations locales musculaires, peuvent également s'associer des manifestations systémiques, d'autres maladies auto-immunes ou des cancers, ce qui les rapprochent des autres maladies auto-immunes telles que la PR, le lupus ou la sclérodermie; d) comme pour la plupart des maladies auto-immunes, le traitement reste encore non spécifique, s'appuyant sur la corticothérapie et les immunosuppresseurs, mais le démembrement des processus physiopathologiques impliqués ouvre de nouveaux horizons et peut faire espérer le développement d'une immunothérapie ciblée.

Dans ce document, nous avons présenté les liens et interactions, à la fois *in vivo* dans le tissu musculaire, et *in vitro* sur culture cellulaire, entre les composants de l'immunité innée, récepteurs spécifiques et voie IFN de type I, et l'immunité adaptative impliquant cellules dendritiques et lymphocytes T. Pour cela, nous avons étudié en particulier les TLRs, véritables passerelles entre l'immunité innée et adaptative, dont l'expression est spécifique des myopathies inflammatoires. Nous avons d'autre part pu démontrer le rôle fondamental des cellules musculaires immatures dans la physiopathologie des myosites, au centre du processus de régénération mais aussi du processus inflammatoire.

Il apparaît ainsi tout au long du document que le tissu musculaire des myopathies inflammatoires est caractérisé par :

- **Une surexpression musculaire des antigènes HLA de classe I**, dont la toxicité musculaire, soit directe, soit liée à la présentation antigénique aux lymphocytes T, reste à préciser.
- **Une surexpression musculaire d'auto antigènes spécifiques des myosites**, qui par leur potentiel immunogènique d'activation de la voie IFN type I et par leur potentiel chémotactique, peuvent participer à l'initiation et la propagation de la réponse auto-immune.

- **Une surexpression musculaire des TLR3 et TLR7**, dont l'activation possible par des ligands endogènes dérivés des cellules nécrotiques, conduit à la production musculaire de cytokines et de chémokines proinflammatoires qui amplifient le processus.
- **Une production musculaire locale d'IFN de type I dépendante en partie de la voie TLR3** qui entretient la boucle inflammatoire en contribuant à la surexpression des antigènes HLA de classe I.

Plus encore, nous avons localisé l'expression de ces différents éléments caractéristiques du tissu musculaire inflammatoire, puisqu'absents du tissu normal ou myopathique non-inflammatoire, aux précurseurs musculaires immatures. Ainsi, le rôle central des cellules musculaires immatures à potentiel de régénération dans la physiopathologie des PM et DM pourrait rendre compte du défaut de réparation associé au processus auto-immun de destruction musculaire.

Enfin, nous avons pu mettre en évidence localement, *in vivo* au sein du tissu musculaire, un processus immunitaire impliquant la voie des Th1 et Th17, et *in v itro* un mécanisme de régulation impliquant de façon différente les cytokines Th1 et Th17.

Finalement, c'est naturellement, qu'après la mise en évidence du rôle central des précurseurs musculaires dans la réaction inflammatoire, les perspectives de ce travail s'orientent vers l'exploration du lien entre inflammation et défaut de réparation tissulaire conduisant à la perte musculaire. Ainsi, la compréhension du rôle des cytokines proinflammatoires, en particulier l'IL6, au cours de la différentiation musculaire, et la régulation du processus de régénération par la balance myostatin/follistatin en conditions inflammatoires sont parmi les objectifs à venir.

Références.

1. Dalakas MC, Hohlfeld R. Polymyositis and dermatomyositis. Lancet. 2003 20;362:971-82.

2. Page G, Chevrel G, Miossec P. Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: Interaction with chemokines and Th1 cytokine-producing cells. Arthritis Rheum. 2004 ;50:199-208.

3. Nagaraju K, Raben N, Loeffler L, Parker T, Rochon PJ, Lee E, Danning C, Wada R, Thompson C, Bahtiyar G, Craft J, Hooft Van Huijsduijnen R, Plotz P. Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies. Proc Natl Acad Sci U S A. 2000;97:9209-14.

4. Sordet C, Goetz J, Sibilia J. Contribution of autoantibodies to the diagnosis and nosology of inflammatory muscle disease. Joint Bone Spine. 2006;73:646-54.

5. Casciola-Rosen L, Nagaraju K, Plotz P, Wang K, Levine S, Gabrielson E, Corse A, Rosen A. Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy.J Exp Med. 2005;201:591-601.

6. Howard OM, Dong HF, Yang D, Raben N, Nagaraju K, Rosen A, Casciola-Rosen L, Härtlein M, Kron M, Yang D, Yiadom K, Dwivedi S, Plotz PH, Oppenheim JJ. Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, autoantigens in myositis, activate chemokine receptors on T lymphocytes and immature dendritic cells. J Exp Med. 2002;196:781-91.

7. Eloranta ML, Barbasso Helmers S, Ulfgren AK, Rönnblom L, Alm GV, Lundberg IE. A possible mechanism for endogenous activation of the type I interferon system in myositis patients with anti-Jo-1 or anti-Ro 52/anti-Ro 60 autoantibodies. Arthritis Rheum. 2007;56:3112-24.

8. Walsh RJ, Kong SW, Yao Y, Jallal B, Kiener PA, Pinkus JL, Beggs AH, Amato AA, Greenberg SA. Type I interferon-inducible gene expression in blood is present and reflects disease activity in dermatomyositis and polymyositis. Arthritis Rheum. 2007;56:3784-92.

9. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 2002;416:603-607.

10. Lau CM, Broughton C, Tabor AS, Akira S, Flavell RA, Mamula MJ, Christensen SR, Shlomchik MJ, Viglianti GA, Rifkin IR, Marshak-Rothstein A. RNA-associated autoantigens

activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. J Exp Med. 2005;202:1171-7.

11. Brentano F, Schorr O, Gay RE, Gay S, Kyburz D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via TOLL-like Receptor 3. Arthritis Rheum. 2005;52:2656-2665.

12. Patole PS, Gröne HJ, Segerer S, Ciubar R, Belemezova E, Henger A, Kretzler M, Schlöndorff D, Anders HJ. Viral double-stranded RNA aggravates lupus nephritis through Toll-like receptor 3 on glomerular mesangial cells and antigen-presenting cells. J Am Soc Nephrol. 2005;16:1326-38.

13. Chevrel G, Page G, Granet C, Streichenberger N, Varennes A, Miossec P. Interleukin-17 increases the effects of IL-1 beta on muscle cells: arguments for the role of T cells in the pathogenesis of myositis. J Neuroimmunol. 2003;137:125-33.

14. Bohan A, Peter JB. Polymyositis and dermatomyositis. N Engl J Med 1975;292:344-347.

15. Dalakas MC. Polymyositis, dermatomyositis, and inclusion-body myositis. N Engl J Med. 1991;325:1487–1498.

16. Van der Meulen MF, Bronner IM, Hoogendijk JE, Burger H, Van Venrooij WJ, Voskuyl AE, Dinant HJ, Linssen WH, Wokke JH, de Visser M. Polymyositis: an overdiagnosed entity. Neurology. 2003;61:316–321.

17. Griggs RC, Askanas V, DiMauro S, et al. Inclusion body myositis and myopathies. Ann Neurol 1995;38:705–13.

18. Hoogendijk JE, Amato AA, Lecky BR, Choy EH, Lundberg IE, Rose MR, Vencovsky J, de Visser M, Hughes RA.119th ENMC international workshop: trial design in adult idiopathic inflammatory myopathies, with the exception of inclusion body myositis, 10-12 October 2003, Naarden, The Netherlands. Neuromuscul Disord. 2004;14:337-45.

19. Troyanov Y, Targoff IN, Tremblay JL, Goulet JR, Raymond Y, Senécal JL. Novel classification of idiopathic inflammatory myopathies based on overlap syndrome features and autoantibodies: analysis of 100 French Canadian patients. Medicine (Baltimore). 2005 ;84:231-49.

20. Benveniste O, Dubourg O, Herson S. La myosite à inclusions. Rev Rhum 2008;75:151-156.

21. **Tournadre A**, Soubrier M. Le rhumatologue face à une myopathie cortisonique. Rev Rhum 2008;75:157-161.

22. **Tournadre A**, Dubost JJ, Soubrier M.. Treatment of inflammatory muscle disease in adults. Joint Bone Spine. 2010 Jun.

23. **Tournadre A,** Porcherot M, Chérin P, Marie I, Hachulla E, Miossec P. Th1 and Th17 balance in inflammatory myopathies: interaction with dendritic cells and possible link with response to high-dose immunoglobulins. Cytokine. 2009;46:297-301.

24. **Tournadre A**, Amarger S, Joly P, d'Incan M, Ristori JM, Soubrier M. Polymyositis and pemphigus vulgaris in a patient: successful treatment with rituximab. Joint Bone Spine. 2008;75:728-9.

25. Dalakas MC. Intravenous immunoglobulin in autoimmune neuromuscular diseases. JAMA. 2004;291:2367-75.

26. Dalakas MC, Rakocevic G, Schmidt J, Salajegheh M, McElroy B, Harris-Love MO, Shrader JA, Levy EW, Dambrosia J, Kampen RL, Bruno DA, Kirk AD. Effect of Alemtuzumab (CAMPATH 1-H) in patients with inclusion-body myositis. Brain. 2009;132:1536-44.

27. Horber FF, Scheidegger JR, Grünig BE, Frey FJ. Evidence that prednisone-induced myopathy is reversed by physical training. J Clin Endocrinol Metab. 1985;61:83-8.

28. Kuang S, Rudnicki MA. The emerging biology of satellite cells and their therapeutic potential. Trends Mol Med. 2008;14:82-91.

29. Lundberg I, Ulfgren AK, Nyberg P, Andersson U, Klareskog L. Cytokine production in muscle tissue of patients with idiopathic inflammatory myopathies. Arthritis Rheum. 1997;40: 865-874.

30. Hohlfeld R, Engel AG. Coculture with autologous myotubes of cytotoxic T cells isolated from muscle in inflammatory myopathies. Ann Neurol 1991; 29: 498–507.

31. Bender A, Ernst N, Iglesias A, Dornmair K, Wekerle H, Hohlfeld R. T cell receptor repertoire in polymyositis: clonal expansion of autoaggressive CD8 T cells. J Exp Med 1995; 181:1863–68.

32. Emslie-Smith A.M, Arahata K, Engel AG Major histocompatibility complex class I antigen expression, immunolocalization of interferon subtypes, and T cell-mediated cytotoxicity in myopathies. Hum. Pathol. 1989;20:224-231.

33. Bartoccioni E, Gallucci S, Scuderi F, Ricci E, Servidei S, Broccolini A, Tonali P. MHC class I, MHC class II and intercellular adhesion molecule-1 expression in inflammatory myopathies. Clin. Exp. Immunol. 1994;95:166-172.

34. Pavlath GK. Regulation of class I MHC expression in skeletal muscle: deleterious effect of aberrant expression on myogenesis. J Neuroimmunol. 2002;125:42-50.

35. Nagaraju K, Raben N, Merritt G, Loeffler L, Kirk K, Plotz P. A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli. Clin. Exp. Immunol. 1998 ; 113 : 407-414.

36. Plotz PH, Dalakas M, Leff RL, Love LA, Miller FW, Cronin ME. Current concepts in the idiopathic inflammatory myopathies: polymyositis, dermatomyositis, and related disorders. Ann Intern Med. 1989;111:143-57.

37. Nyberg P, Wikman AL, Nennesmo I, Lundberg I. Increased expression of interleukine 1α and MHC class I in muscle tissue of patients with chronic, inactive polymyositis and dermatomyositis. J. Rheumatol. 2000 ; 27 : 940-948.

38. Englund P, Lindroos E, Nennesmo I, Klareskog L, Lundberg I. skeletal muscle fibers express major histocompatibility complex class II antigens independently of inflammatory infiltrates in inflammatory myopathies. Am. J. Pathol. 2001 ; 159 : 1263-1273.

39. Englund P, Nennesmo I, Klareskog L, Lundberg IE. Interleukin-1alpha expression in capillaries and major histocompatibility complex class I expression in type II muscle fibers from polymyositis and dermatomyositis patients: important pathogenic features independent of inflammatory cell clusters in muscle tissue. Arthritis Rheum. 2002;46:1044-55.

40. Allison J, Campbell IL, Morahan G, Mandel TE, Harrison LC, Miller JFAP. Diabetes in transgenic mice resulting from over-expression of class I histocompatibility molecules in pancreatic β cells. Nature. 1988 ; 333 : 529-533.

41. Turnley AM, Morahan G, Okano H, Bernard O, Mikoshiba K, Allison J,Bartlett PF, Miller JFAP. Dysmyelination in transgenic mice resulting from expression of class I histocompatibility molecules in oligodendrocytes. Nature 1991 ; 353 : 566-569.

42. Frauman AG, Chu P, Harrison LC. Nonimmune thyroid destruction results from transgenic overexpression of an allogeneic major histocompatibility complex class I protein. Mol. Cell. Biol. 1993 ; 13 : 1554-1564.

43. Nagaraju K, Casciola-Rosen L, Lundberg I, Rawat R, Cutting S, Thapliyal R, Chang J, Dwivedi S, Mitsak M, Chen YW, Plotz P, Rosen A, Hoffman E, Raben N. Activation of the endoplasmic reticulum stress response in autoimmune myositis: potential role in muscle fiber damage and dysfunction. Arthritis Rheum. 2005;52:1824-35.

44. Guttridge DC, Mayo MW, Madrid LV, Wang CY, Baldwin AS Jr. NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. Science. 2000;289:2363-6.

45. De Bleecker JL, Engel AG. Expression of cell adhesion molecules in inflammatory myopathies and Duchenne dystrophy. J. Neuropathol. Exp. Neurol. 1994;53:369-376.

46. Lundberg I, Kratz A.K, Alexanderson H, Patarroyo M. Decreased expression of interleukine-1α, interleukine-1β, and cell adhesion molecules in muscle tissue following corticosteroid treatment in patients with polymyositis and dermatomyositis. Arthritis Rheum 2000;43:336-348.

47. Goebels N, Michaelis D, Wekerle H, Hohlfeld R. Human myoblasts as antigen-presenting cells. J. Immunol. 1992;49: 661-667.

48. Michaelis D, Goebels N, Hohlfeld R. Constitutive and cytokine-induced expression of human leukocyte antigens and cell adhesion molecules by human myotubes. Am. J. Pathol. 1993;143:1142-1149.

49. Marino M, Scuderi F, Mazzarelli P, Mannella F, Provenzano C, Bartoccioni E. Constituve and cytokine-induced expression of MHC and intercellular adhesion molecule-1 on human myoblasts. J. Neuroimmunol. . 2001;116: 94-101.

50. Bernasconi P, Confalonieri P, Andreetta F, Baggi F, Cornelio F, Mantegazza R. The expression of co-stimulatory and accessory molecules on cultured human muscle cells is not dependent on stimulus by proinflammatory cytokines : relevance for the pathogenesis of inflammatory myopathy. J. Neuroimmunol. 1998;85:52-58.

51. Murata K, Dalakas MC. Expression of the costimulatory molecule BB-1, the ligands CTLA-4 and CD28, and their mRNA in inflammatory myopathies. Am J Pathol. 1999;155:453-60.

52. Wiendl H, Mitsdoerffer M, Schneider D, Chen L, Lochmüller H, Melms A et al. Human muscle cells express a B7-related molecule, B7-H1, with strong negative immune regulatory potential: a novel mechanism of counterbalancing the immune attack in idiopathic inflammatory myopathies.FASEB J. 2003;17:1892-4.

53. Greenberg SA, Pinkus JL, Pinkus GS, Burleson T, Sanoudou D, Tawil R et al. Interferon-alpha/beta-mediated innate immune mechanisms in dermatomyositis. Ann Neurol. 2005;57:664-78.

54. López de Padilla CM, Vallejo AN, McNallan KT, Vehe R, Smith SA, Dietz AB et al. Plasmacytoid dendritic cells in inflamed muscle of patients with juvenile dermatomyositis. Arthritis Rheum. 2007;56:1658-68.

55. Greenberg SA, Pinkus GS, Amato AA, Pinkus JL. Myeloid dendritic cells in inclusion-body myositis and polymyositis. Muscle Nerve. 2007;35:17-23.

56. Baechler EC, Bauer JW, Slattery CA, Ortmann WA, Espe KJ, Novitzke J, Ytterberg SR, Gregersen PK, Behrens TW, Reed AM. An interferon signature in the peripheral blood of dermatomyositis patients is associated with disease activity. Mol Med. 2007;13:59-68.

57. Dastmalchi M, Grundtman C, Alexanderson H, Mavragani CP, Einarsdottir H, Helmers SB, Elvin K, Crow MK, Nennesmo I, Lundberg IE. A high incidence of disease flares in an open pilot study of infliximab in patients with refractory inflammatory myopathies. Ann Rheum Dis. 2008;67:1670-7.

58. Halloran PF, Urmson J, Van der Meide PH, Autenried P. Regulation of MHC expression in vivo. II. IFN-alpha/beta inducers and recombinant IFN-alpha modulate MHC antigen expression in mouse tissues. J Immunol. 1989;142:4241-7.

59. Miller FW, Waite KA, Biswas T, Plotz PH. The role of an autoantigen, histidyl-tRNA synthetase, in the induction and maintenance of autoimmunity. Proc Natl Acad Sci U S A 1990;87: 9933–7.

60. Miller FW, Twitty SA, Biswas T, Plotz PH. Origin and regulation of a disease-specific autoantibody response: antigenic epitopes, spectrotype stability, and isotype restriction of anti-Jo-1 autoantibodies. J Clin Invest 1990;85:468–75.

61. Stone KB, Oddis CV, Fertig N, Katsumata Y, Lucas M, Vogt M, Domsic R, Ascherman DP. Anti-Jo-1 antibody levels correlate with disease activity in idiopathic inflammatory myopathy. Arthritis Rheum. 2007;56:3125-31.

62. Levine SM, Rosen A, Casciola-Rosen LA. Anti-aminoacyl tRNA synthetase immune responses: insights into the pathogenesis of the idiopathic inflammatory myopathies. Curr Opin Rheumatol. 2003;15:708-13.

63. Bell D, Young JW, Banchereau J. Dendritic cells. Adv. Immunol. 1999;72: 255-324.

64. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol 2003;21:685–711.

65. Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, Di Pucchio T, Connolly J, Fay JW, Pascual V, Palucka AK, Banchereau J. Dendritic cell subsets in health and disease. Immunol Rev. 2007;219:118-42.

66. **Tournadre A,** Miossec P. Chemokines and dendritic cells in inflammatory myopathies. Ann Rheum Dis. 2009;68:300-4.

67. Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. Am J Physiol Regul Integr Comp Physiol 2002;283:R7– R28.

68. Dieu MC, Vanbervliet B, Vicari A, Bridon JM, Oldham E, Aït-Yahia S, Brière F, Zlotnik A, Lebecque S, Caux C. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. J Exp Med. 1998;188:373-86.

69. Page G, Lebecque S, Miossec P. Anatomic localization of immature and mature dendritic cells in an ectopic lymphoid organ: correlation with selective chemokine expression in rheumatoid synovium. J Immunol 2002;168:5333-5341.

70. Ngo VN, Tang HL, Cyster JG. Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. J Exp Med 1998;188:181-191.

71. Civatte M, Bartoli C, Schleinitz N, Chetaille B, Pellissier J.F, Figarella-Branger D. Expression of the beta chemokines CCL3, CCL4, CCL5 and their receptors in idiopathic inflammatory myopathies. Neuropathol Appl Neurobiol. 2005;31:70-9.

72. **Tournadre A**, Miossec P. Cytokine response in inflammatory myopathies. Curr Rheumatol Rep. 2007;9:286-90.

73. Grundtman C, Salomonsson S, Dorph C, Bruton J, Andersson U, Lundberg IE. Arthritis Rheum. 2007;56:674-87. Immunolocalization of interleukin-1 receptors in the sarcolemma and nuclei of skeletal muscle in patients with idiopathic inflammatory myopathies.

74. Shreeniwas R, Koga S, Karakurum M. Hypoxia-mediated induction of endothelial cell interleukine-1 alpha. An autocrine mechanism promoting expression of leukocyte adhesion molecules on the vessel surface. J. Clin. Investigation. 1992;90: 2333-2339.

75. Chevrel G, Granet C, Miossec P. Contribution of tumour necrosis factor alpha and interleukin (IL) 1beta to IL6 production, NF-kappaB nuclear translocation, and class I MHC expression in muscle cells: in vitro regulation with specific cytokine inhibitors. Ann Rheum Dis. 2005;64:1257-62.

76. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med. 2009;361:888-98.

77. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441:235-8.

78. Li YP, Reid MB. Effect of tumor necrosis factor-alpha on skeletal muscle metabolism. Curr Opin Rheumatol. 2001;13:483-7.

79. Austin L, Burgess AW. Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines. J Neurol Sci. 1991;101:193-7.

80. Austin L, Bower J, Kurek J, Vakakis N. Effects of leukaemia inhibitory factor and other cytokines on murine and human myoblast proliferation. J Neurol Sci. 1992 ;112:185-91.

81. Ebisui C, Tsujinaka T, Morimoto T, Kan K, Iijima S, Yano M, Kominami E, Tanaka K, Monden M. Interleukin-6 induces proteolysis by activating intracellular proteases (cathepsins B and L, proteasome) in C2C12 myotubes. Clin Sci (Lond). 1995;89:431-9.

82. Lundberg I, Brengman JM, Engel AG. Analysis of cytokine expression in muscle in inflammatory myopathies, Duchenne dystrophy, and non-weak controls. J Neuroimmunol 1995;63:9–16.

83. Lepidi H, Frances V, Figarella-Branger D, Bartoli C, Machado-Baeta A, Pellissier JF. Local expression of cytokines in idiopathic inflammatory myopathies. Neuropathol Appl Neurobiol 1998;24:73–9.

84. Tucci M, Quatraro C, Dammacco F, Silvestris F. Interleukin-18 overexpression as a hallmark of the activity of autoimmune inflammatory myopathies. Clin Exp Immunol 2006;146:21–31.

85. Zrioual S, Toh ML, **Tournadre A**, Zhou Y, Cazalis MA, Pachot A, Miossec V, Miossec P IL-17RA and IL-17RC receptors are essential for IL-17A-induced ELR+ CXC chemokine expression in synoviocytes and are overexpressed in rheumatoid blood. J Immunol. 2008;180:655-63

86. Zrioual S, Ecochard R, **Tournadre A**, Lenief V, Cazalis MA, Miossec P.Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. J Immunol. 2009;182:3112-20.

87. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 2003;21:335-76.

88. Rifkin IR, Leadbetter EA, Busconi L, Viglianti G, Marshak-Rothstein A. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. Immunol Rev. 2005;204:27-42.

89. Ospelt C, Brentano F, Rengel Y, Stanczyk J, Kolling C, Tak PP,Gay RE, Gay S, Kyburz D: Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis. Arthritis Rheum 2008, 58:3684-3692.

90. Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, van Lieshout AW, Sprong T, van den Hoogen FH, van den Berg WB, Radstake TR: The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. Arthritis Rheum 2005, 52:2313-2322.

91. Goldstein RS, Bruchfeld A, Yang L, Qureshi AR, Gallowitsch-Puerta M, Patel NB, Huston BJ, Chavan S, Rosas-Ballina M, Gregersen PK, Czura CJ, Sloan RP, Sama AE,

Tracey KJ: Cholinergic anti-inflammatory pathway activity and high mobility group box-1 (HMGB1) serum levels in patients with rheumatoid arthritis. Mol Med 2007, 13:210-215.

92. Sacre SM, Andreakos E, Kiriakidis S, Amjadi P, Lundberg A, Giddins G, Feldmann M, Brennan F, Foxwell BM: The Toll-like receptor adaptor proteins MyD88 and Mal/TIRAP contribute to the inflammatory and destructive processes in a human model of rheumatoid arthritis. Am J Pathol 2007, 170:518-525.

93. **Tournadre A**, Lenief V, Miossec P. Expression of TLR3 and TLR7 in muscle is characteristic of inflammatory myopathy and is differentially regulated by Th1 and Th17 cytokines. Arthritis Rheum. 2010;62:2144-51.

94. Schreiner B, Voss J, Wischhusen J, Dombrowski Y, Steinle A, Lochmuller H et al. Expression of toll-like receptors by human muscle cells in vitro and in vivo: TLR3 is highly expressed in inflammatory and HIV myopathies, mediates IL-8 release and up-regulation of NKG2D-ligands. FASEB J. 2006;20:118-20.

95. Valladeau J, Duvert-Frances V, Pin JJ, Kleijmeer MJ, Ait-Yahia S, Ravel O, Vincent C, Vega F Jr, Helms A, Gorman D, Zurawski SM, Zurawski G, Ford J, Saeland S. Immature human dendritic cells express asialoglycoprotein receptor isoforms for efficient receptor-mediated endocytosis. J Immunol. 2001;167:5767-74.

96. Kerrigan AM, Brown GD. C-type lectins and phagocytosis. Immunobiology. 2009;214:562-75.

97. Garofalo R, Mei F, Espejo R, Ye G, Haeberle H, Baron S, Ogra PL, Reyes VE. Respiratory syncytial virus infection of human respiratory epithelial cells up-regulates class I MHC expression through the induction of IFN-beta and IL-1 alpha. J Immunol. 1996 ;157:2506-13.