IL - 17 et réponse inflammatoire systémique : focus sur le foie et le muscle

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RESUME

L'interleukine (IL)-17 et le TNFα sont deux cytokines pro-inflammatoires jouant un rôle important dans diverses maladies inflammatoires systémiques et auto-immunes affectant différents organes et tissus comme le foie et les muscles. Cependant, les rôles de l'IL-17 et du TNFα restent encore mal compris dans les muscles et le foie, qui est impliqué dans la réponse en phase aiguë. En utilisant des cultures de myoblastes, d'hépatocytes et de cellules stellaires hépatiques humains, nous avons trouvé que l'IL-17 et le TNFα augmentent en synergie la sécrétion de la cytokine pro-inflammatoire IL-6 et de plusieurs chimiokines. Dans les myoblastes, l'IL-17 et le TNFα induisent un stress oxydatif et une dérégulation de calcium montrant ainsi que les processus pathologiques immuns et non-immuns interagissent. Dans les hépatocytes, en augmentant en synergie les niveaux de la CRP et des transaminases, l'IL-17 et le TNFα participent à l'inflammation systémique et aux dommages cellulaires. Etant donné que des infiltrats de cellules immunitaires sont retrouvés lors d'atteintes inflammatoires, les interactions cellulaires contribuent certainement à la chronicité de l'inflammation. Des cellules mononuclées du sang périphérique activées ou non ont ainsi été placées en co-cultures avec les myoblastes, les hépatocytes et les cellules stellaires. Par comparaison aux monocultures, les productions de l'IL-6 et des chimiokines IL-8 et/ou CCL20 étaient augmentées dans les co-cultures. L'IL-17 et le TNFα contribuaient partiellement à ces effets. Les effets systémiques de l'IL-17 et du TNFα en font donc des cibles thérapeutiques attrayantes pour le traitement des nombreuses maladies inflammatoires systémiques.

Mots clés : interleukine-17, tumor necrosis factor-α, inflammation, interactions cellulaires, hépatocytes, cellules stellaires hépatiques, myoblastes

RESUME SUBSTANTIEL

L'interleukine (IL)-17 et le TNFα sont deux cytokines pro-inflammatoires jouant un rôle important dans de nombreuses maladies inflammatoires systémiques et auto-immunes comme le psoriasis ou la polyarthrite rhumatoïde. Ces maladies systémiques sont caractérisées par une atteinte anormale et persistante du système immunitaire menant à une altération de la fonction de plusieurs organes et tissus comme par exemple le foie et les muscles. Des inhibiteurs de l'IL-17 et du TNFα sont actuellement disponibles pour le traitement de certaines de ces pathologies. L'IL-17 peut coopérer avec le TNFα pour agir en synergie sur plusieurs gènes inflammatoires dans divers types cellulaires. Cependant, leurs rôles restent encore mal compris dans le foie, qui joue un rôle central dans l'inflammation systémique en produisant la plupart des protéines de la phase aiguë de l'inflammation, ainsi que dans certains troubles musculaires comme les myopathies inflammatoires idiopathiques caractérisées par des mécanismes pathologiques immuns et non-immuns. De plus, étant donné que l'inflammation locale est caractérisée par une infiltration de cellules immunitaires, les interactions locales avec les cellules infiltrées peuvent jouer un rôle central dans la chronicité de l'inflammation. La contribution de ces types d'interactions dans la réponse inflammatoire nécessite donc d'être étudiée.

Les objectifs de ces travaux de thèse étaient ainsi de :

- Déterminer les effets de l'IL-17 et du TNFα dans la réponse inflammatoire hépatiques par l'utilisation d'hépatocytes et de cellules stellaires hépatiques humains
- D'évaluer le rôle de l'IL-17 et du TNFα dans les mécanismes pathogéniques immuns et non-immuns des myosites par l'utilisation de myoblastes humains
- Etudier les interactions cellulaires entre les cellules mononuclées du sang périphérique et les hépatocytes, les cellules stellaires hépatiques ou les myoblastes dans la réponse inflammatoire et la contribution de l'IL-17 et du TNFα dans ces interactions

La combinaison IL-17 et TNFα amplifie la réponse inflammatoire hépatique

Les effets de l'IL-17 et du TNFα ont été étudiés dans des cultures cellulaires d'hépatocytes primaires humains ainsi que dans les lignées humaines d'hépatocytes HepaRG et de cellules stellaires hépatiques LX-2. Dans ces cultures, la coopération IL-17/TNFα augmentait en synergie la production de l'IL-6, connu comme un inducteur majeur de la production hépatique de protéines de la phase aiguë. L'IL-17 et le TNFα agissaient ainsi sur l'inflammation systémique et les dommages cellulaires hépatiques en augmentant la CRP et les niveaux de transaminases via l'induction de l'IL-6 dans les cultures d'hépatocytes. Indépendamment de l'IL-6, l'IL-17 amplifiait l'effet du TNFα sur l'induction de l'expression des chimiokines IL-8, CCL20 et MCP-1. Étonnamment, l'exposition en premier de l'IL-17, mais pas du TNFα, était crucial pour l'initiation de l'effet synergique IL-17/TNFα sur la sécrétion de l'IL-6 et de l'IL-8 par les hépatocytes. Le blocage de l'IL-17 et/ou du TNFα pourrait être une stratégie thérapeutique potentiellement intéressante pour contrôler à la fois l'inflammation systémique et l'attraction des cellules dans le foie.

L'IL-17 et le TNFα pourraient perturber la fonction musculaire en agissant sur la réponse inflammatoire et l'influx calcique de type SOCE dans les myoblastes humains

Les myopathies inflammatoires idiopathiques (ou myosites) sont des pathologies auto-immunes caractérisées par une dégénérescence des tissus musculaires et une infiltration de cellules mononuclées. La pathogénèse des myosites comprend des mécanismes immuns avec des niveaux élevés d'IL-17 et de TNFα et des mécanismes non-immuns incluant notamment un stress oxydatif et un dérèglement de l'homéostasie du calcium. En utilisant des cultures de myoblastes humains, nous avons trouvé que l'IL-17 et le TNFα agissaient sur l'état inflammatoire et le recrutement des cellules immunitaires en augmentant en synergie la sécrétion de l'IL-6 et de la chimiokine CCL20 par les myoblastes. L'IL-17 et le TNFα induisaient aussi la production de dérivés actifs de l'oxygène (ROS), un stress du réticulum endoplasmique et l'influx calcique de type SOCE (store-operated calcium entry). L'utilisation d'inhibiteurs de SOCE réduisait la production de l'IL-6 induite par l'IL-17 et le TNFα. Ainsi les processus immuns et non-immuns des maladies inflammatoires idiopathiques peuvent

interagir. Le ciblage de cytokines pro-inflammatoires comme l'IL-17 et/ou le TNFα pourrait être une stratégie thérapeutique prometteuse dans les myosites afin de contrôler à la fois l'état inflammatoire et certains mécanismes non-immuns pathologiques, en particulier, le dérèglement de l'homéostasie du calcium qui est crucial dans la fonction musculaire.

L'IL-17 et le TNFα contribuaient à l'induction de la sécrétion de l'IL-6 et de certaines chimiokines par les interactions cellulaires entre les cellules mononuclées du sang périphériques et les hépatocytes, les cellules stellaires hépatiques ou les myoblastes

En produisant des chimiokines comme CCL20 et MCP-1, les hépatocytes, les cellules stellaires hépatiques LX-2 et les myoblastes peuvent recruter des cellules immunitaires, notamment des lymphocytes T, des monocytes ou des cellules dendritiques, localement dans le foie et les muscles contribuant à la chronicité de l'inflammation. Afin d'évaluer le rôle inflammatoire des interactions cellule-cellule, les hépatocytes HepaRG, les cellules stellaires hépatiques LX-2 ou les myoblastes ont été placés en culture avec des cellules mononuclées du sang périphérique (PBMC) et ont été activées ou non *in vitro* par la phytohémagglutinine. La production de l'IL-6, des chimiokines CCL20 et IL-8 a été augmentée dans les co-cultures par comparaison aux monocultures. L'activation des PBMC avec la phytohémagglutinine augmentait la sécrétion de l'IL-6 dans les co-cultures comprenant les cellules HepaRG et/ou LX-2 mais pas avec les myoblastes. Le blocage de l'IL-17 et/ou du TNFα dans les co-cultures diminuait la libération de l'IL-6, de l'IL-8 et de CCL20 dans les co-cultures. Ainsi, l'IL-17 et/ou du TNFα participent à l'induction de la réponse inflammatoire induite par les interactions cellulaires.

L'inflammation est ainsi un processus dynamique dans lequel les interactions cellulaires jouent un rôle important et sont certainement déterminantes dans l'issue de l'état inflammatoire. Dans ces interactions, l'échange de facteurs solubles pro-inflammatoires comme l'IL-17 et le TNFα mais aussi les contacts directs cellule-cellule contribuent à la réponse inflammatoire. En induisant en synergie l'expression et la sécrétion de chimiokines et

de cytokines inflammatoires systémiques par les hépatocytes, les cellules stellaires hépatiques et les myoblastes, l'IL-17 et le TNFα peuvent amplifier la réponse inflammatoire aigüe mais aussi chronique en augmentant localement le recrutement de cellules immunitaires et la production de médiateurs inflammatoires systémiques. Ces effets peuvent à leur tour induire la production de l'IL-17 et le TNFα menant à une boucle de rétroaction pro-inflammatoire. Les effets systémiques et pro-inflammatoires de l'IL-17 et du TNFα, en font donc des cibles thérapeutiques attrayantes pour le traitement des nombreuses maladies inflammatoires systémiques.

IL-17 and Systemic Inflammatory Response: Focus on Liver and Muscle

SUMMARY

Interleukin-17A (IL-17) and tumor necrosis factor-α (TNFα) are two pro-inflammatory cytokines playing an important role in various systemic inflammatory and autoimmune disorders affecting different organs and tissues including the liver and the muscles. However, the roles of IL-17 and TNFα are not fully understood in the muscles and also in liver, which is crucial in the acute phase response. By using cultures of human myoblasts, primary human hepatocytes, human HepaRG cells and LX-2 hepatic stellate cells, we found that IL-17 and TNFα increase in synergy the production of the pro-inflammatory cytokine IL-6 and chemokines (IL-8, CCL20, MCP-1). In myoblasts, the IL-17 and TNFα stimulation induces endoplasmic reticulum stress and calcium dysregulation showing that immune and non-immune pathogenic mechanisms interplay. In hepatocytes, IL-17 and TNFα mediate systemic inflammation and cell damage by increasing in synergy the CRP acute-phase protein and transaminase levels through the induction of IL-6. Since active liver and muscle disorders are characterized by inflammatory infiltrates of immune cells, the cell interactions play certainly an important role in the chronicity of the inflammation. Peripheral blood mononuclear cells activated or not were therefore co-cultured with myoblasts, hepatocytes and/or hepatic stellate cells to assess the inflammatory role of the cell-cell interactions. Co-cultures enhance the production of IL-6, IL-8 and/or CCL20 compared to monocultures. IL-17 and TNFα contribute partially to these inductions. The systemic effects of IL-17 and/or TNFα make them attractive therapeutic targets for the treatment of various systemic inflammatory disorders.

Key words: interleukin-17, tumor necrosis factor-α, inflammation, cell interactions, hepatocytes, hepatic stellate cells, myoblasts

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ABBREVIATIONS

α-SMA	Alpha Smooth Muscle Actin
APC	Antigen Presenting Cell
ASAT	Aspartate Amino-Transferase
Ca^{2+}	Calcium
CCL	Chemokine (C-C motif) Ligand
CCR	C-C motif chemokine Receptor
CD	Cluster of Differentiation
CFSE	CarboxyFluorescein diacetate succinimidyl Ester
CHB	Chronic Hepatitis B
Con A	Concanavalin A
COX	Cyclooxygenase
CRP	C-Reactive Protein
CXCL	Chemokine (C-X-C motif) Ligand
CXCR	C-X-C motif chemokine Receptor
DC	Dendritic Cell
DM	Dermatomyositis
ECM	Extracellular Matrix
ER	Endoplasmic Reticulum
HCC	Hepatocellular Carcinoma
HSC	Hepatic Stellate Cell
IBM	Inclusion Body Myositis
IFN	Interferon
IIM	Idiopathic Inflammatory Myopathy
IL	Interleukin
IL-6R	IL-6 Receptor
JAK	Janus Kinase
LPS	Lipopolysaccharide
LSEC	Liver Sinusoidal Endothelial Cells
MAPK	Mitogen Activated Protein Kinase

MCP-1	Monocyte Chemoattractant Protein 1
MHC	Major Histocompatibility Complex
MIP-3α	Macrophage Inflammatory Protein-3α
MMP	Matrix Metalloproteinase
NFκB	Nuclear Factor κB
NK	Natural Killer
PBL	Peripheral Blood Lymphocyte
PBMC	Peripheral Blood Mononuclear Cell
PD	Programmed cell Death
PGE	Prostaglandin E
PHA	Phytohemagglutinin
PHH	Primary Human Hepatocyte
PI3K	PhosphatidylInositiol-4,5-biphosphate 3-Kinase
PM	Polymyositis
PRR	Pattern Recognition Receptors
ROS	Reactive Oxygen Species
SAA	Serum Amyloid A
SOCE	Store-Operated Calcium Entry
SR	Sarcoplasmic Reticulum
STAT	Signal Transducer and Activator of Transcription
STIM1	Stromal Interaction Molecule 1
TCR	T Cell Receptor
TGFβ	Transforming Growth Factor β
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll Like Receptors
TNFα	Tumor Necrosis Factor α
TNFR	TNFα receptor
TRADD	TNFR-Associated Death Domain

GENERAL INTRODUCTION

1 Inflammation

Inflammation is an essential biological reaction of the body to protect tissues from pathogens, foreign bodies or tissue injury. It is a tightly regulated cascade that is orchestrated by soluble immune signaling molecules called cytokines. The inflammatory response can further be described as local and/or systemic where cytokines are key players (Figure 1).

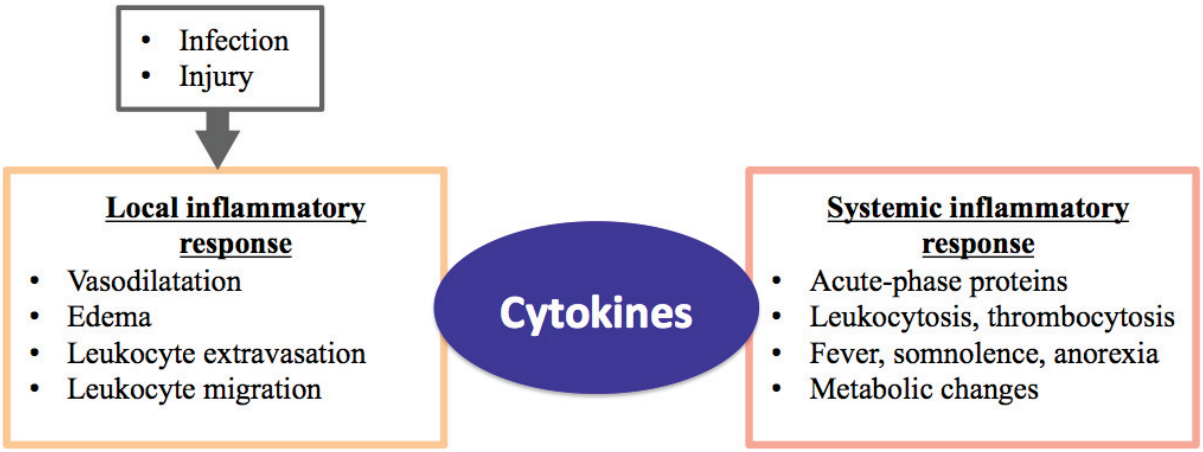

FIGURE 1: Cytokines are key mediators, inducers and regulators of the local and systemic inflammatory response

1.1 Local inflammatory response

Five cardinal signs characterize the local inflammation: redness *(rubor)*, heat *(calor)*, swelling *(tumor)*, pain *(dolor)* and loss of function *(functio laesa)*. These signs are secondary to a local vasodilatation and a series of complex cellular and biochemical processes. Inflammation is initiated by a tissue-destroying process that involves the recruitment of plasma proteins, fluid and leukocytes into perturbed tissue leading to edema and leukocyte extravasation. This migration is facilitated by alterations in the local vasculature characterized by vasodilation, enhanced vascular permeability and increased blood flow.

The first step of the inflammation activation involves recognition of infection or damage by the detection of pathogen-associated molecular patterns (PAMPs) from circulating micro-

organisms (“strangers”) or alarmins/damage-associated molecular patterns (DAMPs) from tissue damage (“dangers”). PAMPs and DAMPs are recognized by pattern recognition receptors (PRRs), which mediate the up-regulation of inflammatory genes. The local release of pro-inflammatory cytokines and chemokines promotes effector functions of inflammation including the recruitment of neutrophils and monocytes to injured site. Neutrophils are the fist innate immune cells recruited; they are professional phagocytes with oxidative and non-oxidative pathogen killing mechanisms (degranulation and production of neutrophil extracellular traps). Neutrophil infiltration is followed by the recruitment of monocytes-macrophages that remove cellular debris, bacteria and senescent cells. Finally, lymphocytes are attracted to the site of inflammation for the activation of the adaptive immune response. The resident cells and the infiltrated immune cells can interact with each other and undergo the activation with the release of pro-inflammatory cytokines and other cellular mediators including leukotrienes, prostanglandins, thromboxanes or the platelet-activating factor.

Although acute inflammation is initiated as a protective response, chronic inflammation is persistent and detrimental phenomenon for the tissue function. Inflammation resolution is the reestablishment of normal homeostasis with apoptosis and clearance of activated inflammatory cells and tissue healing. Persistent inflammatory stimuli or dysregulation of mechanisms of the resolution phase results in chronic inflammation. Chronic inflammation is a major cause of common human diseases such as asthma, type 2 diabetes, cardiovascular diseases or arthritis.

The inflammatory response is therefore a dynamic process controlled by cell interactions, the concentrations and kinetics of production and degradation of soluble mediators. The outcomes of the inflammatory response are dependent on the nature of the trigger, the duration of the activation and the environmental factors.

1.2 Systemic inflammatory response

Pro-inflammatory cytokines, such as interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)α or interferon (IFN)γ, produced locally in inflamed tissues can spill into the circulation and initiate a systemic inflammatory response. This systemic response following local

inflammation is known as the acute-phase response and drives a wide range of mechanisms throughout the body. They include leukocytosis in the bone marrow, changes in the brain leading to pyrexia and altered consciousness as well as metabolic disturbances such as cachexia, changes of lipid metabolism and decreased gluconeogenesis. The acute-response also results in changes in concentrations of some plasma proteins called acute-phase proteins (Gabay and Kushner, 1999).

The acute-phase protein has been defined as one whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at least 25% during inflammatory disorders (Gabay and Kushner, 1999). In human, the most induced acute-phase proteins include C-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin. In contrast, plasma concentrations of albumin and transferrin decrease during the acute-phase response. Many of these proteins are multifunctional providing a variety of different actions. Some of them are components of the complement system or of the coagulation cascade. Others are protease inhibitors, transport proteins or secreted PRRs. Although not all functions of the acute-phase proteins are know, their modulations are crucial for the control of systemic inflammation and appear beneficial for the response of the body to infectious insults and inflammation (Bode et al., 2012; Gabay and Kushner, 1999).

The acute-phase proteins are mainly produced by hepatocytes in response of a variety of different cytokines released during the inflammatory response including IL-6, IL-1β, TNFα and IFNγ. IL-6 is the major regulator of the hepatic acute-phase proteins. IL-1β and TNF-α can modulate the production of some acute-phase proteins by inducing the synthesis of IL-6 by hepatocytes and also by enhancing the intracellular signaling of the IL-6-induced acute phase protein production (Bode et al., 2012; Kramer et al., 2008; Yoshizaki, 2011). The pro-inflammatory cytokines are therefore the chief stimulators of the systemic inflammatory response.

1.3 Pro-inflammatory cytokines

Cytokines are bioactive proteins with low molecular weights (8,000 to 30,000 daltons) that act as mediators and modulators of the immunological response. Almost all nucleated cells are

capable of synthesizing cytokines and, in turn, of responding to them. They modulate the function and activity of cells around them to coordinate and control the inflammatory response. The cytokines act in networks, they can interact with each other to mediate additive, antagonistic or synergistic effects. The cytokine production is tightly regulated. The relative concentrations of cytokines are often associated to the physiological effects of cytokines. Some cytokines initiate and amplify the inflammatory response, others sustain or attenuate it and some of them cause it to resolve. Based on their main biological activities, cytokines can be divided into pro-inflammatory (e.g. IL-6, TNFα or IL-1β) or anti-inflammatory (e.g. IL-10 or transforming growth factor (TGF)β) cytokines.

IL-6, TNFα and IL-17 are three pro-inflammatory cytokines contributing to the chronic inflammatory state of many autoimmune and inflammatory disorders. They are therefore attractive therapeutic targets. Inhibitors of IL-6, TNFα or IL-17 pathways are now available on the drug market. For this reason, this study focuses particularly on these three cytokines.

1.3.1 Interleukin-6

IL-6 is a four-helix protein of 184 amino acids with pleiotropic activities. IL-6 is synthetized and secreted by monocytes, macrophages, T cells, fibroblasts and endothelial cells. IL-6 binds the IL-6 receptor (IL-6R), which is not signaling competent. Indeed, IL-6 signaling is initiated upon association of the IL-6/IL-6R complex with a second receptor named glycoprotein (gp) 130 that thereupon dimerizes (Figure 2). Dimerization of gp130 leads to activation of the tyrosine kinase janus kinases (JAKs), which stimulate several intracellular signaling pathways including signal transducer and activator of transcription (STAT) 1 and STAT3 pathways and mitogen-activated protein kinase (MAPK) and phosphatidylinositiol-4,5-biphosphate 3-kinase (PI3K) pathways (Calabrese and Rose-John, 2014).

However, IL-6R is only expressed on few cell types including some leukocytes, hepatocytes, some epithelial cells (e.g. biliary epithelial cells) and non-epithelial cells (e.g. hepatic stellate cells) (Schmidt-Arras and Rose-John, 2016). However, because a soluble form of IL-6R (sIL-6R) can be generated from IL-6R expressing cells with the same affinity for IL-6, the IL-6–sIL-6R complex could stimulate cells that do not express IL-6R (e.g. endothelial cells and

smooth-muscle cells) (Calabrese and Rose-John, 2014). This increases the spectrum of IL-6 target cells. This signaling mode is called IL-6 trans-signaling and the IL-6 signaling through the membrane-bound IL-6R is the IL-6 classic signaling (Figure 2). Exploration of these two IL-6 signaling suggests that the IL-6 classic signaling is important for regenerative and protective functions of IL-6 whereas IL-6 trans-signaling is associated with the IL-6 pro-inflammatory activities (Scheller et al., 2011).

In human blood, a soluble form of gp130 (sgp130) is also present and acts as a natural inhibitor of IL-6 trans-signaling without affecting the IL-6 classic signaling. High levels of sgp130 are founded in the circulation of healthy individuals (250-400 ng/mL) compared to the levels of sIL-6R (40-60 ng/mL) and IL-6 that is even lower (1-5 pg/mL). Therefore, sIL-6R and sgp130 constitute an effective blood buffer for IL-6 (Calabrese and Rose-John, 2014). Interestingly, individuals carrying a polymorphism on the IL-6R gene increasing the sIL-6R levels in blood are protected from several autoimmune diseases and cardiovascular diseases (Ferreira et al., 2013; Rafiq et al., 2007; Scheller and Rose-John, 2012). The level of sIL-6R is consequently crucial in the IL-6 overall activity since the increase of the sIL-6R level increases the capacity of the IL-6 buffer in blood.

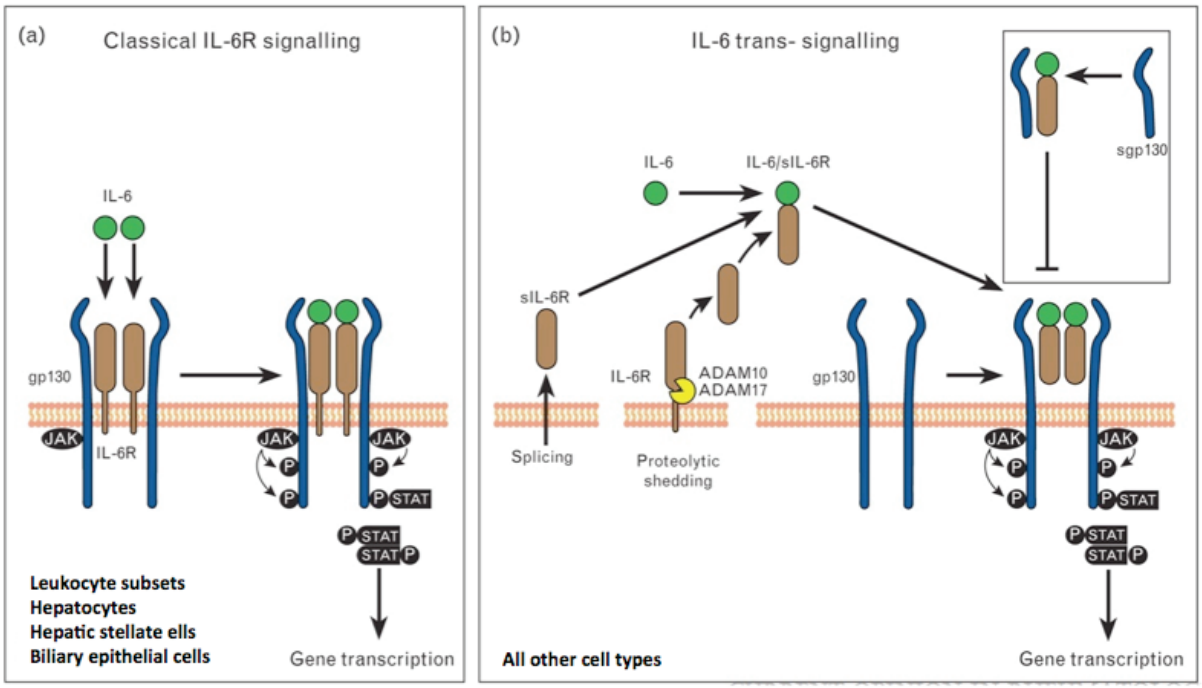

FIGURE 2: Signaling of IL-6 via the membrane-bound and soluble IL-6 receptor

(a) In classic IL-6 signaling, IL-6 binds the membrane-bound IL-6R on hepatocytes and some leukocytes. The IL-6–IL-6R complex then associates with the signal transducing protein gp130, which is ubiquitously expressed. This association induces dimerization and IL-6 signal transduction. (b) In trans-signaling, IL-6 binds the soluble IL-6 receptor (sIL-6R) generated by translation from an alternatively spliced mRNA or the cleavage of membrane-bound IL-6R by the metalloprotease ADAM10 or ADAM17. The IL-6–sIL-6R complex associates with the membrane-bound gp130 on cells that do not express the membrane bound IL-6R and induces dimerization and IL-6 signaling pathway. Soluble gp130 (sgp130), present in the circulation in healthy conditions, blocks the IL-6 trans-signaling by binding the IL-6–sIL-6R complex without affecting the classical signaling. Adapted from Liu et al., 2016 - DOI: 10.1097/BOR.0000000000000255, with permission from RightsLink / Wolters Kluwer Health, Inc; License Number: 4390181057524

IL-6 is a pleiotropic cytokine with multiple functions in the body. It contributes to host defense against pathogens but plays also an important role in various autoimmune and inflammatory diseases such as rheumatoid arthritis (Tanaka et al., 2012). By acting on a wide variety of cells, IL-6 exerts multiple biological activities. IL-6 is the major inducer of the hepatic acute-phase proteins (Heinrich et al., 1990; Schmidt-Arras and Rose-John, 2016). IL-6 promotes also T cell differentiation toward Th17 cells and immunoglobulin synthesis in activated B cells (Tanaka et al., 2012). IL-6 is also involved in the chronicity of the inflammatory response by inducing mononuclear cell recruitment to the site of inflammation (Gabay, 2006). In the bone marrow, IL-6 enhances the production of platelets and the activation of hematopoietic stem cells. Moreover, IL-6 acts on synovial fibroblasts to increase osteoclast differentiation and angiogenesis. IL-6 also stimulates the collagen production by dermal fibroblasts (Tanaka et al., 2012).

However, IL-6 has an important role in the regenerative response of intestinal epithelial cells and hepatocytes to injury (Scheller et al., 2011; Schmidt-Arras and Rose-John, 2016). In addition to its effect on hepatocyte regeneration, IL-6 acts on liver metabolic functions. This cytokine is therefore crucial in the liver homeostasis (Hassan et al., 2014; Schmidt-Arras and Rose-John, 2016). These anti-inflammatory effects of IL-6 can have important consequences on the use of IL-6 inhibitors for the treatment of chronic inflammatory diseases. Indeed, blockade of IL-6 is associated with gastrointestinal perforations (Calabrese and Rose-John, 2014; Taniguchi et al., 2015), transaminase elevation (Genovese et al., 2017) and adverse lipid changes (Strang et al., 2013).

1.3.2 Tumor necrosis factor α

TNFα is a pleiotropic cytokine that participates in a variety of inflammatory, infectious and malignant conditions. Activated monocytes and macrophages are the main sources of TNFα but a wide range of cells can also produce TNFα including mast cells, T cells, natural killer (NK) cells and non-immune cells such as endothelial cells (Sedger and McDermott, 2014). TNFα is synthesized as a 26 kDa membrane bound protein which can be cleaved into a soluble 17 kDa form by the TNFα-converting enzyme (TACE also known as ADAM17). Both membrane-associated and soluble TNFα are active and mediate their downstream signal by binding to TNFα receptor 1 (TNFR1) and TNFR2 (Bradley, 2008). TNFR1 (also known as CD120a) is expressed ubiquitously and activated by both transmembrane and soluble TNFα. However, TNFR2 (also known as CD120b) is restricted to specific cell types including immune cells or endothelial cells and binds preferentially the transmembrane form of TNFα in the context of cell-cell interactions (Figure 3). These two receptors contain distinct intracellular domains. TNFR2 lacks a death domain and thus is unable to induce programmed cell death directly whereas TNFR1 signals by the recruitment of TNFR-associated-death domain protein (TRADD) (Kalliolias and Ivashkiv, 2016).

Trimeric TNFα binding to TNFRs leads to receptor trimerization and assembly of distinct signaling complexes: complexes I, IIa, IIb and IIc (Figure 3). The formation of the complex I induces MAPK signaling cascades and the activation of nuclear factor κB (NFκB) pathway leading to expression of genes involved in inflammation, host defense and cell proliferation and survival. In contrast, activation of complexes IIa and IIb (also known as ripoptosome) by the TNFα-TNFR1 binding mediates cell apoptosis through the activation of a caspase cascade. Furthermore, assembly of complex IIc (necrosome) activates the necroptosis effector mixed lineage kinase domain-like protein (MLKL) that results in necroptosis and inflammation (Figure 3). Indeed, necroptosis is characterized by cell membrane rupture leading to the release of intracellular contents triggering local inflammation, in contrast to apoptosis where cells are rapidly phagocytized (Kalliolias and Ivashkiv, 2016).

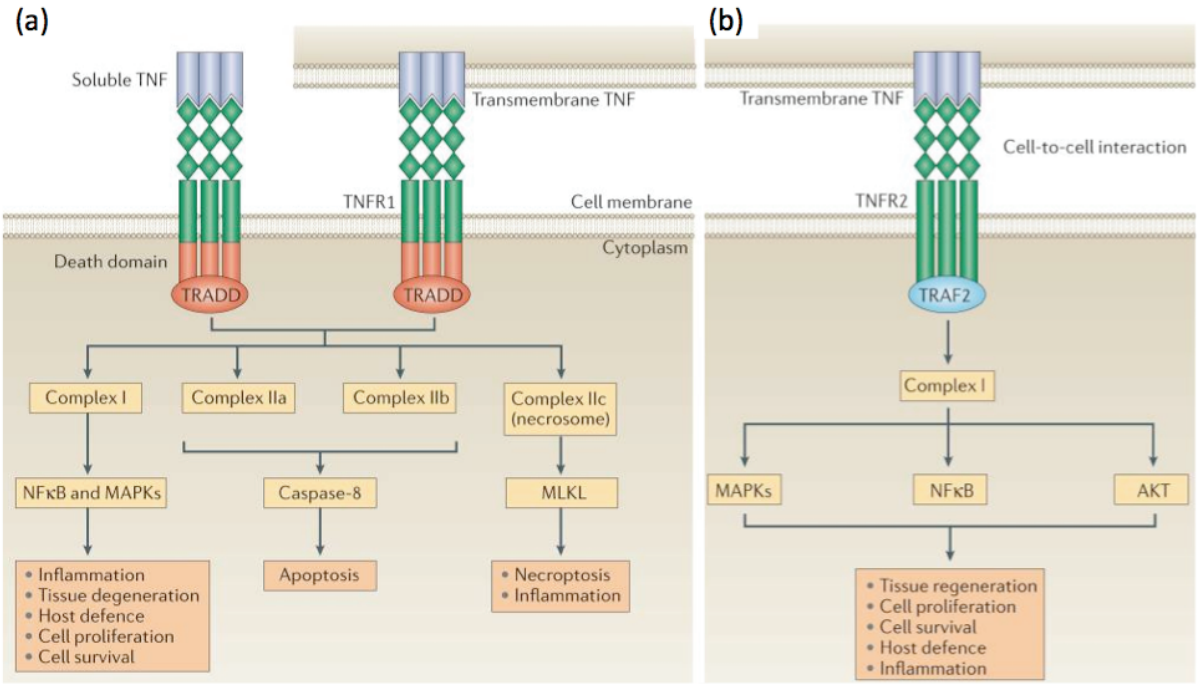

FIGURE 3: TNFα signaling pathways via TNFα receptor 1 and 2

(a) Both soluble and transmembrane TNFα can activate TNFα receptor (TNFR) 1 signaling. TNFR1 bears a death domain recruiting the TNFR-associated-death domain protein (TRADD). Ligand binding to TNFR1 leads to complex I assembly, which induces mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NFκB) driving to inflammation, tissue degeneration, host defense and cell proliferation and survival. In contrast, other signaling pathways are associated with programmed cell death: assembly of Complex IIa and IIb induces apoptosis whereas activation of complex IIc results in necroptosis and inflammation. (b) TNFR2 is preferentially activated by transmembrane TNFα. Ligation of TNFR2 leads to the recruitment of TNFR-associated factor 2 (TRAF2), which triggers assembly of complex I, and activation of MAPK, NFκB and AKT pathways. This mediates homeostasis effects including tissue regeneration, cell proliferation and survival as well as inflammatory and host defense effects. MLKL, mixed lineage kinase domain-like protein. Adapted from Kalliolas and Ivashkiv, 2016 - DOI: 10.1038/nrrheum.2015.169, with permission from RightsLink / Springer Nature; License Number: 4401830967157

TNFα can therefore trigger multiple signaling pathways involved in inflammation, host defense, proliferation and cell death. One of the major biological functions of TNFα is in the immune response to bacterial, viral and parasitic infections. TNFα is a key regulator of the local inflammatory immune response by initiating the release of a cascade of inflammatory mediators, promoting thrombosis and increasing vascular permeability, which enhances immune cell recruitment in the site of infection (Bradley, 2008). TNFα is an attractive and current therapeutic target for a wide range of inflammatory diseases including rheumatoid arthritis, inflammatory bowel diseases, ankylosing spondylitis or psoriasis.

The pro-inflammatory cytokine IL-17 is presented in the part 2 of the introduction.

1.4 Pro-inflammatory chemokines

Chemokines are small soluble proteins (8-12 kDa) playing an important in tissue homeostasis by orchestrating leukocyte trafficking. Indeed, these chemoattractant cytokines induce cell recruitment, activation of leukocyte movements and cellular adhesion. This study focuses on three key chemokines: IL-8, monocyte chemoattractant protein 1 (MCP-1) and chemokine C-C motif ligand (CCL)20.

IL-8 (or chemokine C-X-C motif ligand (CXCL)8) is produced by a wide range of cell types such as monocytes/macrophages or hepatocytes and binds the C-X-C motif chemokine receptor (CXCR)1 and CXCR2. This chemokine is traditionally associated with neutrophil activation and recruitment into injured tissue. However, CXCR1 and CXCR2 are not only expressed on neutrophils, but also on other leukocytes and non-immune cells including fibroblasts, endothelial cells, smooth muscle cells and hepatocytes. By acting on these cells, IL-8 may also contribute to fibrosis, angiogenesis and tumor growth in addition to host defense (Russo et al., 2014).

MCP-1 (also known as CCL2) is secreted by many cells including monocytes, T cells, fibroblasts or endothelial cells. MCP-1 interacts with C-C motif chemokine receptor (CCR)2 on monocytes to induce monocyte chemotaxis and extravasation through vascular endothelium. This chemokine is also involved in the recruitment of memory T cells and NK cells. Since MCP-1 is overexpressed in autoimmune diseases (e.g. rheumatoid arthritis), atherosclerosis and angiogenesis, this chemokine may participate to the pathogenesis of these disorders (Melgarejo et al., 2009).

CCL20 alternatively named macrophage inflammatory protein-3α (MIP-3α) binds CCR6 expressed by Th17 and Treg cells but also by a wide variety of other leukocyte subsets such as dendritic cells (DCs). CCL20 controls both effectors and regulators of many immune responses and may participate to the pathogenesis of autoimmune and inflammatory diseases including multiple sclerosis or rheumatoid arthritis (Comerford et al., 2010).

2 IL-17 in chronic inflammation

IL-17A, also known as IL-17, is another pro-inflammatory cytokine playing an important role in host defense and several inflammatory disorders. The family, the cellular sources and the biology of IL-17 as well as the recent findings on the therapeutic strategies for targeting the IL-17 pathway and the clinical results are summarized in this following review:

Beringer A, Noack M and Miossec P. IL-17 in chronic inflammation: from discovery to targeting. *Trends Mol Med.* 2016;22(3):230-41.

CellPress

Review

IL-17 in Chronic Inflammation: From Discovery to Targeting

Audrey Beringer,[1] Melissa Noack,[1] and Pierre Miossec[1,*]

Interleukin-17 (IL-17) is a cytokine which elicits protection against extracellular bacterial and fungal infections and which plays important roles in inflammation. However, when produced in excess, it contributes to chronic inflammation associated with many inflammatory and autoimmune disorders. This has made IL-17 an attractive therapeutic target. The present review describes the structure of the IL-17 family, the IL-17 receptor complex, and the cells producing IL-17. The contributions of IL-17 to disease as well as new IL-17-based treatment options are discussed. Finally, the results of IL-17 or IL-17 receptor inhibitors in clinical trials are detailed. With a fruitful outlook, drug registration has now been granted for psoriasis psoriatic arthritis and ankylosing spondylitis, and also bears great potential in a growing number of conditions.

Introduction

The proinflammatory cytokine IL-17 was described fairly recently and is becoming an important therapeutic target for a growing number of chronic inflammatory diseases [1]. IL-17 plays a key role in host defense against extracellular bacterial and fungal infections [2]. Excess contribution of IL-17 has been associated with several inflammatory disorders including psoriasis, psoriatic arthritis (PsA), rheumatoid arthritis (RA), and ankylosing spondylitis (AS). A first antibody against IL-17 (anti-IL-17) was approved in 2015 for the treatment of psoriasis. Other IL-17 pathway inhibitors are currently being tested for an increasing number of clinical indications relevant to various conditions [3].

This review first describes the structure and signaling pathways of IL-17 and IL-17-producing cells. The key functions of IL-17 are analyzed in the context of disease to introduce the treatment strategies. Finally, we highlight the benefits and limitations of inhibitors targeting IL-17 and the IL-17 receptor (IL-17R).

This discussion is timely, as it represents a good example of 'translational research', highlighting recent advances. It shows how quickly information from the discovery of IL-17 and T helper 17 (Th17) cells has been translated into the development of a successful therapy.

IL-17 and IL-17R Family Members and IL-17 Signaling: The Basics

IL-17 Family

Human cytotoxic T lymphocyte-associated antigen 8 (CTLA8) was identified in 1993 and named IL-17 in 1995 [4]. The first bioactivity of human IL-17 was described in 1996 by showing the production of IL-6 and IL-8 from RA synoviocytes in response to IL-17. This immediately linked IL-17 to inflammation through IL-6 and to neutrophil recruitment through IL-8 [5].

Sequence screening identified an IL-17 family comprising six members from IL-17A (the first described IL-17) to IL-17F (Figure 1). IL-17A and F are the closest members, with 50% homology. They are secreted as IL-17A and IL-17F homodimers and as IL-17A/F heterodimers [6,7].

Trends

Interleukin-17 (IL-17) is a proinflammatory cytokine that plays a key role in host defense against extracellular bacterial and fungal infections.

T helper 17 (Th17) cells play a key role in the production of IL-17.

Increased production and contribution of IL-17 have been associated with several inflammatory disorders, including psoriasis, psoriatic arthritis, rheumatoid arthritis, and ankylosing spondylitis.

The first antibody against IL-17 was approved by the FDA and EMA in 2015 for the treatment of psoriasis.

Other IL-17 inhibitors are under development for a growing number of clinical indications. These include bispecific molecules targeting IL-17A and IL-17F as well as tumor necrosis factor (TNF) and IL-17A.

Inhibitors of Th17 differentiation include those that target IL-23 and the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt).

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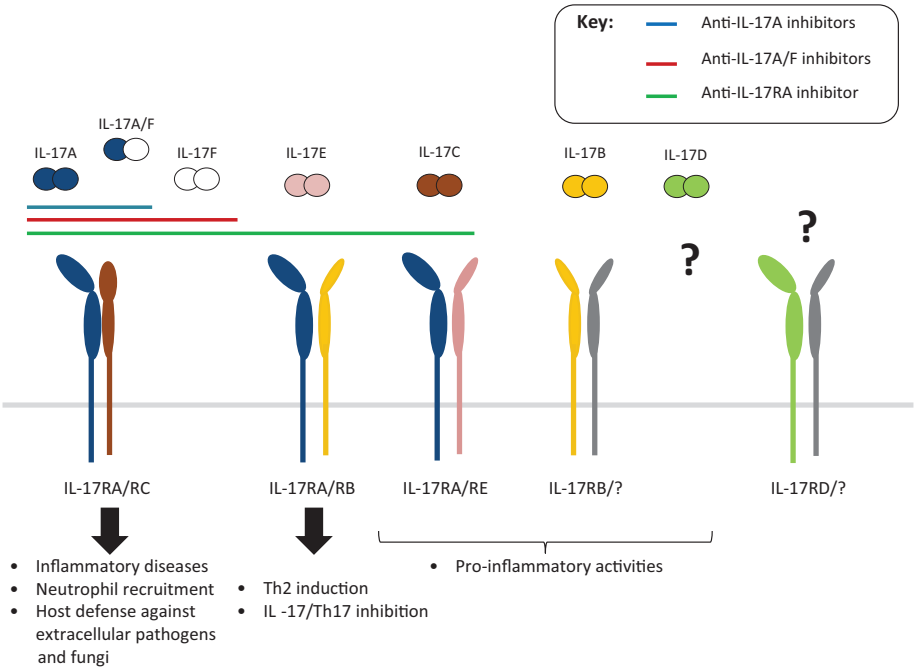


Figure 1. Interleukin (IL)-17 Cytokine and Receptor Family. IL-17 homodimer or heterodimer ligands bind various receptor complexes. IL-17A and IL-17F bind the IL-17 receptor (IL-17R)A and IL-17RC complex. IL-17E or IL-25 binds the IL-17RA and IL-17RB complex. Bars represent the various antibodies in clinical development that target IL-17A (blue), IL-17A and IL-17F (red), or IL-17RA (green).

They share most of their activities, with IL-17A being more potent than IL-17F and IL-17A/F having an intermediate activity [8]. IL-17B, IL-17C, and IL-17D are classified as proinflammatory cytokines but their role is not fully known. By contrast, IL-17E, also known as IL-25, has the lowest homology and is involved in Th2 cell responses against parasites and allergy [7]. IL-25 regulates IL-17 function and this could possibly occur via competition at the receptor level [9].

IL-17R Family

IL-17R was identified in 1995 as a new type of cytokine receptor [10]. The IL-17R family was later described with five subunits, from IL-17RA to IL-17RE (Figure 1). IL-17A, IL-17F, and IL-17A/F bind the same receptor complex comprising IL-17RA and IL-17RC subunits [11,12]. IL-17RA is also a receptor subunit of the receptor for IL-25, comprising IL-17RA and IL-17RB. This is important when targeting IL-17RA, which blocks the proinflammatory pathways mediated by IL-17A, -17F, and -17A/F but also the anti-inflammatory response mediated by IL-25 (Figure 1).

IL-17R Signaling

All receptor subunits have a single transmembrane domain and the binding of IL-17A to the IL-17RA/RC complex recruits the ubiquitin ligase Act1 via the SEF/IL-17R (SEFIR) domain [11]. Act1 recruits tumor necrosis factor (TNF) receptor-associated factor 6 (Traf6) leading to the activation of nuclear factor kappa B (NF-κB) and the mitogen-activated protein (MAP) kinase pathways. Such activation upregulates many inflammatory genes, particularly the neutrophil-specific CXC chemokines [13,14].

IL-17-Producing Cells

Th17 Cells

The production of IL-17 by a subset of T cells was discovered in 1999 using T cell clones from the joints of RA patients [15]. The results were then confirmed in mice and the term Th17 subset was introduced in 2005 in the mouse as a T helper subset distinct from Th1 and Th2 cells [16–18]. IL-12 had been identified as the key cytokine for the production of interferon gamma (IFNγ), the signature cytokine of the Th1 pathway. The new cytokine IL-23 was found to be associated with the Th17 pathway [19]. Th17 cell differentiation involves an initiation step in the presence of transforming growth factor beta (TGF-β) and IL-21 or IL-6, which induce the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt) (human counterpart RORC). Then amplification occurs with IL-1β and IL-6 or IL-21, which induces the expression of IL-23R. This leads to the final step of stabilization with IL-23 [20]. The key cytokines produced by human activated Th17 cells are IL-17A, IL-17F, IL-21, and IL-22.

Th17 cell differentiation is also linked to the differentiation of $CD4^+$ regulatory T cells (Tregs). Because of their opposite effects on the immune response, the Th17/Treg balance is critical in maintaining immune homeostasis. In inflammatory conditions, Tregs are defective and can be converted into Th17 cells [21,22].

Other Sources of IL-17

IL-17 is also produced in both humans and mice by innate immune cells in peripheral tissues such as lungs, intestinal mucosa, and skin [23]. They control the immediate IL-17 response to stress or tissue injury. Their list keeps growing and includes $CD8^+$ T cells, γδ T cells, invariant natural killer T cells (iNKT), natural killer (NK) cells, natural Th17 cells, lymphoid tissue inducer (LTi) cells, and group 3 innate lymphoid (ILC3) cells [13,23,24].

Macrophages, neutrophils, and mast cells in both humans and mice have been reported as another source of IL-17 [7,23]. However, this remains controversial. Although mast cells may stain positive for IL-17 in sections of inflamed tissue, demonstration of active IL-17 mRNA expression has been difficult, suggesting that mast cells might engulf IL-17, serving as a local reservoir [25].

The Biology of IL-17A

IL-17 has pleiotropic effects on multiple cell types. It plays a key role in host defense against infections but also in the development and chronicity of inflammatory disorders.

Role of IL-17 in Host Defense

Th17 cells and other IL-17-producing cells protect the host against extracellular bacterial and fungal infections at epithelial and mucosal surfaces. To control infection, IL-17 promotes granulopoiesis leading to neutrophilia by increasing granulocyte colony-stimulating factor (G-CSF) and migration in response to neutrophil chemoattractants such as IL-8/CXCL8 [5]. The production of chemoattractants for lymphocytes, dendritic cells, and monocytes is also induced by IL-17. CCL20 is an important chemokine that attracts Th17 cells by binding to CCR6, the receptor for CCL20, which is also a marker of Th17 cells [26].

Overexpression of IL-17 in the lung enhances *Klebsiella pneumoniae* clearance and mouse survival [27]. Mice deficient in IL-17 and/or IL-17RA have a higher susceptibility to several extracellular bacteria, specifically *Staphylococcus aureus* [28,29]. IL-17 is also essential in controlling fungal infections [30]. After skin infection with *Candida albicans*, IL-23- and IL-17-deficient mice show delayed skin healing and a higher fungal burden, which are improved by exogenous administration of IL-17A [31].

In humans, this phenotype is reproduced in patients with hyper-IgE syndrome caused by a genetic mutation in the STAT3 gene leading to a reduced number of Th17 cells, defective production of IL-17, and Th2 activation leading to increased production of IgE. They suffer severe *S. aureus* and *C. albicans* infections [32]. Moreover, chronic mucocutaneous candidiasis has been associated with genetic deficiencies of IL-17RA, IL-17F, Act1, IL-17RC, and RORC and occurs in patients with autoantibodies against IL-17A, IL-17F, and IL-22 [32–34].

IL-17 and Inflammation

IL-17A and IL-17F act on various isolated cells in both humans and mice, such as endothelial cells, macrophages, fibroblasts, osteoblasts, and chondrocytes. This increases the production of proinflammatory cytokines from monocytes [TNFα, IL-1β, IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), G-CSF] [1]. IL-17 acts on mesenchymal cells from synovium and skin to induce chemokines leading to neutrophil (IL-8/CXCL8), lymphocyte (CCL20), and macrophage recruitment [35–37]. CCL20 drives the recruitment of Th17 and dendritic cells to the inflammatory site [26]. In turn, Th17 cells are activated and produce inflammatory mediators leading to chronic inflammation [38]. IL-17 contributes to cartilage destruction by stimulating the expression of cartilage-degrading enzymes [39] and to bone destruction by enhancing the expression of receptor activator of NF-κB ligand (RANKL) on osteoblasts that activate RANK-positive osteoclasts [40].

IL-17 alone is often poorly active but it can synergize with other inflammatory cytokines such as TNFα, IL-1β, IL-22, IFNγ, and GM-CSF, leading to increased production of inflammatory mediators such as IL-6 and IL-8 [6,37,41]. This results from a combination of mechanisms. IL-17 stabilizes the mRNA expression activated by TNFα, leading to increased and prolonged protein production [42]. In addition, IL-17 induces TNFα receptor II expression, which increases the response to TNFα [41]. As a consequence, a combination of IL-17 and TNFα inhibitors has greater efficacy in arthritis progression than the monotherapies in mouse models [43]. Similar results have been obtained with *ex vivo* cultures of explants taken from the synovium and bone of arthritis patients [40].

The interactions between IL-17 and TNFα that lead to increased inflammation are the main basis for targeting both IL-17 and TNFα, either with a single bispecific molecule or with two inhibitors. This approach could be interest in patients with an inadequate response to TNFα inhibitors [44].

The Role of IL-17 in Inflammatory Diseases

IL-17 thus has two opposite contributions. Its deficiency leads to reduced control of infections, but its overproduction can lead to several chronic inflammatory diseases.

Psoriasis

Psoriasis is an immunological skin disease characterized by chronic inflammation with proliferation of keratinocytes and accumulation of immune cells, specifically Th17 cells. Expression and production of IL-17 by skin-infiltrating cells is increased in psoriatic skin lesions [45]. It is well known that IL-17 acts on keratinocytes to induce the expression of several chemokines leading to the recruitment and accumulation of neutrophils, T cells, and dendritic cells.

Rheumatic Diseases

PsA is a chronic inflammatory arthritis leading to distal joint destruction. It affects 20–30% of psoriasis patients but can also be observed in the absence of skin manifestations. High levels of Th17 cells and $CD8^+$ $IL\text{-}17^+$ T cells are found in human peripheral blood and synovial fluid, correlating with disease activity [46]. *In vitro*, IL-17 treatment of PsA synoviocytes induces higher levels of IL-6, IL-8, and matrix metalloproteinase 3 (MMP-3) than that of osteoarthritis synoviocytes, suggesting a link between IL-17 and local tissue changes in the joint [47].

RA is another chronic inflammatory disease characterized by synovitis and destruction of bone and cartilage. The first demonstration of the production of IL-17 at the site of a human disease was shown in 1999 using RA synovium explants [35]. The addition of an anti-IL-17 antibody to supernatants of these RA synovium cultures reduced the production of IL-6 by synoviocytes exposed to these supernatants. Moreover, elevated levels of IL-17A have been found in serum and synovial fluid of RA patients, correlating with disease activity [48]. Antibodies against IL-17A or IL-17RA have been shown to reduce synovial inflammation and prevent joint destruction in the collagen-induced arthritis mouse model as well as in human synovium and bone explants [40,49].

AS affects the sacroiliac joints and the spine. In contrast to the destructive aspects of RA and PsA, AS leads to ectopic bone formation in the spine, referred to as syndesmophytes. Elevated levels of IL-17 and IL-23 are found in the serum of AS patients [50]. High levels of Th17 cells are also found in their peripheral blood and synovial fluid [51]. In the sacroiliac joint, IL-17 may be produced by cells other than Th17 cells [25,52]. ILC3 cells, which express high levels of IL-17 and IL-22, are expanded in the peripheral blood, synovial fluid, gut, and bone marrow of AS patients, indicating that overall systemic changes occur in these patients [53].

Multiple Sclerosis (MS)

MS is a chronic inflammatory autoimmune disease of the central nervous system (CNS) characterized by the destruction of myelin by autoreactive pathogenic T cells. Early results showed increased expression of IL-17 in the brain at autopsy. IL-17-expressing cells are abundant in active CNS lesions in MS models and patients [54,55].

Crohn's Disease (CD)

CD is an inflammatory bowel disease characterized by local mucosal inflammation in the intestine. The contribution of IL-17 in CD remains unclear. IL-17 production results in intestinal inflammation in some mouse studies but is protective in others [56,57]. In some but not other studies of CD patients, the number of Th17 cells and the expression of their related cytokines have been reported to be increased in intestinal biopsies and to correlate with disease activity [58,59]. Another study has indicated that local inflammation leads to greater conversion of Tregs into Th17 cells [60]. Together these results suggest that IL-17 could play a dual role in disease activity and protection from mucosal damage.

Other Diseases

An increasing number of diseases has been associated with the IL-17 pathway, including asthma, chronic obstructive pulmonary disease (COPD), lupus, hidradenitis suppurativa, polymyalgia rheumatica, giant cell arteritis, Behçet disease, dry-eye syndrome, and Sjögren's syndrome. However, for most it remains unclear whether the association is pathogenic and whether it could justify attempting IL-17 targeting.

Tools to Target the IL-17 Pathway

Studies using cell systems as well as samples from patients and animal models have provided a strong justification for targeting IL-17 in human diseases, with the goal of controlling the harmful (and/or painful) manifestations associated with chronic inflammation. Two major options are currently being developed to target the IL-17 pathway, one acting directly on IL-17A and IL-17F or IL-17RA and the other acting upstream on the differentiation of Th17 cells (Table 1 and Figure 2, Key Figure).

Direct Targeting of IL-17A, IL-17F, and IL-17RA

Targeting the cytokine or its receptor with monoclonal antibodies (mAbs) is the most direct and specific strategy. The first two anti-IL-17A antibodies tested in the clinic were secukinumab

Table 1. Drug Candidates Targeting IL-17 or its Receptor IL-17RA and Their Current Clinical Status

Drug	Manufacturer					
		Psoriasis	PsA	AS	RA	Other Indications
IL-17A Inhibitors						
Secukinumab (AIN457), Consentyx™	Novartis	Approved	Approved	Approved	Phase III	
Ixekizumab (LY2439821)	Lilly	Submitted	Phase III		Phase II	
CNTO 6785	Janssen				Phase II	COPD (Phase II)
CJM112	Novartis	Phase I/II				Hidradenitis suppurativa (Phase II)
BCD 085	Biocad					Healthy subjects (Phase I)
IL-17A and IL-17F Inhibitors						
Bimekizumab (UCB-4940)	UCB	Phase I	Phase I		Phase II	
ALX-0761 (MSB 0010841)	Merck Serono Ablynx	Phase I				
IL-17A and TNFα Inhibitors						
ABT-122	AbbVie		Phase II		Phase II	
COVA322	Janssen/ Covagen	Phase I/II	Preclinical	Preclinical	Preclinical	
IL-17RA Inhibitors						
Brodalumab (AMG 827)	Valeant Pharmaceuticals	Phase III		Phase III	Phase II	

Clinical status is based on data from Clinicaltrials.gov.

(AIN457, Consentyx™), a fully human IgG1κ anti-IL-17A mAb, and ixekizumab (LY2439821), a humanized IgG4 antibody (Table 1). Several other antibodies, such as CNTO 6785, CJM112, and BCD085, are now in clinical trials.

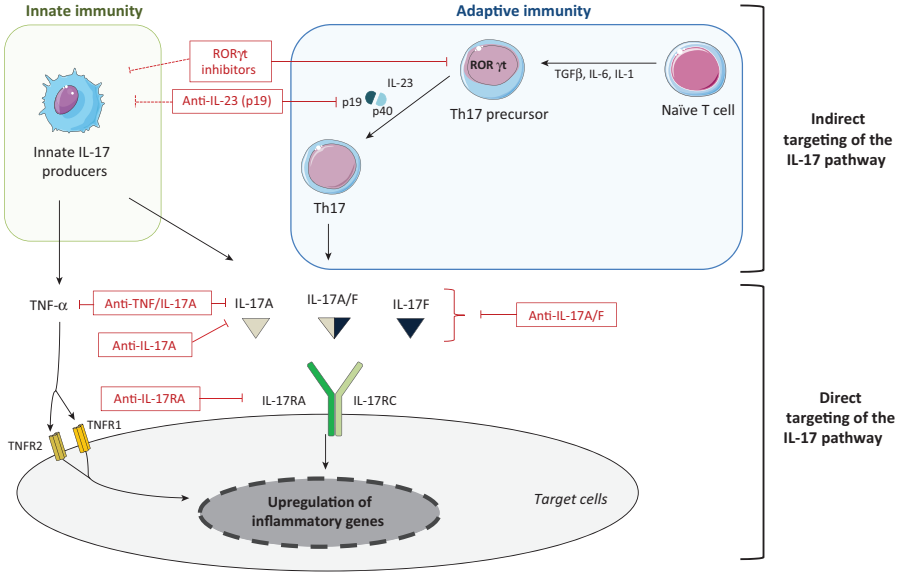
Based on the contribution of IL-17F to inflammation in addition to IL-17A, biomolecules targeting common motifs shared by IL-17A and IL-17F are now in clinical development, including the nanobody ALX-0761 and the mAb bimekizumab [61].

To target the IL-17 receptor, brodalumab (AMG 827) is a fully human IgG2 that selectively blocks signaling through the IL-17RA chain of the IL-17 receptor. This antibody inhibits human IL-17A, IL-17F, and IL-17C but also IL-25 (Figure 1).

Based on the synergistic interactions between TNFα and IL-17 described above, bispecific molecules are now in clinical development. ABT-122 is a dual variable domain immunoglobulin (DVD-Ig™) molecule with one site binding TNFα and the other IL-17A [62,63]. COVA322 is a fusion molecule comprising the fully human anti-TNFα antibody adalimumab with an anti-IL-17A fynomer [64].

Key Figure

Therapeutic Strategies for Targeting the Interleukin (IL)-17 and T helper 17 (Th17) Pathways



Antibody against IL-17A (Anti-IL-17A)	Antibody against IL-17A and IL-17F (Anti-IL-17A/F)	Antibody against TNFα and IL-17A (Anti-TNFα/IL-17A)	Antibody against IL-17 receptor type A (Anti-IL-17RA)
Secukinumab (Novartis)	Bimekizumab (UCB)	ABT-122 (AbbVie)	Brodalumab (Valeant)
Ixekizumab (Lilly)	ALX-122 (Merck Serono/Ablynx)	COVA322 (Janssen/Covagen)	
CNTO 6785 (Janssen)			
CJM112 (Novartis)			
BCD 085 (BIOCAD)			

Trends in Molecular Medicine

Figure 2. The IL-17 pathway can be targeted directly using monospecific antibodies against IL-17A (anti-IL-17A) and IL-17 receptor (IL-17R)A. Bispecific antibodies can target IL-17A and tumor necrosis factor alpha (TNFα) or IL-17A and IL-17F. The IL-17 pathway can be targeted indirectly by acting on the differentiation of Th17 cells with inhibitors of IL-23 using a specific antibody against the IL-23 p19 subunit and with inhibitors of the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt).

Indirect Targeting of the IL-17 Pathway

IL-23 acts upstream of IL-17. It is a heterodimeric ligand comprising the IL-23-specific p19 subunit and the common p40 subunit shared with IL-12. Specific inhibitors of IL-23 described as IL-23p19 antibodies include tildrakizumab (MK-3222, SCH-900222), guselkumab (CNTO 1959), AMG 139, LY3074828, and BI 655066 [61]. These inhibitors act on IL-17A, IL-17F, IL-21, and IL-22 production.

RORγt controls the differentiation of Th17 cells and its targeting will reduce the production of IL-17A, IL-17F, IL-21, and IL-22. Several small molecules such as the synthetic ligand SR1001 can bind RORγt and suppress its transcriptional activities *in vitro* and in mouse models such as experimental autoimmune encephalomyelitis [65–67]. They are now at an early stage of development.

Clinical Results with Inhibitors of IL-17 or IL-17R

A summary of the clinical results with antibodies directly targeting the IL-17 pathway is shown in Table 1.

Psoriasis

The key clinical marker for psoriasis response is the Psoriasis Area and Severity Index (PASI), which measures changes in skin lesion area from baseline. Secukinumab has been evaluated in psoriasis, with the largest number of clinical trials. The first report, published in 2010, showed that the percentage of patients achieving PASI 75 at week 12 was higher with secukinumab than with placebo [68]. Importantly, and for the first time, a drug could achieve a PASI 100 response. Using the same read-out, secukinumab was found to be superior to two registered drugs for psoriasis: etanercept, a TNFα inhibitor [69], and ustekinumab, an inhibitor of the p40 common chain shared by IL-12 and IL-23 [70]. The FDA and EMA approved this anti-IL-17 in 2015 for the treatment of adult patients with moderate to severe plaque psoriasis, with an initial dose of 300 mg subcutaneously at weeks 0, 1, 2, 3, and 4 followed from week 8 by 300 mg once monthly.

The first results with ixekizumab (anti-IL-17A mAb), published in 2012, showed that 40% of patients on the drug achieved PASI 100 versus 0% with placebo [71]. Two recent Phase III clinical studies confirmed these results compared with placebo and etanercept [72]. The first results with brodalumab (anti-IL-17RA antibody), published in 2012, showed that 75–80% of patients on the drug achieved PASI 90 versus 0% with placebo [73]. The results were independent of the presence of arthritis [74]. In a recent Phase III trial, a PASI 100 response rate was possibly more common with brodalumab than with ustekinumab [75]. Based on these results, a dossier for registration has been submitted for ixekizumab and brodalumab.

Regarding the inhibition of IL-23, rather similar results have been seen with guselkumab, which was later found to be more active than adalimumab, a TNFα inhibitor [76,77]. Tildrakizumab showed efficacy against placebo in Phase I and II trials [78,79].

PsA

In addition to the PASI score for skin, PsA response is evaluated in part with the American College of Rheumatology 20 response rate (ACR20), which measures percentage change in disease activity from baseline.

In the first Phase II trial, the response to secukinumab did not meet the ACR20 primary end point [80]. However, this was not the case in two larger Phase III trials, which also showed an effect on radiographic joint damage [81,82]. Now, secukinumab is EMA and FDA-approved for PsA results with ixekizumab and with brodalumab also demonstrated a higher ARC 20 response rate with the drug [83].

AS

In AS, response to treatment is based on the level of improvement of the Assessment of Spondyloarthritis International Society criteria (ASAS20, 50, 70), which measures percentage change in disease activity from baseline.

In a Phase II study, the response to secukinumab was rapid, since at week 6 the ASAS20 response rate was 59% with secukinumab versus 24% with placebo [84]. In a longer follow-up of up to 2 years, sustained clinical improvement was accompanied by regression of spinal inflammation [85]. Secukinumab is now EMA and FDA-approved for AS.

RA

RA response rates to treatment are mostly evaluated using ACR and Disease Activity Score 28 (DAS28) response rates.

The first results on secukinumab in RA, published in 2010, showed positive results based on the ACR20 clinical response [68]. In another Phase II trial, the same primary efficacy end point was not achieved at week 16 [86]. In a 52-week trial, RA patients who failed to respond to methotrexate and other biologics showed improvement after long-term treatment with secukinumab, with better patient-reported outcomes [87,88]. Analysis of individual response rates showed a high degree of heterogeneity. In a subanalysis, genetic markers associated with the MHC type I polymorphism *HLA-DRB1** shared epitope and with high rheumatoid factor levels (but not with anti-CCP antibody positivity) were linked to a better clinical response [89].

Ixekizumab was administered in a first in-human Phase I trial in patients with RA. The first results, published in 2010, showed better ACR and DAS28 indices with the drug than with placebo [90]. Similar conclusions were reached in a Phase II study in biologic-naïve patients and in patients with an inadequate response to TNFα inhibitors [91]. By contrast, no response was seen with brodalumab [92,93]. No explanation has been proposed for the lack of clinical effect of anti-IL-17RA brodalumab, but it is possible that, unlike anti-IL-17 antibodies, it might result in inhibition of the anti-inflammatory cytokine IL-25.

MS

The use of cytokine inhibitors in MS has been limited as the initial proof-of-concept trials with TNFα inhibitors in MS showed increased inflammatory lesions [94]. Although there are many studies employing the experimental autoimmune encephalomyelitis MS mouse model to support the inhibition of IL-17 [95], only secukinumab has been tested in MS. A Phase II trial showed a 60% decrease of new MRI lesions compared with placebo, with a trend of reduction of the annual relapse rate [96,97].

CD

Secukinumab was tested for CD in two Phase II studies. No positive effect was found; rather, treatment resulted in increased disease activity and a higher rate of serious adverse events in some patients [98–100]. Two Phase II trials with brodalumab reached the same conclusion [84]. These negative results may be explained by the protective function of IL-17 in the intestine and the differential contributions of IL-23 and IL-17, as suggested by mouse models of colitis [101].

Other Diseases

The extent of information on other conditions is limited. The effects of secukinumab and brodalumab on various symptoms, including lung function in asthma, do not support an important contribution of IL-17 in clinical improvement [102]. Secukinumab did not appear to influence the severity of dry-eye syndrome [103].

Adverse Events

As predicted from the role of IL-17 in host defense and neutrophil biology, the rate of mild or moderate infections in patients treated with IL-17 inhibitors has been generally higher [99]. Also as predicted, *Candida* infections have been more common in patients treated with IL-17A and IL-17RA inhibitors [69,72]. However, the severity has been documented as being lower than in patients with genetic defects affecting the IL-17 pathway [33]. Cases of reduced neutrophil count have also been reported [73]. Importantly, cases of tuberculosis reactivation, a problem noted with TNF inhibition, have not been reported with IL-17 inhibition [99].

Cases of stroke and myocardial infarction have been observed in a recent study of secukinumab in PsA but not in other diseases, or on treatment with other drugs [82]. In addition, suicidal ideation and behaviors were observed in patients taking brodalumab for psoriasis [104]. No mechanism has been proposed for this unpredicted adverse event, which has not been seen with the other IL-17A inhibitors. Only post-authorization safety studies and real-life use of the

drugs in large numbers of patients will allow a full assessment of the safety profile and the risk: benefit ratio.

Concluding Remarks

Inhibition of IL-17A and IL-17RA has already provided a major improvement in the care of psoriasis, achieving a level of response not seen before. Drug registration has been obtained for PsA and AS. These are already impressive achievements for a molecule discovered in 1995 and identified as a clinical target in 1999.

Other options based on IL-17 biology are now being tested, including bispecific antibodies against IL-17A and IL-17F and against TNFα and IL-17A, which may be of interest in cases of observed lack or loss of response to anti-TNFα therapy [43]. In parallel, molecules targeting Th17 cells and related pathways are under active development. Differences in efficacy and tolerance are already emerging among these options.

It is clear, however, that a better understanding of patient heterogeneity will be needed to achieve accurate and improved personalized medicine (see Outstanding Questions). Moreover, additional research is needed to identify patients with IL-17-driven diseases as well as the various components that contribute to those diseases [105]. Nevertheless, the process has begun.

Outstanding Questions

What are the respective contributions of Th17 cells versus other IL-17-producing cells in an inflammatory response?

What are the positive and negative long-term consequences of IL-17 pathway inhibition?

As the IL-17R antibody brodalumab blocks several IL-17 family cytokines, including the anti-inflammatory IL-25, will this inhibitor show a gain of effectiveness and/or more side effects compared with anti-IL-17A antibodies?

What are the positive and negative consequences of IL-17A versus IL-17A and IL-17F inhibition?

The bispecific anti-TNFα/IL-17 antibodies are promising biotherapies because of the common synergistic interactions between these two cytokines. Will blocking both TNFα and IL-17 exacerbate the risk of infection? As the bispecific anti-TNFα/IL-17 antibodies are already in clinical trials, is there a benefit to combining an anti-TNFα drug with an anti-IL-17 drug to modify the dose or sequence of administration?

The therapeutic response of IL-17 pathway inhibitors appears to be heterogeneous in diseases such as RA. Regarding safety and efficacy, which criteria or biomarkers would be useful in selecting patients for IL-17 pathway inhibitor trials?

What is the contribution of IL-17 in other diseases such as lupus, scleroderma, and vasculitis? Could IL-17 pathway inhibitors be useful in these disorders?

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3 Role of IL-17 and cell interactions in hepatic inflammation

3.1 The liver, a major organ for the immune system

The liver provides a multitude of functions to the organism. It is usually perceived as a non-immunological organ, central for metabolic activities, nutriment storage and detoxification. However, by producing most of the acute-phase proteins, the liver plays an important role in the acute phase response (as seen in part 1.2). The balance between acute immune responses and tolerance in liver is also essential to the overall health (Crispe, 2009; Kubes and Jenne, 2018). Receiving 80% of its blood supply from the gut and the spleen, the liver is constantly exposed to environmental toxins, dietary and commensal bacterial products with inflammatory potential via the portal venous blood (Robinson et al., 2016). Mechanisms to resolve inflammation are essential to maintain liver homeostasis and disruption of one of them or dangerous stimuli leads to chronic pathological inflammation. Therefore the liver is a central intersection point of the immune system (Bode et al., 2012).

The different functions of the liver are tightly linked to the cell composition and structural organization. The sinusoids are lined by fenestrated monolayer of liver sinusoidal endothelial cells (LSECs). The low-pressure blood and the fenestrated endothelium allow rapid exchanges between blood and hepatocytes as well as non-parenchymal cells of the liver including hepatic stellate cells (HSCs) and liver macrophages (also known as Kupffer cells). This also facilitates the interactions between immune cells in liver sinusoids and some liver resident cells in the space of Disse (or sub-endothelial compartment) (Racanelli and Rehermann, 2006).

The structural-functional organization of the liver, the hepatic cell repertoire and its “buffer” function between the gut content and the systemic inflammation create a unique microenvironment. This microenvironment determines the balance between tolerance and inflammation (Robinson et al., 2016). In healthy liver, an active and complex cytokine milieu exists including pro-inflammatory and anti-inflammatory cytokines. The inflammatory state modulates the expression of various cytokines in the liver. These cytokines are the driving force in the fibrosis and cirrhosis processes (Tilg et al., 2006).

3.2 IL-17 and IL-17-producing cells in liver disorders

Because liver cells express ubiquitously the IL-17 receptors (Lafdil et al., 2010; Meng et al., 2012), the pro-inflammatory cytokine IL-17 can mediate a broad effect in the liver. Moreover, the direct pathogenic contribution of IL-17 in some hepatic disorders is emerging. Recent data of both *in vitro* and *in vivo* studies on the potential role of IL-17 in liver diseases are summarized and discussed in this following review:

Beringer A and Miossec P. IL-17 and IL-17-producing cells in liver diseases, with focus on autoimmune liver diseases. *Autoimmun Rev.* 2018;17(12):1176-85.

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IL-17 and IL-17-producing cells and liver diseases, with focus on autoimmune liver diseases

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ABSTRACT

The pro-inflammatory cytokine interleukin(IL)-17 and IL-17-producing cells are important players in the pathogenesis of many autoimmune / inflammatory diseases. More recently, they have been associated with liver diseases. This review first describes the general knowledge on IL-17 and IL-17 producing cells. The second part describes the *in vitro* and *in vivo* effects of IL-17 on liver cells and the contribution of IL-17 producing cells to liver diseases. IL-17 induces immune cell infiltration and liver damage driving to hepatic inflammation and fibrosis and contributes to autoimmune liver diseases. The circulating levels of IL-17 and the frequency of IL-17-producing cells are elevated in a variety of acute and chronic liver diseases. The last part focuses on the effects of IL-17 deletion or neutralization in various murine models. Some of these observed beneficial effects suggest that targeting the IL-17 axis could be a new therapeutic strategy to prevent chronicity and progression of various liver diseases.

1. Introduction

The liver is an essential metabolic and immunological organ [1,2]. It plays a central role in immunosurveillance but also in systemic inflammatory reaction as the main organ producing acute-phase proteins. Dysregulation of immune cell homeostasis and inflammation in liver are major features of almost all types of liver diseases. The infiltrated immune cells can interact with liver cells and induce liver damage. If not resolved, chronic liver inflammation can drive to liver fibrosis, cirrhosis and then hepatocellular carcinoma.

IL-17A, also known as IL-17, is a more recently described cytokine, with dual effects on the immune response. It plays a key role in the control of bacterial and fungal infections. It is also an important inflammatory cytokine with direct contribution to various autoimmune / inflammatory diseases [3]. Antibodies targeting IL-17 are now available to treat psoriasis, psoriatic arthritis and ankylosing spondylitis. Over the last ten years, the number of studies on the role of IL-17 in liver injury and inflammation has drastically increased. In this review, the structure and the signaling pathway of IL-17 and the IL-17 producing cells are first described. The second part will analyze the *in vitro* effects of IL-17 on liver resident cells. The third part will focus on the *in vivo* involvement of IL-17 and IL-17 producing cells in liver diseases with a focus on autoimmune liver diseases (AILDs). Finally, the different strategies to inhibit the IL-17 axis already on the market or in development will be presented.

2. Interleukin-17

2.1. General knowledge on IL-17

IL-17A, also known as IL-17, is the first member of the IL-17 family of cytokines composed of six members from IL-17A to IL-17F. IL-17A and IL-17F share the strongest homology of 50%. They are secreted as IL-17A and IL-17F homodimers and as IL-17A/F heterodimers. IL-17A is more potent than IL-17F. IL-17B, IL-17C and IL-17D are classified as proinflammatory cytokines but their effects remain poorly understood. In contrast, IL-17E, also known as IL-25, with the lowest homology with IL-17A, has anti-inflammatory effects, acting as an inhibitor of IL-17-

Abbreviations: ALF, acute liver failure; ACLF, acute-on-chronic liver diseases; αGalCer, α-galactosylceramide; α-SMA, α-smooth muscle actin; AIH, autoimmune hepatitis; AILD, autoimmune liver diseases; BEC, Biliary epithelial cells; CCL20, chemokine C—C motif ligand 20; CHB, chronic hepatitis B; CLD, chronic liver diseases; ConA, Concanavalin A; CRP, C-reactive protein; DILI, drug-induced liver injuries; HCC, hepatocellular carcinoma; IL, interleukin; IL-17-R, IL-17 receptor; KC, kupffer cells; LPS/GaIN, lipopolysaccharide/D-galactosamine; LSEC, liver sinusoidal endothelial cells; MAIT, mucosal-associated invariant T; MCP-1, monocyte chemoattractant protein-1; PBC, primary biliary cirrhosis; PSC, primary sclerosing cirrhosis; SEC, sinusoidal endothelial cell; SC, stellate cells; TGF-β, requires transforming growth factor-β

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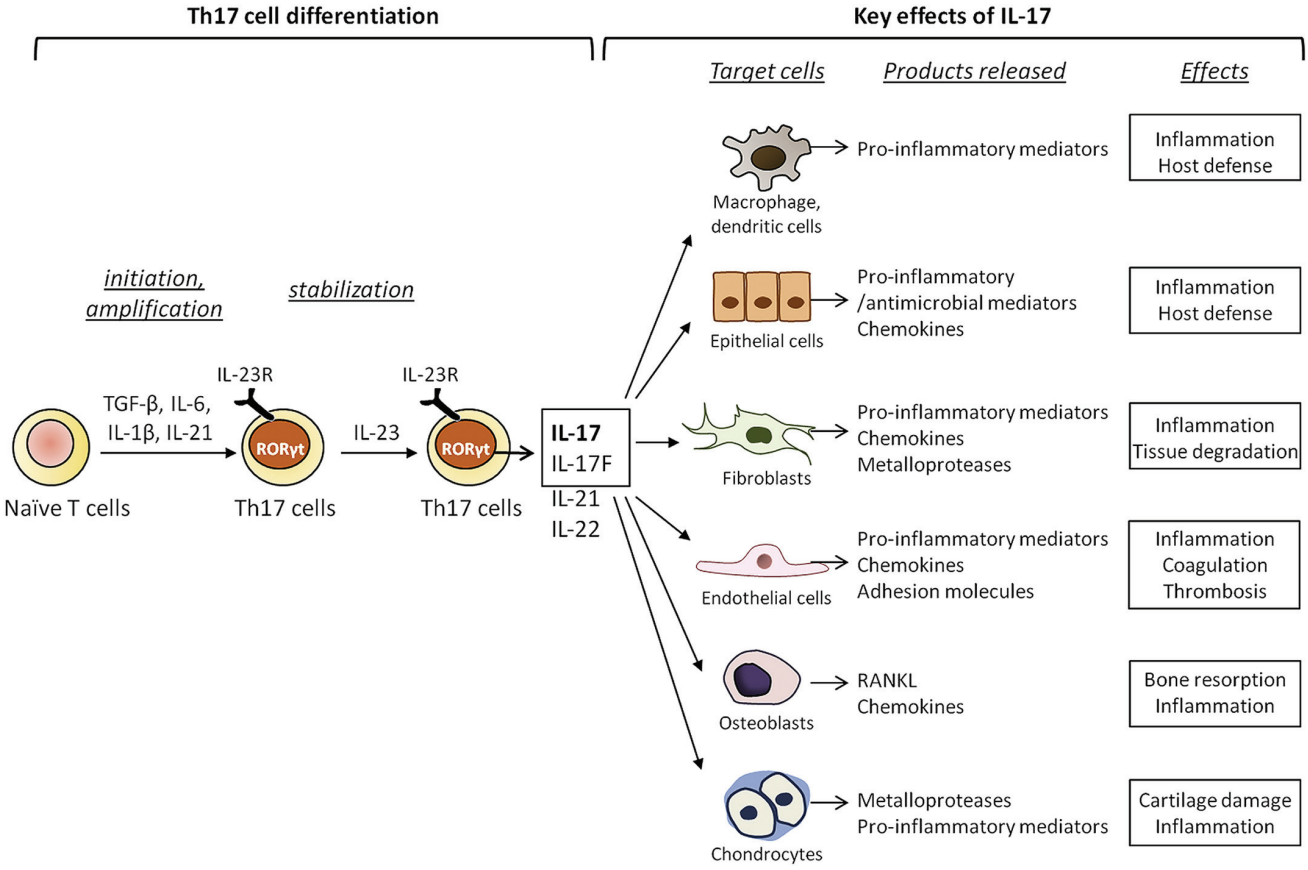

Fig. 1. Differentiation of Th17 cells and key biological effects of IL-17.
Th17 differentiation is initiated by TGF-β and IL-6 or IL-21, which induce ROR transcription factor. IL-1β and IL-6 or IL-21 amplify the Th17 cell differentiation and induce the expression of IL-23 receptor (IL-23R). IL-23 stabilizes the Th17 cell phenotype. IL-17, IL-17F, IL-21 and IL-22 are the main Th17 cytokines. IL-17 acts on multiple cell types to promote inflammation and host protection. By inducing chemokine production, IL-17 attracts neutrophils locally, which exert both protective and destructive effects. The induction of matrix metal- loprotease and RANKL by IL-17 leads to tissue damage as observed in joint inflammation. By acting on endothelial cells, IL-17 promotes coag- ulation and thrombosis. IL-23R, IL-23 receptor; RANKL, receptor activator of nuclear fac- tor-κB ligand.

driven inflammation [4].

The IL-17 receptor (IL-17R) family includes five receptor subunits from IL-17RA to IL-17RE. IL-17A and IL-17F signaling is activated after IL-17 binding to the IL-17RA/IL-17RC dimeric receptor complex [3]. This binding leads to the recruitment of the adaptor protein ACT1, which activates nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [5]. IL-17RA can also form a complex with IL-17RB to mediate IL-17E/IL-25 signaling. Because IL-17RA and IL-17RC are ubiquitously expressed by all cells including liver cells, IL-17 may act on all these cells resulting in liver inflammation [6,7].

2.2. IL-17 producing cells

$CD4^+$ Th17 cells were the first identified IL-17 producing cells. The differentiation of Th17 from naïve $CD4^+$ T cells can be divided into three steps. The initiation step requires transforming growth factor-β (TGF-β) and the proinflammatory cytokines IL-21 or IL-6, which induce the expression of the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt in mice and RORc in humans). Then IL-1β and IL-6 or IL-21 amplify the differentiation of Th17 precursor cells and induce the expression of IL-23R. During the third step, IL-23 stabilizes the Th17 cell phenotype. IL-17, IL-17F, IL-21 and IL-22 are the main secreted Th17 cytokines [3]. However, in the absence of inflammation, TGF-β induces the expression of the transcription factor FoxP3, the marker of $CD4^+$ regulatory T cells (Treg) leading to Treg instead of Th17 differentiation [8]. Because Th17 and Treg cells have opposite effects on the immune response, the Th17/Treg balance is crucial in the maintenance of immune homeostasis. Indeed, elimination of Treg cells enhances the hepatic Th17 cell response and the severity of liver fibrosis in mice [9]. The increase of Th17/Treg ratio in a liver fibrosis mouse model is associated with the progression of fibrosis [10]. Moreover, there is an important plasticity between Th17 and Treg cells, and Treg can be converted into Th17 cells and *vice versa*, under inflammatory changes [11].

Other immune cells can produce IL-17 in response to stress, injury or pathogens. These cells include mucosal-associated invariant T (MAIT) cells, γδ T cells, $CD8^+$ T cells, invariant natural killer T (iNKT) cells, natural killer (NK) cells, lymphoid tissue inducer (LTi) cells and group 3 innate lymphoid cells (ILC3) [12]. In the liver, MAIT cells represent more than 60% of the IL-17^+ cells following PMA/ionomycin activation [13]. Th17 cells and IL-17^+ $CD8^+$ T cells are commonly reported as the major IL-17 secreting cells in several inflammatory liver diseases [14,15]. However, MAIT cells represent more than 60% of the IL-17+ cells in liver following PMA/ionomycin activation. MAIT cells are certainly an important source of IL-17 at the early phase of response in liver. By secreting CCL20, the MAIT cells can promote the recruitment of Th17 cells [13,16].

2.3. IL-17 biology

IL-17 plays a key protective role in host defense against extracellular bacterial and fungal pathogens at the epithelial and mucosal barriers.

To control such infections, IL-17 increases the secretion of inflammatory mediators leading to neutrophil recruitment to the site of infection to eliminate the pathogen [3]. Mice deficient in IL-17 and/or IL-17RA and human patients with genetic deficiencies of Th17 cells and IL-17 have an increased susceptibility to several bacterial and fungal infections, specifically *Staphylococcus aureus* and *Candida albicans* [17–21]. This point must be kept in mind when it comes to targeting IL-17 and will be discussed later.

IL-17 is involved in both acute and chronic inflammation. At the early-phase, IL-17 plays a key role by inducing the recruitment of neutrophils to the site of inflammation through IL-8/CXCL8 chemokine production. An uncontrolled and thus chronic IL-17 production leads to a chronic infiltration of immune cells resulting in autoimmunity and tissue damage. Several chronic inflammatory diseases, including psoriasis, rheumatoid arthritis and ankylosing spondylitis, are associated with such IL-17 overproduction. The effects of IL-17 are amplified by acting on a large variety of cells and through synergistic interactions with other inflammatory cytokines. For example, TNFα cooperates with IL-17 to induce in synergy a massive production of IL-6 and IL-8 by endothelial cells, skin and synovial fibroblasts as well as hepatocytes [22–25]. This synergistic effect involves several mechanisms. IL-17 and TNFα co-stimulate the activation of transcription factors involved in the gene expression of pro-inflammatory mediators [26,27]. In synovial fibroblasts, IL-17 up-regulates TNF receptor II expression and enhances TNFα response [28]. In addition, IL-17 increases mRNA stability of cytokines induced by TNFα which can prolong the protein production [29].

These general aspects of the biology of IL-17 are summarized in Fig. 1. We will now consider the effects of IL-17 on isolated resident liver cells.

3. *In vitro* effects of interleukin-17 on liver resident cells

Hepatocytes are the most abundant parenchymal cell population in the liver. The non-parenchymal cells include liver sinusoidal endothelial cells (LSECs), biliary epithelial cells (BECs), stellate cells (SCs), Kupffer cells (KCs) and resident lymphocytes [1]. The main interactions of IL-17 with liver resident cells on the induction of inflammation and fibrosis are presented in Fig. 2.

3.1. Hepatocytes

Hepatocytes represent about 80% of the liver mass; they ensure many metabolic functions and produce acute-phase proteins such as CRP. *In vitro*, IL-17 induces the production of IL-6 by human hepatoma cell lines and primary hepatocytes with a synergistic effect when TNFα or free fatty acid are combined to IL-17 [25,30–33]. This increase of IL-6 induces the production of CRP and the pro-invasive factors matrix metalloproteinase-2 and vascular endothelial growth factor [30]. IL-8, monocyte chemoattractant protein-1 (MCP-1/CCL2) and chemokine C−C motif ligand 20 (CCL20/MIF-3α) are key chemokines up-regulated by the IL-17 and TNFα synergistic interaction, but independently of IL-6 [25]. By acting on IL-8, IL-17 and TNFα enhance neutrophil recruitment as seen in acute hepatitis whereas MCP-1/CCL2 and CCL20 up-regulation is associated with the infiltration of monocytes, T cells, specifically Th17 cells, and dendritic cells as seen in chronic hepatitis, including autoimmune hepatitis. The IL-17 and TNFα combination has a role in fibrosis by inducing in synergy periostin expression in hepatocytes, leading to fibroblast activation and collagen production [34]. During cholestasis, IL-17 may enhance the bile acid-induced production of inflammatory mediators by hepatocytes including IL-23, which in turn, induces Th17 cell expansion in a positive feedback loop [35]. IL-17 influences also insulin sensitivity *in vitro*. In primary human hepatocytes, IL-17 reduces hepatic insulin signaling, the insulin-mediated suppression of glucose production and the stimulatory effect of insulin on glycolysis [36]. In summary, in hepatocytes, IL-17 mediates systemic inflammation, immune / inflammatory cell recruitment, fibrosis and insulin resistance.

3.2. Liver sinusoidal endothelial cells

LSECs are the second most abundant non-parenchymal liver cells. They are involved in the exchange of cellular mediators between sinusoids and hepatocytes. LSECs have also antigen presentation properties [2]. Expression of adhesion molecules on LSECs leads to T-cell trapping and activation [1]. *In vitro,* murine LSECs inhibit the Th1 and Th17 cytokine secretion through IL-10 and programmed cell death protein 1. This mechanism may contribute to hepatic tolerance [37]. However, LSECs also contribute to IL-17 producing cell recruitment and migration. LSECs stimulation by IFN-γ and TNFα increases Th17 cell and T $CD8^+$ $IL\text{-}17^+$ cell adhesion to endothelium through expression of the chemokine receptor CXCR3, intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 [16].

3.3. Biliary epithelial cells

BECs are cholangiocytes forming bile ducts to drain the bile secreted by hepatocytes. Stimulation with IL-17 up-regulates IL-6, IL-1β and IL-23 expression in human BECs. These cytokines induce Th17 differentiation. Moreover, IL-17 increases the release of several chemokines including CCL20 and MCP-1/CCL2, which attract Th17 cells [38]. By acting on BECs, IL-17 itself can therefore promote Th17 cell differentiation and attraction around the bile ducts and could contribute to chronic cholangitis. In a human cell line of intrahepatic BECs, IL-17 induces also epithelial-mesenchymal transition and fibroblast-like morphological changes [39]. This may contribute to fibrosis development and BEC damage.

3.4. Hepatic stellate cells

HSCs are in the space of Disse in contact with hepatocytes and LSECs. Because activated HSCs are the major source of collagen and extracellular matrix, these cells are crucial in liver fibrosis. HSCs also amplify inflammation by the secretion of pro-inflammatory mediators. The LX-2 human HSC cell line and HSCs isolated from mice respond to IL-17A and/or IL-17F stimulation by up-regulating IL-17 receptors, pro-inflammatory cytokines (IL-6, IL-1β and TNFα) and profibrotic mediators (TGF-β and α-smooth muscle actin (α-SMA)) leading to collagen production [7,40–42]. The blockade of the IL-6 pathway in murine HSCs stimulated with IL-17 slightly reduces the expression of profibrotic genes but does not eliminate the IL-17 effect [7]. Moreover, blocking IL-17R in human HSC and MAIT cell co-culture decreases the pro-inflammatory IL-1β, IL-8 and CCL2 gene expression showing that the IL-17 pathway contributes to HSC activation [43]. Stimulation of human HSCs by IL-17 promotes HSC proliferation and the production of growth related oncogen-α (GROα) and IL-8, which then induces the recruitment of neutrophils [44,45]. Moreover, α-SMA production by mouse HSCs is increased in the presence of Th17 cells and reduced with Treg cells [10]. IL-17 plays also a cooperative role with TGF-β in the development of fibrosis. IL-17 up-regulates TGF-β1 and TGF-β-RII expression and induces TGF-β-RII signaling in LX-2 cells, which enhances HSCs response to TGF-β and activates fibrosis [7,46]. Therefore, IL-17 induces HSCs activation and amplifies the TGF-β-effect on the induction of liver fibrosis.

3.5. Kupffer cells

KCs are resident liver macrophages [2]. They are located in the sinusoid space but can also infiltrate the space of Disse and be in contact with hepatocytes. IL-17RA and IL-17RC are expressed by mouse KCs and up-regulated by IL-17A and/or IL-17F. IL-17A and/or IL-17F stimulation of KCs increases their own expression and that of pro-

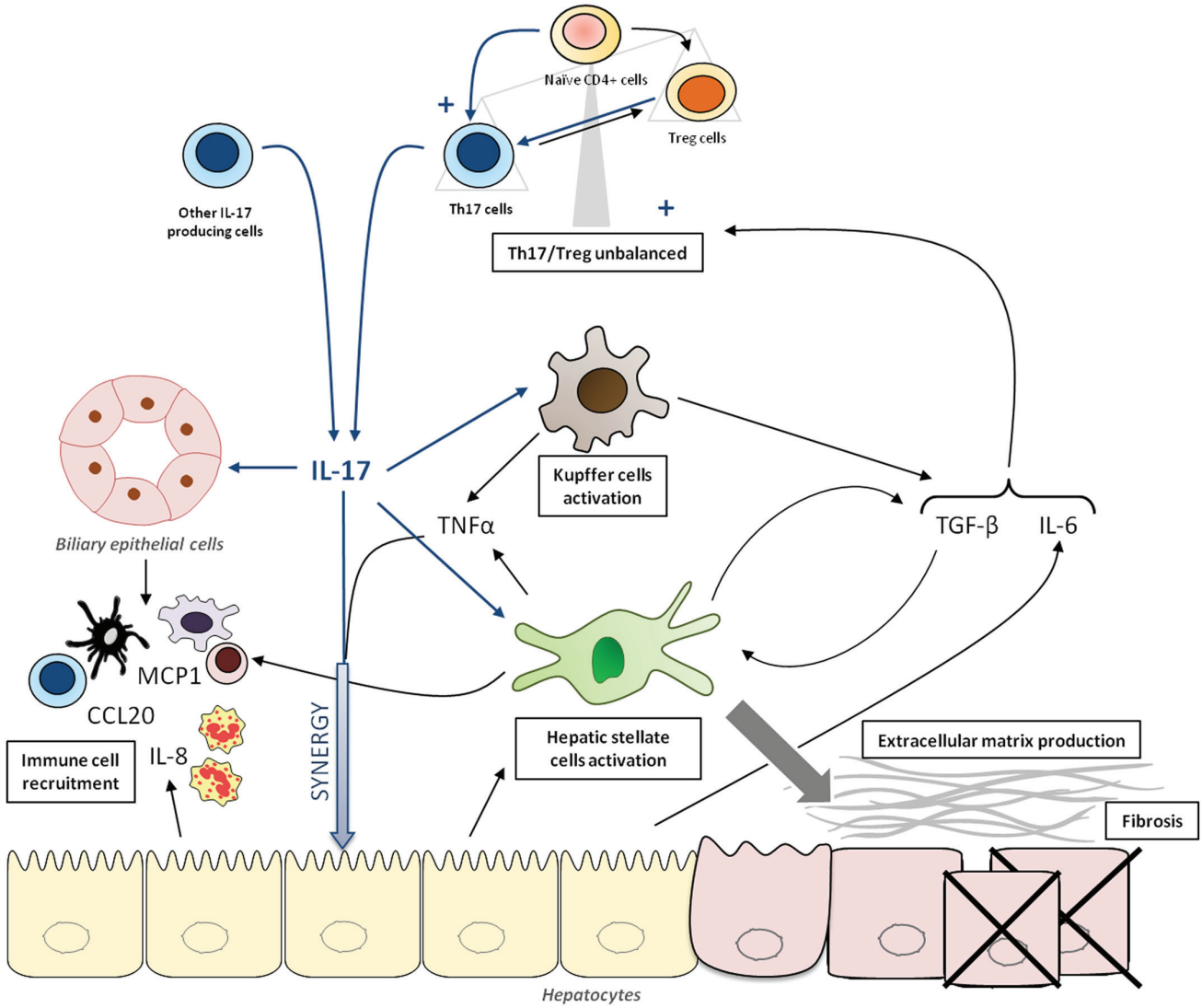

Fig. 2. Main effects of IL-17 on liver resident cells.
The induction of pro-inflammatory and pro-fibrotic cytokines by IL-17 and the Th17 cells expansion results in a vicious cycle that leads to chronic inflammation. IL-17 produced by Th17 cells or other IL-17 pro- ducing cells (e.g. γδ T cells, NK cells or group 3 innate lymphoid cells) induces TNFα, IL-6 and TGF-β release by Kupffer cells (KC) and hepatic stellate cells (HSC). The IL-17 and TNFα combination acts in synergy on hepatocytes to induce the production of IL-8, IL-6 and the profibrotic mediator periostin, which activates HSCs. IL-17 increases HSCs response to TGF-β by enhancing TGF-β and TGF-β-receptor expression in a posi- tive feedback loop. HSC activation by IL-17, TGF-β and periostin leads to collagen production and liver fibrosis. In addition, IL-6 and TGF-β co- operate to activate Th17 differentiation leading to Th17/Treg imbalance and uncontrolled inflammation. By acting on hepatocytes and biliary epithelial cells (BEC), IL-17 up-regulates various chemokine expression including MCP-1 and CCL20 involved in the recruitment of Th17 cells, dendritic cells and monocytes. IL-8 produced by HSCs, hepatocytes and BECs following IL-17 stimulation mediates neutrophil attraction.

inflammatory cytokines (IL-6, IL-1β, TNFα) and the profibrotic cytokine TGF-β [7,47]. KCs from a mice model of hepatitis induce Th17 differentiation from naïve $CD4^+$ T cells in co-cultures [48]. KC and HSC co-cultures from wild type (WT) or IL-17RA−/− mice show that the percentage of collagen-α1 is lower in WT KC – IL-17RA−/− HSC or IL-17RA−/− KC – WT HSC co-cultures than WT KC – WT HSC co-cultures, and this reduction is even higher in IL-17RA−/− KC – IL-17RA−/− HSC co-cultures [7]. IL-17 may therefore contribute to collagen production by acting both on KCs and HSCs. The increase of TGF-β production by KCs following IL-17 stimulation in turn activates HSCs and collagen production [7].

4. Contribution of interleukin-17 and interleukin-17-producing cells to liver diseases

Animal models of liver injury and samples from human subjects with liver diseases have been used to better understand the involvement of IL-17 in acute liver failure (ALF), chronic liver diseases (CLD) with a focus on AILDs and then in acute-on-chronic liver diseases (ACLF). A summary of the effects of IL-17 in healthy conditions, ALF, and CLDs is shown in Fig. 3.

4.1. Acute liver diseases

ALF or fulminant hepatitis is a rapid loss of liver function that occurs in patients without preexisting liver conditions. Since neutrophils

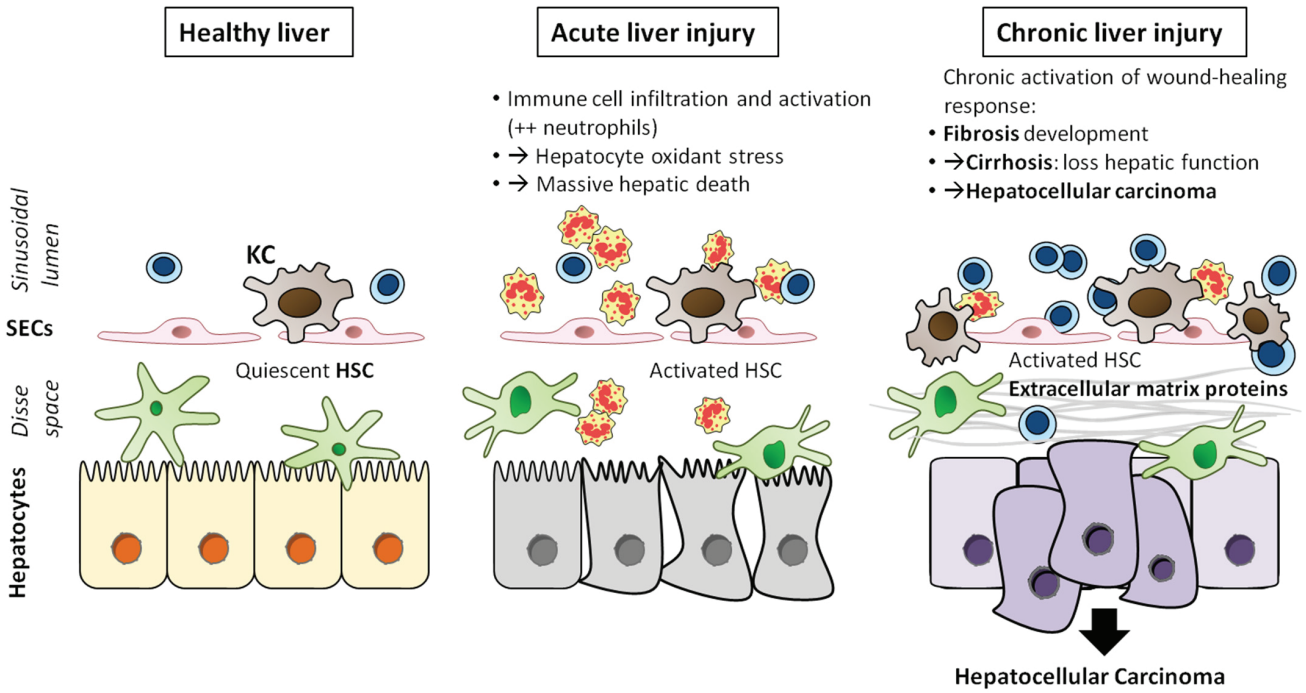

Fig. 3. Liver cell composition in healthy conditions, acute-liver injury and chronic liver injury.
In healthy conditions, the hepatic stellate cells (HSCs) in space of Disse are quiescent and Kupffer cells (KCs) stay within the liver sinusoid space. Acute-liver injury is characterized by the infiltration first of neutrophils that respond quickly to tissue injury, cellular stress or systemic inflammation. Neutrophil and hepatic cell interactions lead to neutrophil activation and hepatocyte oxidant stress that cause hepatic death. In chronic liver injury, chronic activation of infiltrated immune cells and HSCs induces inflammatory damage and matrix deposition with fibrosis progression. Fibrosis causes architecture changes and loss of hepatic function driving to cirrhosis. Cirrhosis can progress to hepatocellular carcinoma. KCs, Kupffer cells; HSCs, hepatic stellate cells; SECs, sinusoidal en- dothelial cells.

are early responders to tissue injury, cellular stress or systemic inflammation, they play an important role in ALF (Fig. 3) [49]. There are many causes of ALF, the most frequent being drug-induced liver injuries (DILI), and viral infections [50,51].

The role of IL-17 in ALF was studied in several animal models with hepatitis induced by chemicals such as Concanavalin A (ConA) or lipopolysaccharide/D-galactosamine (LPS/GaIN) (Table 1). In the mouse models of hepatitis, the induction of hepatitis is associated with an increased IL-17 level correlated with liver damage [47,48,52–55]. Although IL-17 deletion or neutralization attenuates liver injury in most models, a protective role of IL-17 is seen with the α-galactosylceramide (αGalCer)-induced hepatitis model. This surprising effect could be linked to the model itself [56]. αGalCer-induced hepatitis leads to a specific activation of the innate immune response *via* NKT cells with an hepatitis of moderate intensity. Conversely, the ConA model is a model of severe hepatitis characterized by the activation of the adaptive immune system with massive hepatic $CD4^+$ cell infiltration [56]. The cellular sources of IL-17, the timing, the duration and the nature of the injury may therefore influence the final effect of IL-17 on liver inflammation.

Concerning DILIs, the effect of IL-17 was investigated in drug-administrated mice and DILI patients (Tables 1 and 2). IL-17 level is enhanced in drug overdose administration in mice and IL-17 neutralization reduces hepatic damage, in line with the contribution of IL-17 to DILI development [57–60]. In human IL-17, elevated IL-17 level is associated with the severity of the acute hepatic injury [61] and with poor prognosis [62]. These animal and human studies demonstrate the involvement of IL-17 in the induction or exacerbation of ALF. Targeting IL-17 at the initiation of the ALF may prevent neutrophil infiltration and liver injury and therefore improve prognosis.

4.2. Chronic liver diseases

CLDs are characterized by chronic inflammation and the development of fibrosis possibly followed by cirrhosis and hepatocellular carcinoma. The causes of CLDs are many, including viral infections, chronic toxic/drug exposure (mainly alcohol), metabolic, autoimmune, or cholestatic disorders. The hepatic tissue damage results from the chronic activation of the wound-healing response leading to fibrosis progression (Fig. 3).

The studies on the IL-17 and IL-17-producing cell contribution in murine models of CLD and patients with CLDs are presented in Tables 1 and 2, respectively. An increase in $IL\text{-}17^+$ cell frequency and Th17 cell-related cytokine levels are commonly reported in both murine and human studies on CLDs. In human liver with CLD, the $IL\text{-}17^+$ cells represent around 2–3% of the $CD3^+$ T cell infiltrate, with $CD4^+$ Th17 cells present at slightly higher frequency than T $CD8^+$ $IL\text{-}17^+$ cells [16]. Because BECs may attract Th17 cells through CCL20 chemokine up-regulation in inflammatory conditions, $IL\text{-}17^+$ cells are detected next to bile ducts within inflamed portal tracts in CLD patients [16]. IL-17 local expression is increased in liver from patients with liver fibrosis compared to patients without, and IL-17RA expression correlates positively with the stage of liver fibrosis or cirrhosis [7]. Inhibition of IL-17 signaling in immune cells, KCs or HSCs attenuates liver fibrosis in mice with induced liver injury, indicating that IL-17 acts both on resident and non-resident liver cells to induce fibrosis [7]. Conversely, IL-17E/IL-25 administration in a model of liver fibrosis reduces its development [7,63]. IL-17 and IL-17E/IL-25 have therefore opposite effects on liver fibrosis.

The mechanisms of increased Th17 cell response vary according to CLD etiology. In chronic hepatitis B (CHB) patients, the increase of Th17 response may be related to that of the HBV antigen [64,65] and serum level of HMGB1 [66,67]. The hepatic release of thymic stromal

Table 1
IL-17 and Th17 cells contribution to in *in vivo* murine models of liver injury.αGalCer, α-galactosylceramide; AST, aspartate transaminase; BDL, bile duct ligation; ConA, Concanavalin A; MCDD, methionine and choline deficient diet; NAFLD, non-alcoholic fatty liver disease; WT; wild-type.

Murine models	Results	Ref.
Chemical compounds-induced hepatitis models		
Carbon tetrachloride (CCl4)-induced liver fibrosis mice	Increase of plasma IL-17 level and splenic Th17 cell frequency following CCl4 injection. IL-17RA deletion or IL-17 neutralization attenuates plasma and hepatic levels of inflammatory cytokines, hepatic fibrosis and hepatocellular necrosis.	[10,40,103]
Concanavalin A (ConA)-induced hepatitis mice	ConA injection increases IL-17 liver expression, which correlates with severity of liver injury. IL-17$^{-/-}$ mice or IL-17 blockade ameliorates hepatitis.	[47,48,52,53]
Poly I:C-induced acute hepatitis mice	Poly I:C induces IL-17 production by hepatic γδ T cells following IL-23 release by Kupffer cells. IL-17 neutralization decreases inflammatory cytokine levels and necrotic lesions.	[54]
Lipopolysaccharide/D-galactosamine (LPS/GaIN)-induced hepatitis mice	IL-17 deletion reduces serum inflammatory cytokine levels, hepatic neutrophil accumulation and mortality in LPS/GaIN-induced fulminant hepatic injury mice.	[55]
α-galactosylceramide (αGalCer)- induced hepatitis mice	αGalCer injection in mice induces IL-17 production by NKT cells. Neutralization of IL-17 before αGalCer injection exacerbates hepatitis and IL-17 administration ameliorates αGalCer-induced hepatitis.	[56]
Viral hepatitis models		
Adenovirus	Adenovirus increases hepatic IL-17A and IL-17F production, predominantly by γδ T cells. IL-17R deletion or IL-17 neutralization reduces adaptive T cell responses and infiltration and liver injury.	[104,105]
Mouse hepatitis virus	Mouse hepatitis virus increases IL-17 level which is associated with liver damage, elevated level of inflammatory cytokines and death. IFN-γ regulates negatively virus induced-Th17 cell response by increasing Th17 apoptosis.	[106]
Drug-induced liver injury models		
Diclofenac overdose	Up-regulation of the hepatic expression of the Th17 cell-related factors RORγt and STAT3.	[107]
Acetaminophen overdose	IL-17 serum level is increased and associated with the induction of neutrophil recruitment in liver and hepatotoxicity.	[59,60]
Halothane	Increase of plasma IL-17 level and neutrophil infiltration. IL-17 neutralization suppresses the hepatotoxic effect.	[57]
Triptolide oral gavage	Increase of plasma IL-17 level and hepatic Th17 cell frequency. IL-17 neutralization reduces the hepatic damage.	[58]
Alcoholic liver disease models		
Chronic-binge alcohol	Chronic-binge alcohol exposure reduces Treg cell frequency but increases Th17 cell number and serum IL-17 levels.	[108]
Alcoholic liver disease	IL-17 neutralization reduces steatosis by suppressing IL-17-related fatty acid metabolism in alcoholic liver disease group.	[109]
Non-alcoholic fatty liver disease (NAFLD) models		
High fat	High fat diet increases liver and circulating Th17 cell frequency. Recombinant IL-17A induces fibrosis and liver injury whereas blocking IL-17A reduces steatosis and liver injury and prevents hepatocellular carcinoma.	[33,110,111]
Methionine and choline deficient diet (MCDD)	MCDD increases hepatic Th17 cell infiltration, IL-17RA expression and IL-17A/IL-17F production. IL-17$^{-/-}$ mice on MCDD are protected from increased pro-inflammatory cytokine expression, immune cell infiltration and hepatocellular damage.	[112,113]

lymphopoietin is associated to CD4$^+$ T cell polarization toward Th17 cells in HCV patients [68]. Therefore, the mechanisms of induction of the Th17 cell response are linked to each disease etiology but all these CLDs have in common an inflammatory condition in favor of Th17 cell expansion and IL-17 secretion.

Because IL-17 and Th17 cells are involved and now targeted in several autoimmune diseases, they may also play a crucial role in the pathogenesis of AILDs. Circulating Th17 cell frequency and Th17/Treg

Table 2
IL-17 and Th17 cells contribution in human liver diseases other than autoimmune liver diseases ALD, alcoholic liver disease; AIH, autoimmune hepatitis; CHB, chronic hepatitis B; CHC, chronic hepatitis C; DILI, drug-induced liver injury; HCC, hepatocellular carcinoma; HBV, Hepatitis B virus;; NAFL, non-alcoholic fatty liver; NASH, nonalcoholic steatohepatitis.

Human liver diseases	Results in humans	Ref.
Drug-induced liver injury (DILI)	Circulating Th17 frequency is increased in DILI patients and correlates with plasma transaminase level. Elevated IL-17 level is associated with poor prognosis.	[62] [32,71,72]
Alcoholic liver disease (ALD)	Increase of IL-17 plasma level and liver IL-17$^+$ cell infiltrates in ALD patients. The IL-17-secreting cell infiltrates are associated with fibrosis score and the Th17/Treg imbalance with poor prognosis.	[45,114]
Non-alcoholic fatty liver (NAFL) disease	Increase of IL-17$^+$ cells and Th17 cell-related gene expression in liver tissue from nonalcoholic steatohepatitis (NASH). IL-17A levels correlate positively with steatosis. Progression from NAFL to NASH is marked by an increase of Th17 cell accumulation in liver and a higher Th17/Treg ratio in peripheral blood.	[33,111,115]
Chronic hepatitis B (CHB)	Circulating Th17 frequency is increased in CHB patients and correlates positively with plasma HBV load and liver injury. Plasma levels of pro-inflammatory cytokines are elevated in CHB patients. The increase of IL-17$^+$ cells accumulation in liver in CHB patients is associated with neutrophil infiltration and liver fibrosis.	[41,64,81,87,103,116,117] [118]
Chronic hepatitis C (CHC)	IL-17 and IL-17-producing cell implications are not clear and may play a dual role (protective or destructive). Intrahepatic Th17 cell number increases in CHC patients and correlates with the severity of liver injury. IL-17-producting CD8$^+$ T cells are associated with lower inflammatory activity.	[15,68,119–122] [118]
Hepatocellular carcinoma (HCC)	IL-17-producing cells and neutrophils are enriched in HCC tissue and their levels correlates with disease progression and poor survival. By increasing the CXC chemokines expression in liver epithelial cells, IL-17 may promote the migration of neutrophils into HCC and then neutrophils can stimulate the proangiogenic activity of tumor cells.	[123,124]

ratio increase in AILD patients and this increase is higher at the active stage than the remission stage of AILD [69]. The Th17/Treg imbalance may therefore play an important role in the pathogenesis of AILD. The possible contribution of IL-17 and Th17 cell in AILDs will be discussed for the three main AILDs: autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC).

4.2.1. Autoimmune hepatitis

Autoimmune hepatitis (AIH) is a chronic liver disease characterized by hepatitis, with markers of autoimmunity with hyper-gammaglobulinemia and various autoantibodies. AIH is often associated with another autoimmune disease such as Sjogren's syndrome. IL-17 and IL-23 levels and Th17 cell frequency are increased in peripheral blood of AIH patients by comparison to CHB patients and healthy subjects [32,70]. Moreover, the hepatic expression of Th17 cells and Th17 cell-related cytokines are elevated in the AIH patients. Interestingly, the number of Th17 cell infiltration correlates with the degree of hepatic inflammation and the fibrosis grade [32]. In a mouse model of experimental AIH, IL-17 expression in serum and liver is increased whereas the use of IL-17 neutralizing antibody reduces the histological inflammatory grade score and the serum ALT levels [70]. These results are strong argument for the role of IL-17 in the pathogenesis of AIH.

Treg cell impairment has been observed in AIH and may contribute to the loss of immune tolerance. This defect of Treg number and function and the increase of Treg plasticity toward effector Th17 cells may contribute to the abnormal autoimmune reaction in AIH patients [71]. The AIH patients show also a higher proportion of IL-17$^+$ and RORC$^+$ cells in the newly generated Treg (nTreg CD25$^-$) by comparison to healthy subjects. The elimination of IL-17 leads to the development of ngTreg toward a stable Treg phenotype with suppressive function [72]. Therefore, IL-17 contributes to the reduced Treg function. Taking together, the blockade of IL-17 axis or/and the use / induction of Treg cells could be an interesting therapeutic strategy to restore the immune homeostasis in AIH.

4.2.2. Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by the progressive destruction of small bile ducts of the liver leading to cholestasis and the presence of auti-mitochondrial antibodies. As previously described above, by acting on BECs, IL-17 induces BEC morphological changes and a microenvironment promoting Th17 induction *in vitro* [38,39]. In PBC patients, pro-Th17 cytokines and IL-17 mRNA expression as well as serum IL-23, IL-6, IL-1β and IL-17 protein levels are elevated by comparison to healthy and disease controls [73,74]. Circulating Th17 cell frequency is increased in PBC whereas Treg frequency is decreased, resulting to a Th17/Treg imbalance [44,74]. IL-17 producing cells accumulate in the inflamed portal area and their number increases in liver of advanced stage PBC [38,44,73,75]. The Th1/Th17 balance shifts to Th17 in advanced PBC, suggesting that Th1 cells play an important role at the onset, whereas Th17 cells contribute to the perpetuation of the PBC disease [76]. In a xenobiotic-induced murine model of PBC, IL-17A deletion reduces the level of autoantibodies and biliary damage [77]. The therapeutic blockade of IL-17 may therefore be considered in PBC.

4.2.3. Primary Sclerosing Cholangitis

PSC is a chronic cholestatic liver disease characterized by inflammation and fibrosis of the bile ducts resulting in liver cirrhosis and end-stage liver disease. Its etiology remains unknown but autoimmunity appears as one of the pathogenic mechanisms. Results on the IL-17/Th17 cell contribution to human PSC are limited. In PSC patients, the IL-17-producting cells aggregate within periductal areas [78]. Moreover, peripheral blood mononuclear cells from PSC patients induce a higher Th17 cell frequency *in vitro* in response to pathogens, which are frequently found in the bile fluid of these patients [78]. During bile duct ligation in mice, a cholestatic model of liver injury, levels of serum and hepatic IL-17 and IL-17R are increased [7,35]. The induction of IL-23 production by bile acid observed in mice with bile duct ligation or fed with bile acid, can induce Th17 cell expansion and IL-17 production. In turn, IL-17 may act in synergy with bile acid to increase liver inflammation, since IL-17 and bile acids interact to promote *in vitro* the production of inflammatory mediators by hepatocytes [35]. IL-17 and/or IL-17RA deletion or IL-17 neutralization reduces hepatic neutrophil accumulation, liver fibrosis and liver damage in mice with bile duct ligation [7,35,42]. However, it is still difficult to find PSC models with all PSC attributes, and experimental biliary obstruction in mice presents some limitations [79]. Therefore, further studies in PSC patients or in other PSC mouse models are required to confirm the IL-17 and Th17 cell contribution in PSC.

4.3. Acute-on-chronic liver failure

ACLF is defined as an acute deterioration of pre-existing CLDs, leading to a rapid and progressive liver failure. The acute insults are many and include viral hepatitis, alcohol or hepatotoxic drugs [80].

Th17 cells may contribute to the acute deterioration of liver function in chronic HBV infected patients. Liver and blood Th17 cell frequencies and IL-17 serum levels are higher in HBV-related ACLF than CHB or asymptomatic chronic HBV carriers [81–87]. High blood Th17 cell frequency and high IL-17 serum level are associated with poor prognosis in ACLF patients [81,83,85]. The Th17/Treg ratio is also dramatically higher in ACLF HBV patients and is inversely associated with patient survival [83,86,88,89]. In summary, the immune-mediated liver injury effects of Th17 cells and the Th17/Treg imbalance may have an important role in the CLD progression to ACLF.

5. Targeting the interleukin-17 axis

Both *in vitro* and *in vivo* studies provide evidence for the involvement of IL-17 in the pathogenesis of liver diseases by increasing chemokine and cytokine production, immune cell recruitment and fibrosis. Targeting the IL-17 axis could therefore be considered as a promising therapeutic strategy to prevent the induction and the progression of liver diseases. Antibodies targeting IL-17 and IL-17RA are now approved for the treatment of several inflammatory diseases [90].

5.1. Direct targeting of IL-17

To inhibit directly the IL-17 pathway, antibodies against IL-17 and IL-17R are the most straight-forward options (Fig. 4). Monoclonal antibodies against IL-17A are the most specific therapeutic option to target the IL-17 pathway [90]. IL-17RA blockade might be less specific because this receptor subunit is also part of the anti-inflammatory IL-17E/IL-25 signaling pathway. Two anti-IL-17A inhibitors (secukinumab and ixekizumab) and one IL-17RA inhibitor (brodalumab) have been recently approved for the treatment of psoriasis, psoriatic arthritis and ankylosing spondylitis. Since IL-17 and TNFα often work in synergy, bispecific inhibitors blocking both IL-17 and TNFα are in clinical development [90,91]. Another option is the targeting of both IL-17A and IL-17F with bispecific antibodies [92].

5.2. Indirect targeting of the IL-17 pathway with inhibitors of Th17 cell generation

Blocking Th17 cell generation is another therapeutic option to reduce the contribution of this subset and of the production of Th17 cell-related cytokines, which include IL-17A and IL-17F but also IL-21 and IL-22. Because IL-6 and IL-1β are involved in the Th17 cell differentiation, the inhibition of the IL-6 pathway (by anti-IL-6R or JAK inhibitors) or the IL-1β pathway (by anti-IL-1β or IL-1R antagonist) may reduce in part the Th17 cell pathway (Fig. 4) [93–95]. Targeting IL-23 is another option to inhibit the IL-23-IL-17 axis since IL-23 stabilizes the

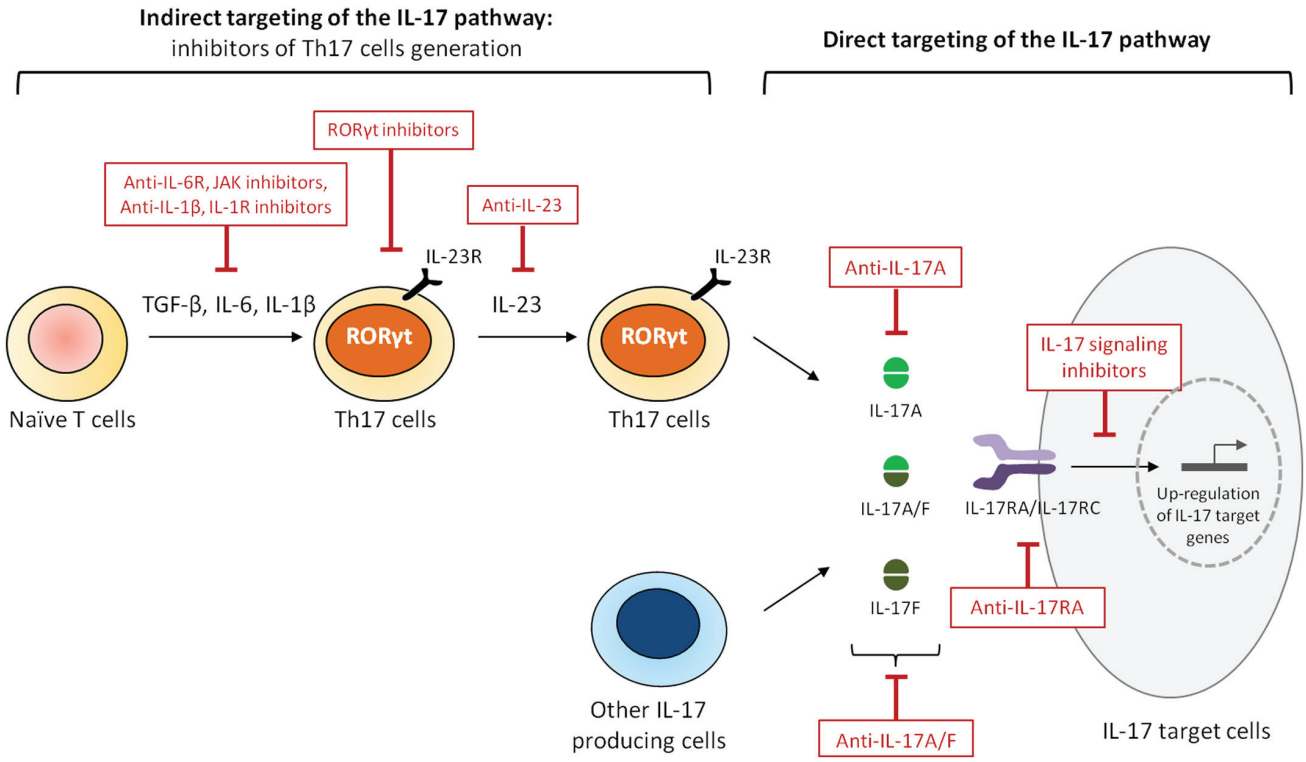

Fig. 4. Therapeutic strategies to target IL-17 pathway and Th17 cell generation.
IL-17 pathway is targeted directly with antibodies against IL-17 and IL-17 receptor or by inhibiting IL-17 signal transduction. Inhibiting Th17 cell generation is another strategy to target indirectly the IL-17 pathway. Inhibitors against the IL-6 pathway (anti-IL-6R or JAK in- hibitors) or IL-1 pathway (IL-1R antagonist or anti-IL-1β) may inhibit the initiation of Th17 cell generation and anti-IL-23 the stabilization of the Th17 cell phenotype. RORγt inhibitors reduce the Th17 cell differentiation. IL-6R, IL-6 receptor; IL-1R, IL-1 receptor; JAK, janus kinase; RORγt, retinoic acid receptor-related orphan nuclear receptor gamma t

Th17 cell phenotype [90]. Different pathway inhibitors of IL-6 (tocilizumab or the anti-JAK tofacitinib), IL-1β (anakinra or canakinumab) and IL-23 (tildrakizumab or guselkumab) are now tested. Targeting the Th17 cell transcription factor RORγt/ RORc can also restore the Treg/ Th17 cell balance and therefore reduces the Th17 cell-related cytokine production. Several small molecules inhibiting RORc are in development [96,97].

5.3. Adverse events and safety

Despite the prominent involvement of IL-17 in inflammatory and autoimmune diseases, IL-17 has also a protective role in different bacterial and fungal infections [17–19,21]. In mouse liver, IL-17 contributes to protection against *Listeria monocytogenes* and *Salmonella enterica* infections [98,99]. Adverse events of the IL-17 inhibitors in humans are as expected bacterial infections and localized Candida infections. Induction and reactivation of Crohn's disease have also been observed, possibly as a result of loss of the protective effect of IL-17 against *Candida albicans* colonization [90,100–102]. The therapeutic benefit/risk balance of the IL-17 inhibitors in liver diseases still needs to be assessed to determine if IL-17 targeting could be a valid treatment option in liver diseases.

6. Conclusion

As IL-17 and IL-17 producing cells rapidly initiate an inflammatory response by the recruitment and activation of neutrophils, they play a key role in host defense but also in liver inflammation and damage in acute liver dieases. At a later stage, they contribute to the recruitment of immune cells and fibrosis in CLDs. However, further investigations are still required on the cellular sources of IL-17 at the early and late phases of the inflammatory response and the role of the other IL-17 family members in liver biology and disease.

Since the role of IL-17 in liver diseases is becoming established, the IL-17 axis appears as an interesting therapeutic target for liver diseases. Various drugs are already on the market. IL-17 blockade may be helpful in very acute situations, where the primary end-point is easy to establish. Obviously, the situation is more complex in CLDs. As always, acting early may prevent the establishment and the progression of acute and chronic liver inflammation and dysfunction.

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3.3 Role of cell interactions in hepatic inflammation and fibrosis

Chronic inflammation is a dynamic process where leukocytes and stromal cells interact together by paracrine and contact-dependent interactions. The soluble factor exchanges and the direct cell-cell contacts may modulate the behavior of hepatocytes and HSCs but also leukocyte recruitment, survival and polarization. Therefore, the interactions between infiltrating leukocytes and stromal cells appear critical in the induction, the maintenance and the resolution of liver inflammation and fibrosis. They determine most likely the outcome of liver injury.

Hepatocytes, HSCs and peripheral blood mononuclear cells (PBMCs) are three key players in the chronically inflamed liver because:

- Hepatocytes are the most abundant cell population in liver and can modulate HSC and immune cell responses by secreting damaged hepatocyte-derived mediators and cytokines/chemokines
- Chronic inflammation leads to HSC activation, proliferation and transdifferentiation into myofibroblasts resulting in excessive deposition of extracellular matrix (ECM) and fibrotic remodeling
- PBMCs infiltrate the liver in chronic inflammatory conditions

On the basis of *in vitro* studies, the roles of the interactions between hepatocytes-HSCs (1), hepatocytes-PBMCs (2) and HSCs-PBMCs (3) in liver homeostasis are discussed in this section.

3.3.1 Hepatocyte - Hepatic stellate cell interactions

By their localization, HSCs in the space of Disse can have a close link with hepatocytes. Co-culture systems were used to study the interactions between HSCs and hepatocytes. Microarray analysis on McA-RH7777 hepatocellular carcinoma cells from rats detected 28,728 genes, 573 of which were up- or down-regulated more than 2-fold when McA-RH7777 were co-cultured with activated HSCs from rats with a cell culture insert avoiding

direct cell contacts (Wang et al., 2014). Among the genes with altered expression levels, 432 were up-regulated and 141 genes were down-regulated. The up-regulated genes included CXCL1, MCP-1 and CXCL10 chemokines. The production of matrix metalloproteinase (MMP)-2, MMP-9, hepatocyte growth factor and IL-6 increased in co-cultures compared to McA-RH7777 cell monocultures. In contrast, the levels of TNFα and TGFβ were no affected by the presence of HSCs (Wang et al., 2014). Genome-wide expression profiling were also performed in the human hepatic HepaRG cell line and the human LX-2 HSCs separated by a culture cell insert (Coulouarn et al., 2012). In HepaRG cells, the expression of 212 genes were modulated by the presence of LX-2 cells with 83% of these genes regulated positively. These up-regulated genes comprised genes related to cell chemotaxis (e.g. IL-8, MCP-1, CCL20 and CXCL2 chemokines), pro-inflammatory cytokines (e.g IL-1β and IL-6) and acute-phase proteins (e.g. SAA). For the LX-2 cells, the co-culture condition altered the expression of 123 genes including the up-regulation of master genes involved in extra cellular matrix remodeling and angiogenesis (Coulouarn et al., 2012).

Concerning the effects of hepatocyte-HSC interactions on the main pro-fibrotic genes, the presence of the Huh7 hepatoma cells decreased the expression of procollagen-α1 and TGFβ whereas the key HSC activator marker α-smooth muscle actin (α-SMA) expression increased in primary human HSCs (Sancho-Bru et al., 2010). Interestingly, LX-2 cell exposure to conditioned medium from FHCC-98 human hepatoma cell cultures promoted the LX-2 cell activation with an increase expression of α-SMA, collagen I and tissue inhibitor of metalloproteinase (TIMP) (Ma et al., 2015). Hepatocellular carcinoma cells can thus secrete soluble mediators, which promote the activation of HSCs and vice versa.

Therefore, a bidirectional paracrine crosstalk exists between hepatocytes and HSCs driving to a pro-inflammatory and pro-fibrotic microenvironment. However, some mediators, such as IL-8, are not regulated in the same way according to the different studies (Coulouarn et al., 2012; Sancho-Bru et al., 2010). This differential effect could be due to the culture conditions such as the culture medium, which can influence the activation of HSCs. The different cell response can also be related to the cellular model used. For example, the mRNA expression of IL-6 is up-regulated in HepaRG cells, down-regulated in Huh7 cells and unchanged in HepG2

cells and HuGB biliary cells in presence of the LX-2 HSCs (Coulouarn et al., 2012). However, all the studies reported an induction of a pro-angiogenic and pro-migratory microenvironment by the hepatocyte-HSC interactions (Coulouarn et al., 2012; Sancho-Bru et al., 2010; Wang et al., 2014). As only non-contact co-cultures are used in these studies, the role of the direct cell-cell contacts remain unknown.

3.3.2 Hepatocyte - Peripheral blood mononuclear cell interactions

In the liver, hepatocytes interact and establish cell-cell contacts with T cells through fenestrations in liver sinusoidal endothelial cells (Warren et al., 2006). Because hepatocytes express major histocompatibility complex (MHC) class I and class II molecules in inflammatory conditions (Franco et al., 1988; Herkel et al., 2003), hepatocytes have the capacity to act as antigen-presenting cells (APC) and therefore to contribute to T cell activation and immune regulation in the liver. Hepatocytes from mice can activate $CD4^+$ and $CD8^+$ T cells *in vitro* through antigen-dependent activation but were not able to sustain this activation and to create an effective immune response (Bertolino et al., 1998; Herkel et al., 2003). In contrast, in co-cultures with peripheral blood lymphocytes (PBLs), the human hepatic cancer cell line HepG2 or immortalized human hepatocytes enhanced T cell proliferation through antigen-independent activation signals. Indeed, antibodies against MHC class II molecules did not abolish T cell proliferation. Moreover, this effect was mainly cell contact dependent and required the presence of accessory cells since the use of culture cell inserts or the use of pure $CD3^+$ T cells instead of PBLs reduced strongly T cell proliferation induced by hepatocytes (Correia et al., 2009). The interaction with HepG2 cells or immortalized human hepatocytes increased also PBL survival. Unlike the hepatocyte-induced T cell proliferation effect, the soluble factors released by hepatocytes were sufficient to decrease T cell death. In addition, the induction of T cell survival by hepatocytes was maintained in $CD3^+$ T cell-HepG2 cell co-cultures (Correia et al., 2009). Therefore, hepatocytes may play an important role in local T cell homeostasis by acting on T cell proliferation and survival.

Hepatocytes also contribute to immune regulation and tolerance in liver. Interactions between $CD4^+$ T cells and hepatocytes from murine liver increased the IL-10 secretion by $CD4^+$ T cells following T cell receptor (TCR) stimulation in a cell-cell contact-dependent manner via Notch signaling. This increase was even more pronounced with hepatocytes from regenerated livers of mice pretreated with the lectin concavanalin A (Con A). The $CD4^+$ T cells primed by hepatocytes were able to suppress proliferation of responder T cells upon TCR stimulation. Interestingly, most of the $CD4^+$ $IL\text{-}10^+$ cells co-express IFNγ but not Foxp3 (Burghardt et al., 2013). However, the same research team shows that murine hepatocytes in the presence of TGFβ promoted Foxp3 expression within $CD4^+$ T cells and the generation of Treg cells with suppressive capacity upon TCR stimulation. This induction was dependent on Notch signaling (Burghardt et al., 2014). By these mechanisms, hepatocytes may play a pivotal role maintaining immunological tolerance in liver.

The immunoregulatory effects of the hepatocyte-PBMC interactions were also studied in the context of hepatocellular carcinoma (HCC) by using primary human hepatocyte and autologous PBMC co-cultures (Doumba et al., 2013). The presence of PBMCs increased the expression of MHC class II molecules on HCC and non-HCC hepatocytes from liver resection specimens of patients operated for HCC. The viability of MHC II-expressing HCC hepatocytes was also increased in co-cultures. However, the MHC II expression on hepatocytes from donors with no liver diseases remained unchanged when hepatocytes were co-cultured with PBMCs. Therefore, hepatocytes from HCC patients have a higher MHC II expression and may act as APC when they are in contact with PBMCs. In addition, co-cultures with HCC but not non-HCC hepatocytes induced the expression of MHC class II on PBMCs. In this way, HCC hepatocytes may modulate PBMC activation whereas PBMC apoptosis and necrosis were not affected by the presence of hepatocytes (Doumba et al., 2013). Concerning the activated $CD8^+$ T cells, the $CD8^+$ T cell death increased in co-cultures with murine or human HCC hepatocytes but not with non-HCC hepatocytes (Bertolino et al., 1998; Doumba et al., 2013). Therefore, PBMC-HCC hepatocyte interactions promote the APC ability of HCC hepatocytes and the PBMC activation. This effect can provide help to immune cells, which are defective in the tumor environment. In contrast, the elevated viability

of MHC II expressing HCC hepatocytes and $CD8^+$ T cell necrosis in PBMC-HCC hepatocyte co-cultures may contribute to HCC survival and escape from immune attack (Doumba et al., 2013). Interactions between hepatoma cells and immune cells thus contribute to the immune response, which is critical for cancer regression or progression.

Because hepatocytes can modulate immune cell activation, proliferation, polarization and survival, as described above, hepatocyte-PBMC interactions play certainly a crucial role in liver tolerogenic effects but also in the induction and the outcome of hepatitis. The effect of hepatocytes on the immune response appears to dependent on the nature of the injury and the pathological or non-pathological environment.

3.3.3 Hepatic stellate cell - Peripheral blood mononuclear cell interactions

HSCs are crucial in liver fibrosis by producing fibrotic mediators leading to extracellular matrix deposition. Alteration of liver homeostasis by the presence of infiltrated immune cells and soluble inflammatory mediators may promote HSC activation and vice-versa. A recent paper shows that oncostatin M, mainly produced by macrophages and neutrophils, had a crucial role in the HSC fibrogenic activity. Indeed, oncostatin M suppressed directly fibrolysis through the up-regulation of TIMP1 expression in HSCs and promoted indirectly fibrogenesis by inducing the release of soluble factors from profibrotic macrophages (Matsuda et al., 2018). Conversely, HSCs have also an immunoregulatory activity; they can modulate the macrophage/monocyte phenotype. In co-cultures, activated primary human HSCs or the human LX-2 cell line can reprogram monocytes to an immunosuppressive phenotype characterized by the up-regulation of immunosuppressive cytokines (IL-10 and TGFβ1) and the down-regulation of pro-inflammatory cytokines (TNFα and IL-1β) (Ji et al., 2015) and MHC class II (Höchst et al., 2013). This effect required direct cell-cell contacts and was mediated via CD44 (Höchst et al., 2013; Ji et al., 2015). As HSCs are activated during chronic inflammation, this monocyte polarization to an immunosuppressive M2 macrophage or myeloid derived suppressor cell phenotype may represent a negative feedback loop to attenuate local inflammation.

HSCs may also regulate the hepatic immune response by influencing $CD4^+$ T cell survival. Without stimulation, HSCs express low MHC class II and T cell co-stimulatory molecules (CD40 and CD80) (Charles et al., 2013; Dangi et al., 2012). In addition, human HSCs induced a very low allogenic T cell proliferative response indicating they are not professional APCs (Charles et al., 2013). However, HSCs reduced significantly the T-cell proliferative response in a dose-dependent manner and induced the apoptosis of activated T cells (Charles et al., 2013; Zhao et al., 2012). Because the separation of HSCs and T cells by a pored membrane inhibited largely the HSC ability to suppress the T cell proliferative response, this inhibitory effect was cell-cell contact dependent. The induction of activated T cells apoptosis was mediated via B7-H1 (programmed death-ligand 1 (PD-L1)) - PD1 ligation in HSC-T cell co-cultures incubated with anti-CD3/CD28 coated beads (Charles et al., 2013) and via Fas-Fas ligation in lipopolysaccharide (LPS)-stimulated HSC-$CD4^+$ T cell co-cultures (Dangi et al., 2012). In addition, tumor-specific HSCs but not quiescent HSCs promoted T-cell hypo-responsiveness and apoptosis by inducing DC-derived immunoglobulin receptor 2 (DIgR2) expression in DCs (Xia et al., 2017).

However, HSCs have differential effects on the $CD4^+$ T cell population, they can induce at the same time $CD4^+$ T cell apoptosis and Treg cell expansion (Dangi et al., 2012). Indeed, incubation of T cells with HSCs increased the number of Treg cells (Li et al., 2017; Zhao et al., 2012) and this effect was enhanced when HSCs were pretreated with LPS (Dangi et al., 2012; Kumar et al., 2017). HSC-expanded Treg cells retained a suppressive phenotype and function. Separation of HSCs from Treg cells by the use of a permeable culture inserts prevented Treg cell expansion showing that a direct cell-cell contact is required. Because LPS increased MHC class II and CD80 expression and the MHC class II blockade reduced the HSC-induced proliferation of Treg cells, the MHC class II-TCR signaling has certainly a predominant role in this effect (Dangi et al., 2012). In addition, LPS increased also the activity of the immunoregulatory enzyme indoleamine-pyrrole 2,3-dioxygenase (IDO1) in HSCs, which is associated not only to Treg expansion but also with a strong increase of Foxp3 expression (Kumar et al., 2017). In turn, Treg cells reduced the release of pro-inflammatory cytokines (IL-6, TNFα and IL-1β) by HSCs whereas the production of the anti-

inflammatory cytokine IL-10 was increased by the presence of Treg cells in LPS-stimulated HSC cultures (Dangi et al., 2012). By producing IL-8 and TGFβ1, Treg cells may also down-regulate pivotal ligands for activating NK cell receptors on HSCs and therefore reduce the anti-fibrotic activity of NK cells (Langhans et al., 2015).

The effect of HSCs on T cell response was also investigated in other $CD4^+$ T cell subtypes. Interactions between activated HSCs from rats and rat $CD4^+$ T cells reduced the Th1/Th2 ratio *in vitro* by promoting apoptosis and by inhibiting IFNγ production in Th1 cells whereas the Th2 cell response was enhanced. By inhibiting the Th1 cell response, activated HSCs may facilitate the shift from a Th1 to a Th17 cell response and promote liver fibrosis (Xing et al., 2015). Recently, the effects of Th17 cell and HSC interactions were studied in co-cultures with $CD4^+$ T cells from patients with chronic hepatitis B (CHB). The interactions between $CD4^+$ T cells from CHB patients and activated HSCs enhanced the proliferation of $CD4^+$ T cells, the frequency of Th17 cells and the secretion of the Th17 cell-related cytokines including IL-17A, IL-21 and IL-22 (Liu et al., 2017). Another study showed that the exposure of $CD4^+$ T cells from CHB patients to LX-2 cell and primary HSC supernatants increased the Th17 cell frequency (Li et al., 2017). Soluble factor exchanges between HSCs and $CD4^+$ T cells appear therefore sufficient to induce the polarization of T cells toward Th17 cells.

In turn, Th17 cells can modulate HSC activation. Murine HSCs in co-cultures with Th17 cells enhanced the expression of α-SMA in proportion to the amount of Th17 cells whereas Treg cells had opposite effects (Sun et al., 2014). IL-17-activated PBLs or monocytes up-regulated also α-SMA expression in LX-2 cells *in vitro* (Sun et al., 2012). In addition, the human primary HSC and Th17 cell interactions increased the secretion of TGFβ1 as well as the release of IL-6 and IL-1β when Th17 cells were purified from patients with CHB. Gene expressions of Collagen-III, MMP2, TIMP1 and the pro-fibrotic cytokines TGFβ1, CTGF, EGF and PEGF-BB were also up regulated in HSC-Th17 cell co-cultures compared to HSC monocultures. Interestingly, these effects on HSC activation mediated by Th17 cells were completely reversed by the addition of an anti-IL-17A (Liu et al., 2017). The induction of HSC activation by Th17 cells is therefore mainly mediated by IL-17. Moreover, IL-17 participated to the increased secretion of growth related oncogen α, involved in neutrophil

infiltration, when human HSCs were cultured with phytohemagglutinin (PHA)-stimulated PBMC supernatant (Lemmers et al., 2009). Blocking IL-17R in human HSC and mucosal-associated invariant T cell co-cultures decreased also the pro-inflammatory IL-1β, IL-8 and CCL2 gene expressions (Böttcher et al., 2018) showing that IL-17 participates to HSC activation and HSC-mediated immune cell infiltration.

When activated HSCs were co-cultured with Th17 cells from CHB patients, HSCs induced the generation of a highly pathogenic Th17 phenotype with the up-regulation of most Th17 cell key gene signatures (IL-17A, IL-23R, CCL20, CCR6 and RORc). Neutralization of IL-6 and/or IL-1β inhibited significantly the frequency of IL-17A-producing $CD4^+$ T cells and the secretion of IL-17 in activated T $CD4^+$ from CHB patients-HSC co-cultures separated by a permeable membrane (Liu et al., 2017). The IL-17 production and the frequency of IL-17-producing $CD4^+$ T cells were also reduced by the use of a selective COX-2 (cyclooxygenase-2) inhibitor in the co-culture system. Consequently, the COX-2 inhibition decreased also the release of cytokines associated with Th17 cells (IL-17A, IL-21) and HSCs (IL-6, IL-1β and TGF-β1) as well, in activated Th17 cells from CHB patients–HSC co-cultures (Liu et al., 2017). In contrast, PGE2 (prostaglandin E2) or agonist of PGE2 receptors-EP2 and EP4 enhanced the frequency of Th17 cells in $CD4^+$ T cells (Li et al., 2017). The activation of COX-2/PGE2 pathway and the PGE2 secretion by HSCs may thus act on PGE2 receptors on $CD4^+$ T cells and promote the differentiation of hepatic Th17 cells. Therefore, a pro-inflammatory and pro-fibrotic feedback loop between Th17 cells and HSCs may exist in CHB patients (Figure 4).

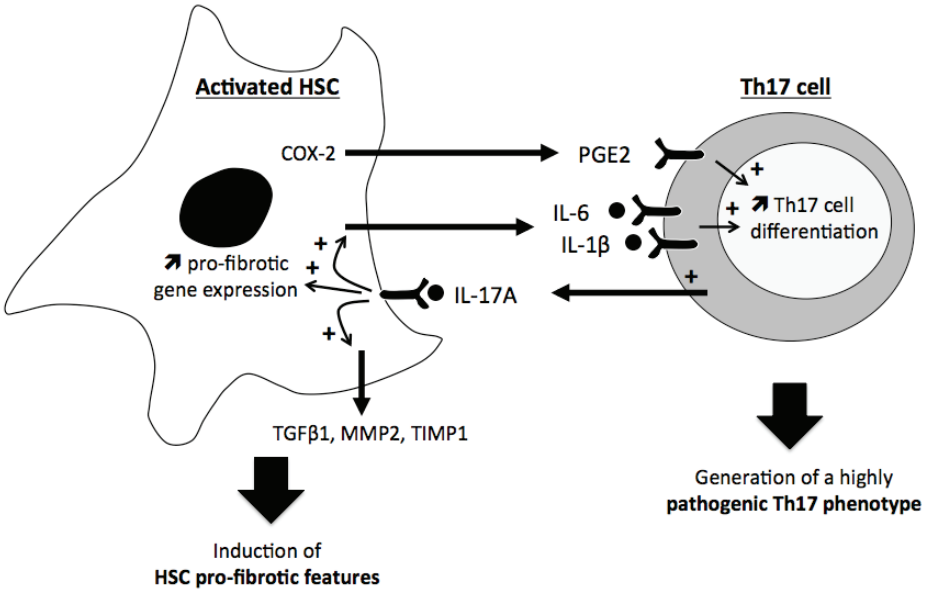

FIGURE 4: Pro-inflammatory and pro-fibrotic bidirectional crosstalk between HSCs-Th17 cells in chronic hepatitis B.

Activated HSCs enhance Th17 cell differentiation and IL-17 secretion via COX-2/PGE2, IL-6 and IL-1β pathways. In turn, IL-17 promotes the activation of HSCs by inducing the expression of pro-fibrotic genes (α-SMA, Collagen-III, TGFβ1, CTGF, EGF and PEGF-BB) and the secretion of IL-6, IL-1β, TGFβ1, MMP2 and TIMP1.

The immunoregulatory role of activated HSCs on immune cells is complex and appears to depend on the *in vitro* cell model used and the cell stimulation. In chronically inflamed liver, HSCs are activated and trans-differentiate into pro-fibrogenic myofibroblasts. By inducing immunosuppressive functions of Treg cells, monocytes and DCs, activated HSCs can exert regulatory immune functions (Dangi et al., 2012; Höchst et al., 2013; Ji et al., 2015; Kumar et al., 2017; Xia et al., 2017; Zhao et al., 2012). This may prevent excessive liver injury but also facilitates immunologic escape of cancer cells. However, the role of the HSC immunoregulatory effects in the progression of fibrosis remains controversial. Treg cells may alter the control of hepatic fibrogenic activity (Langhans et al., 2015). TGFβ produced by Treg cells or M2 macrophage may worsen fibrosis by activating HSCs. In contrast, the release of IL-10 can inhibit HSC activation directly but also indirectly by reducing the effector function of other intrahepatic T cells (Liu et al., 2017). Further studies are necessary to evaluate the role of Treg cells or M2 macrophage phenotype in the liver microenvironment.

In hepatitis B virus (HBV)-related liver fibrosis, the frequency of IL-17-producing cells in liver is elevated in patients with in advanced stage of fibrosis (Li et al., 2017). HBV infection induces certainly a Th17-polarizing liver environment (Liu et al., 2017). In turn, the interplay between Th17 cells and HSCs appears critical to sustain a profibrotic loop and chronic inflammation. Because IL-17 and IL-17 producing cells seem to be involved in the pathology of other liver diseases including autoimmune hepatitis or alcoholic liver diseases (as seen in part 3.2), the pro-inflammatory and profibrotic feedback loop described during HBV infection could be found in other liver disorders.

4 IL-17 in myositis

4.1 Idiopathic inflammatory myopathies

The idiopathic inflammatory myopathies (IIMs), also called myositis, are a group of chronic muscle diseases comprising polymyositis (PM), dermatomyositis (DM) and sporadic inclusion body myositis (IBM). The clinical features of these diseases include symmetrical skeletal muscle weakness, fatigue and elevated muscle enzymes in serum. Histologically, muscle tissues of IIM patients are characterized by mononuclear cell infiltration and myofiber degeneration (Dalakas and Hohlfeld, 2003). The presence of autoreactive lymphocytes and autoantibodies suggests that the autoimmune response has a significant contribution to the disease pathogenesis. For this reason, the IIMs are treated with either glucocorticoids or other immunosuppressive drugs. However, the innate immune activation and the non-immune intrinsic defects in IIMs also contribute certainly to muscle damage and dysfunction (Rayavarapu et al., 2013). Therefore, IIM pathogenesis comprises complex pathways and their relative contributions and inter-relationships are still unclear. Because a wide range of cells produced cytokines and, in turn, respond to them, they may have a central role in the interplays between immune and non-immune mechanisms in IIM pathogenesis by acting on both immune cells and muscle cells (Figure 5).

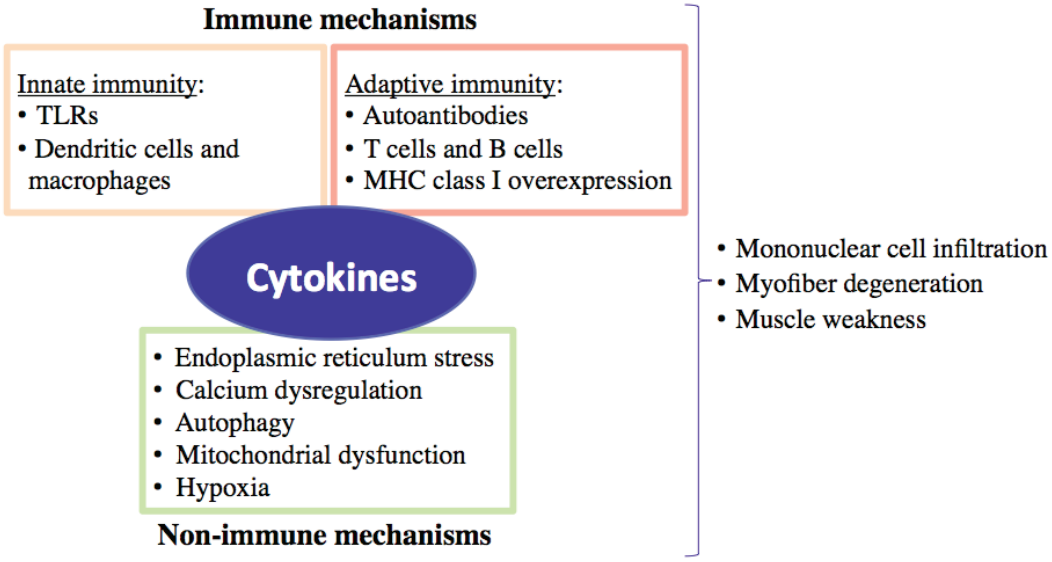

FIGURE 5: Potential central role of cytokines in the relationship between immune and non-immune pathological mechanisms in idiopathic inflammatory myopathies

4.1.1 Innate immune mechanisms

Emerging evidence indicates that the innate immune response is involved in IIMs, as suggested the presence of macrophages and DCs and the overexpression of Toll-like receptors (TLRs) in muscle tissues of myositis patients (Page et al., 2004; Rayavarapu et al., 2013). Indeed, increased expression of TLR-2, TLR-3, TLR-4, TLR-7 and TLR-9 are found in IIM muscular biopsies and TLR pathways participate to the induction of pro-inflammatory cytokines leading to an inflammatory environment in affected muscles (Brunn et al., 2012; Kim et al., 2010; Schreiner et al., 2006; Tournadre et al., 2010).

4.1.2 Adaptive immune mechanisms

The over-expression of MHC class I molecules on the surface of muscle cells, the presence of autoreactive T lymphocytes and myositis-specific autoantibodies used for diagnosing support an important contribution of the adaptive immune response in IIMs. Both immature and mature DCs have been found in muscle biopsies of IIM patients (Page et al., 2004). By priming and activating T cells, DCs are a crucial link between the innate and adaptive immune systems and the amplification of the inflammatory process in IIMs (Coutant and Miossec, 2016). DM is considered as a $CD4^+$ T cells-mediated disease in which the dysregulation of the humoral mechanisms and the activation of the complement are responsible for the vascular endothelium attack leading to skin and muscle injury. In contrast, in PM and IBM, $CD8^+$ T cells are predominant and induce a cytotoxic attack resulting in myofiber necrosis (Dalakas and Hohlfeld, 2003; Rayavarapu et al., 2013).

4.1.3 Non-immune mechanisms

Recent data in IIMs suggests that both immune and non-immune processes contribute to muscle weakness and damage in myositis. The endoplasmic reticulum (ER) stress, the metabolic disturbances, the autophagy and the hypoxic conditions have been observed in skeletal muscles of IIM patients (Coley et al., 2012; Henriques-Pons and Nagaraju, 2009; Rayavarapu et al., 2013). Because the ER is intimately connected to other cellular components and performs crucial tasks including calcium (Ca^{2+}) release, posttranslational

maturation, protein folding or lipid biosynthesis, the ER can modulate many cellular functions. In myositis, uncontrolled ER stress leads to a cross-talk with mitochondria and the formation of autophagosomes leading to the activation of cell death pathways (Rayavarapu et al., 2012). The ER stress may also affect muscle function by modulating Ca^{2+} regulation. The sarcoplasmic reticulum (SR) is a specialized form of ER found in muscle cells. SR is a Ca^{2+} storage depot and regulates Ca^{2+} release during muscle contraction. The ER and SR can rapidly refill Ca^{2+} store by inducing Ca^{2+} entry from the extracellular environment via the Ca^{2+} sensors stromal interaction molecule (STIM1) and the Ca^{2+} channel Orai (Henriques-Pons and Nagaraju, 2009). Abnormalities of this Ca^{2+} pathway, known as store-operated Ca^{2+} entry (SOCE), can impair Ca^{2+} homeostasis and therefore the regulation of muscle contraction leading to skeletal myopathies (Stiber et al., 2008).

Interestingly, the immune pathways interplay with the non-immune mechanisms in IIMs. For instance, the pro-inflammatory cytokines IL-1α and IL-1β can cause mitochondrial dysfunction. Moreover, overexpression of the MHC class I molecule in skeletal muscle can induce ER stress (Rayavarapu et al., 2012, 2013). Conversely, ER stress can activate the master regulator of inflammation NF-κB leading to the transcription of pro-inflammatory cytokines and chemokines (Henriques-Pons and Nagaraju, 2009).

4.2 Role of IL-17 in inflamed muscle tissues

Traditionally, the Th1 cell response is considered as the predominant driver of IIM immunopathogenesis (Moran and Mastaglia, 2014). However, the presence of IL-17-producing cells in inflamed muscle tissues and the *in vitro* effects of IL-17 on muscle cells suggest a pathogenic contribution of the IL-17 pathway in IIMs.

4.2.1 Expression of IL-17 in myositis

Several studies have investigated the presence of IL-17 in muscle biopsies and blood in patients with IIMs. In muscle tissue, the expression of IL-23p19 and IL-17 mRNA was increased in myositis muscles and the elevated IL-17 gene expression was associated with increased mRNA levels of TLR-4 and TLR-9 (Brunn et al., 2012; Kim et al., 2010; Kondo et

al., 2009). This suggests that activation of TLRs is connected to the Th17 cell response and the increase of IL-17 expression in IIMs. In addition, IL-17-producing cells were detected in lymphocyte infiltrates of both DM and PM muscle biopsies in contrast to healthy muscle biopsies (Chevrel et al., 2003; Page et al., 2004; Tournadre et al., 2009). However, the number of IL-17-producing cells was low compared to IFNγ-positive cells (Tournadre et al., 2009).

Overexpression of IL-17 transcripts was also found in the whole blood of DM and PM patients. Interestingly, a subset of DM patients had an enhanced IL-17 gene signature rather than a type I IFN gene signature (Higgs et al., 2012). One study reports an increased of IL-17 serum level in DM and PM patients (Szodoray et al., 2010) whereas another study detected similar levels between the patients with IIMs and healthy controls (Allenbach et al., 2014). The frequency of $CD3^+$ $CD4^+$ $IL\text{-}17^+$ cells was not higher in IBM patients than healthy controls (Allenbach et al., 2014). In contrast, the frequency of $CD3^+$ $CD4^+$ $IL\text{-}17^+$ cells and the serum levels of cytokines involved in the Th17 differentiation increased in DM patients. The serum level of the muscle enzyme creatine kinase, an indicator of the severity of muscle severity, correlates also positively with the frequency of Th17 cells (Tang et al., 2013). Additionally, upon anti-CD3/CD28 stimulation, PBMCs from patients with early DM and PM produced higher levels of IL-17 than PBMCs from healthy controls or patients with established DM and PM diseases (Shen et al., 2011).

4.2.2 Effects of IL-17 on muscle cells

A limited number of studies have explored the direct effects of IL-17 on muscle cells. First, IL-17 could be involved in muscle destruction by inhibiting myogenic differentiation *in vitro* with the reduction of myoblast migration through the activation of ERK pathway and the inhibition of urokinase type plasminogen activator expression (Kocić et al., 2012, 2013). IL-17 has also pro-inflammatory activities on human myoblasts by increasing the production of IL-6 in a dose dependent manner. In addition, IL-17 can amplify the effect of IL-1β on the IL-6 and CCL20 secretion in myoblast cultures and muscle tissue samples. IL-17 promotes also the expression of HLA class I with a higher effect when IL-17 and IL-1β were combined

(Chevrel et al., 2003). IL-17, but not IFNγ, can further act in synergy with the activation of the TLR-3 pathway for the production of IL-6 and CCL20 (Tournadre et al., 2010). By amplifying the release of IL-6 and CCL20 from muscle cells, IL-17 can promote mononuclear cell migration and differentiation in muscle tissue. Indeed, IL-6 is a key cytokine in the Th17 differentiation and CCL20 is involved in the recruitment of immature DCs as well as Th17 cells. The cytokine microenvironment comprising IL-17 and cell-cell interactions contributes therefore to DC homing and maturation. In turn, DCs can interact with T cells to amplify the inflammatory process in IIMs (Page et al., 2004). IL-17 within a complex network of interactive cytokines may thus contribute to the pathogenesis of various inflammatory diseases including the IIMs.

RESULTS

IL-17 is a major systemic pro-inflammatory cytokine playing an important role in many autoimmune and inflammatory disorders. IL-17 can cooperate with other cytokines such as TNFα to amplify their pro-inflammatory effects. However, the role of these two cytokines remains poorly understood in the liver and the muscle, which can be affected in some systemic inflammatory disorders. In addition, as the tissue inflammation is characterized by immune cell infiltration, the local cell-cell interactions play certainly an important role in the induction and the outcome of the inflammatory response. To determine the effects of the IL-17 and TNFα combination and the contribution of the cell-cell interactions in the inflammatory response, *in vitro* culture systems with human cells from liver and muscles are used.

The objectives of this thesis project are:

1) To investigate the effects of IL-17 and TNFα on the inflammatory response in hepatocytes
2) To assess the role of the PBMC-hepatocyte interactions on the immune response by using a co-culture system
3) To determine the effects of IL-17 and TNFα on HSCs and the role of the interactions between HSCs, hepatocytes and/or PBMCs on the inflammatory and fibrotic processes
4) To establish the role of IL-17 and TNFα in immune and non-immune processes in myoblasts

1 Synergistic effect of IL-17 and TNFα on inflammatory response in hepatocytes through IL-6-dependent and independent pathways

Beringer A, Thiam N, Molle J, Bartosch B and Miossec P. Synergistic effect of interleukin-17 and tumor necrosis factor-α on inflammatory response in hepatocytes through interleukin-6-dependent and independent pathways. *Clin Exp Immunol.* 2018;193(2):221-233.

1.1 Background & Objective

The liver plays an important role in the acute-phase response by producing acute-phase proteins such as CRP or SAA. IL-6 is the master regulator of the acute-phase protein production. However, other cytokines such as TNFα, IL-1β or IL-17 are also involved in the acute-phase protein response and the production of IL-6 itself (Kramer et al., 2008; Patel et al., 2007; Yoshizaki, 2011). IL-17 and TNFα cooperate to induce in synergy the secretion of IL-6 and IL-8 in various cell types including synovial or skin fibroblasts (Chiricozzi et al., 2011; Hot et al., 2012; Katz et al., 2001; Osta et al., 2015). However, the effects of the IL-17 and TNFα combination in the hepatic inflammatory response need to be clarified. Primary human hepatocytes (PHH) and three human hepatoma cell lines (HepaRG, HepG2 and Huh7.5 cells) were used in this study.

Objective: To investigate the role of IL-17 and TNFα in the hepatic inflammatory response and the contribution of IL-6 in the effects triggered by IL-17 and TNFα.

1.2 Results

The IL-17 and TNFα combination increases in synergy the expression and production of IL-6 in PHH and HepaRG cell cultures. This effect is not observed in the HepG2 and Huh7.5 cell lines.

IL-17 and/or TNFα stimulation indirectly enhances CRP and aspartate aminotransferase (ASAT) level through the induction of IL-6. Indeed, the IL-6 pathway blockade reduces strongly the IL-17 and/or TNFα effect on the CRP and ASAT levels in PHH and HepaRG cell cultures.

The IL-17 and TNFα combination increases in synergy the expression and/or production of IL-8, MCP-1 and CCL20 chemokines independently of the IL-6 pathway. As the use of the anti-IL-6R does not inhibit the induction of these chemokines following the IL-17/TNFα exposure, the IL-17 and TNFα effect on IL-8, MCP-1 and CCL20 is mainly independent of the IL-6 pathway.

IL-17 initiates the IL-17 and TNFα synergistic effect on the IL-6 and IL-8 production. Indeed, pre-incubation first with IL-17 followed by the addition of TNFα induces two-fold higher IL-6 and IL-8 release than the pre-incubation first with TNFα, then with IL-17.

IL-17 enhances IL-6 mRNA stabilization. This effect increases the IL-6 transcript abundance and, in turn, probably the levels of IL-6 secreted. This could participate in the IL-17 and TNFα synergistic effect on IL-6 in hepatocytes.

IL-17 and TNF-α increase the hepatic IL-6 and IL-8 secretion in synergy via the activation of ERK and/or PI3K/Akt signaling pathways and/or NF-κB transcription factor. ERK inhibition reduces both IL-6 and IL-8 production induced by IL-17 and TNFα whereas the activation of PI3K/Akt and NF-κB pathways are only involved in the synthesis of IL-6 but not IL-8.

1.3 Conclusion

IL-17 and TNFα cooperate to increase systemic inflammation and hepatic damage by inducing CRP and ASAT levels in hepatocyte cultures through the induction of IL-6. Independently of the IL-6 pathway, IL-17 and TNFα induce in synergy the expression and/or production of IL-8, MCP-1 and CCL20 chemokines. Blocking IL-17 and/or TNFα could be a promising therapeutic strategy to control systemic inflammation but also the local cell recruitment and associated liver cell injury.

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Synergistic effect of interleukin-17 and tumour necrosis factor-α on inflammatory response in hepatocytes through interleukin-6-dependent and independent pathways

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Summary

The proinflammatory cytokines interleukin (IL)-17 and tumour necrosis factor (TNF)-α are targets for treatment in many chronic inflammatory diseases. Here, we examined their role in liver inflammatory response compared to that of IL-6. Human hepatoma cells (HepaRG, Huh7.5 and HepG2 cells) and primary human hepatocytes (PHH) were cultured with IL-6, IL-17 and/or TNF-α. To determine the contribution of the IL-6 pathway in the IL-17/TNF-α-mediated effect, an anti-IL-6 receptor antibody was used. IL-17 and TNF-α increased in synergy IL-6 secretion by HepaRG cells and PHH but not by Huh7.5 and HepG2 cells. This IL-17/TNF-α synergistic cooperation enhanced the levels of C-reactive protein (CRP) and aspartate aminotransferase (ASAT) in HepaRG cell and PHH cultures through the induction of IL-6. IL-17/TNF-α also up-regulated IL-8, monocyte chemoattractant protein (MCP)-1 and chemokine (C-C motif) ligand 20 (CCL20) chemokines in synergy through an IL-6-independent pathway. Interestingly, first exposure to IL-17, but not to TNF-α, was crucial for the initiation of the IL-17/TNF-α synergistic effect on IL-6 and IL-8 production. In HepaRG cells, IL-17 enhanced IL-6 mRNA stability resulting in increased IL-6 protein levels. The IL-17A/TNF-α synergistic effect on IL-6 and IL-8 induction was mediated through the activation of extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase, nuclear factor-κB and/or protein kinase B (Akt)–phosphatidylinositol 3-kinase signalling pathways. Therefore, the IL-17/TNF-α synergistic interaction mediates systemic inflammation and cell damage in hepatocytes mainly through IL-6 for CRP and ASAT induction. Independently of IL-6, the IL-17A/TNF-α combination may also induce immune cell recruitment by chemokine up-regulation. IL-17 and/or TNF-α neutralization can be a promising therapeutic strategy to control both systemic inflammation and liver cell attraction.

Keywords: hepatocyte, inflammation, interleukin-6, interleukin-17, tumour necrosis factor-α

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Introduction

Interleukin (IL)-6 is a systemic proinflammatory cytokine playing a pivotal role in the acute-phase response to tissue injury, infection or inflammation [1,2]. This response is characterized by changes in the hepatic production of acute-phase proteins such as increased C-reactive protein (CRP) production. However, the acute-phase protein response and the production of IL-6 itself are also induced by a long list of cytokines, such as tumour necrosis factor-α (TNF-α), IL-1β and IL-17A, also known as IL-17 [3,4].

Clinical inhibition of IL-6 with an anti-IL-6 receptor (anti-IL-6R) antibody has shown beneficial effects on the joint manifestations of rheumatoid arthritis, with a massive decrease of CRP levels [5–7]. However, IL-6 also has anti-inflammatory functions [8]. Indeed, IL-6-deficient mice developed liver inflammation, steatosis and insulin

resistance [9]. Moreover, IL-6 blockade has been associated with colon perforation [7,10], transaminase elevation [11] and adverse lipid changes [12,13], probably related to liver changes. Therefore, the IL-6 effects on hepatocytes remain not well known and some of the liver changes in chronic inflammation may reflect the effects of other cytokines, independently or not of IL-6.

Liver diseases are characterized by immune cell infiltrates following chemokine release [14]. Neutrophils are key players in the initiation of the inflammatory response. Because IL-17 is known to induce neutrophil-attracting chemokines such as IL-8, IL-17 contributes to liver inflammation by inducing the local recruitment of neutrophils [15]. As shown in various cells, including endothelial cells or skin and synovial fibroblasts, IL-17 can cooperate with TNF-α to induce *in-vitro* IL-8 and IL-6 secretion in synergy [16–19]. These two cytokines are also involved in several liver disorders [15,20–22]. In the liver, IL-17 was also able to activate hepatic stellate cells and CRP production by hepatocytes independently of IL-6 [3,23].

The objective of this study was to clarify the effects of IL-17 and TNF-α on the induction of the inflammatory response in hepatocytes and to determine the contribution of IL-6 in these effects. Because primary human hepatocytes (PHH) are from native liver, they are considered to be the gold standard approach to reflect the specific functionality and mediators of the human organ. Therefore, PHH and human hepatoma cell lines were used. IL-17 and TNF-α cooperated to increase CRP expression and aspartate aminotransferase (ASAT) level in hepatocyte cultures through the activation of the IL-6 pathway. Independently of the IL-6 pathway, IL-17 and TNF-α induced IL-8, monocyte chemoattractant protein-1 (MCP-1) and chemokine (C-C motif) ligand 20 (CCL20) expression and/or production synergistically. These differences may help understanding of the liver situation in chronic inflammation.

Materials and methods

Cell cultures

The human hepatoma Huh7.5, HepG2 and HepaRG cells were cultured as described previously [24,25]. Proliferative HepaRG cells were used after 15 days post-plating and differentiated HepaRG cells were maintained in the same standard medium supplemented by dimethylsulphoxide (DMSO) 2% for 2 more weeks. PHH were isolated from surgical liver resections and cultured as reported [24]. The samples were collected according to the local ethical committee and the Ministry of Research, which approved the study (reference number: AC-2010-1164).

Culture conditions

Hepatocytes were exposed to IL-6 5 ng/ml (R&D Systems, Minneapolis, MN, USA) or IL-17A 50 ng/ml (Dendritics, Lyon, France) and/or TNF-α 1 ng/ml (R&D systems). To block the IL-6, IL-17 or TNF-α pathways, tocilizumab (Roche, Welwyn, UK), anti-IL-17A (R&D Systems) and infliximab (MSD, Courbevoie, France) were used at 10 μg/ml. A monoclonal antibody against the BetV1 allergen (Dendritics) was used as a control antibody at the same concentration. Exposures to nuclear factor-kappaB (NF-κB) inhibitor pyrrolidine dithiocarbamate, phosphoinositide 3-kinase (PI3K) inhibitor LY294002, protein kinase B (Akt) inhibitor A6730 (all from Sigma, St Louis, MO, USA) and mitogen-activated protein kinase (MAPK) inhibitors SP6000125 [c-Jun N-terminal kinase (JNK) inhibitor], SB203580 (p38 inhibitor), U0125 [mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) inhibitor] (all from Calbiochem, San Diego, CA, USA) at 1, 10, 20 and/or 100 μM were added 1 h prior to cytokine addition. Cells were treated for 12 and 24 h for mRNA expression, 24 h for cytokine production and 120 h for CRP and transaminase levels.

mRNA stability

HepaRG cells were treated with IL-17 and/or TNF-α for 12 h. Cells were then washed and incubated with 5 μg/ml actinomycin D (Orphan Europe, Puteaux, France) to inhibit further transcription. Total mRNA was extracted following 0, 1, 2 and 3 h incubation with actinomycin D. Results were presented as % mRNA remaining compared with the steady-state level.

Quantitative real time PCR

Total RNA was purified using an RNeasy® Plus Mini kit (Quiagen, Hilden, Germany). cDNA was synthesized using the iScript™ kit (Bio-Rad, Hercules, CA, USA). Polymerase chain reaction (PCR) amplification was performed using the CFX96™ real-time system instrument (Bio-Rad) with the iTaq™ universal SYBR® green supermix (Bio-Rad) and the Qiagen QuantiTect® primers. Expression of the genes of interest was normalized to the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene.

Enzyme-linked immunosorbent assays (ELISA)

Supernatant IL-6 and IL-8 concentrations were quantified with human ELISA kits, according to the manufacturer's instructions (R&D Systems).

Western blotting

Proliferative HepaRG cells were exposed to high concentrations of TNF-α alone (10 ng/ml) or a combination of IL-17 (50 ng/ml) and TNF-α (1 ng/ml) for 30 min. Cells were lysed using the Halt™ protease and phosphatase inhibitor cocktail kit (Thermo Scientific, Rockford, IL, USA) and the protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Equal

amounts of protein (50 μg) were loaded and separated by 15% sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred on nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% non-fat milk or 5% bovine serum albumin (BSA). Blots were incubated overnight at 4°C with specific antibodies against total and phosphorylated ERK1/2, Akt and NF-κB inhibitor α (IκBα) (Cell Signaling Technology, Leiden, the Netherlands). Blots were washed three times and incubated with a secondary antibody for 1 h at room temperature. Protein bands were detected with an enhanced chemiluminescence (ECL) detection kit (Bio-Rad). Blots were scanned and analysed using the Gel Doc™ XR+ Gel system (Bio-Rad) and Image Lag 5.2 software. Cyclophilin B quantification was used as loading controls. Results are shown as the ratio of the total and phosphorylated forms.

Laboratory automated analyser

The CRP level was quantified by the automated analyser BN ProSpec® (Siemems, Erlangen, Germany) and transaminases by the Architect analyser (Abbot Diagnostics, Chicago, IL, USA).

Statistical analysis

Calculations were performed with GraphPad Prism version 5.01 software. Data are the mean of at least three independent experiments ± standard error of the mean (s.e.m.). Statistical differences were analysed using the Wilcoxon paired *t*-test. *P*-values less than 0·05 were considered significant.

Results

IL-17 and TNF-α combination increases IL-6 expression and production synergistically

IL-17 and TNF-α have been shown previously to induce a massive IL-6 production by various cell types in synergy [16,17]. Here, the IL-17/TNF-α effect on IL-6 was studied in hepatic cell lines and PHH. In HepaRG cells and PHH, the IL-17/TNF-α combination exposure increased the IL-6 secretion synergistically compared to IL-17 or TNF-α alone. However, the IL-6 production by Huh 7.5 and HepG2 cell lines was not affected by IL-17/TNF-α exposure (Fig. 1a). Based on these results, the HepaRG cell line was used for the following experiments. The IL-17/TNF-α synergistic effect was also observed on IL-6 mRNA in proliferative HepaRG cells, which was up-regulated by up to 44-fold (Fig. 1b).

Up-regulation of IL-17 and TNF-α receptors may contribute to this IL-17/TNF-α synergistic effect. IL-17 binds the heterodimer receptor complex, composed of the IL-17 receptor subunit A (IL-17RA) and the IL-17RC, whereas TNF-α acts through two independent receptors, the TNF receptor I (TNF-RI) and the TNF-RII. Only the TNF-RII was up-regulated in HepaRG cells following IL-17/TNF-α stimulation (Fig. 1c and data not shown). IL-17R and TNF-R expressions were also compared between the HepG2 and Huh7.5 cells, which did not produce IL-6 after the IL-17/TNF-α stimulation, and the proliferative HepaRG cells, which released IL-6 following the IL-17/TNF-α stimulation (Fig. 1a). The expression levels of TNF-RI, IL-17RA and IL-17RC were similar between the HepG2 and the HepaRG cell lines (data not shown). However, the TNF-RII mRNA levels were much lower in HepG2 (Fig. 1c) and Huh7.5 (data not shown) cell lines than in HepaRG cells. Therefore, TNF-RII may have a crucial role in the IL-6 production by IL-17/TNF-α.

To determine whether IL-6 may regulate its own mRNA, proliferative HepaRG cells were treated with different IL-6 concentrations and in the presence or not of an anti-IL-6R antibody. The specific effect of the anti-IL-6R antibody was verified with a control antibody which had no effect on the IL-6 mRNA and protein levels induced by IL-6 or IL-17/TNF-α (Fig. 1e and data not shown). Increasing IL-6 concentrations up-regulated IL-6 expression dose-dependently (Fig. 1d) and the IL-6R blockade inhibited this effect in proliferative HepaRG cells (Fig 1f), indicating that IL-6 regulated its own mRNA expression positively and directly. However, the anti-IL-6R did not reduce the IL-6 up-regulation-induced IL-17/TNF-α significantly (Fig. 1f). The contribution of the IL-6-positive feedback loop in the IL-6 induction by IL-17/TNF-α was therefore very weak compared to the IL-17/TNF-α direct effect on IL-6. The IL-6 supernatant levels in hepatocyte cultures stimulated with IL-6 or IL-17/TNF-α were similar or slightly higher in the presence of the anti-IL-6R antibody (Fig. 1g). Because the anti-IL-6R blocks competitively the IL-6 binding to its receptor, the IL-6 free fraction level in supernatant increased in the presence of the anti-IL-6R antibody. This increase can be balanced by the IL-6 positive-feedback loop effect occurring in the absence of anti-IL-6R antibody.

Induction of the IL-6-dependent CRP and ASAT level following IL-17 and/or TNF-α stimulation

IL-6 was shown to control CRP production [2,4]. Here, CRP expression was up-regulated significantly by IL-6 (19-fold), IL-17 (6-fold) and the synergistic IL-17/TNF-α combination (37-fold) *versus* control in proliferative HepaRG cells. The IL-6 pathway blockade reduced strongly the CRP mRNA level induced by IL-17 and/or TNF-α (Fig. 2a,b). Moreover, CRP production by PHH from different donors following IL-17/TNF-α stimulation correlated strongly with the PHH ability to produce IL-6 (Fig. 2c,d). Therefore, CRP up-regulation by IL-17 and/or TNF-α was mainly IL-6-dependent.

Liver inflammation may induce liver damage, as reflected by transaminase activity elevation in the clinic. In HepaRG cells and PHH cultures, ASAT levels increased after 5 days of IL-6 and IL-17/TNF-α stimulation. These

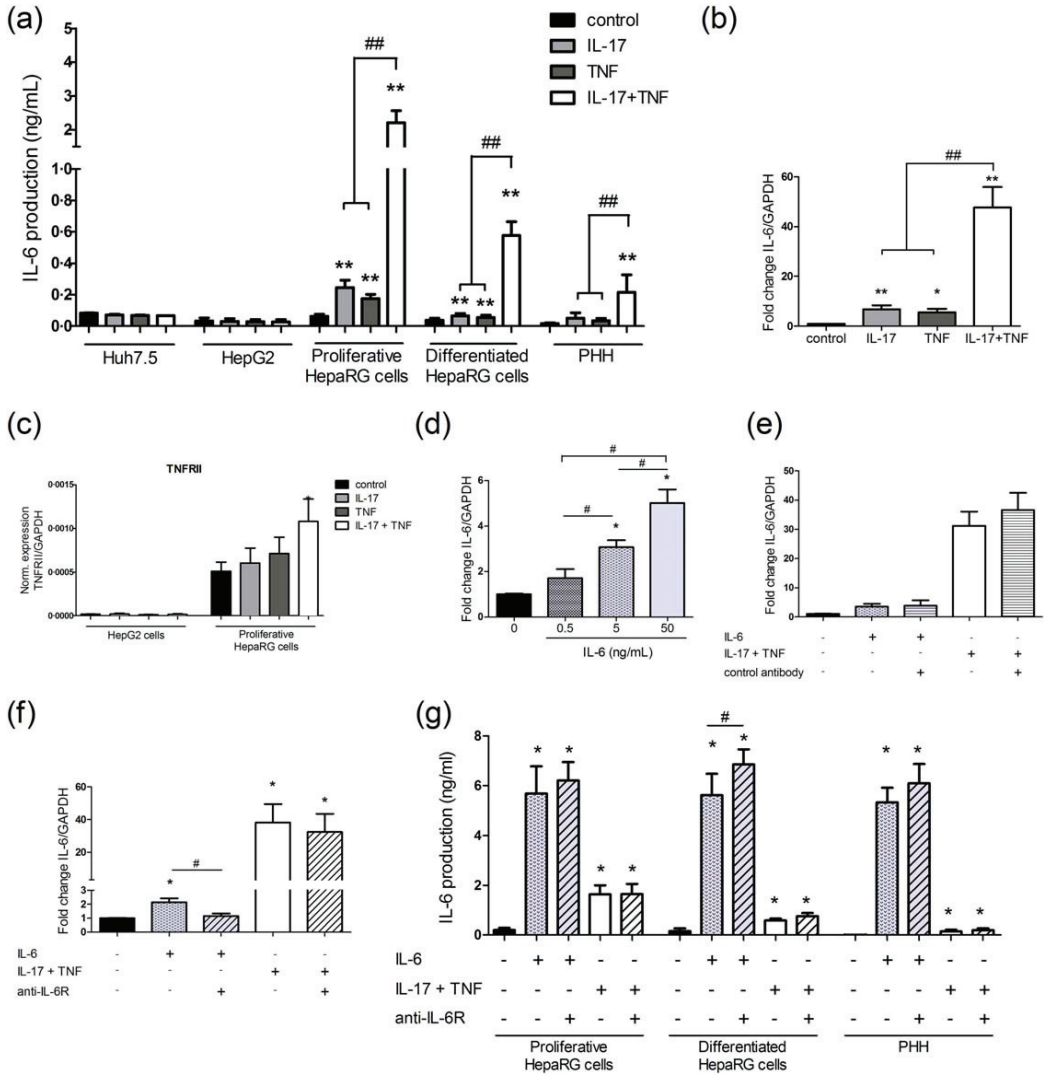

Fig. 1. Interleukin (IL)-17 and tumour necrosis factor (TNF)-α combination increases IL-6 expression and production synergistically. Hepatocytes were exposed to IL-17 and/or TNF-α or IL-6 with/without the anti-IL-6R. (a,g) IL-6 production by hepatocytes was quantified by enzyme-linked immunosorbent assay (ELISA). (b,d,e,f) IL-6 expression in proliferative human HepaRG cells at 12 h was expressed as fold changes compared to control. (c) TNF receptor II (TNF-RII) expression at 12 h was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The control antibody had no effect on IL-6 mRNA and protein levels following IL-6 or IL-17/TNF-α stimulation in HepaRG cells (e, data not shown). Data are the mean of three to 18 independent experiments ± standard error of the mean (s.e.m.); $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ *versus* control; $\#P < 0{\cdot}05$ and $\#\#P < 0{\cdot}01$ *versus* other cytokine conditions.

elevated ASAT levels returned to control level when an anti-IL-6R antibody was added (Fig. 2e,f). In these cultures, alanine aminotransferase (ALAT) level was lower than the detection limit (< 6 UI/l) (data not shown). The increase in ASAT level was therefore mainly IL-6-dependent.

IL-17 and TNF-α increase in synergy IL-8 expression and production independently of the IL-6 pathway

Cell recruitment is crucial for the inflammatory response. Liver biopsies in patients with active liver disease are characterized by the presence of inflammatory infiltrates [26]. IL-8 is associated with neutrophil recruitment involved in the acute-phase inflammatory response, as in acute hepatitis [15]. The IL-17/TNF-α co-operation enhanced IL-8 production by HepaRG cells and PHH *versus* IL-17 alone, TNF-α alone and control (Fig. 3a). IL-8 mRNA expression in proliferative HepaRG cells was up-regulated by IL-17 alone (5-fold) and TNF-α alone (5-fold), with a synergistic effect of both (24-fold) (Fig. 3b). The IL-17/TNF-α synergistic interactions also increased IL-8 mRNA levels by up to 14-fold in PHH (Fig. 3b).

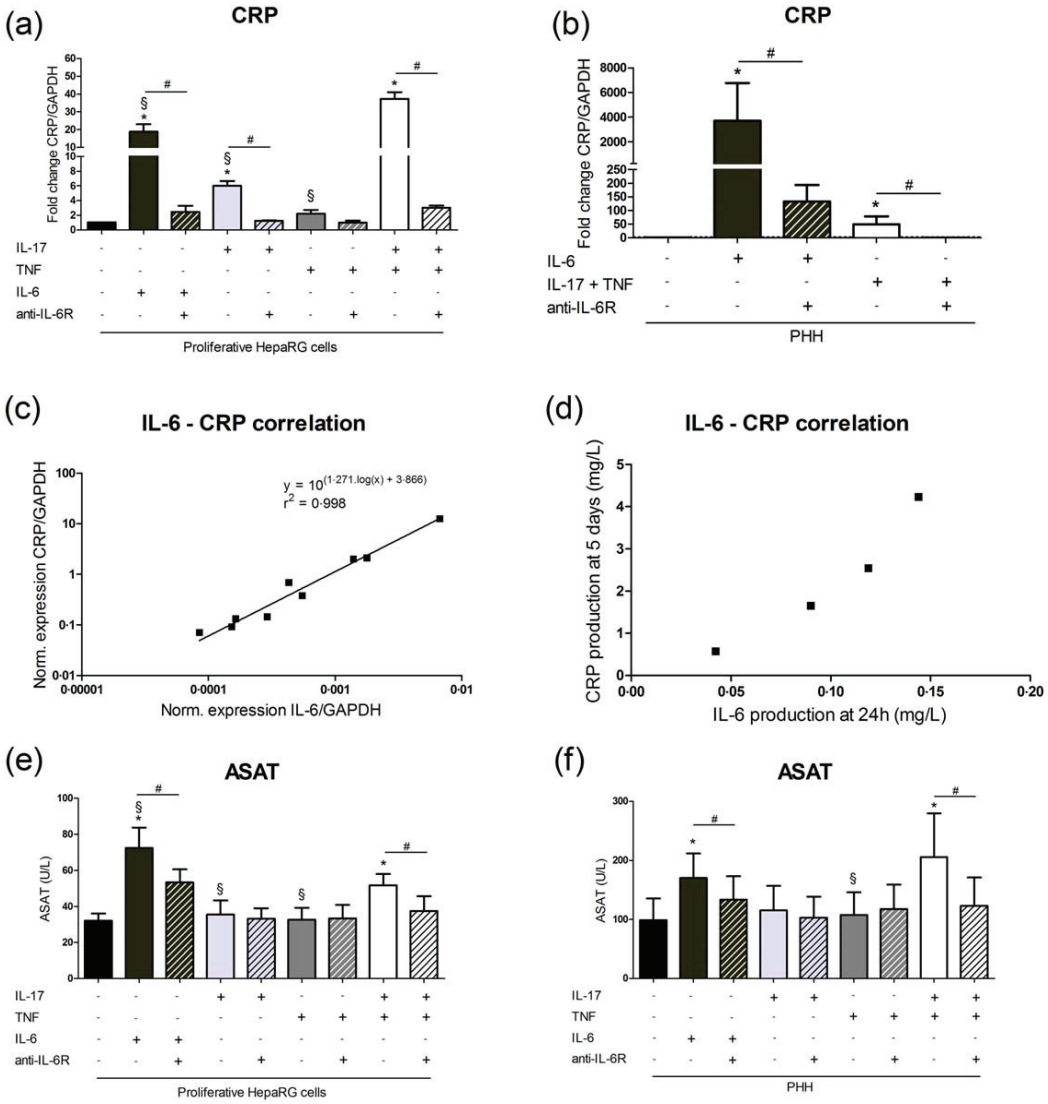

Fig. 2. Induction of the interleukin (IL)-6-dependent C-reactive protein (CRP) expression and aspartate aminotransferase (ASAT) activity level following IL-17 and/or tumour necrosis factor (TNF)-α stimulation. Hepatocytes were treated with IL-17 and/or TNF-α or IL-6 with/without the anti-IL-6R. (a,b) CRP expression in the human HepaRG cell line and primary human hepatocytes (PHH) at 24 h was expressed as fold changes compared to control. The control antibody had no effect on CRP mRNA levels following IL-6 or IL-17/TNF-α stimulation in HepaRG cells (data not shown). (c) Correlation between IL-6 and CRP mRNA levels in PHH stimulated with IL-17/TNF-α for 24 h. mRNA levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (d) Correlation between IL-6 and CRP production in PHH cultures treated with IL-17/TNF-α. (e,f) ASAT supernatant levels were quantified at 120 h. Data are the mean of four to six independent experiments ± standard error of the mean (s.e.m.); $*P < 0{\cdot}05$ *versus* control, $\S P < 0{\cdot}05$ *versus* IL-17/TNF-α condition, $\#P < 0{\cdot}05$ *versus* other cytokine conditions.

However, the IL-8 mRNA and protein level was unchanged after IL-6 exposure. Moreover, the IL-6 pathway inhibition had no effect on the induction of IL-8 expression and production by IL-17/TNF-α (Fig. 3c,d). Therefore, IL-8 induction by IL-17 and TNF-α was not mediated through IL-6. The IL-17 and TNF-α combination may thus have a key role in the migration of neutrophils to the liver in the context of acute hepatitis.

IL-17 and TNF-α increase in synergy MCP-1 and CCL20 expression mainly through an IL-6-independent pathway

MCP-1 and CCL20 are two chemokines acting on mononuclear cells involved in the chronicity of the inflammatory response [27–29]. MCP-1 and CCL20 mRNA levels increased in the presence of IL-17 or TNF-α, with a clear

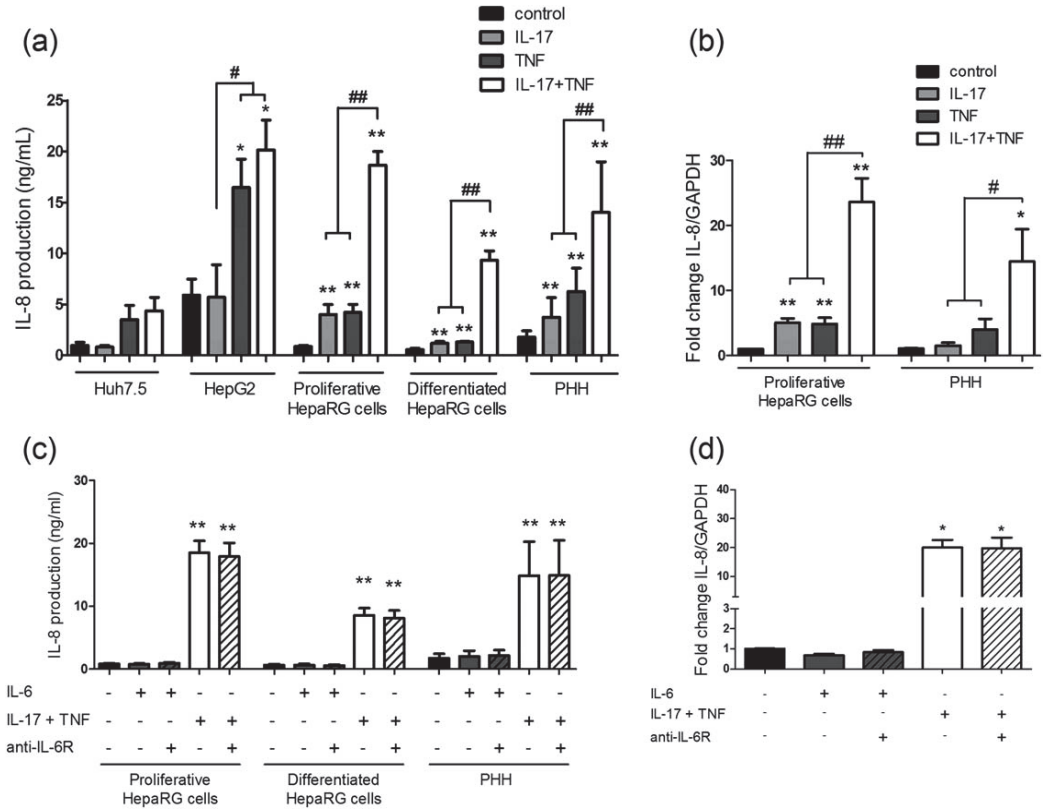

Fig. 3. Interleukin (IL)-17 and tumour necrosis factor (TNF)-α combination increases IL-8 expression and production synergistically independently of the IL-6 pathway. Hepatocytes were exposed to IL-17 and/or TNF-α or IL-6 with/without an anti-IL-6R antibody. (a,c). IL-8 production was quantified by enzyme-linked immunosorbent assay (ELISA). (b,d) IL-8 expression at 12 h was expressed as fold change compared to control. The control antibody had no effect on the IL-8 mRNA and protein levels following IL-6 or IL-17/TNF-α stimulation in human HepaRG cells (data not shown). Data are the mean of three to 18 independent experiments ± standard error of the mean (s.e.m.); $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ *versus* control; $\#P < 0{\cdot}05$ and $\#\#P < 0{\cdot}01$ *versus* other cytokine conditions.

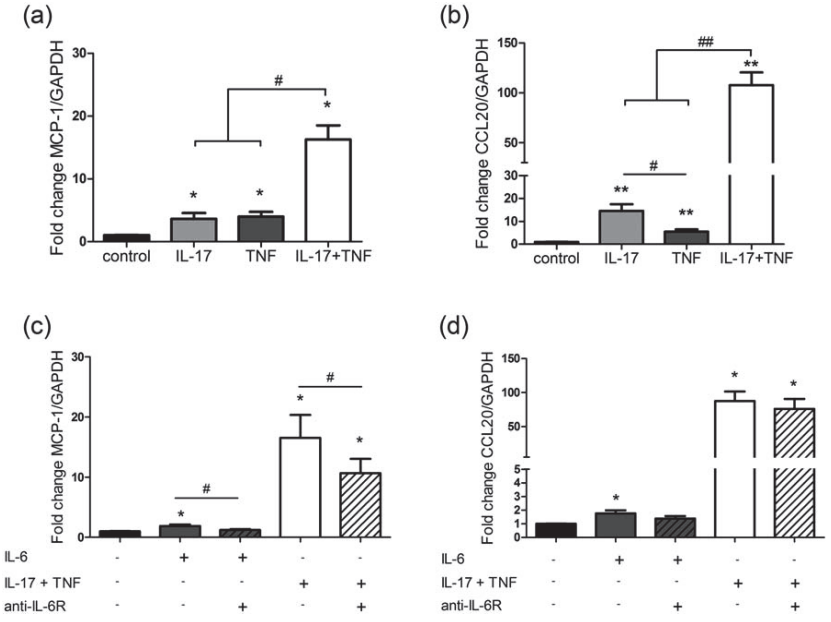

Fig. 4. Interleukin (IL)-17 and tumour necrosis factor (TNF)-α combination increases monocyte chemoattractant protein (MCP)-1 and chemokine (C-C motif) ligand 20 (CCL20) chemokine expression synergistically, mainly through an IL-6-independent pathway. Proliferative human HepaRG cells were exposed to IL-17 and/or TNF-α or IL-6 with/without an anti-IL-6R antibody. (a–d) MCP-1 and CCL20 expression at 12 h in proliferative HepaRG cells was expressed as fold change compared to control. The control antibody had no effect on the MCP-1 and CCL20 mRNA levels following IL-6 or IL-17/TNF-α stimulation (data not shown). Data are the mean of seven to eight independent experiments ± standard error of the mean (s.e.m.); $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ *versus* control; $\#P < 0{\cdot}05$ and $\#\#P < 0{\cdot}01$ *versus* other cytokine conditions.

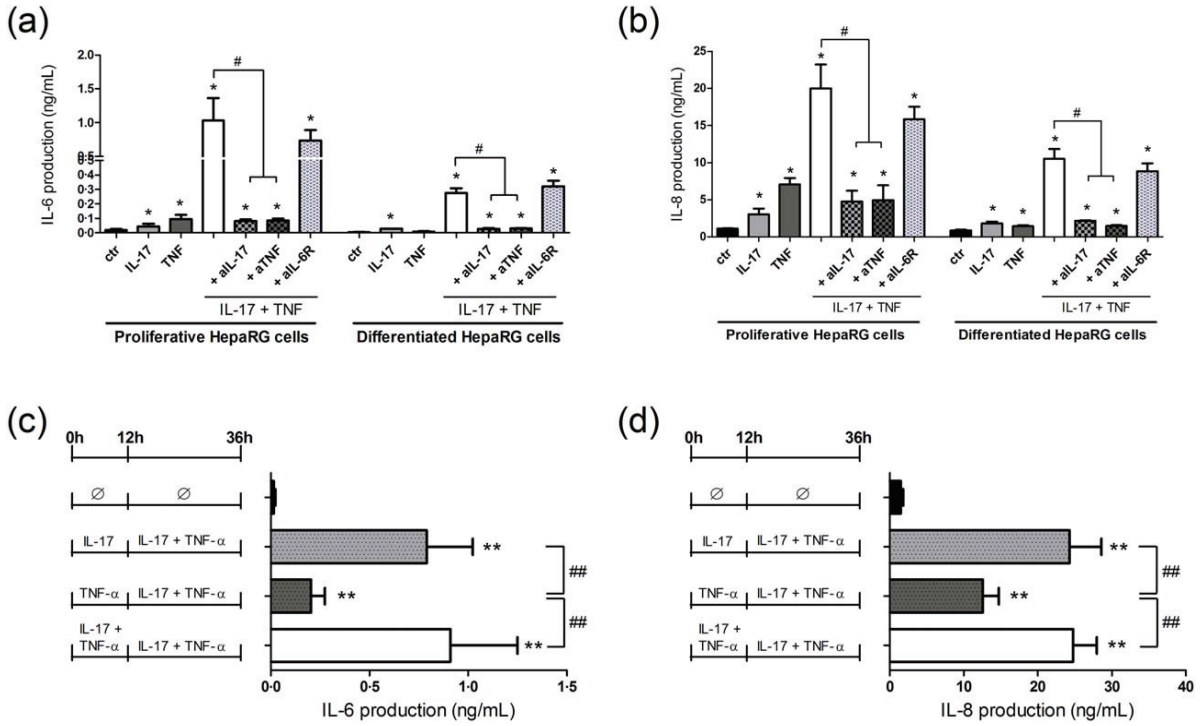

Fig. 5. Interleukin (IL)-17 initiates the IL-17 and tumour necrosis factor (TNF)-α synergistic effect on IL-6 and IL-8 production. (a,b) To evaluate the contribution of IL-17, TNF-α and IL-6 pathways in the IL-17/TNF-α synergistic effect, human hepatoma cells (HepaRG) cells were treated with IL-17 and/or TNF-α with/without anti-IL-17A (aIL-17), anti-TNF-α (aTNF) or anti-IL-6R (aIL-6R) antibody. The control antibody had no effect on the IL-6 and IL-8 protein levels following IL-17/TNF-α stimulation (data not shown). (c,d) Proliferative HepaRG cells were pre-exposed to IL-17 and/or TNF-α overnight (for 12 h) before IL-17 and/or TNF-α addition to have both cytokines in the culture medium (except for the control (Ø)). The IL-6 and IL-8 production was measured by enzyme-linked immunosorbent assay (ELISA). Data are the mean of five to seven independent experiments ± standard error of the mean (s.e.m.); $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$ *versus* control; $\#P < 0{\cdot}05$ and $\#\#P < 0{\cdot}01$ *versus* other cytokine conditions.

synergistic effect of both cytokines (16- and 108-fold, respectively, $P < 0{\cdot}01$) *versus* control (Fig. 4a,b).

To determine whether the IL-17/TNF-α effect on MCP-1 and CCL20 expression was mediated through the IL-6 pathway, IL-6 and an anti-IL-6R antibody were used. By comparison with IL-17/TNF-α stimulation, IL-6 had a very minimal effect on MCP-1 and CCL20 mRNA levels. Similarly, the anti-IL-6R antibody did not abrogate the MCP-1 and CCL20 expression induced by the IL-17 and TNF-α combination in proliferative HepaRG cells (Fig. 4c,d). Therefore, IL-17 and TNF-α can act independently of the IL-6 signalling pathway on the induction of MCP-1 and CCL20 expression. IL-17/TNF-α may thus have a key role in the migration of immune cells, including T helper type 17 (Th17) cells, to the liver in the context of chronic hepatitis.

IL-17 initiates the IL-17 and TNF-α synergistic effect on IL-6 and IL-8 production

To understand more clearly the contribution of each cytokine on the IL-17 and TNF-α synergistic effect, antibodies blocking the IL-17, TNF-α and IL-6 pathways were used. Anti-IL-17 or anti-TNF-α antibody exposure in conjunction of the IL-17/TNF-α combination reduced the synergistic effect on IL-6 and IL-8 production in HepaRG cells, whereas the IL-6R inhibition had no significant effect on IL-6 and IL-8 supernatant levels (Fig. 5a,b).

We next investigated whether first exposure to IL-17, TNF-α or both initiated the IL-17 and TNF-α synergistic effects on IL-6 and IL-8 release. Proliferative HepaRG cells were pre-exposed overnight to IL-17 and/or TNF-α and then IL-17 and/or TNF-α were added to both cytokines in the culture medium. Pre-incubation first with IL-17 followed by the addition of TNF-α induced two-fold higher IL-6 and IL-8 production at 24 h than pre-incubation with TNF-α, then with IL-17 (Fig. 5c,d). First exposure to IL-17, but not to TNF-α, was thus crucial for initiation of the IL-17/TNF-α synergistic effect.

IL-17 enhanced the stability of IL-6 mRNA

Post-transcriptional regulations could contribute to the IL-17 and TNF-α synergistic effect. To determine the IL-17

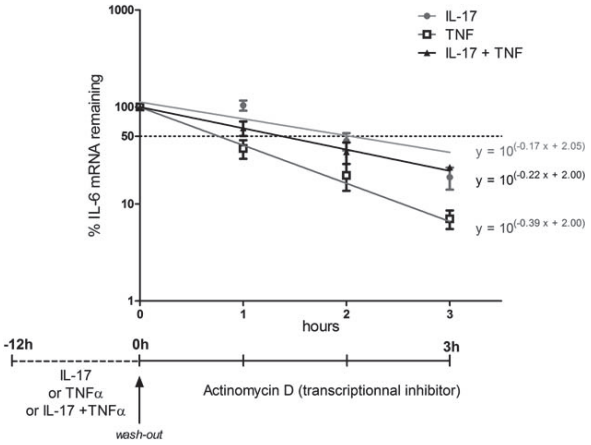

Fig. 6. Interleukin (IL)-17 enhances the stability of IL-6 mRNA. Proliferative human HepaRG cells were incubated with IL-17 (filled circles), tumour necrosis factor (TNF)-α (empty squares) or the IL-17/TNF-α combination (filled triangles) for 12 h. Actinomycin D was added to inhibit further transcription. The IL-6 and IL-8 expression during the next 3 h were quantified. Results are presented as % of mRNA remaining over time compared with the steady-state level (at 0 h). Data are the mean of four independent experiments ± standard error of the mean (s.e.m.).

and TNF-α effect on mRNA stabilization, HepaRG cells were treated with IL-17 and/or TNF-α for 12 h and then transcription was inhibited. TNF-α-induced IL-6 transcripts had a half-life of 46 min, whereas IL-17 and the IL-17/TNF-α combination increased the half-life up to 124 and 82 min, respectively (Fig. 6). IL-17 may thus increase IL-6 mRNA stabilization in hepatocytes.

IL-17 and TNF-α increase IL-6 and IL-8 production in synergy via the activation of ERK and/or PI3K/Akt signalling pathways and/or NF-κB transcription factor

The effect of the TNF-α/IL-17 combination on the downstream signalling pathways was then investigated, focusing on the MAPKs (JNK, p38, ERK), NF-κB and Akt/PI3K pathways [22,30,31]. Results are shown as the ratio of the total and phosphorylated forms (Fig. 7a). In HepaRG cells, the IL-17/TNF-α association induced phosphorylation of IκBα (leading to NF-κB release and activation) and ERK. The IL-17/TNF-α effect of Akt phosphorylation was weaker (Fig. 7a). Proliferative HepaRG cells were therefore treated with chemical inhibitors of these pathways. Quantification of transaminase secretion and cell viability were monitored to select the concentrations that did not induce cell death and other cytotoxic effects (data not shown).

The IL-17/TNF-α synergistic effect on IL-6 and IL-8 production was reduced slightly using JNK or p38 MAPK inhibitors. In contrast, ERK inhibition decreased strongly the production of IL-6 (29 and 70% of inhibition for 1 and 10 μM, respectively) and IL-8 (31 and 69% of inhibition for 1 and 10 μM, respectively) in a dose-dependent manner induced by the IL-17/TNF-α combination (Fig. 7b,c). IL-6 production induced by the IL-17/TNF-α combination was also inhibited in the presence of the NF-κB inhibitor (60% of inhibition), the Akt inhibitor (more than 70% of inhibition for 10 and 20 μM) and the PI3K inhibitor (40 and 65% of inhibition for 1 and 10 μM, respectively), whereas IL-8 production was not impacted significantly (Fig. 7d–g). Activation of ERK, NF-κB and/or PI3K/Akt signalling pathways was therefore involved in the IL-17/TNF-α synergistic effect on IL-6 and/or IL-8 production.

Discussion

This study shows how the IL-17/TNF-α synergistic interactions mediate a hepatic inflammatory response mainly through IL-6 for CRP and ASAT induction, and independently of IL-6 for IL-8, MCP-1 and CCL20 chemokine up-regulation.

IL-6 is a systemic inflammatory mediator, which plays a key role in triggering the acute-phase response to injury or inflammation. IL-17 was shown previously to induce IL-6 production by the human hepatoma Huh7, HepG2 and Hep3B cell lines [3,32,33]. Here, IL-17 and TNF-α increased the production of IL-6 in HepaRG cells and PHH in synergy. Because IL-6 promotes the generation and differentiation of Th17 cells [28], the main IL-17-producing cells, the increase of IL-6 production by the IL-17/TNF-α synergistic effect and IL-6 autoinduction could exacerbate the inflammatory IL-6/Th17/IL-17 amplification. Interestingly, IL-6 was also able to up-regulate directly its own expression *in vitro* leading to a positive feedback loop of IL-6. However, the contribution of this positive autoregulation of IL-6 in the IL-6 induction following the IL-17/TNF-α synergistic interaction was very weak at 12 h (Fig. 1g) and 24 h (data not shown) compared to the direct IL-17/TNF-α effect. Because an anti-IL-6R antibody blocks the IL-6 binding to its receptor competitively, a higher IL-6 protein level was expected in supernatants of hepatic cultures exposed to the anti-IL-6R antibody. For this reason, the IL-6-positive feedback loop after IL-6 treatment was observed only on IL-6 mRNA levels and not on IL-6 protein levels in supernatants.

High CRP levels are associated with an increased risk of cardiovascular events [34]. *In-vitro* and *in-vivo* studies have demonstrated the potent role of IL-6 on CRP production [3,7]. Here, IL-17 and/or TNF-α enhanced CRP expression in HepaRG cells, showing a synergistic effect with the combination. The IL-6 pathway blockade inhibited CRP induction strongly by IL-17 and/or TNF-α. In PHH treated with the IL-17/TNF-α combination, CRP levels correlated perfectly with those of IL-6. Moreover, CRP mRNA level was lower at 12 than 24 h (data not shown), indicating that IL-17 and TNF-α act first on the induction of IL-6 production, which leads in turn to CRP up-regulation. Therefore, the induction of CRP appears mainly IL-6-dependent.

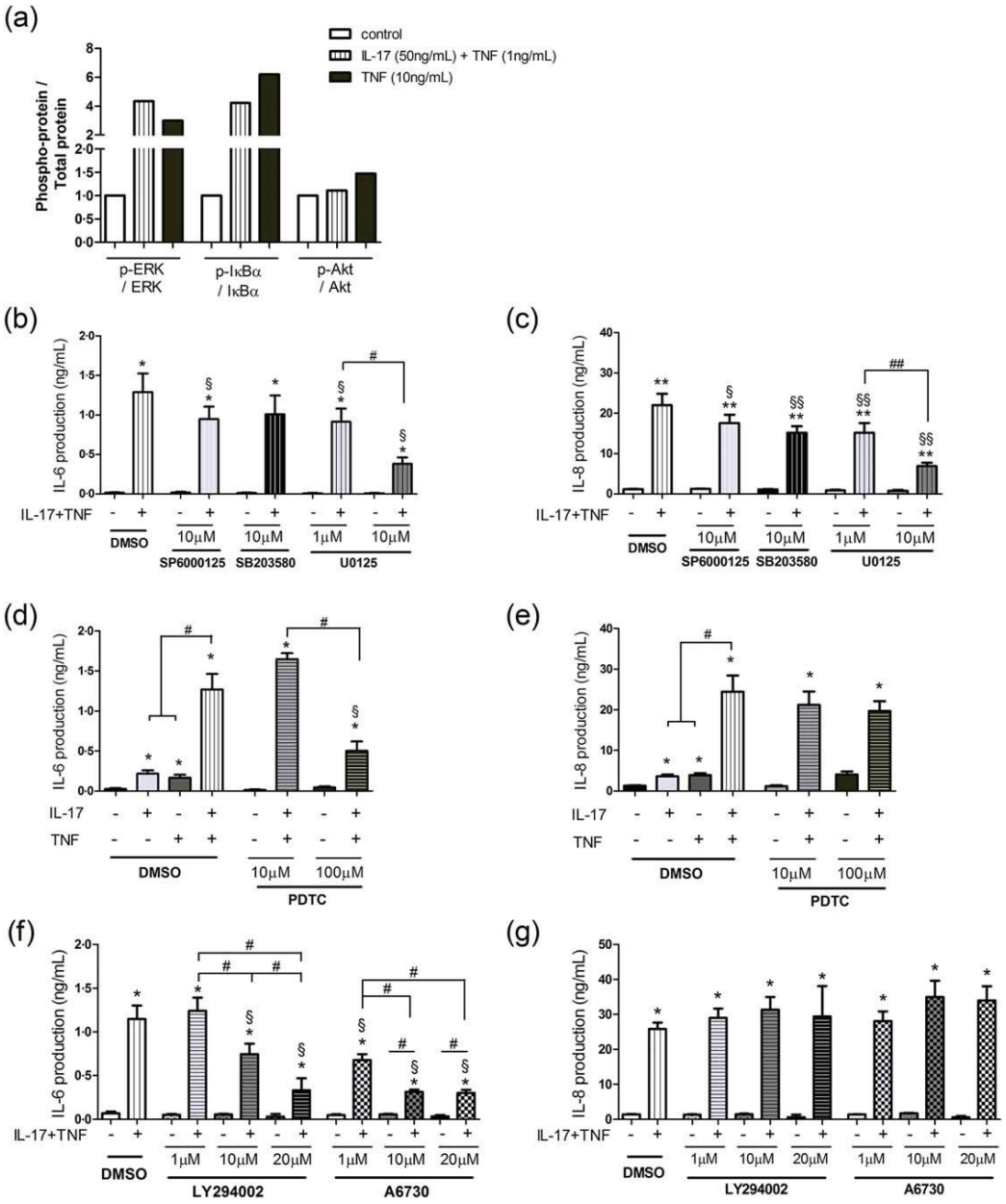

Fig. 7. Interleukin (IL)-17 and tumour necrosis factor (TNF)-α combination increases IL-6 and IL-8 production synergistically through the activation of extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK) and/or phosphatidylinositide 3-kinase/protein kinase B (PI3K/Akt) signalling pathways and nuclear factor kappa B (NF-κB) transcription factor. (a) Proliferative human HepaRG cells were incubated with IL-17 and/or TNF-α for 30min. The total and phosphorylated forms of ERK, NF-κB inhibitor α (IκBα) and Akt were quantified by Western blotting and their densitometry values were normalized to the cyclophilin content. Data are presented as the fold induction change of the ratio of phosphorylated to total protein in control samples. One representative experiment is shown. (b–g) Proliferative HepaRG cells were pre-exposed to MAPKs [Janus kinase (JNK), p38, ERK], NF-κB, Akt and PI3K pathway inhibitors for 1 h followed by the IL-17 and/or TNF-α addition. IL-6 and IL-8 production at 24 h was measured by enzyme-linked immunosorbent assay (ELISA). SP6000125: JNK inhibitor, SB203580: p38 MAPK inhibitor, U0125: MEK/ERK inhibitor; pyrrolidine dithiocarbamate (PDTC): NF-κB inhibitor; LY294002: PI3K inhibitor and A6730: Akt inhibitor. Data are the mean of at least four to eight independent experiments ± standard error of the mean (s.e.m.); $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ *versus* control in dimethylsulphoxide (DMSO) condition, §$P < 0{\cdot}05$ and §§$P < 0{\cdot}01$ *versus* IL-17/TNF-α condition in DMSO medium; #$P < 0{\cdot}05$ and ##$P < 0{\cdot}01$ *versus* other inhibitor conditions.

However, one study has demonstrated that IL-17 can stimulate CRP expression independently of IL-6 in Hep3B cells [3], suggesting that a possible minor pathway independent of IL-6 could exist.

In chronic inflammatory diseases, liver changes are common and may lead to transaminase elevation [35]. The IL-17/TNF-α combination increased the ASAT activity level through the IL-6 pathway. These results appear consistent with our *in-vivo* studies, which showed that TNF-α neutralization decreased serum levels of transaminase in mice and patients with autoimmune hepatitis [35,36]. Moreover, IL-17 deficiency or IL-17 neutralization reduced transaminase levels in various mouse models of liver injury [37,38]. Therefore, controlling IL-17 and TNF-α levels and functions may be protective and reduce liver damage.

Hepatic infiltration of neutrophils is an early response to systemic inflammation crucial to initiate liver injury [39,40]. The link between IL-17, neutrophil recruitment and hepatic necrosis was demonstrated in several mouse models [37,41]. IL-8 has a key role in the neutrophil mobilization and activation [15]. IL-17 may stimulate IL-8 production through IL-6 up-regulation in Huh7 cells [33]. In our study, IL-8 production was increased by the IL-17/TNF-α synergistic interaction independently of the IL-6 pathway, as the exposure to an anti-IL-6R antibody had no effect (Fig. 3c,d). IL-6 could nevertheless act *in vivo* on the IL-8 level by promoting the Th17–IL-17 cells axis leading to an increase of IL-17 production and, in turn, of IL-8.

Chronic hepatitis is characterized by the liver infiltration by various immune cells attracted by chemokines, such as CCL20 and MCP-1. Here, IL-17 and TNF-α were able to up-regulate CCL20 and MCP-1 expression synergistically through an IL-6-independent pathway. In HepG2 culture and an autoimmune hepatitis mouse model, TNF-α induced CCL20 expression and this up-regulation was associated with the progression of fatal inflammation [27,36]. MCP-1 was described as a central co-ordinator of hepatocyte-mediated inflammation [42,43]. MCP-1 expression was up-regulated in primary hepatocytes by activation of the IL-6-mediated signalling cascade [44]. In turn, MCP-1 induced IL-6 production in mouse hepatocytes, suggesting a possible positive feedback loop between IL-6 and MCP-1 [42]. Taken together, CCL20 and MCP-1 induction by IL-17/TNF-α can increase the accumulation of a Th17-driven response and lead to a chronic inflammatory state.

Although the IL-17/TNF-α synergistic effect has been well described in several cell types [16–19,45,46], the mechanism of this effect on IL-6 and IL-8 production has not yet been studied in hepatocytes. Various mechanisms may act at several levels: at a receptor level, at a post-transcriptional level and at a promoter level. In synoviocytes, the TNF receptor II (TNF-RII) contributed to the IL-17 and TNF-α synergistic effect on CCL20 production, and IL-17 treatment alone up-regulated its expression [47]. Here, in HepaRG cells, stimulation with the IL-17/TNF-α combination but not with IL-17 alone increased TNF-RII expression. Regulation of the TNF-RII expression may be different between synoviocytes and HepaRG cells. However, the TNF-RII mRNA level was much lower in HepG2 and Huh7.5 cells than in HepaRG cells (data not shown). This could explain the lack of response of the HepG2 and Huh7.5 cells to the IL-17/TNF-α synergistic effect on IL-6 and IL-8 production.

Post-transcriptional regulation is important to control cellular transcript abundance and, in turn, the levels of the secreted proteins. Several studies have reported that the IL-17 and TNF-α co-operation modulates mRNA stability [45,46,48–50]. In HepaRG cells, IL-17 could enhance IL-6 mRNA stability (Fig. 6). As the 3'-untranslated region of the IL-6 mRNA contains adenylate and uridylate (AU)-rich elements, IL-17 may promote the binding of stabilizing AU-binding proteins over that of destabilizing AU-binding proteins, prolonging IL-6 mRNA half-life [51]. Activation of the ERK MAPK pathway may contribute to this IL-6 mRNA stabilization [52].

To investigate the potential signalling pathways involved in IL-17 and TNF-α synergistic stimulation on hepatic IL-6 and IL-8 production, several chemical pathway inhibitors were used. MAPK, PI3K and its downstream mediator Akt were involved in the IL-6 signalling, and also in the IL-17-induced production of IL-6, IL-8 and MCP-1 in several cell types, including human hepatocellular carcinoma cell lines [32,33,53–55]. In HepaRG cells, activation of the ERK MAPK pathway in the IL-17–TNF-α interaction appears crucial, as the ERK pathway inhibition reduced both IL-6 and IL-8 production. The PI3K/Akt signalling pathway contributes to the synergistic effect of IL-17/TNF-α on the induction of IL-6 but not of IL-8. Because the MAPK and PI3K/Akt pathways can be activated by IL-6, their contribution on the IL-6 production can be related to the positive autoregulation of IL-6 (Fig. 1c). However, the anti-IL-6R antibody failed to reduce IL-6 expression induced by IL-17/TNF-α (Fig. 1f) in HepaRG cells. Therefore, the IL-17/TNF-α combination could activate the ERK MAPK and PI3K/Akt pathways directly to induce IL-6 and/or IL-8 production.

Transcription factor-binding sites for NF-κB and C/EBP (CCAAT/enhancer-binding protein) in the IL-6 promoter were both involved in the IL-17 and TNF-α synergistic effect on IL-6 in an osteoblastic cell line [56]. Part of the IL-17/TNF-α synergistic effect on IL-6 may occur at the gene transcription level through the up-regulation of C/EBPδ by IL-17/TNF-α and the increase of C/EBPδ recruitment to the promoter by TNF-α [31,56,57]. In primary murine hepatocytes, NF-κB contributed to the activation of many IL-17 target genes related to inflammation [58]. Here, in HepaRG cells, the NF-κB pathway appears to be involved in IL-6 release induced by the IL-17/TNF-α combination (Fig. 7d). Both IL-17 and TNF-α signalling

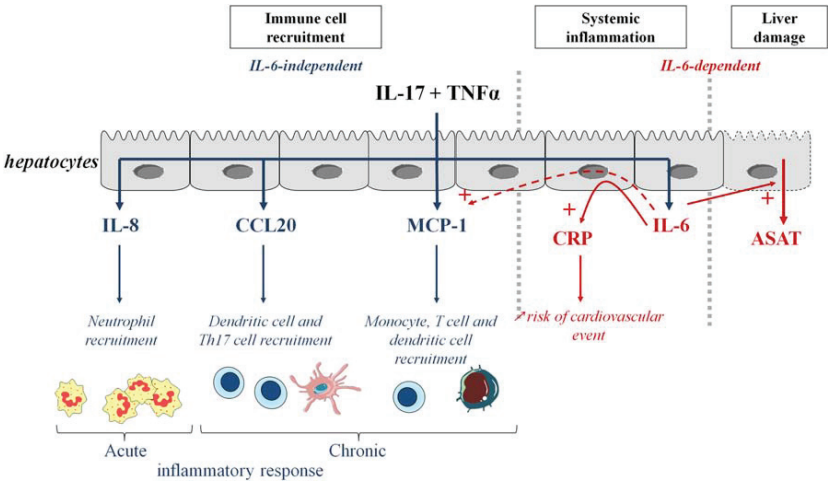

Fig. 8. Summary of the effects of interleukin (IL)-17 and tumour necrosis factor (TNF)-α on hepatocytes. IL-17 and TNF-α have synergistic interactions on cytokine production by hepatocytes, with IL-17 acting first to increase the effect of TNF. IL-17 and TNF-α induce C-reactive protein (CRP) through the induction of IL-6. IL-17 and TNF-α induce chemokine production independently of IL-6.

pathways promote NF-κB activation. However, the IL-17/TNF-α combination may induce a further increase of NF-κB activation through IκBζ, which acts as a NF-κB co-activator [57,59]. In primary murine hepatocytes, IL-17 and TNF-α cooperate to enhance the IκBζ mRNA expression in synergy [58].

In conclusion, targeting IL-17 and/or TNF-α could be a promising therapeutic strategy to control systemic inflammation, as seen with IL-6 inhibition, but also the local cell recruitment and associated liver cell injury. These results are summarized in Fig. 8. Furthermore, control of the CRP level could be critical to reduce the cardiovascular risks that represent a major cause of death in patients with chronic inflammatory diseases.

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Disclosure

None.

Author contributions

A. B. conducted the experiments and writing the manuscript; N. T., A. C. and B. B. developed the assays and experiments; and P. M. was responsible for the concept and writing the manuscript.

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2 Two-phase kinetics of the inflammatory response through hepatocyte-peripheral blood mononuclear cell interactions

Two-phase kinetics of the inflammatory response through hepatocyte-peripheral blood mononuclear cell interactions *(in preparation).*

Keywords*:* hepatocytes; immune cells; cell-cell interactions; inflammation; tolerance

Abstract

Active liver diseases are characterized by the infiltration of inflammatory immune cells, which can interact locally with hepatocytes. Co-cultures between non- and PHA-activated human PBMCs and human hepatoma HepaRG cells were used to determine the role of theses cell interplays on the inflammatory response. The PBMC-HepaRG cell interactions increased the mRNA expression and/or secretion of IL-6, IL-8, CCL-20 and MCP-1 partially through direct cell contact and the induction was higher in PHA-activated conditions. The pro-inflammatory cytokines IL-17 and/or TNFα contributed also to the increase of IL-6 and IL-8 secretion. HepaRG cells modulated T cell polarization by increasing the Th1 cell transcription factor expression and by reducing the $CD3^+$ $CD4^+$ $IL\text{-}17^+$ cell frequency when PBMCs were activated with PHA. Moreover, the presence of HepaRG cells inhibited the PHA-induced HLA-DR expression on PBMCs and PBMC proliferation. In contrast, the presence of skin fibroblasts had no effect on the PBMC proliferation induced by PHA. After a first pro-inflammatory phase, the PBMC-HepaRG cell interactions may therefore down-regulate the immune response. The PBMC-hepatocyte interplays can thus participate in the initiation of hepatitis and also in the maintenance of immune tolerance in liver.

2.1 Introduction

The liver is traditionally perceived as a metabolic organ but it is also a central intersection point of the immune system. Receiving 80% of its blood supply from the gut, the liver is constantly exposed to environmental toxins, dietary and bacterial products via the portal vein (Robinson et al., 2016). The liver plays also an important role in the initiation of the acute-phase response by producing most of the acute-phase proteins. The structural organization of

the liver, the hepatic cell repertoire and its "buffer" function between the gut content and systemic inflammation create an unique environment which determines the balance between inflammation and immunosuppression (Robinson et al., 2016).

Because liver transplantations are commonly well-tolerated and required low levels of immunosuppressive therapy compared to other allogeneic grafts such as skin graft, the liver is often perceived as an immunologically tolerant organ (Calne et al., 1969; Lerut and Sanchez-Fueyo, 2006). In addition, the liver can mediate systemic tolerance since the liver graft can protect other transplanted organs from rejection (Kamada et al., 1981; Simpson et al., 2006; Wang et al., 1998).

During liver injury and inflammation, immune cells infiltrated the liver (Quintin et al., 2010) and can interact with liver resident cells by paracrine and contact-dependent interactions. Leukocyte and stromal cell interplays within liver appear crucial in the outcome of liver injury (Holt et al., 2008). Theses interactions are facilitate by the low-blood pressure and the fenestrated endothelium within the liver allowing direct cell-cell interactions between cells in liver sinusoids and hepatocytes (Racanelli and Rehermann, 2006; Warren et al., 2006). By secreting damaged hepatocyte-derived mediators and cytokines, hepatocytes, which represent the most abundant cell population in liver, can also modulate the immune cell response (Petrasek et al., 2015).

The objective of this study was to assess the role of the immune cell-hepatocyte interactions on the inflammatory response by using co-cultures between human PBMCs and the human hepatoma HepaRG cells. The PBMC-HepaRG cell interactions increased the mRNA expression and/or secretion of IL-6 as well as several chemokines partially through direct cell contacts and this effect was higher when PBMCs were activated with PHA. Hepatocytes can then modulate the PBMC immune response by acting on T cell polarization and cytokine secretion and also by reducing the PHA-induced PBMC proliferation and the antigen presenting cell capacity of the PBMCs. The PBMC-hepatocyte interplays can thus participate in the initiation of hepatitis and also in the maintenance of immune tolerance in liver.

2.2 Materials and methods

Cell culture

The human hepatoma HepaRG cells were grown in William's E medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, USA),

2 mM L-glutamine (Eurobio, Les Ulis, France), 5 µg/mL insulin (Sigma), 50 µM hydrocortisone hemisuccinate (Serb, Paris, France), 50 U/mL penicillin and 50 µg/mL streptomycin (Eurobio). HepaRG cells were used after 15 days post-plating. Human skin fibroblasts were obtained from biopsies of non-lesional skin as previously described (Noack et al., 2016). Fibroblasts were maintained in Dulbecco's modified Eagle's medium (Eurobio) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Eurobio), 100 U/mL penicillin and 100 µg/mL streptomycin (Eurobio).

PBMC isolation and co-culture assays

Whole blood samples were obtained from the Etablissement français du Sang. PBMCs were isolated by Ficoll-Hypaque (Eurobio) density gradient centrifugation. Cells were maintained in RPMI 1640 medium (Eurobio) supplemented with 10% human AB serum (Etablissement Français du Sang, La Plaine Saint-Denis, France), 2 mM L-glutamine (Eurobio). PBMCs were activated or not with 5 μg/ml phytohemagglutinin (PHA) (Sigma-Aldrich) and added on HepaRG cells or skin fibroblasts at a ratio of 5 PBMCs for 1 HepaRG cell or 1 skin fibroblast. This ratio was based on data from the literature (Doumba et al., 2013; Noack et al., 2016). For cell culture insert assays, HepaRG cells were cultured at the bottom of a culture plate well and PBMCs were placed in Falcon® cell-culture inserts (Corning, NY, USA) with a small-pored membrane (0.4μm) preventing direct cell-cell contacts but allowing the circulation of soluble factors. For IL-17 and/or TNFα neutralization assays, PBMCs activated or not with PHA for 24h were exposed to anti-IL-17 secukinumab (Novartis, Basel, Switzerland) at 10 μg/mL and/or anti-TNFα infliximab (Merck, Kenilworth, USA) at 10 μg/mL during 2h before being added to HepaRG cells. A monoclonal antibody against the BetV1 allergen (Dendritics, Lyon, France) was used as a control antibody at the same concentration.

Quantitative real time-PCR

Total RNA was purified using an RNeasy® Plus Mini kit (Quiagen, Hilden, Germany). cDNA was synthetized using the iScript™ kit (Bio-Rad, Hercules, CA, USA). PCR amplification was performed using the CFX96™ Real time system instrument (Bio-Rad) with the iTaq™ universal SYBR® green supermix (Bio-Rad) and the Qiagen QuantiTect® primers.

The expression of the genes of interest was normalized to the expression of the housekeeping GAPDH gene.

Enzyme-linked immunosorbent assays

Supernatant cytokine concentrations were quantified with human ELISA kits according to the instructions of the manufacturer. IL-6 and IL-8 ELISA kits from Diaclone (Besancon, France) and CCL20, IL-17, TNFα, IFNγ, IL-1β and IL-10 ELISA kits from R&D system (Minneapolis, USA) were used.

Flow Cytometry

Cell phenotyping was performed on Navios flow cytometer (Beckman Coulter, Indianapolis, IN, USA). For $CD3^+$ $CD4^+$ $IL\text{-}17^+$ cell staining, eFluor 450 labeled anti-CD3 antibody (clone UCHT1, 48-0038, eBiosciences, San Diego, CA, USA) and phytoerythrin (PE)-Cyanine7 labeled anti-CD4 antibody (clone RPA-T4, 25-0049, eBiosciences) were used for surface staining. After cell fixation with 2% paraformaldehyde (Sigma) for 15 min and cell permeabilization with 0.5% saponin (Sigma) for 20min, the allophycocyanin (APC) labeled anti-IL-17A (clone eBio64DEC17, 17-7179, eBiosciences) were used for intracellular staining. For the other analysis, the PBMC population and the HepaRG cell population were distinguished by the use of the pacific blue (PB) labeled anti-CD45 (clone HI30, 304029, Biolegend, San Diego, CA, USA). PE labeled anti-HLA-DR (clone immu357, IM1639, Beckman Coulter) was used to stain HLA Class II. Corresponding isotypic antibodies labeled with the same fluorophores and from the same suppliers were used as controls. To track PBMC proliferation, PBMCs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described by Quah B et al (Quah et al., 2007). Briefly, PBMCs (10.10^6 cells/mL) were labelled with 1 μM CFSE for 5min at room temperature, PBMCs were then washed and cultured for 3 days in presence or not of PHA. To investigate cell viability the fluorescein isothiocyanate (FITC) annexin V apoptosis detection kit (556547, BD, Franklin Lakes, NJ, USA) were performed according to the manufacturer's instructions. Data were analyzed using Kaluza solftware (version 1.2, Beckman Coulter).

Statistical analysis

Calculations were performed with GraphPad Prism version 5.01 software. Data are the mean of at least 3 independent experiments ± SEM. Statistical differences were analyzed using the Mann Whitney test. P values less than to 0.05 were considered significant.

2.3 Results

PBMC-hepatocyte interactions induce the IL-6 synthesis in activated conditions

Acute-phase proteins are mainly produced by hepatocytes in response of a variety of different pro-inflammatory cytokines in which IL-6 is the chief stimulator (Schmidt-Arras and Rose-John, 2016). In liver, hepatocytes can interact and establish cell-cell contracts with PBMCs through fenestrations in liver endothelial cells (Warren et al., 2006). The role of PBMC-HepaRG cell interactions on the IL-6 mRNA expression and secretion was studied by using a co-culture system (Figure 6A). No contribution of alloreactivity has been detected in the same type of co-culture model (Correia et al., 2009). When PBMCs and HepaRG cells were cultured together, the IL-6 mRNA levels were higher (>8.0-fold, $p<0.05$) than when the cells were cultured separately and the increase of the IL-6 mRNA expression was even stronger in PHA activated conditions (>19-fold, $p<0.05$) (Figure 6B). However, the IL-6 supernatant level was not significantly different between PBMCs alone and the co-cultures in non-stimulated PBMC conditions. In contrast, PHA activation enhanced significantly the IL-6 release in PBMC monocultures with a strong induction in co-cultures. Indeed, the IL-6 supernatant level was 18-fold higher in co-cultures versus PBMCs alone in PHA-stimulated cultures. The use of cell culture inserts avoiding direct cell-cell contacts but allowing the circulation of soluble factors reduced by 72% ($p<0.01$) the IL-6 secretion compared to co-cultures without inserts (Figure 6C). The PBMC-HepaRG cell interactions thus induced a strong IL-6 secretion in PHA-activated conditions and the direct cell-cell contacts have an important contribution in this effect.

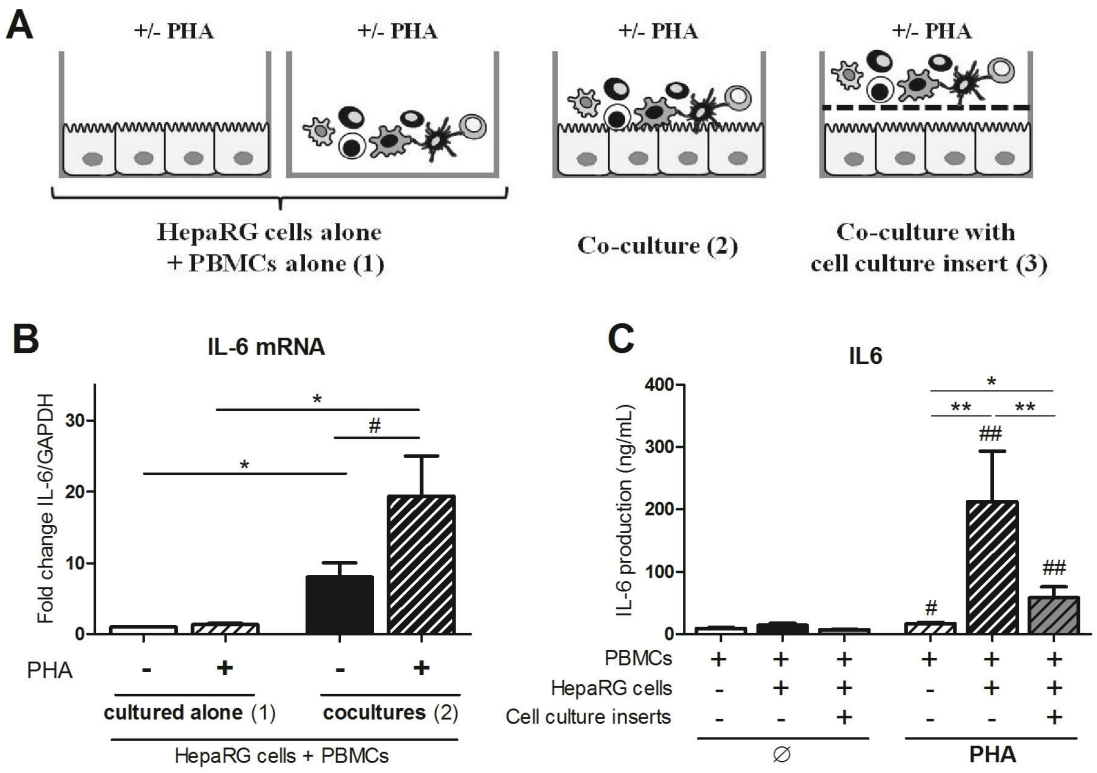

FIGURE 6: PBMC-HepaRG cell interactions increase the expression and/or secretion of IL-6 in activated condition mainly through direct cell-cell contacts

(A) Human PBMCs and HepaRG cells were cultured alone or in co-cultures with or without cell culture inserts at a ratio of 5 PBMCs : 1 HepaRG cell in presence or not of phytohemagglutinin (PHA). (B) IL-6 mRNA expression at 24h in PBMCs and HepaRG cells were expressed as fold changes compared to non-activated conditions with PBMCs and HepaRG cells cultured alone. (C) IL-6 supernatant levels were quantified by ELISA at 48h in PBMC monocultures, PBMC-HepaRG cell co-cultures with or without cell culture inserts. Data are the mean of 7 to 9 independent experiments ± SEM; Mann Whitney test, *p<0.05, **p<0.01 versus co-culture conditions; #p<0.05 and ##p<0.01 versus non-activated conditions.

PBMC-hepatocyte interactions increase the expression and/or secretion of IL-8, CCL20 and MCP-1 chemokines

Chemokine release induces immune cell recruitment that is crucial for the inflammatory response. Inflammatory infiltrates are founded in patients with active liver diseases (Quintin et al., 2010). The effect of PBMC-HepaRG cell interactions on IL-8, CCL20 and MCP-1 chemokines was thus investigated. mRNA expression of IL-8, CCL20 and MCP-1 was up-regulated (<4.9-; 4.6- and 3.8-fold, $p<0.05$) in PBMC-HepaRG cell co-cultures compared to PBMCs and HepaRG cells cultured alone (Figures 7A-C). PHA activation increased the MCP-1 mRNA levels by 1.8-fold ($p<0.05$) in co-cultures (Figure 7C). In contrast, IL-8 and CCL20 mRNA levels in co-cultures were no significantly different between non-activated and PHA-activated co-cultures (Figures 7A, B). The quantification of the IL-8 and CCL20 supernatant levels confirmed the induction of IL-8 and CCL20 by the PBMC-HepaRG cell interactions compared to PBMCs alone ($p<0.01$) and HepaRG cells alone (17.5 ng/mL for IL-8 and 3.6 ng/mL for CCL20, $p<0.01$, data not shown) in both non- and PHA-activated conditions (Figures 7D, E). PHA activation enhanced significantly the IL-8 and CCL20 secretion in co-cultures ($p<0.05$). The inhibition of the direct PBMC-HepaRG cell contacts by the use of cell culture inserts reduced the IL-8 and CCL20 release by 45% and 19%, respectively ($p<0.05$) in activated conditions (Figure 7D, E). The contribution of the direct cell-cell contacts on the IL-8 and CCL20 release was lower compared to the IL-6 secretion for the same conditions (72%) (Figure 6B). Therefore, soluble factor exchanges between PBMCs and HepaRG cells had a major contribution in the release of IL-8 and CCL20 induction.

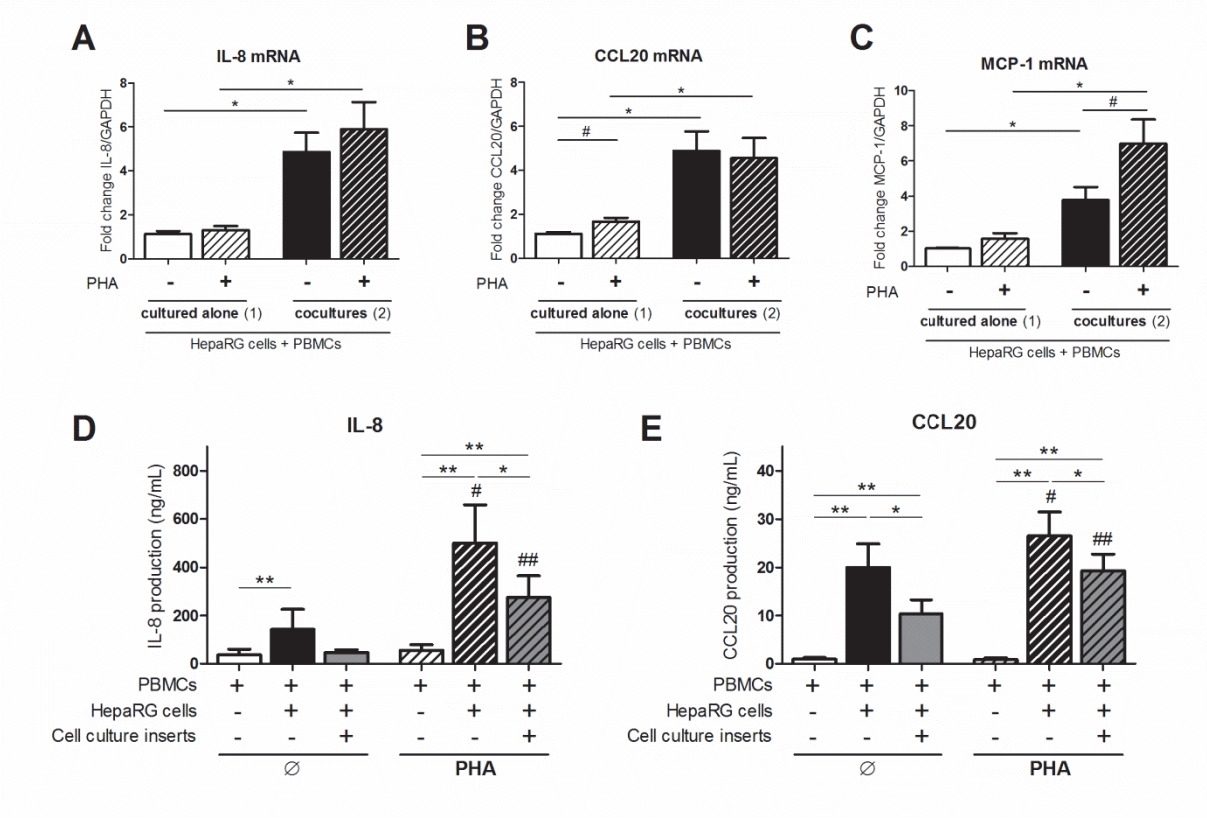

FIGURE 7: PBMC-HepaRG cell interactions increase the expression and/or secretion of IL-8, CCL20 and MCP-1 chemokines

Human PBMCs and HepaRG cells were cultured alone or in co-cultures with or without cell culture inserts at a ratio of 5 PBMCs : 1 HepaRG cell in presence or not of phytohemagglutinin (PHA). (A-C) IL-8 and CCL20 mRNA expression at 24h and MCP-1 mRNA expression at 8h in PBMCs and HepaRG cells were expressed as fold changes compared to non-stimulated conditions with PBMCs and HepaRG cells cultured alone. (D, E) IL-8 and CCL20 supernatant levels were quantified by ELISA after 48h of culture in PBMC monocultures, PBMC-HepaRG cell co-cultures with or without cell culture inserts. Data are the mean of 7 to 8 independent experiments ± SEM; Mann Whitney test, *p<0.05, **p<0.01 versus co-culture conditions; #p<0.05 and ##p<0.01 versus non-stimulated conditions.

IL-17 and TNFα contribute to the induction of the IL-6 and IL-8 secretion by the PBMC-HepaRG cell interactions

IL-17 and TNFα are two pro-inflammatory cytokines which induced in synergy the IL-6 and IL-8 production by hepatocytes (Beringer et al., 2018). As shown in Figures 6C and 7D, PBMC-HepaRG cell interactions enhanced the secretion of IL-6 and IL-8 and a part of this effect was mediated through the soluble factor exchanges. To determine the contribution of IL-17 and TNFα produced by the PBMCs on the IL-6 and IL-8 release in co-cultures, PBMCs

activated with PHA for 24h were exposed to specific inhibitors of IL-17 and/or TNFα and then added in the HepaRG cell cultures. This PBMC pre-incubation step was to better mimic the *in vivo* conditions in chronic inflammatory disorders in which PBMCs are certainly pre-activated before being in contact with resident tissue cells. As expected, interactions between pre-incubated PBMCs and HepaRG cells increased significantly the IL-6 and IL-8 secretion compared to PBMCs alone or HepaRG cells alone (Figures 8A, B). Neutralization of IL-17, TNFα or both reduced significantly the production of IL-6 by 18%, 38% and 39% and IL-8 by 26%, 39% and 44%, respectively versus the condition with the control antibody. IL-6 and IL-8 secretion was lower in presence of the anti-TNFα alone or the combination of anti-IL-17 and anti-TNFα compared to the anti-IL-17 alone ($p<0.01$ for IL-6 and $p<0.05$ for IL-8) (Figures 8C, D). Therefore, the use of both anti-IL-17 and anti-TNFα had no additive or synergistic inhibitory effects on the IL-6 and IL-8 release. Consistent with our prior experiments with HepaRG cell monocultures (Beringer et al., 2018), the blockade of IL-6 pathway had no effect on the IL-8 release in co-cultures (Figure 8D). IL-17 and TNFα thus contributed to the induction of IL-6 and IL-8 secretion in PBMC-HepaRG cell co-cultures but the combination of IL-17 and TNFα inhibitors had no additive effects on the IL-6 and IL-8 inhibition.

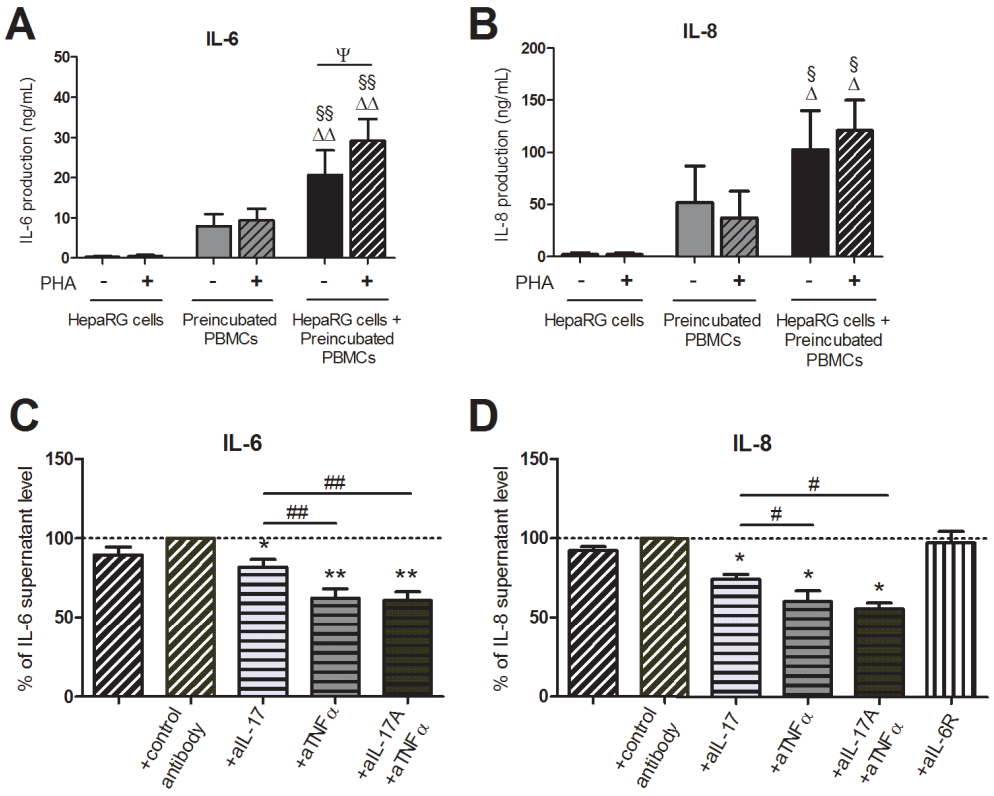

FIGURE 8: IL-17 and TNFα contribute to the induction of IL-6 and IL-8 secretion by the PBMC-HepaRG cell interactions

Human PBMCs were incubated for 24h in presence or not of phytohemagglutinin (PHA) and then exposed or not to the anti-IL-17 (aIL-17a) and/or the anti-TNFα (aTNFα) or the anti-IL-6 receptor (aIL-6R) or the control antibody during 2h before being added to the HepaRG cells. IL-6 and IL-8 supernatant levels were quantified by ELISA after 48h of co-cultures. (A, B) Co-cultures between HepaRG cells and pre-incubated PBMCs increased the IL-6 and IL-8 secretion compared to HepaRG cells alone or pre-incubated PBMCs alone. (C, D) Data are expressed as IL-6 or IL-8 supernatant level percentages compared to the PHA-activated PBMC–HepaRG cell co-cultures in presence of the control antibody. Data are the mean of 7 to 8 independent experiments ± SEM; Mann Whitney test, $\Delta p<0.05$ and $\Delta\Delta p<0.01$ versus HepaRG cells alone; $\S p<0.05$ and $\S\S p<0.01$ versus preincubated PBMCs alone; $\Psi p<0.05$ versus PHA condition; $*p<0.05$ and $**p<0.01$ versus the PHA-stimulated co-culture conditions with the control antibody; $\#p<0.05$ and $\#\#p<0.01$ versus the PHA-stimulated co-culture conditions with the anti-IL-17.

PBMC-hepatocyte interactions increase Tbet expression whereas RORc expression and $CD3^+$ $CD4^+$ $IL\text{-}17^+$ cell frequency decreased in PHA-activated conditions

As IL-17, a signature cytokine of the Th17 cell, contributed to the induction of IL-6 and IL-8 in co-cultures, PBMC-HepaRG cell interactions may act on T cell polarization and potentially on the secretion of specific T cell cytokines. The transcription factor mRNA expressions of Treg cells (FoxP3), Th1 cells (T-bet) and Th17 cells (RORc) were therefore quantified in

PBMCs and HepaRG cells cultured alone or together. PHA activation increased FoxP3 mRNA levels in both separate cultures and co-cultures ($p<0.05$) (Figure 9A). In contrast, T-bet mRNA expression was significantly up-regulated whereas the RORc mRNA level was down-regulated in PHA-activated co-cultures compared to the other conditions ($p<0.05$) (Figures 9B, C). The frequency of $CD3^+$ $CD4^+$ $IL\text{-}17^+$ cells being lower in PBMC-HepaRG cell co-cultures compared to PBMCs alone in presence of PHA confirms this effect on Th17 cells (Figure 9D). Hepatocytes therefore contributed to the T cell polarization in PHA-activated conditions by increasing Tbet expression and by reducing $CD3^+$ $CD4^+$ $IL\text{-}17^+$ cell frequency.

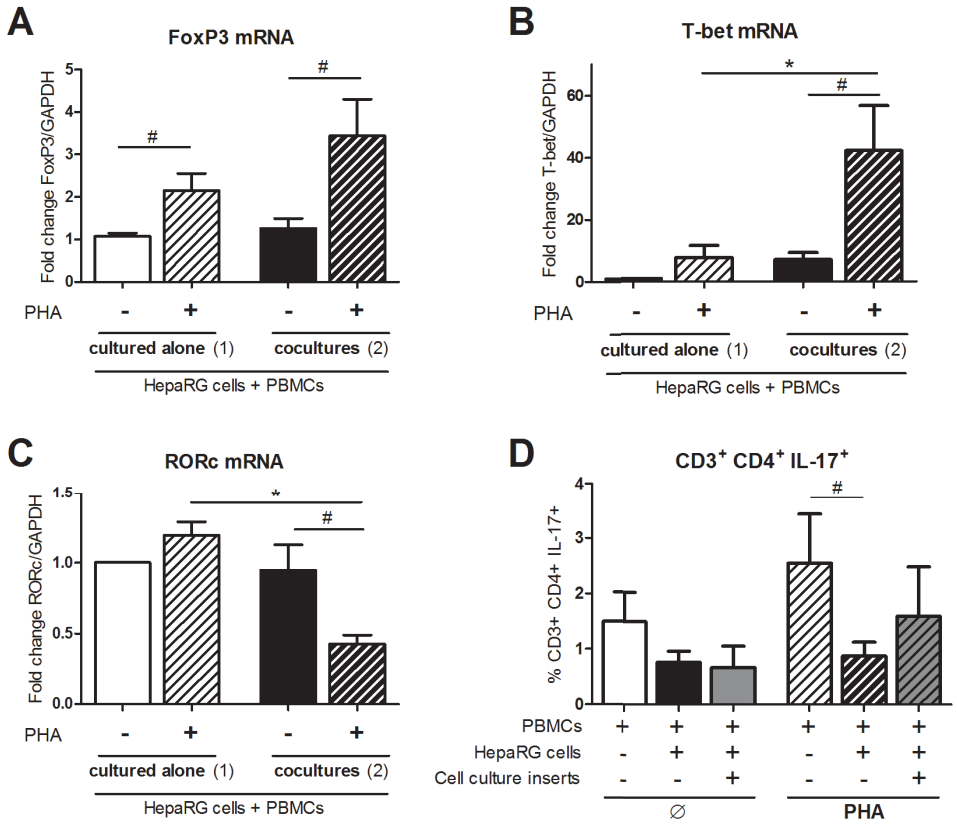

FIGURE 9: PBMC-HepaRG cell interactions up-regulate Tbet expression whereas RORc expression and $IL\text{-}17^+$ $CD4^+$ $CD3^+$ cell frequency decreased in PHA-activated conditions

Human PBMCs and HepaRG cells were cultured alone or in co-cultures with or without cell culture inserts at a ratio of 5 PBMCs : 1 HepaRG cell in presence or not of phytohemagglutinin (PHA). (A-C) FoxP3, T-bet and RORc mRNA expression at 24h was expressed as fold changes compared to non-activated conditions with PBMCs and HepaRG cells cultured alone. (D) Frequency of IL-17 positive CD4 T cells after 48h of cultures. Cells were first gated on CD3 and CD4 expression. Data are the mean of 6 to 7 independent experiments ± SEM; Mann Whitney test, *$p<0.05$ versus co-culture conditions; #$p<0.05$ versus non-activated conditions.

PBMC-hepatocyte interactions decrease the secretion of TNFα but not the IL-10, IL-1β, IL-17 and IFNγ release

Because hepatocytes can act on T cell polarization, mRNA and/or culture supernatant levels of the pro-inflammatory cytokines IL-1β, IL-17, IFNγ and TNFα and of the anti-inflammatory cytokine IL-10 were quantified in PBMC cultures in presence or not of HepaRG cells. mRNA levels of IL-10 and IL-1β were increased by the PBMC-HepaRG cell interactions with a higher induction in PHA-stimulated condition versus non-activated conditions ($p<0.05$) (Figures 10A, B). IL-17 mRNA level was strongly up-regulated in PHA-activated co-cultures compared to other conditions ($p<0.05$) (Figure 10C). Cytokine supernatant levels were then quantified in PBMC monocultures and PBMC-HepaRG cell co-cultures. In HepaRG cell cultures, cytokine production was too low compared to other culture conditions or not detected (data not shown). Supernatant levels of IL-10, IL-1β, IL-17 and IFNγ were higher in PHA-conditions ($p<0.05$) but similar between PBMCs alone and in co-cultures (Figures 10D, E, F, G). In contrast, TNFα secretion was enhanced by the PHA activation only when PBMCs were cultured alone ($p<0.05$) (Figures 10H). Therefore, PBMC-HepaRG cell interactions induced gene expressions of IL-10, IL-1β and IL-17 but not their secretion in culture supernatants in PHA-activated conditions.

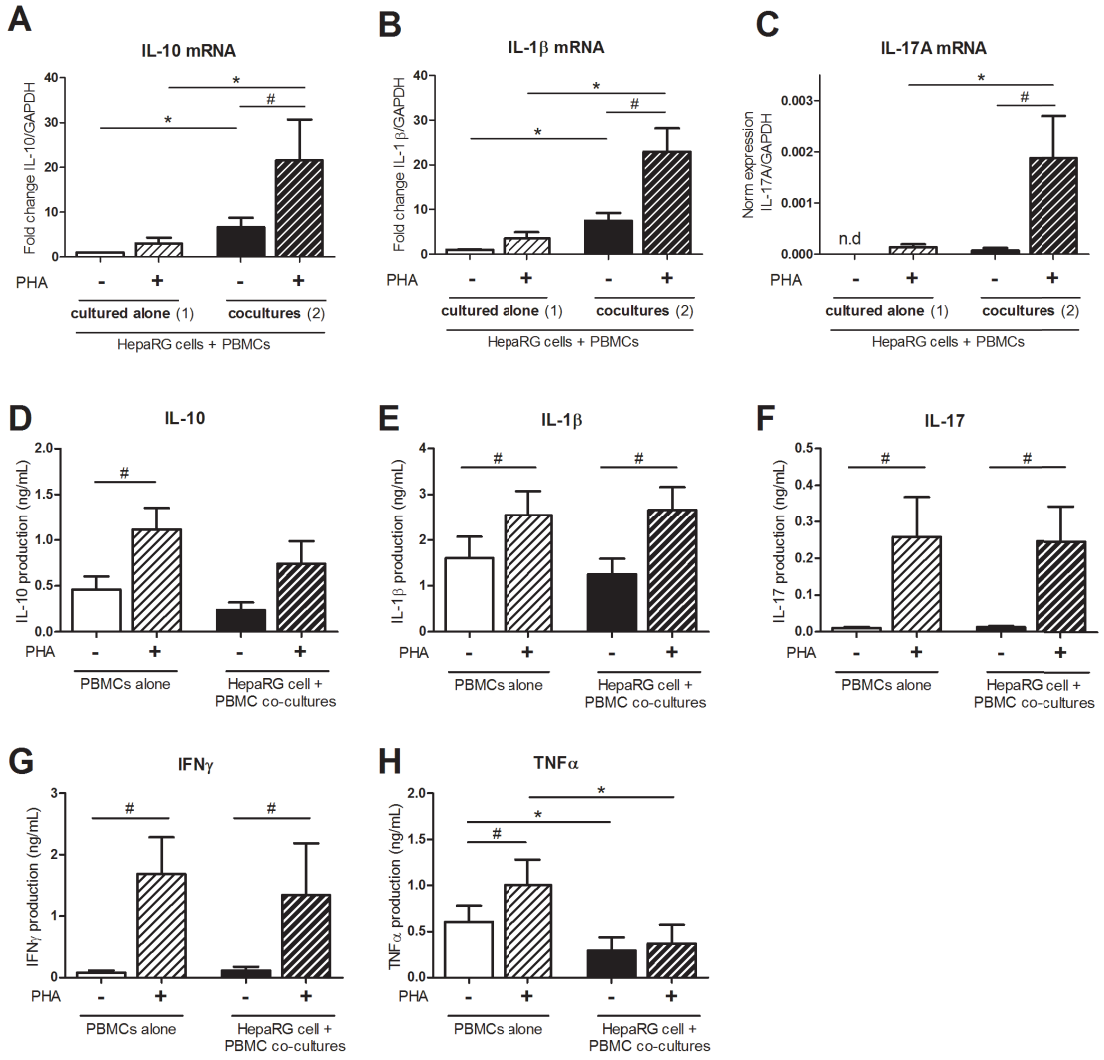

FIGURE 10: PBMC-HepaRG cell interactions decrease the secretion of TNFα and had no effect on the IL-10, IL-1β, IL-17 and IFNγ release in PHA-activated condition

Human PBMCs and HepaRG cells were cultured alone or in co-cultures at a ratio of 5 PBMCs : 1 HepaRG cell in presence or not of phytohemagglutinin (PHA). (A, B) IL-10 and IL-1β mRNA expressions at 24h were expressed as fold changes compared to non-activated condition with PBMCs and HepaRG cells cultured separately. (C) IL-17A expression at 24h was normalized to that of glyceraldehydes 3-phosphate dehydrogenase (GAPDH). (D-H) IL-10, IL-1β, IL-17, IFNγ and TNFα supernatant levels were quantified by ELISA after 48h of culture. Data are the mean of 6 to 11 independent experiments ± SEM; Mann Whitney test, *p<0.05 versus co-culture conditions; #p<0.05 and ##p<0.01 versus non-stimulated conditions.

PBMC-hepatocyte interactions inhibit the antigen presenting cell capacity of PBMCs and the PBMC proliferation induced by PHA

The effect of PBMC-HepaRG cell interactions on the antigen presenting cell capacity to $CD4^+$ T cells was next determined by looking at the human leukocyte antigens (HLA)-DR

expression on both PBMCs and HepaRG cells as hepatocytes express major histocompatibility complex (MHC) class II in inflammatory conditions (Franco et al., 1988; Herkel et al., 2003). To distinguish PBMC and HepaRG cell population by cytometry, cells were stained with anti-CD45 antibody (Figure 12A). In the PBMC population, the PBMC-HepaRG cell interactions enhanced HLA-DR expression by 1.4-fold ($p<0.05$) versus PBMCs alone in non-activated conditions. In contrast, HLA-DR expression of PBMCs was down-regulated in PHA-activated co-cultures compared to other conditions ($p<0.05$) (Figure 11A). In the HepaRG cell population, PHA activation increased HLA-DR expression in both PBMC mono- and co-cultures ($p<0.05$) whereas the PBMC-HepaRG cell interactions decreased slightly the HLA-DR expression compared to HepaRG cells cultured alone in non-activated conditions ($p<0.05$) (Figure 11B). The potential effect of the PBMC-HepaRG cell interactions on PBMC proliferation was then investigated by using the CFSE staining. As expected the PHA activation enhanced the percentage of dividing cells ($p<0.05$). However, this effect was suppressed in presence of HepaRG cells (Figures 11C, D). To determine whether this effect is specific of hepatocytes, skin fibroblasts were co-cultured with PBMCs. Interestingly, the PBMC-skin fibroblast interactions had no effect on the PHA-induced PBMC proliferation (Figures 11E, F). To verify if the HepaRG cell inhibitory effect on the PBMC division was related to cell death, the cell viability was also determined. PBMC viability was slightly reduced in PHA-activated PBMC monocultures ($p<0.01$) but not in co-cultures (Figure 12B). This lower frequency of PBMC viability in PHA-activated PBMC monocultures was most likely offset by the strong induction of PBMC proliferation induced by PHA. HepaRG cell viability was also modulated by the presence of PBMCs activated or not with PHA. The viability of HepaRG cells was especially decreased in PHA-activated co-cultures (Figure 12B). Therefore, the HepaRG cells can reduce the antigen presenting cell capacity of the PBMCs in PHA conditions as well as the PBMC proliferation induced by PHA without affecting the PBMC viability.

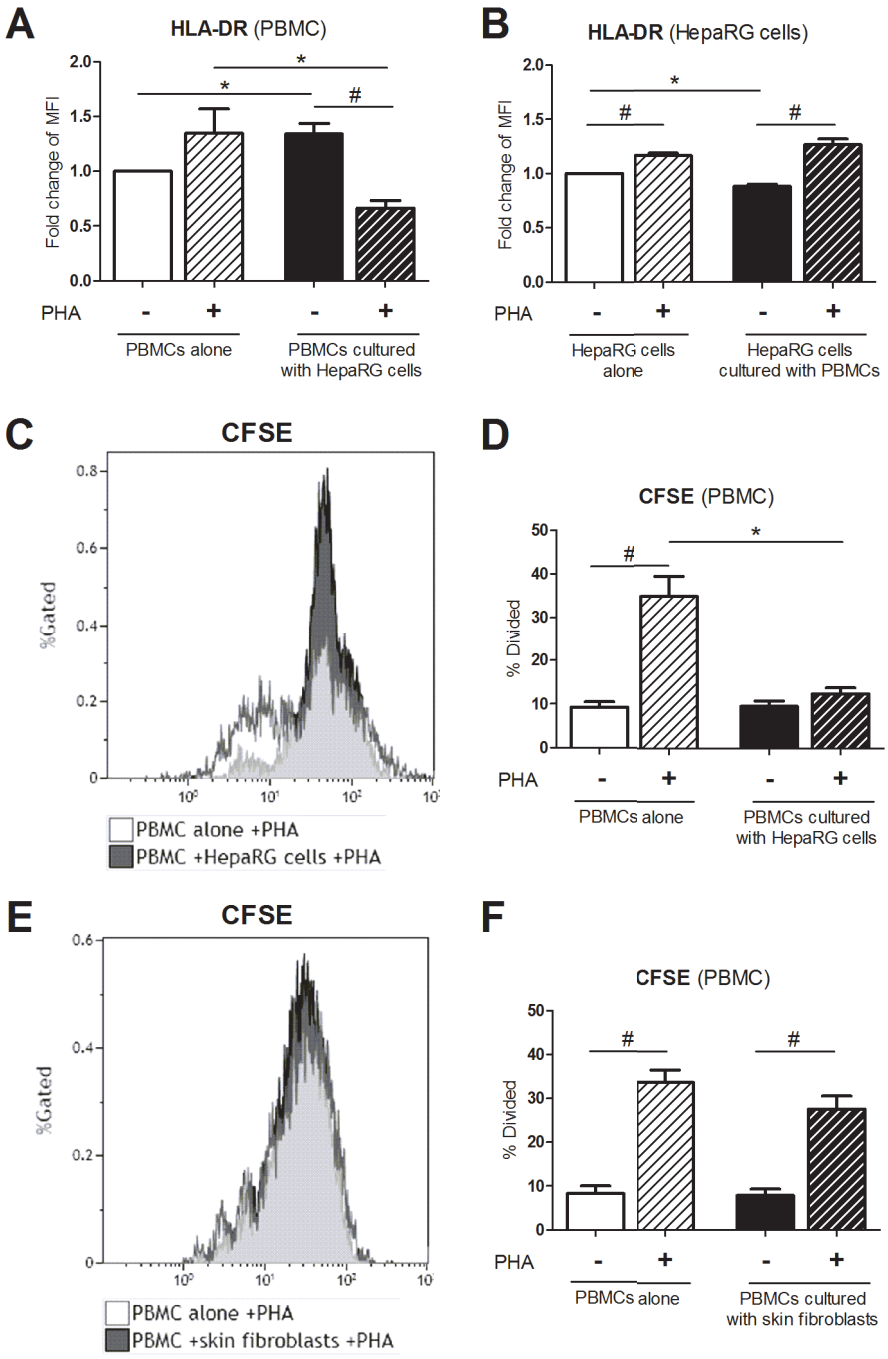

FIGURE 11: PBMC-HepaRG cell interactions reduce the HLA-DR expression on PBMCs and the PHA-induced PBMC proliferation

Human PBMCs and HepaRG cells or skin fibroblasts were cultured alone or in co-cultures at a ratio of 5 PBMCs : 1 HepaRG cell or 1 skin fibroblast in presence or not of phytohemagglutinin (PHA). (A, B) HLA-DR expression was measured in HepaRG cells and PBMCs after 48h of cultures by flow cytometry. (C-E) PBMCs were first labeled with CFSE and then cultured with/without HepaRG cells or skin fibroblasts and in presence or not of PHA for 3 days. Data are the mean of 6 to 7 independent experiments ± SEM; Mann Whitney test, *$p<0.05$, vs. co-culture conditions; #$p<0.05$ vs. unstimulated conditions.

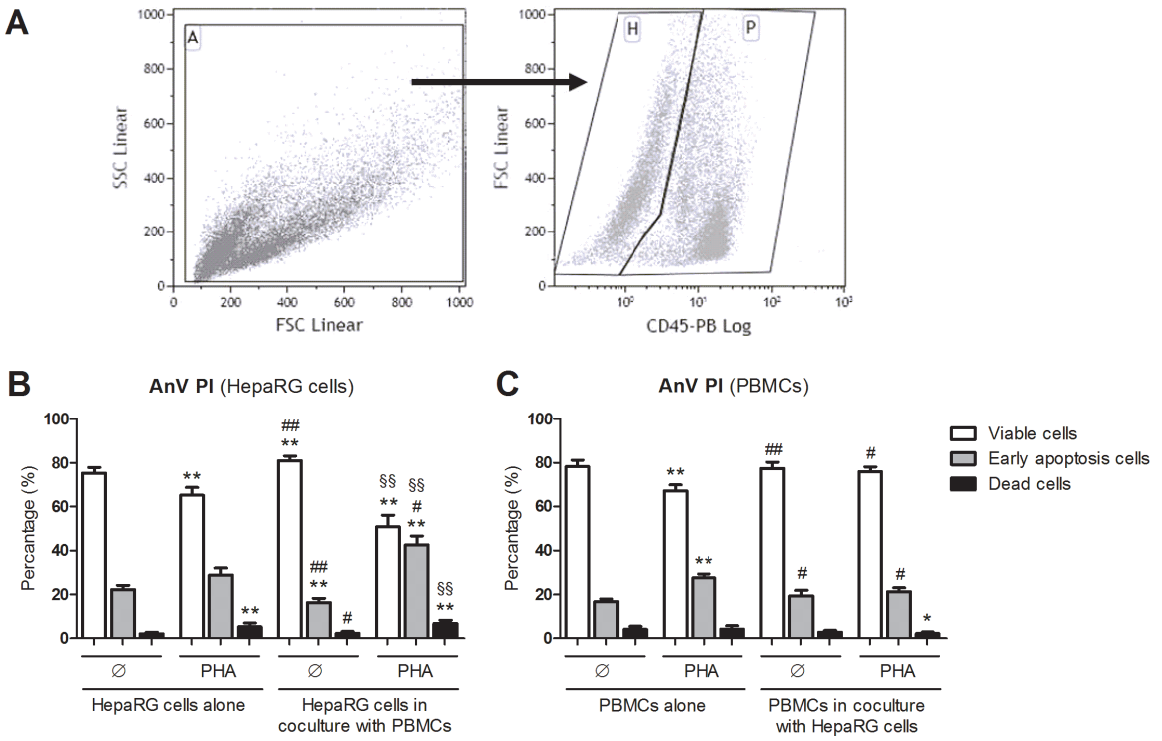

FIGURE 12: Effect of PBMC-HepaRG cell interactions on cell viability

PBMCs and HepaRG cells were cultured alone or in co-cultures at a ratio of 5 PBMCs : 1 HepaRG cell in presence or not of phytohemagglutinin (PHA) for 48h. (A) Cells were then labeled with pacific blue anti-CD45 antibody to distinguish by flow cytometry the PBMC population ($CD45^+$) and the HepaRG cell population ($CD45^-$). (C, D) Cells were stained with Annexin V (AnV)-FITC and propidium iodide (PI) to quantify the percentage of viable (AnV^- PI^-), early apoptosis (AnV^+ PI^-) and dead (AnV^+ PI^+) cells by flow cytometry. Data are the mean of 8 independent experiments ± SEM; Mann Whitney test, *p<0.05 and **p<0.01, vs. monoculture conditions without PHA; #p<0.05, ##p<0.01 vs. monoculture conditions with PHA; §§p<0.01 vs. co-culture conditions without PHA.

2.4 Discussion

This study shows that PBMC-HepaRG cell interactions may enhance immune cell recruitment and inflammation in liver by inducing IL-6 as well as IL-8, CCL20 and MCP-1 chemokine mRNA expression and/or secretion. In addition, the hepatocytes modulated the PBMC immune response by acting on T cell polarization and by reducing the PBMC antigen presenting cell capacity and the PBMC proliferation in PHA-activated conditions. The PBMC-HepaRG cell interactions appear therefore to promote first the inflammatory response and then hepatocytes may inhibit the PBMC immune response.

The systemic inflammatory cytokine IL-6 is the major regulator of the release of acute-phase proteins that are mainly produced by the liver (Heinrich et al., 1990; Schmidt-Arras and Rose-

John, 2016). The liver plays therefore an important role in the acute phase response. To mimic the infiltration of immune cells during liver injury and inflammation, conventional and non-contact co-cultures between non-activated and activated PBMCs and HepaRG cells were used. PBMC-HepaRG cell interactions increased the IL-6 secretion mainly through direct cell-cell contact in PHA-activated conditions. However, the expression of CRP, a major acute-phase protein, was no significantly enhanced by the PBMC-HepaRG cell interactions.

Both acute-phase and chronic inflammatory responses are characterized by the attraction of immune cells induced by the local chemokine release. IL-8 is important in the mobilization and activation of neutrophils (Lemmers et al., 2009) that are mainly involved in the early response. CCL20 and MCP-1 chemokines attract various immune cells, which contribute to the chronicity of the inflammation. Co-cultures between the human monocyte cell line THP-1 and the human hepatoma HepG2 cells up-regulated the gene expression of IL-8 and MCP-1 compared to THP-1 cell alone (Honda and Inagawa, 2016). Here, the PBMC-HepaRG cell interactions enhanced the IL-8, CCL20 and MCP-1 expression and/or production in both non- and PHA-activated co-cultures. The IL-8 and CCL20 induction was partially mediated through direct cell-cell interactions. By their effects on the chemokine levels, the PBMC-HepaRG cell interactions may increase immune cell recruitment and therefore amplify the cell interactions.

IL-17 and TNFα are two pro-inflammatory cytokines which has been implicated in several liver diseases (Beringer and Miossec, 2018; Schwabe and Brenner, 2006; Yang and Seki, 2015). As IL-17 and TNFα induced in synergy the IL-6 and IL-8 release by HepaRG cells independently of the IL-6 pathway (Beringer et al., 2018), their potential contributions on the induction of IL-6 and IL-8 secretion in activated PBMC–HepaRG cell co-cultures were assessed. To better mimic the *in vivo* conditions during chronic liver injury, PBMCs were pre-activated for one day and then co-cultured with the HepaRG cells. Neutralization of IL-17 and/or TNFα inhibited the IL-6 and IL-8 production in co-cultures without an additional inhibitory effect of the blockade of both IL-17 and TNFα. As previously demonstrated (Beringer et al., 2018), inhibition of the IL-6 pathway did not reduce the IL-8 secretion. Soluble factor exchanges between PBMCs and HepaRG cells such as IL-17 and TNFα are therefore important in the induction of IL-8 but also IL-6 to a less extent.

Hepatocytes can influence the immune cell phenotype and the cytokine expression. Indeed, the human monocyte cell line THP-1 and HepG2 interactions increased the mRNA expression

of IL-1β, TNFα and IL-10 compared to THP-1 cultured alone (Honda and Inagawa, 2016). In addition, the interactions between $CD4^+$ T cells and hepatocytes from murine livers increased the IL-10 secretion by $CD4^+$ T cells following T cell receptor stimulation in a cell-cell contact-dependent manner via Notch signaling (Burghardt et al., 2013). Hepatocytes enhanced also the frequency $CD4^+$ $FoxP3^+$ Treg cells in presence of TGFβ through Notch signaling (Burghardt et al., 2014). The induction of IL-10 and $FoxP3^+$ Treg cells was even more pronounced when $CD4^+$ T cells where co-cultured with hepatocytes from regenerated livers of mice pretreated with the lectin concavanalin A (Burghardt et al., 2013, 2014). The $CD4^+$ T cells primed by hepatocytes were able to suppress proliferation of responder T cells upon T cell receptor stimulation (Burghardt et al., 2014). In this study, the Treg transcription factor FoxP3 expression and IL-10 production were enhanced in PHA-activated cultures without difference between PBMCs cultured alone or with HepaRG cells. In contrast, the Tbet expression was increased in PHA-activated co-cultures compared to PBMC monocultures whereas the secretion of IFNγ, known as a signature cytokine of Th1 cells, was similar between these two conditions. Surprising, the PBMC-HepaRG cell interactions decreased the RORc expression and the frequency of $CD3^+$ $CD4^+$ $IL\text{-}17^+$ cells whereas the IL-17 mRNA expression increased in PHA-activated conditions. There are fewer Th17 cells in PHA-activated co-cultures but they express higher quantity of IL-17 mRNA. Furthermore, the IL-17 secretion remained similar between PBMC monocultures and co-cultures activated with PHA whereas the TNFα release was lower in co-cultures. In addition, gene expressions of IL-10, IL-1β and IL-17 were up-regulated in PHA-activated co-cultures but not the IL-10, IL-1β and IL-17 secretion. Hepatocytes can thus act both at transcriptional and post-transcriptional levels by enhancing the transcription of several cytokines without increasing their release.

Because hepatocytes express MHC I and II molecules in inflammatory conditions as well as co-stimulatory molecules (Franco et al., 1988; Herkel et al., 2003), hepatocytes can act as antigen-presenting cells and therefore contribute to T cell activation and immune regulation in liver. Murine hepatocytes activated $CD4^+$ and $CD8^+$ T cells *in vitro* through antigen-dependent activation but were not able to sustain this activation and to create an effective immune response (Bertolino et al., 1998; Herkel et al., 2003). Autologous co-cultures between human PBMCs and primary human hepatocytes increased the MHC II expression when hepatocytes were from patients operated for hepatocellular carcinoma but not from donors without liver diseases (Doumba et al., 2013). Here, the HLA-DR expression of the HepaRG cells decreased slightly in presence of non-activated PBMCs. In contrast, PHA-

activation increased the HLA-DR expression of HepaRG cells cultured alone or with PBMCs. The antigen-presenting cell ability of PBMCs to $CD4^+$ T cells was also assessed. HLA-DR expression of PBMCs was up-regulated in non-activated co-cultures and down-regulated in PHA-activated co-cultures compared to monocultures. In activated conditions, HepaRG cells can thereby inhibit the initiation of the antigen-specific immune response by reducing the MHC II molecules of PBMCs. At the same time, the MHC II expression on HepaRG cells was induced by the PHA activation. However, this induction of antigen presentation by hepatocytes may influence the inflammatory response but was not sufficient to produce an effective immune response (Bertolino et al., 1998; Herkel et al., 2003) and was not associated with the development of liver autoimmune diseases (Herkel et al., 2003).

As PBMCs proliferate upon activation, the effect of hepatocytes on PBMC proliferation was thus investigated. Murine hepatocytes were able to induce $CD8^+$ T cell proliferation but fail to promote their survival (Bertolino et al., 1998). Soluble factors released by murine hepatocytes enhanced also the expansion of hematopoietic precursor cells and their differentiation to natural killer cells (Bordoni et al., 2004). In addition, co-cultures of the human HepG2 cell line or nontumoral immortalized human hepatocytes with peripheral blood lymphocytes enhanced T cell survival and proliferation independently of the MHC class II molecules. Interestingly, this effect on the T cell proliferation was mainly cell contact dependent and required the presence of accessory cells since the use of culture cell inserts or the use of pure $CD3^+$ T cells instead of peripheral blood lymphocytes reduced strongly the T cell proliferation (Correia et al., 2009). Here, in non-stimulated conditions, the PBMC-HepaRG cell interactions had no effect on PBMC proliferation and death after two and three days of cultures. A longer time of culture is certainly necessary to observe the effects described by Correia *et al* in which hepatocytes and peripheral blood lymphocytes where co-cultured for seven days (Correia et al., 2009). However, as expected, PHA-stimulation enhanced PBMC proliferation but the presence of HepaRG cells removed the PHA-induced PBMC proliferation without affecting PBMC death. In contrast, the PBMC proliferation induced by PHA was not impacted by the PBMC-skin fibroblast interactions. Hepatocyte and skin fibroblasts have thus different effects on the PBMC response that could be crucial in graft rejection. Indeed, liver transplantations are commonly well tolerated compared to skin graft (Calne et al., 1969; Lerut and Sanchez-Fueyo, 2006). These immunosuppressive effects of hepatocytes on the PBMCs can therefore contribute to the maintenance of immune tolerance in liver.

Cell interactions may also influence cell survival and cell death. Increased survival of peripheral blood lymphocytes has been observed in presence of hepatocytes through the release of soluble factors (Correia et al., 2009). In this study, PBMC viability was lower in PHA-activated PBMC monocultures compared to other conditions. However, this lower percentage of viable cells in PHA-activated PBMC monocultures was certainly offset by the PHA-induced PBMC proliferation. HepaRG cell viability was also impacted by the presence of PHA and/or PBMCs. Indeed, PHA reduced slightly the viability of the HepaRG cells cultured alone whereas the presence of PBMCs increased the frequency of viable HepaRG cells in non-activated co-cultures. Nevertheless, PHA-activated PBMCs decreased the viability HepaRG cells. Therefore, long-term exposure to activated PBMCs could lead to a massive HepaRG cell death and, consequently, a loss of the tolerogenic HepaRG cell activities. This situation probably occurs in chronic liver diseases that are characterized with hepatocyte death, sustained inflammation and the development of fibrosis driving to liver cirrhosis (Yang and Seki, 2015).

Since the PHA-induced PBMC proliferation was inhibited in presence of HepaRG cells, the PBMC/HepaRG cell ratio was higher when the PHA-activated PBMCs were cultured alone compared to other culture conditions. This different cell ratio is an important point to take account for all the analysis of this study.

Because hepatocytes act on immune cell recruitment, activation, proliferation and polarization, the complex and bidirectional hepatocyte-PBMC interplays play certainly a crucial role in the onset of hepatitis but also in liver tolerance. The interactions with other liver resident cells such as hepatic stellate cells, liver endothelial sinusoidal cells or Kupffer cells may also interfere with the PBMC-hepatocyte cross talk. A better understanding of the role of the cell interactions in the hepatic immune response could lead to the identification of new therapeutic targets.

3 The IL-17 and TNFα cooperation enhances the hepatic stellate cell pro-inflammatory response

The IL-17 and TNFα cooperation contributes to the pro-inflammatory response of LX-2 hepatic stellate cells. ***(in preparation)***

Keywords*:* hepatic stellate cells, interleukin-17, tumor necrosis factor-α, inflammation, fibrosis, cell interactions

Abstract

HSCs have a central role in liver fibrosis by producing inflammatory and fibrotic mediators. Their activation is regulated through direct cell-cell interactions but also through systemic and local effects of soluble factors. The role of the pro-inflammatory cytokines IL-17 and TNFα as well as the cell interactions with the hepatocytes on HSC activation were assessed. The human LX-2 HSCs and HepaRG hepatoma cells were exposed to IL-17 and/or TNFα. IL-17 and TNFα contribution was determined in a co-culture model including non- or PHA-activated PBMCs, LX-2 cells and/or HepaRG cells. IL-17 enhanced TNFα effects on the induction of IL-6 and IL-1β pro-inflammatory cytokines and IL-8, CCL20 and MCP-chemokines expression/secretion in LX-2 cell cultures. LX-2 cell-HepaRG cell interactions did not enhance IL-6, IL-8 and CCL20 production compared to HepaRG cells alone. However, LX-2 cell-HepaRG cell interactions increased the mRNA CRP expression. Moreover, IL-17 and/or TNFα had no direct pro-fibrotic effects in collagen I α1, TIMP and MMP2 gene expression whereas MMP3 mRNA levels are up-regulated in LX-2 cells. Nevertheless, IL-17 and TNFα could act indirectly on fibrosis at later time points through the induction of TGFβ. Lastly, IL-17 and TNFα contributed to the strong increase of IL-6 and IL-8 production induced by PBMC, LX-2 cell and/or HepaRG cell interactions. As chronic liver inflammation leads to liver fibrosis, IL-17 and/or TNFα neutralization can of interest to control liver inflammation and therefore its effect on fibrosis.

3.1 Introduction

HSCs are crucial in liver fibrosis by producing inflammatory and fibrotic mediators leading to extracellular matrix deposition. The transdifferentiation of HSCs into activated

myofibroblasts is regulated through direct cell-cell interactions but also through soluble factor exchanges with hepatocytes and immune cells (Barbero-Becerra et al., 2015; Coulouarn et al., 2012; Pellicoro et al., 2014).

The pro-inflammatory cytokine IL-17 and the IL-17-producing cells are associated with several liver diseases where IL-17-secreting cells infiltrated the liver (Beringer and Miossec, 2018). Because HSCs express IL-17 receptors, IL-17 can act directly on HSCs (Meng et al., 2012; Sun et al., 2012). IL-17 stimulation promoted HSC proliferation and enhanced *in vitro* the expression of pro-inflammatory cytokines, chemokines and profibrotic mediators in HSCs (Lemmers et al., 2009; Meng et al., 2012; Shi et al., 2015; Sun et al., 2012; Tan et al., 2013). In addition, IL-17 synergized with TGFβ, the master profibrotic cytokine, to induce the human HSC activation (Fabre et al., 2014). Since IL-17 enhanced the TNFα production by Kupffer cells and the TNFα mRNA expression in HSCs, TNFα can in turn act with IL-17 on local hepatic cells such as HSCs (Hara et al., 2013; Meng et al., 2012). As shown in various cells including hepatocytes or skin and synovial fibroblasts, IL-17 can cooperate with TNFα to up-regulate in synergy some pro-inflammatory genes (Beringer et al., 2016, 2018; Katz et al., 2001; Zrioual et al., 2009). TNFα has various effects on HSCs and appears involved in the HSC activation (Osawa et al., 2013; Pradere et al., 2013; Tarrats et al., 2011; Yang and Seki, 2015).

By their localization, the HSCs are in close contact with hepatocytes and therefore they can interact with each other. The bidirectional exchange of soluble factors between HSCs and hepatocytes were associated with the generation of a pro-inflammatory and pro-fibrotic microenvironment (Coulouarn et al., 2012; Wang et al., 2014). Furthermore, by acting on hepatocytes, IL-17 and TNFα induced in synergy the release of periostin which can in turn activate fibroblast and collagen production (Amara et al., 2015).

The objective of this study was to assess the effects of the IL-17 and TNFα combination on the inflammatory and fibrosis response of the human LX-2 HSC line. Co-cultures between the LX-2 cells and the human hepatoma HepaRG cells were also performed to determine whether cell-cell interactions might amplify the IL-17 and TNFα effects. Finally, the IL-17 and TNFα contribution was determined in a co-culture model including PBMCs, LX-2 cells and/or HepaRG cells. The IL-17 exposure enhanced the TNFα effects on the induction of pro-inflammatory cytokines and chemokines. Moreover, IL-17 and TNFα contributed to the strong increase of IL-6 and IL-8 production induced by the PBMC, LX-2 cell and/or HepaRG cell interactions.

3.2 Materials and methods

Cell lines

The human LX-2 HSCs were cultured in DMEM (Eurobio, Les Ulis, France) supplemented with 2% fetal bovine serum (Life Technologies, Carlsbad, USA), 2 mM L-glutamine (Eurobio) and 50 U/mL penicillin and 50 µg/mL streptomycin (Eurobio). For the monoculture assays, LX-2 cells were serum-starved overnight in DMEM with L-glutamine prior to cytokine exposure in serum-free conditions. The human hepatoma HepaRG cells were grown in William's E medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Eurobio), 5 µg/mL insulin (Sigma), 50 µM hydrocortisone hemisuccinate (Serb, Paris, France), 50 U/mL penicillin and 50 µg/mL streptomycin (Eurobio). HepaRG cells were used after 15 days post-plating.

PBMC isolation

Whole blood samples were obtained from the Etablissement français du Sang. PBMCs were isolated by Ficoll-Hypaque (Eurobio) density gradient centrifugation.

Culture conditions

LX-2 cells were exposed to IL-17A 50 ng/mL (Dendritics, Lyon, France) and/or TNFα 0.5 ng/mL (R&D systems, Minneapolis, MN, USA). To block the IL-6 and TGFβ1 pathways, tocilizumab (Roche, Welwyn, UK) and anti-TGFβ1 (R&D systems) were used at 10 µg/mL. A monoclonal antibody against the BetV1 allergen (Dendritics) was used as a control antibody at the same concentration. For the co-culture assays, cells were maintained in DMEM supplemented with 2% fetal bovine serum (Life Technologies) and 2 mM L-glutamine (Eurobio) at a ratio of 25 PBMCs for 5 HepaRG cells for 1 LX-2 cell. This ratio was chosen according to data from the literature (Barbero-Becerra et al., 2015; Doumba et al., 2013). LX-2 cell-HepaRG cell co-cultures were stimulated or not with the combination of IL-17A 50 ng/mL and TNFα 0.5 ng/mL. For cell culture insert assays, HepaRG cells were cultured at the bottom of a culture plate well and LX-2 cells were placed in Falcon® cell-culture inserts (Corning, NY, USA) with a small-pored membrane (0.4μm) preventing direct cell-cell contacts but allowing the circulation of soluble factors. For the co-cultures with

PBMCs, PBMCs were activated or not with 5 μg/ml phytohemagglutinin (PHA) (Sigma-Aldrich) and exposed to the anti-IL-17 secukinumab (Novartis, Basel, Switzerland) and the anti-TNFα infliximab (Merck, Kenilworth, USA) or the control antibody at 10 μg/mL during 2h before being added to LX-2 cells and/or HepaRG cells.

Quantitative real time-PCR

Total RNA was purified using an RNeasy® Plus Mini kit (Quiagen, Hilden, Germany). cDNA was synthetized using the iScript™ kit (Bio-Rad, Hercules, CA, USA). PCR amplification was performed using the CFX96™ Real time system instrument (Bio-Rad) with the iTaq™ universal SYBR® green supermix (Bio-Rad) and the Qiagen QuantiTect® primers. The expression of the genes of interest was normalized to the expression of the housekeeping GAPDH gene.

Enzyme-linked immunosorbent assays

Supernatant cytokine concentrations were quantified with human ELISA kits according to the instructions of the manufacturers. IL-6 and IL-8 ELISA kits from Diaclone (Besancon, France) and CCL20 ELISA kit from R&D system (Minneapolis, USA) were used.

Statistical analysis

Calculations were performed with GraphPad Prism version 5.01 software. Data are the mean of at least 3 independent experiments ± SEM. Statistical differences were analyzed using the Mann Whitney test. P values less than to 0.05 were considered significant.

3.3 Results

IL-17 amplified the TNFα effect on the induction IL-6 and IL-1β in the LX-2 cells

IL-17 can cooperate with TNFα to increase in synergy the IL-6 secretion in various cell types including hepatocytes, endothelial cells or skin/synovial fibroblasts (Beringer et al., 2018; Hot et al., 2012; Katz et al., 2001). The effects of IL-17 and TNFα were thus investigated in the human LX-2 HSC cultures. IL-17 alone had no or a limited effect on the mRNA expression of IL-6 ($p<0.05$) and IL-1β as well as IL-6 secretion compared to control condition. In contrast, the LX-2 cells stimulation with TNFα alone enhanced strongly the IL-6 and IL-1β mRNA

levels and the IL-6 supernatant level ($p<0.05$). Interestingly, addition of IL-17 to the TNFα induced a further increase of the IL-6 and IL-1β mRNA expression and the IL-6 production versus TNFα alone ($p<0.05$) (Figures 13A-C). Therefore, IL-17 amplified the TNFα effect on the IL-6 and IL-1β induction in the LX-2 cell cultures. Because the LX-2 cells have functional IL-6 and TGFβ receptors (Fabre et al., 2014; Schmidt-Arras and Rose-John, 2016; Schoenherr et al., 2010), a part of the IL-17 and TNFα effects could be mediated indirectly through the HSC autocrine IL-6 and TGFβ secretion. Indeed, an IL-6 positive-feedback loop effect has been described in the human hepatocyte HepaRG cells (Beringer et al., 2018). Here, the blockade of the IL-6 and TGFβ pathways by the use of specific inhibitors had no effect on the induction of IL-6 expression and production whereas the blockade of the IL-6 pathway, but not the TGFβ pathway, reduced by 18% the IL-1β up-regulation induced by IL-17 and TNFα (Figures 13A-C). The induction of IL-6 and IL-1β following the IL-17 and TNFα stimulation is therefore mainly independent of the IL-6 and TGFβ pathway activation.

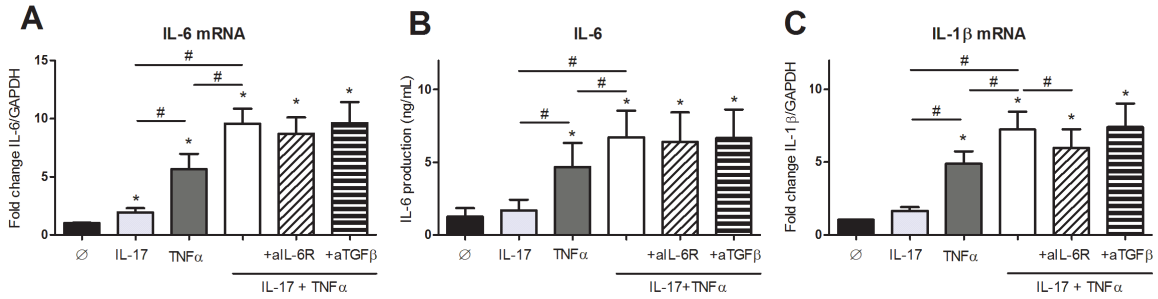

FIGURE 13: IL-17 amplifies the TNFα effect on the induction of IL-6 and IL-1β in the LX-2 cells mainly independently of IL-6 and TGFβ pathways

LX-2 cells were exposed to IL-17 and/or TNFα with/without anti-IL-6R (aIL-6R) or anti-TGFβ (aTGFβ). (A, C) IL-6 and IL-1β mRNA expression at 10h were expressed as fold changes compared to control. (B) IL-6 supernatant level at 48h was quantified by ELISA. The control antibody has no effect on the IL-6 supernatant level following IL-17 and TNFα stimulation (data not shown). Data are the mean of 6 independent experiments ± SEM; Mann Whitney test, *$p<0.05$ versus control, #$p<0.05$ versus other cytokine conditions.

IL-17 amplifies the TNFα effect on the increase of the IL-8, CCL20 and MCP-1 chemokine expression and/or secretion

As immune cell recruitment is crucial for the inflammatory response, the effects of IL-17 and TNFα alone or in combination on the IL-8, CCL20 and MCP-1 (also known as CCL2) chemokines were next investigated in the LX-2 cells. IL-8 is associated with neutrophil recruitment involved in the acute phase response whereas CCL20 and MCP-1 attract mononuclear cells involved in the chronicity of the inflammatory response. TNFα treatment induced the IL-8, CCL20 and MCP-1 mRNA expression by 6.8-, 17- and 9.9-fold, respectively ($p<0.05$) whereas IL-17 had a modest effect on these chemokine mRNA levels. As for IL-6 and IL-1β, IL-17 amplified the TNFα effect on the IL-8, CCL20 and MCP-1 mRNA levels with an increase of 18-, 38.6- and 22-fold, respectively, compared to control condition ($p<0.05$). The higher IL-8 and CCL20 supernatant levels following the IL-17 and TNFα stimulation compared to TNFα alone ($p<0.05$) confirmed that IL-17 potentiated the TNFα effects on the increase of chemokine expression and/or production (Figures 14D, E). In addition, IL-8 induction following IL-17/TNFα was independent of IL-6 and TGFβ pathways since specific inhibitors of IL-6 and TGFβ pathways had no effect on the IL-8 levels induced by IL-17/TNFα (Figures 14A, D). In contrast, the MCP-1 mRNA expression and CCL20 release but not the CCL20 mRNA expression was inhibited by 38% and 26%, respectively ($p<0.05$), by the neutralization of the IL-6 pathway whereas the TGFβ pathway blockade had no effect (Figure 14B, C, E). This IL-17 and TNFα cooperation on the chemokine induction can intensify the local immune cell recruitment mainly through pathways independent of IL-6 and TGFβ.

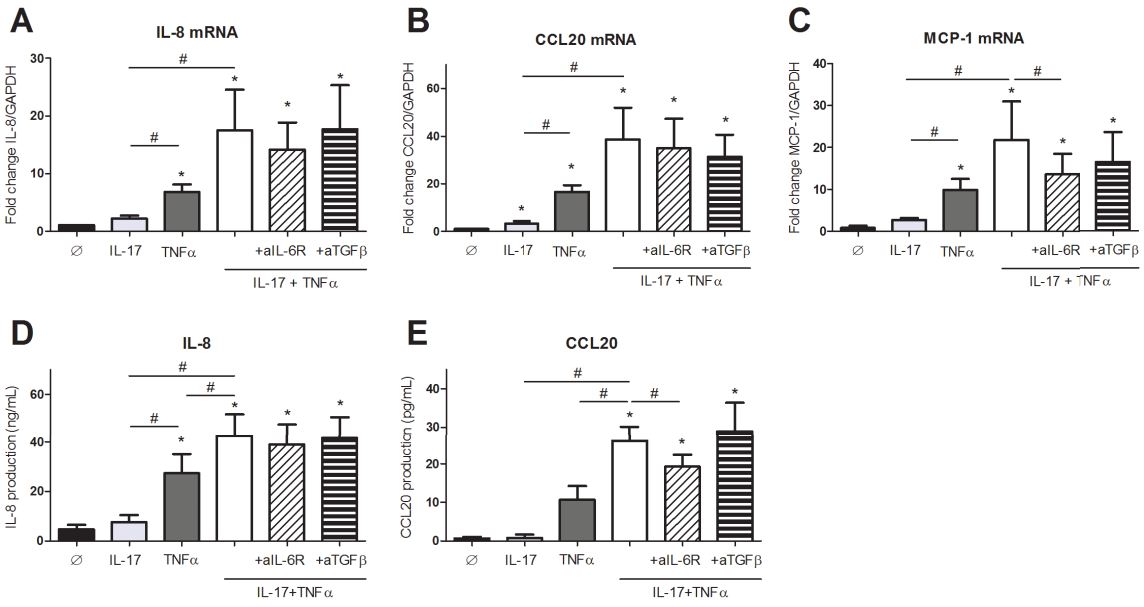

FIGURE 14: IL-17 amplifies the TNFα effect on the increase of the IL-8, CCL20 and MCP-1 chemokine expression and/or secretion

LX-2 cells were exposed to IL-17 and/or TNFα with/without anti-IL-6R (aIL-6R) or anti-TGFβ (aTGFβ). (A-C) IL-8, CCL20 and MCP-1 mRNA expression at 10h were expressed as fold changes compared to control. (D, E) IL-8 and CCL20 supernatant levels at 48h were quantified by ELISA. The control antibody has no effect on the IL-8 and CCL20 supernatant level following the IL-17 and TNFα stimulation (data not shown). Data are the mean of 6 independent experiments ± SEM; Mann Whitney test, *$p<0.05$ versus control, #$p<0.05$ versus other cytokine conditions.

IL-17 and TNFα have no direct effects on pro-fibrotic genes

HSCs have a crucial role in liver fibrosis. Indeed, they can induce fibrogenesis by producing extracellular matrix components such as collagen I. Moreover, they can modulate fibrolysis through the secretion of MMPs and TIMP. The MMP/TIMP balance defects leads to extracellular matrix accumulation. IL-17 and TNFα stimulation increased mRNA expression of the master pro-fibrotic cytokine TGFβ1 by 1.9-fold (non-significant) in LX-2 cells (Figure 15A). Moreover, IL-17 and/or TNFα stimulation had no effect on mRNA levels of collagen I α1, TIMP1 and MMP2 (Figures 15B-D). In contrast, MMP3 mRNA expression was enhanced in presence of the IL-17 and TNFα combination ($p<0.05$) (Figure 15E). IL-6 and TGFβ pathways were not involved in the effects mediated by IL-17 and TNFα since the inhibition of IL-6 or TGFβ pathway had no effect on the gene expression levels in IL-17 and/or TNFα conditions. Therefore, IL-17 and TNFα have no direct pro-fibrotic effects on LX-2 cells.

However, by enhancing MMP3, IL-17 and TNFα could reduce or/and prevent extracellular matrix deposition.

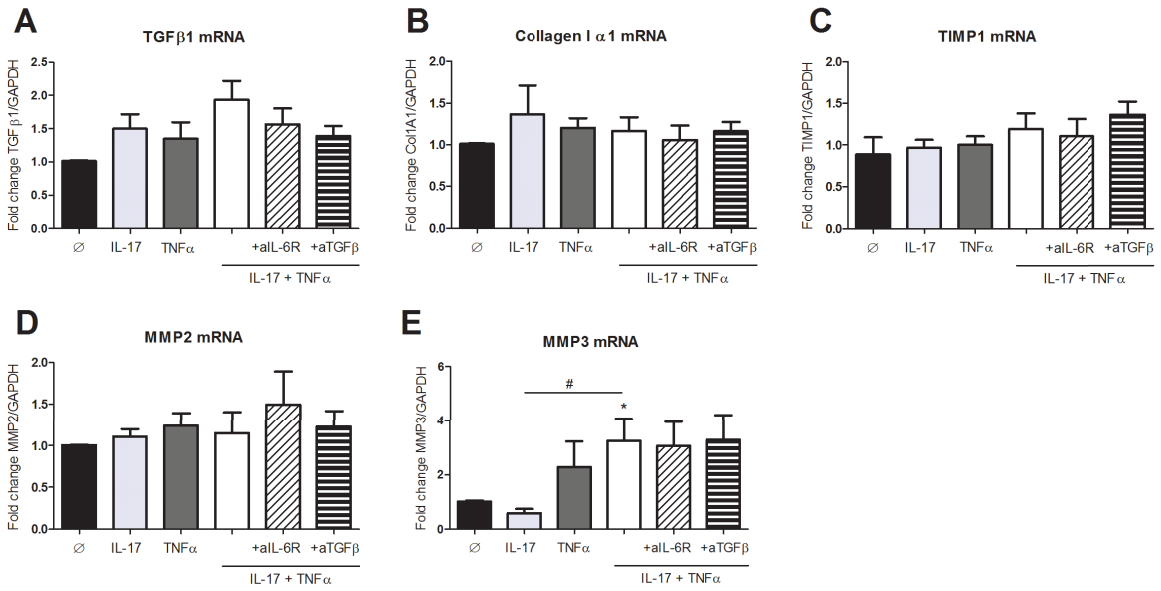

FIGURE 15: IL-17 and TNFα have no direct effects on profibrotic genes
LX-2 cells were exposed to IL-17 and/or TNFα with/without anti-IL-6R or anti-TGFβ. (A-E) TGFβ1 mRNA expression at 10h and collagen I α1, TIMP1, MMP2 and MMP3 mRNA expression at 48h were expressed as fold changes compared to control. Data are the mean of 6 independent experiments ± SEM; Mann Whitney test; #p<0.05 versus other cytokine conditions.

The LX-2 cell-HepaRG cell interactions induce the CRP expression but not the IL-6, IL-8 and CCL20 secretion by HepaRG cells

By their localization, the HSCs and hepatocytes interact together through direct cell contact and paracrine interactions. A bidirectional cross talk between hepatocytes and HSCs has been previously described (Coulouarn et al., 2012; Sancho-Bru et al., 2010; Wang et al., 2014). Here, the effects of the interactions between the human LX-2 HSCs and the HepaRG cells on several pro-inflammatory mediators were studied in non-activated and IL-17/TNFα-activated cultures (Figure 16A). In non-activated conditions, production of IL-6 was slightly enhanced by 1.5-fold in the LX-2 cell-HepaRG cell co-cultures versus the LX-2 cell monocultures ($p<0.05$) (Figure 16A). IL-8 and CCL20 secretion was also higher in the HepaRG cell monocultures and co-cultures compared to LX-2 cells alone ($p<0.05$) (Figures 16C, D). The IL-17 and TNFα stimulation increased the IL-6, IL-8 and CCL20 release in all culture conditions. However, IL-8 and CCL20 supernatant levels in LX-2 monocultures were too low

compared to the levels in HepaRG cell monocultures or co-cultures to observe an induction. IL-8 and CCL20 supernatant concentrations were similar between HepaRG cells alone and the co-cultures; indicating that IL-8 and CCL20 were secreted by the HepaRG cells in co-cultures (Figures 16D, E). In contrast, even if the LX-2 cells were five times less numerous than HepaRG cells, the IL-6 supernatant levels were quite similar between the monocultures of LX-2 cells (9.6 ng/mL) and HepaRG cells (13 ng/mL). Nevertheless, there were no additive effects of the two cell types on the IL-6 production in co-cultures (Figure 16B). However, during the 48h of culture, the LX-2 cell proliferation was certainly higher in monocultures compared to co-cultures whereas the HepaRG cells were not in a proliferation state but in a differentiation state. The use of cell culture inserts, to avoid direct cell contacts but allowing soluble factor exchanges, reduced significantly CCL20 secretion in IL-17/TNFα-activated cultures by 34% but not IL-6 and IL-8 supernatant levels compared to conventional co-cultures (Figures 16B, D, E). mRNA level of the C-reactive protein (CRP), an acute-phase protein commonly induced by IL-6, was also quantified in LX-2 cells and HepaRG cells cultured separately or in co-cultures. LX-2 cell-HepaRG cell interactions up-regulated CRP expression ($p<0.05$) and this effect was further increased in IL-17/TNFα-stimulated conditions (Figure 16C). Therefore, the LX-2 cell-HepaRG cell interactions may have an important role in the CRP induction whereas HepaRG cells but not the LX-2 cells have a major contribution in the chemokine induction in co-cultures exposed to IL-17/TNFα.

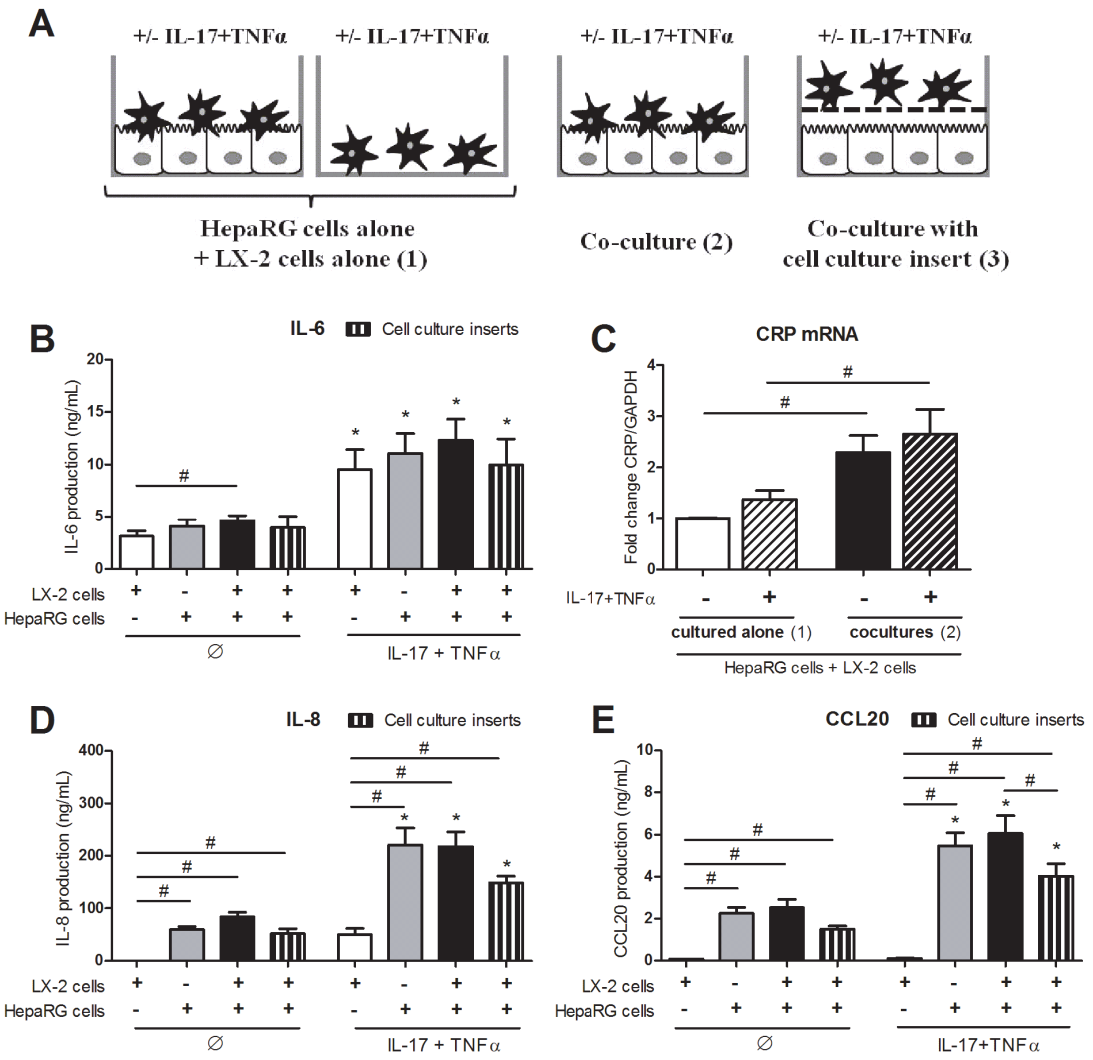

FIGURE 16: LX-2 cell-HepaRG cell interactions enhance CRP expression but not the IL-6, IL-8 and CCL20 secretion by HepaRG cells

LX-2 cells and HepaRG cells were cultured alone or in co-cultures with/without cell culture inserts at a ratio of 5 HepaRG cells : 1 LX-2 cell in presence or not of IL-17 and TNFα (A, C, D) IL-6, IL-8 and CCL20 supernatant levels at 48h was quantified by ELISA. (B) CRP mRNA expression at 48h were expressed as fold changes compared to control. Data are the mean of 6 to 8 independent experiments ± SEM; Mann Whitney test, *p<0.05 versus conditions without cytokines, #p<0.05 other cell compositions of the cultures.

LX-2 cell-HepaRG cell interactions modulate the pro-fibrotic genes expression in IL-17 and TNFα activated conditions

The effects of LX-2 cell–HepaRG cell interactions on fibrotic genes were next assessed with or without IL-17 and TNFα stimulation. Compared to LX-2 cells and HepaRG cells cultured separately, collagen type I α1mRNA expression decreased slightly but no significantly

whereas TIMP1 mRNA levels increased ($p<0.05$) in the LX-2 cell–HepaRG cell co-cultures stimulated with IL-17/TNFα compared to other conditions (Figures 17A, B). MMP2 mRNA levels were similar between the different culture conditions (Figure 17C). As shown in Figure 15E, IL-17 and TNFα exposure increased MMP3 mRNA expression (1.9-fold, non-significant) and this effect was further higher in co-cultures (3.3-fold, non-significant) (Figure 17D). The interplays between HepaRG cells and LX-2 cells may therefore modulate the effects mediated by IL-17 and TNFα on the fibrotic genes.

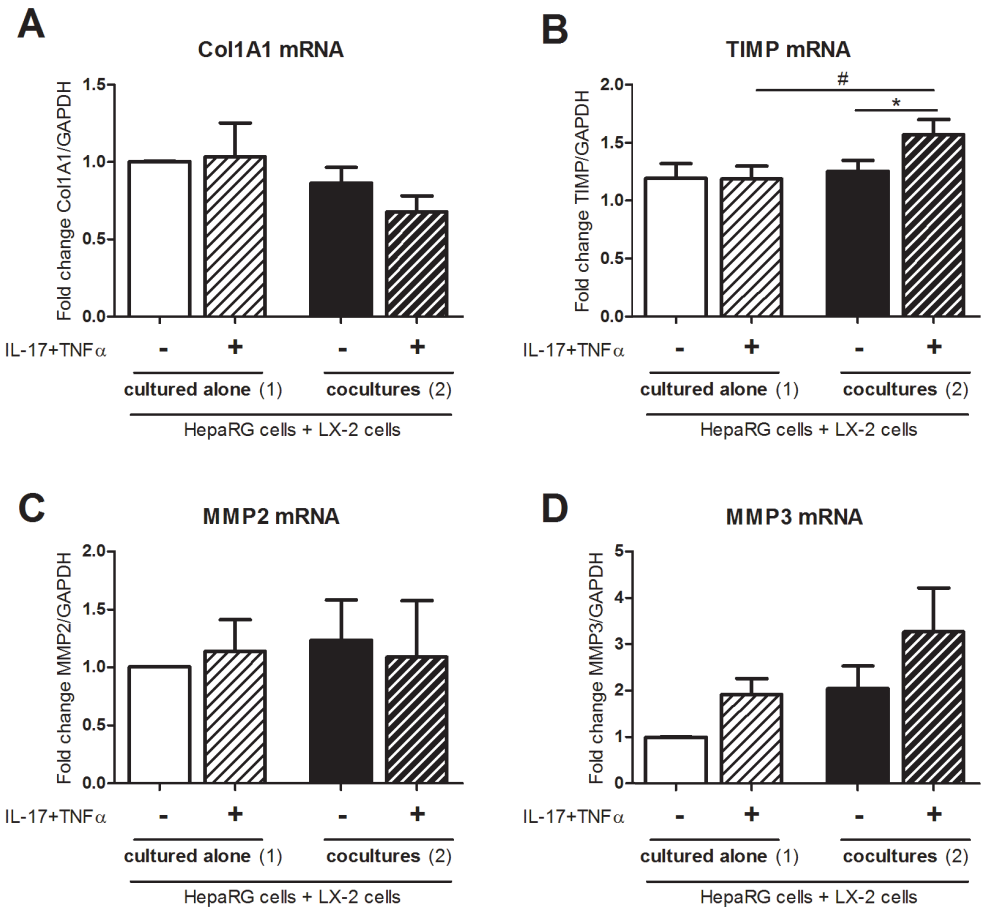

FIGURE 17: LX-2 cell-HepaRG cell interactions modulated Collagen I α1, TIMP and MMP3 expression in IL-17 and TNFα activated conditions

LX-2 cells and HepaRG cells were cultured alone or in co-cultures at a ratio of 5 HepaRG cells : 1 LX-2 cell in presence or not of IL-17 and TNFα. (A-C) Collagen I α1, TIMP1 and MMP3 mRNA expression at 48h were expressed as fold changes compared to control. Data are the mean of 6 independent experiments ± SEM; Mann Whitney test, *$p<0.05$ versus conditions without cytokines, #$p<0.05$ versus co-cultures.

IL-17 and TNFα contribute to the induction of IL-6 and IL-8 production in PBMC-LX-2 cell-HepaRG cell co-cultures in activated conditions

Because inflammatory infiltrates are often observed in liver biopsies of patients with active liver diseases (Quintin et al., 2010), the potential contribution of IL-17 and TNFα was studied in a co-culture model comprising non- or PHA-activated PBMCs, LX-2 cells and/or HepaRG cells. Indeed, inflammation is a dynamic process where the recruited leukocytes interact with parenchymal cells and resident cells inside tissue. Compared to the co-cultures, a low level of IL-6, IL-8 and CCL20 was detected in PBMCs alone as well as in LX-2 cell and HepaRG cell monocultures in presence or not of PHA (Figure 18A-D and data not shown). Interestingly, IL-6 secretion was higher in PBMC-LX-2 cell co-cultures compared to PBMC-HepaRG cell and PBMC-LX-2 cell-HepaRG cell co-cultures in both non- and PHA-activated conditions ($p<0.05$) (Figure 18A). In contrast, the IL-8 supernatant levels were more similar between the different co-cultures (Figure 18B). As seen in LX-2 cell-HepaRG cell co-cultures (Figure 16D), the release of CCL20 was strongly associated with the presence of HepaRG cells since the CCL20 supernatant levels were very low in PBMC-LX-2 cell co-cultures compared to the other co-cultures with HepaRG cells ($p<0.05$) (Figure 18D). PHA activation increased the IL-6 by 1.4- to 1.7-fold in all co-cultures conditions ($p<0.05$) whereas no effect or a slight effect (<1.2-fold) of PHA stimulation was observed for the IL-8 and CCL20 release. Neutralization of IL-17 plus TNFα by the use of specific inhibitors reduced IL-6 production in both non- and PHA-activated co-cultures by 26 to 42% ($p<0.05$) (Figure 18A). IL-8 secretion was also inhibited by the blockade of both IL-17 and TNFα by up to 32% in PHA-activated co-cultures ($p<0.05$) (Figure 18B). IL-17 and TNFα contribution in the CCL20 release was weak in the PBMC-LX-2 cell-HepaRG cell co-cultures (2.2% and 3.0% in non- and PHA-activated condition respectively, $p<0.05$) and was not observed in all the other co-culture conditions (Figure 18D). IL-17 and TNFα were thus involved in the IL-6 and IL-8 secretion induced by the interactions between PBMCs, LX-2 cells and/or HepaRG cells.

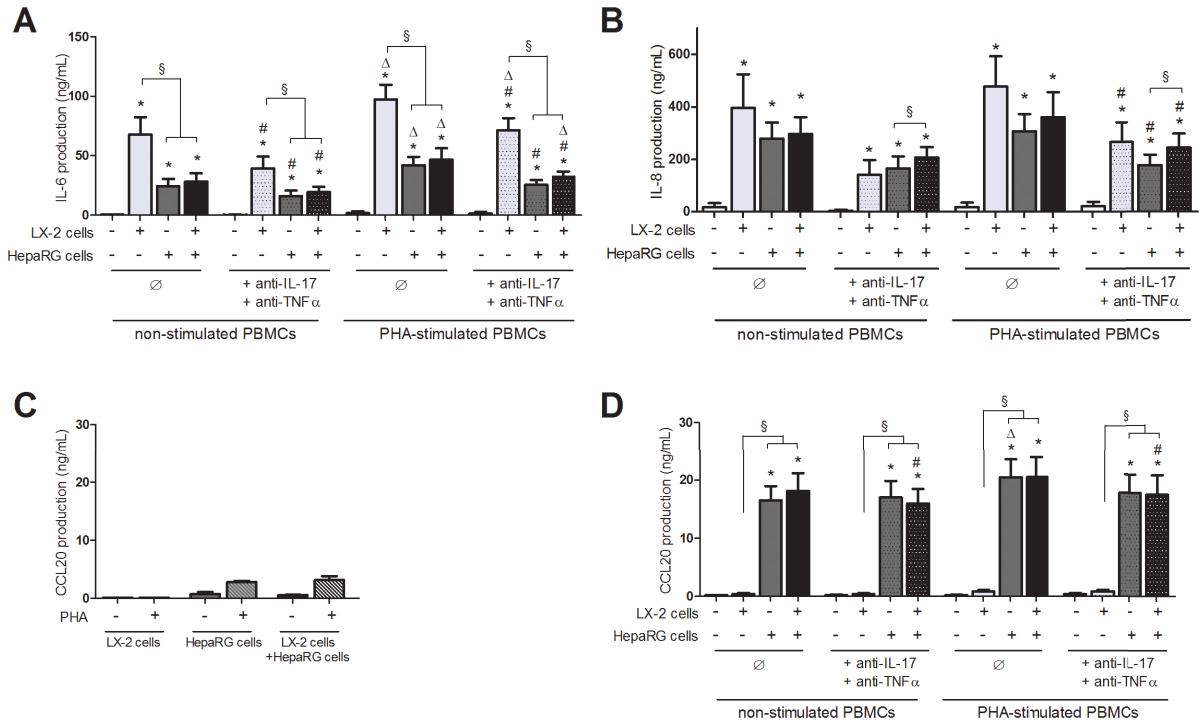

FIGURE 18: IL-17 and TNFα contribute to the induction of IL-6, IL-8 and CCL20 production in LX-2 cell-HepaRG cell-PBMC co-cultures in PHA-activated conditions
PBMCs were stimulated or not with phytohaemagglutinin (PHA) and exposed or not to the anti-IL-17 (aIL-17a) and the anti-TNFα (aTNFα) or control antibody during 2h before being added to the LX-2 cells and/or HepaRG cells at a ratio of 25 PBMCs : 5 HepaRG cells : 1 LX-2 cell. (A-D) IL-6, IL-8 and CCL20 supernatant levels were quantified by ELISA after 48h of cultures. IL-6 and IL-8 were no detected in LX-2 cell and/or HepaRG cell cultures without PBMCs. The control antibody had no effect on the IL-6, IL-8 and CCL20 supernatant levels. Data are the mean of 6 independent experiments ± SEM; Mann Whitney test, *$p<0.05$ versus PBMC alone, §$p<0.05$ versus LX-2 cell-PBMC co-cultures, #$p<0.05$ versus without anti-IL-17 and anti-TNFα, Δ$p<0.05$ versus non-activated conditions.

3.4 Discussion

This study shows that IL-17 amplified the TNFα response on the induction of systemic inflammatory cytokines and chemokines without direct pro-fibrotic effects. LX-2 cell and HepaRG cell interactions had no significant effect on the level of IL-6 secretion following IL-17 and TNFα stimulation but they enhanced the CRP expression. The HepaRG cells had certainly a major contribution in the IL-8 and CCL20 secretions in LX-2 cell – HepaRG cell co-cultures. PBMC, LX-2 cell and/or HepaRG cell interactions induced a strong secretion of IL-6, IL-8 and CCL20. IL-17 and TNFα participated to the increased production of IL-6 and IL-8 in co-cultures.

Chronic liver diseases are characterized by sustained liver inflammation that leads to fibrosis where HSCs play a central role by producing several pro-inflammatory mediators and extracellular matrix. Since HSCs expressed IL-17 receptor (Meng et al., 2012; Sun et al., 2012), the effect of IL-17 on HSCs was therefore studied. IL-17 has been implicated in the HSC proliferation and activation (Hara et al., 2013; Meng et al., 2012; Shi et al., 2015; Sun et al., 2012). IL-17 was also shown to induce the expression and/or production of the systemic inflammatory cytokines IL-6 and IL-1β as well as IL-8 and growth-related oncogene-α involved in neutrophil recruitment in HSCs (Hara et al., 2013; Meng et al., 2012; Shi et al., 2015; Sun et al., 2012; Tan et al., 2013). Here, IL-17 alone had a weak or no significant effect on the induction of IL-6, IL-1β and IL-8 mRNA expression and/or secretion in LX-2 cell cultures compared to the TNFα stimulation. However, IL-17 potentiated the TNFα effects on the IL-6, IL-1β and IL-8 induction. This IL-17 and TNFα cooperation enhanced also the expression of CCL20 and MCP-1 chemokines in LX-2 cells. By inducing CCL20 and MCP-1 which attract T cells, monocytes and dendritic cells, IL-17 and TNFα participate probably to the chronicity of the inflammation in addition to the early response mediated by the induction of IL-8 associated with neutrophil recruitment. Since IL-17 up-regulated IL-6 and TGFβ expression in HSCs (Meng et al., 2012; Sun et al., 2012) and HSCs have functional IL-6 and TGFβ receptors (Fabre et al., 2014; Schmidt-Arras and Rose-John, 2016; Schoenherr et al., 2010), the contribution of these two pathways in the IL-17 and TNFα-mediated effects was assessed. The IL-6 and TGFβ pathways were not involved in the IL-17 and TNFα effects of the pro-inflammatory mediators studied with the exception of the IL-1β and MCP-1 mRNA expression and the CCL20 release that were slightly inhibited by the use of the IL-6 pathway inhibitor in the LX-2 cell cultures. This suggests a main direct effect of IL-17 and TNFα on the induction of the pro-inflammatory mediators studied here. As IL-17 may enhance the local TNFα production in liver (Hara et al., 2013; Meng et al., 2012), TNFα can in turn act in cooperation with IL-17 on HSCs and amplify the inflammatory process by promoting the systemic inflammatory cytokine production and the recruitment of immune cells in liver.

Since inflammation is important for the initiation of liver fibrosis, pro-inflammatory cytokines may promote the pro-fibrotic activities of the HSCs. Because IL-17 up-regulated the expression of the major pro-fibrotic cytokine TGFβ1 as well as collagen type I α1 in HSCs, IL-17 may induce fibrogenesis (Meng et al., 2012; Tan et al., 2013). Another study showed that the effect of IL-17 alone on collagen type I α1 and TIMP1 expression was very weak or

absent compared to TGFβ stimulation. However, IL-17 enhanced the TGFβ response of HSCs by up-regulating its receptor expression at the HSC surface (Fabre et al., 2014). The pro-fibrotic activities of IL-17 can therefore be mediated via the intensification of the TGFβ pathway activation. TNFα was previously associated with matrix degradation through the induction of MMPs. Expressions of MMP1, MMP3 and MMP9 were up-regulated in LX-2 cells by the TNFα stimulation whereas the collagen type I α1, TIMP1 and MMP2 expressions were unchanged (Robert et al., 2016; Tarrats et al., 2011). This matrix breakdown mediated by TNFα could be essential at the early stage of liver injury for the recruitment of inflammatory immune cells and later, TGFβ could decrease the initial TNFα-mediated MMP induction activity leading to the development of liver fibrosis (Knittel et al., 1999). Here, IL-17 and/or TNFα had no effect on the collagen I α1, TIMP1 and MMP2 mRNA expressions in LX-2 cells. However, as expected, the presence of TNFα enhanced MMP3 expression, this could prevent the extracellular matrix accumulation. IL-17 and/or TNFα have thereby no direct pro-fibrotic activities on HSCs. Nevertheless, by inducing the TGFβ1 secretion by Kupffer cells (Hara et al., 2013), IL-17 may have indirect pro-fibrotic effects which can be amplify by the IL-17 and TGFβ cooperation described by Fabre *et al*. (Fabre et al., 2014). The potential indirect effects mediated by TGFβ may occur at later time points and therefore they were no observed in this study.

Hepatocytes are the most abundant cell population in liver. They can modulate the HSC response and vice versa through direct cell-cell and paracrine interactions (Barbero-Becerra et al., 2015; Coulouarn et al., 2012; Ma et al., 2015; Wang et al., 2014). Non-contact LX-2 cell and HepaRG cell co-cultures have demonstrated a bidirectional crosstalk between these two cell types. Indeed, the presence of LX-2 cells up-regulated the gene expressions of IL-6, IL-1β, IL-8, CCL20 and MCP-1 as well as the acute-phase protein serum amyloid A in HepaRG cells (Coulouarn et al., 2012). Here, the LX-2 cell–HepaRG cell co-cultures with and without culture cell inserts did not enhance the secretion of IL-6, IL-8 and CCL20 compared to HepaRG cell cultured alone in both non- and IL-17/TNFα-activated conditions. However, the CRP mRNA expression was up-regulated by the LX-2 cell–HepaRG cell interactions. By this way, the LX-2 cell–HepaRG cell interactions can promote systemic inflammation.

Hepatocytes can also alter the HSC fibrotic activities since the LX-2 cells exposure to the conditioned medium from human hepatocellular carcinoma cells increased HSC proliferation as well as the gene expression levels of collagen I and TIMP (Ma et al., 2015). In this study,

the expression of these two genes was unchanged by the LX-2 cell – HepaRG cell interactions in non-activated conditions. Therefore, the hepatocyte cell line used but more likely the direct cell-cell interactions play certainly an important role. In contrast to non-stimulated conditions, the expression of the fibrotic gene TIMP was enhanced when the two cell types were cultured together in IL-17/TNFα activated conditions. Indeed, by inducing the release of soluble factors such as periostin by hepatocytes, IL-17 and TNFα can promote indirectly fibroblast activation and fibrosis (Amara et al., 2015). However, MMP3 expression was also up-regulated by the presence of IL-17 and TNFα in LX-2 cell-HepaRG cell co-cultures and could therefore counterbalance partially the IL-17/TNFα pro-fibrotic effects.

By secreting chemokines, activated HSCs and hepatocytes recruit immune cells in liver contributing to sustained inflammation and, if not resolved, chronic liver diseases. Therefore, complex interactions between the infiltrated immune cells and the hepatic cells such as HSCs and hepatocytes occur during chronic inflammation in liver. These interplays between the different cell types are mediated through direct cell-cell interactions and soluble factor exchanges. By this way, IL-17 and TNFα can participate to the cell-to-cell communication in liver. Indeed, HSCs exposed to conditioned medium from activated PBMCs increased the growth-related oncogene-α secretion by HSCs and IL-17 contributed to this effect (Lemmers et al., 2009). IL-17-activated monocytes and peripheral blood lymphocytes promoted also HSC activation *in vitro* (Sun et al., 2012). Moreover, co-cultures of human HSCs and mucosal-associated invariant T cells enhanced the expression of the pro-inflammatory genes IL-6, IL-1β, IL-8 and MCP-1 as well as the pro-fibrotic gene TIMP1 via direct cell-cell contact and also partially through IL-17 (Böttcher et al., 2018). TNFα-released from macrophages was also a key factor of HSC activation since the blockage of TNFα decreased mRNA levels of fibrosis markers in HSCs co-cultured with $CCR9^{+}$ macrophages (Chu et al., 2013). In addition, TNFα contributed to the increase of HSC survival induced by the HSC–hepatic macrophage interactions via the activation of NF-κB signaling pathways (Pradere et al., 2013). Here, PBMCs co-cultured with LX-2 cells and/or HepaRG cells induced a strong synthesis of IL-6, IL-8 and CCL20 compared to monocultures. Interestingly, the presence of HepaRG cells appears to reduce the IL-6 secretion observed in PBMC-LX-2 cell co-cultures. By this way, the HepaRG cells may reduce the liver inflammation. In contrast, the PBMC-HepaRG cell interplays and not the PBMC-LX-2 cell interactions induced a strong production. Because IL-17 and TNFα were involved in the induction of IL-6 and IL-8 release

in non- and/or activated co-cultures, IL-17 and TNFα contribute most probably to the perpetuation of the chronic inflammatory state in liver. Since sustained liver inflammation drive to liver fibrosis, neutralization of IL-17 and/or TNFα at the early phase of the liver injury can be a promising therapeutic strategy to control liver inflammation and therefore to prevent fibrosis.

4 IL-17 and TNFα may impair muscle function by acting on myoblast inflammatory reponse and the SOCE calcium pathway in myoblats

Beringer A & Gouriou Y, Lavocat F, Ovize M, and Miossec P. Blockade of store-operated calcium entry reduces cytokine-induced inflammatory response in human myoblasts. ***(In review)***

4.1 Background and Objective

IIMs (or myositis) are chronic muscle diseases characterized by muscle inflammation and dysfunction. Immune cell infiltrates in muscles are observed in IIM patients. Emerging evidences suggest that both immune and non-immune processes contribute IIM pathogenesis. Elevated levels of IL-17 and TNFα are found in IIM muscle samples and their *in vitro* effects on muscle cells suggest that these cytokines contribute to myositis pathogenesis (Chevrel et al., 2003, 2005; De Bleecker et al., 1999; Kuru et al., 2000; Page et al., 2004). Non-immune processes including ER stress, mitochondria dysfunction or Ca^{2+} dysregulation have been observed in the skeletal muscles of IIM patients (Coley et al., 2012; Henriques-Pons and Nagaraju, 2009; Rayavarapu et al., 2013). SOCE is a major pathway of Ca^{2+} and its overexpression was associated with muscular dystrophy (Edwards et al., 2010). SOCE is activated by Ca^{2+} depletion from the ER that triggers STIM1 translocation to Orai leading to store-operated Ca^{2+} channel opening (Figure 19).

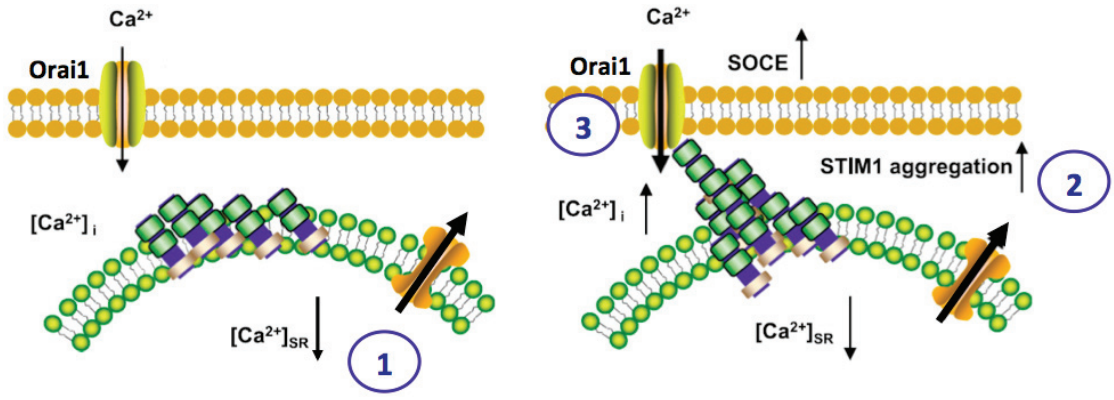

FIGURE 19: Store-operated calcium entry process
The increase of endoplasmic reticulum Ca^{2+} store depletion (1) enhances STIM1 aggregation (2) leading to store-operated channels Ca^{2+} opening (3) and Ca^{2+} entry in cells. Reprinted with the permission of the American Thoracic Society. Copyright© 2018 American Thoracic Society. Jia et al., 2013 - DOI: 10.1165/rcmb.2013-0040OC. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.

Objective: To determine the effects of IL-17 and TNFα on the inflammatory response and SOCE in human myoblasts.

4.2 Results

IL-17 and TNFα increase in synergy IL-6 and CCL20 production by myoblasts. As IL-6 and CCL20 induce Th17 cell differentiation and recruitment respectively, IL-17 and TNFα can contribute to the local Th17 cell induction.

IL-17 and TNFα promote ER stress and mitochondrial ROS stress in myoblasts. Indeed, the IL-17/TNFα combination increases significantly the expression of Grp78 protein, an ER stress marker. Moreover, ROS level in myoblasts is enhanced by IL-17 and/or TNFα.

PBMC-myoblast interaction induces a strong IL-6 and CCL20 release by comparison to myoblasts alone or PBMCs alone. PBMC activation with PHA is not required in these inductions. The use of cell culture inserts, allowing the soluble factor circulation but not the direct cell-cell contacts, reduces strongly the CCL20 secretion whereas the IL-6 production is not affected. Neutralization of TNFα but not IL-17 inhibits the IL-6 and CCL20 release in co-cultures with PBMCs preincubated with PHA. Therefore, the induction of IL-6 production in co-cultures is mainly mediated through soluble factor exchanges between PBMCs and myoblasts, and TNFα contribute to this induction.

IL-17 and TNFα increase SOCE. IL-17 and TNFα up-regulate STIM1 protein expression but not the STIM1 mRNA expression. The induction of STIM1 appears thus to occur at a post-transcriptional level. IL-17 and/or TNFα exposure increase significantly SOCE.

Inhibition of SOCE reduces the IL-6 production induced by IL-17 and TNFα. Two SOCE inhibitors are used to confirm this effect. SOCE interacts therefore with immune processes.

4.3 Conclusion

IL-17 and TNFα play an important role in myoblast inflammatory response by inducing the secretion of pro-inflammatory cytokines. IL-17 and TNFα promote also ER stress, ROS production and SOCE whereas SOCE inhibition reduces the secretion of IL-6 induced by IL-

17/TNFα. Therefore, the immune processes and the calcium dysregulation interplay. Neutralization of IL-17 and/or TNFα may be a promising therapeutic strategy to control both immune and non-immune pathological mechanisms in IIMs.

REVISION

BLOCKADE OF STORE-OPERATED CALCIUM ENTRY REDUCES IL-17/TNF CYTOKINE-INDUCED INFLAMMATORY RESPONSE IN HUMAN MYOBLASTS

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Abstract

Muscle inflammation as in idiopathic inflammatory myopathies (IIM) leads to muscle weakness, mononuclear cell infiltration and myofiber dysfunction affecting calcium channels. The effects of interleukin-17A (IL-17) and tumor necrosis factor-α (TNFα) on inflammation and calcium changes were investigated in human myoblasts. Human myoblasts were exposed to IL-17 and/or TNFα with/without store-operated Ca^{2+} entry (SOCE) inhibitors (2-ABP or BTP2). For co-cultures, peripheral blood mononuclear cells (PBMC) from healthy donors activated or not with phytohemagglutinin (PHA) were added to myoblasts at a 5:1 ratio.

IL-17 and TNFα induced in synergy CCL20 and IL-6 production by myoblasts (>14-fold). PBMC-myoblast co-cultures enhanced CCL20 and IL-6 production in the presence or not of PHA compared to PBMC or myoblast monocultures. Anti-IL-17 and/or anti-TNFα decreased the production of IL-6 in co-cultures ($p<0.05$). Transwell system that prevents direct cell-cell contact reduced CCL20 ($p<0.01$) but not IL-6 secretion. IL-17 and/or TNFα increased the level of the ER stress marker Grp78, mitochondrial ROS and promoted SOCE activation by 2-fold ($p<0.01$) in isolated myoblasts. SOCE inhibitors reduced the IL-6 production induced by IL-17/TNFα. Therefore, muscle inflammation induced by IL-17 and/or TNFα may increase muscle cell dysfunction, which, in turn, increased inflammation. Such close interplay between immune and non-immune mechanisms may drive and increase muscle inflammation and weakness.

Keywords: inflammatory myopathies, interleukin-17, tumor necrosis factor-α, store-operated calcium entry, myoblasts

Introduction

Idiopathic Inflammatory Myopathies (IIM) such as dermatomyositis and polymyositis are chronic muscle diseases characterized by muscle inflammation, skeletal muscle weakness and early sarcopenia. Calcium (Ca^{2+}) dysregulation contributes to muscle cell dysfunction with effects on contractibility (1). Store-operate calcium entry (SOCE) is a major pathway for Ca^{2+}. SOCE is activated by Ca^{2+} depletion from the endoplasmic reticulum (ER) that triggers the store-operated calcium channels (SOCs) opening through stromal-interacting molecule (STIM1) translocation to Orai. SOCE overexpression was associated with muscular dystrophy (2,3).

IIM are characterized by inflammatory / immune cell infiltration (4,5), which contributes to muscle inflammation and dysfunction. Among the local secreted cytokines, TNFα was found upregulated in IIM samples (6–8). Interleukin (IL)-17A, also known as IL-17, was detected in lymphocytic infiltrates in myositis tissues (9,10) and IL-17 serum level was elevated in IIM patients (11–13). In cultured human myoblasts, both IL-17 and TNFα induced massive myoblast inflammatory response (9,14,15). The elevated levels of IL-17 and TNFα in IIM and their in vitro effects suggest that these cytokines play an important role in the pathogenesis of myositis.

Here, the interplay between inflammation and Ca^{2+} dysregulation was studied in human myoblasts exposed to IL-17 and TNFα. Immune cell and myoblast co-cultures were used to mimic the immune cell infiltrate found in IIM and to assess the contribution of these cell-cell contacts. The results indicate that IL-17 and TNFα played an important role in myoblast inflammatory response especially in IL-6 secretion. ER stress, reactive oxygen species (ROS) generation and SOCE were induced by IL-17 and TNFα whereas SOCE inhibition reduced IL-6 production inducted by IL-17/TNFα. Such close interplay between immune and non-immune mechanisms may drive and increase muscle weakness.

Materials and methods

Isolation and culture of muscle cells. Muscle samples were obtained from subjects undergoing orthopedic surgery. Biopsies were performed on m. vastus lateralis (femoral quadriceps) at distance of the joint. Written informed consent was obtained before surgery according to the policies of the local ethical committee and the Ministry of Research, which approved the study (reference number: AC-2010-1164). After surgery, muscle samples were immediately placed in sterile PBS with antibiotics (penicillin and streptomycin, Eurobio, Courtaboeuf, France) and washed. The fat and fibrous tissues were removed. Muscle samples were cut into fragments (1-2mm^3) and incubated at 37°C for 30 minutes with 1mg/mL collagenase (Sigma-Aldrich, St Louis, MO, USA). 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 at 37°C/5% CO_2 in Ham's-F10 medium (Eurobio) supplemented with 20% fetal bovine serum (Life Technologies, Carlsbad, USA), 2% Penicillin-Streptomycin (Eurobio), 1% L-glutamine (Eurobio) and 1% Amphotericin B (Eurobio). After 10 days, adherent cells were detached with trypsin (Eurobio), and myoblasts were purified by positive selection with CD56 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the instructions of the manufacturer. Myoblasts were used between passages 2 and 8.

Myoblast culture exposures. Myoblasts were seeded at a density of 50,000 cells/cm^2. After adhesion, cells were stimulated with 50ng/mL IL-17A (Dendritics, Lyon, France) or 1ng/mL TNFα (R&D Systems, Minneapolis, USA) alone or in combination. To inhibit SOCE, BTP2 (or YM58483) and 2-aminoethyl diphenylborinate (2-APB) inhibitors (Sigma-Aldrich) were used between 10 to 50µM.

PBMC isolation and co-culture assays. Whole blood samples were obtained from the Etablissement Français du Sang. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Eurobio) density gradient centrifugation. Cells were maintained in RPMI 1640 medium supplemented with 10% human AB serum (Etablissement Français du Sang, La Plaine Saint-Denis, France), 2% Penicillin-Streptomycin (Eurobio) and 1% L-glutamine (Eurobio). PBMCs were activated or not with 5µg/mL phytohemagglutinin (PHA) (Sigma-Aldrich) and added on adherent myoblasts at a ratio of 5 PBMCs for 1 myoblast. For cell culture insert assays, myoblasts were cultured at the bottom of a culture plate well and PBMCs were placed in Falcon® cell-culture inserts (Corning, NY, USA) with a small-pored membrane (0.4µm) preventing cell-cell contacts but not the crossing of soluble factors. For the IL-17 and TNFα neutralization assays, PBMCs activated or not with PHA for 24 hours were exposed to an anti-IL-17 antibody (R&D Systems) and/or the anti-TNFα antibody infliximab (Merck, Kenilworth, USA) at 10µg/mL for 3 hours before being added to the HepaRG cells.

Enzyme-linked immunosorbent assay (ELISA). After 48 hours of treatment, supernatants were harvested and the IL-6 and chemokine (C-C motif) ligand 20 (CCL20) productions were quantified with commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions.

Quantitative real time-PCR. Total RNA was purified using an RNeasy® Plus Mini kit (Qiagen, Hilden, Germany). cDNA was synthetized using the iScript™ kit (Bio-Rad, Hercules, CA, USA). PCR amplification was performed using the CFX96™ Real time system instrument (Bio-Rad) with the iTaq™ universal SYBR® green supermix (Bio-Rad) and the Qiagen QuantiTect® primers (QT00083538 for STIM1 and QT01870043 for ORAI1). The expression of the genes of interest was normalized to the expression of the housekeeping GAPDH gene.

Cell lysis and western blotting. Cell lysates were obtained by lysing cells with RIPA buffer supplemented with 1mM Na_3VO_4,1 mM DTT, 20mM NAF, 5mM EDTA and a cocktail of proteases inhibitor. Total protein concentration was determined using Bicinchoninic acid method (BCA, Interchim) and 25µg of protein of each sample was loaded on 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Migration was performed during 15 minutes at 90V followed by 60 minutes at 130V. Proteins were then blotted on a polyvinylidene difluoride (PVDF) membrane by electro transfer (Trans-Blot Turbo Transfer, Bio-Rad). PVDF membrane was incubated at room temperature for 1 hour with 5% milk in PBS for blocking and then incubates overnight at 4°C in the same buffer with the primary antibody (Grp78, sc-376768; STIM1, ab108994; ORAI1, sc68895; Tubulin, sc-5286). Secondary Horse radish peroxidase (HRP) coupled antibodies and ECL (entry-level peroxidase substrate for enhanced chemiluminescence) plus kit and Western Blotting detection system from GE Healthcare were used to reveal the proteins. The protein amount was determined using ImageLab software (Bio-Rad).

Wide-field microscopy for Ca^{2+} live cell imaging. Cells were imaged on an epifluorescence microscope Leica DMI6000B using 40x objective equipped with Orca-Flash4.0 digital camera (Hamamatsu). Myoblasts were double excited at 340 and 380nm and emission was collected at 510nm with identical acquisition parameters. Medium was replaced by a Calcium Containing Buffer (CCB) (in mmol/L: 140NaCl, 5KCl, 10HEPES, $1MgCl_2$, $2CaCl_2$, 10glucose, adjusted to pH7.4) containing 3µmol/L of fura2-AM during 30min at room temperature. Cells were washed twice with calcium free buffer in which 0.1mmol/L EGTA was added and placed under the microscope. For depletion of Ca^{2+} stores, cyclopiazonic acid (CPA*)* (10µM) was used and then 2mM calcium solution (CCB) was added to trigger the SOCE. Fluorescence ratios were calculated

in metaFluor 6.3 (Universal Imaging) and analyzed in Origin Pro (OriginLab) + GraphPad Prism 4 (GraphPad).

Confocal microscopy for oxidative stress detection. Cells were imaged on a confocal microscope Nikon A1r using 40x objective. Myoblasts were excited at 640nm and emission was collected at 665nm. Medium was replaced by a Calcium Containing Buffer (CCB) (in mmol/L: 140NaCl, 5KCl, 10HEPES, 1MgCl2, 2CaCl2, 10glucose, adjusted to pH7.4) containing 2.5µmol/L of CellROX™ Deep Red Reagent during 30min at 37°C. Fluorescence intensity was analyzed in ImageJ Fiji (https://fiji.sc/#, NIH). A threshold at the third quartile of the pixel intensity distribution was applied before the analysis.

Confocal microscopy for STIM1 puncta analysis

Cells were imaged on a confocal microscope Nikon A1r using 40x objective. Myoblasts were excited at 488nm and emission was collected at 510nm. Medium was replaced by a calcium free buffer (CFB) (in mmol/L: 140NaCl, 5KCl, 10HEPES, 1MgCl2, 0.1EGTA, 10glucose, adjusted to pH7.4). Cells were washed twice with calcium free buffer in which 0.1mmol/L EGTA was added and placed under the microscope. For depletion of Ca^{2+} stores, cyclopiazonic acid (CPA*)* (10µM) was used in order to form STIM1 puncta.

For each coverslip, 10 cells were imaged for each experiment and then analyzed with either Image J to calculate the colocalization coefficients or MATLAB® (MathWorks®) to perform Image Correlation spectroscopy (ICS).

For ICS analysis, images of fluorescence channel were filtered and transformed in binary images. The filtering threshold was calculated automatically by the algorithm and determined as the mean value of the fluorescence intensity in each image. The series of images were analyzed with a batch-ICS algorithm adapted from the FICS algorithm developed by Dr Heliot's team (16). Mean and SEM of both surface area and density of the fluorescent clusters were figured out automatically by the batch-ICS algorithm.

Statistical analysis. Data are presented as the mean ± SEM. Statistical differences were analyzed using the non-parametric Wilcoxon paired-test. P-values lower than 0.05 were considered significant.

Results

Synergistic effect of IL-17 and TNFα on CCL20 and IL-6 production by myoblasts

IL-6 is a pro-inflammatory cytokine involved in the differentiation of Th17 cells, the main IL-17-producing cells. By attracting Th17 cells and dendritic cells, CCL20 plays an important role in the local immune cell recruitment (17). IL-17 and TNFα are involved in IL-6 and/or CCL20 production by muscle cells (9,14,15). but the effect of their combination has not yet been investigated. The IL-17/TNFα effect on IL-6 and CCL20 release was studied in human myoblasts. IL-17, TNFα and the IL-17/TNFα combination increased significantly IL-6 production by 4-, 3- and 14-fold respectively compared to untreated condition (Figure 1A). CCL20 secretion by myoblasts was also induced by IL-17 alone (4-fold), TNFα alone (6-fold) ant the IL-17/TNFα combination (29-fold) compared to the control condition ($p<0.05$) (Figure 1B). Therefore, the IL-17/TNFα cooperation increased synergistically the secretion of CCL20 and IL-6. By acting on IL-6 and CCL20 secretion, IL-17 and TNFα may contribute to the local Th17 cell induction.

IL-17 and TNFα increase ER stress and mitochondrial ROS in myoblasts

To study the ER stress triggered by pro-inflammatory cytokine exposure, the expression of BiP/Grp78 protein was quantified. BiP/Grp78 protein controls the activation of the ER stress

sensors and to initiate the ER stress response known as the unfolded-protein response (18). As observed for the secretion of CCL20 and IL-6, IL-17 and TNFα cooperated to increase the expression of BiP/Grp78 protein compared to IL-17 and TNFα alone (Figure 2A and B). In addition to the unfolded-protein response, oxidative stress and accumulation of reactive oxygen species (ROS) initiate and contribute to the inflammatory response. Using confocal microscopy, a 1.5-fold increase in mitochondrial ROS and a 2-fold increase with both cytokines ($p<0.0001$) was observed in TNFα and IL-17 treated myoblasts (Figure 2C and D).

PBMC-myoblast interaction induces a strong IL-6 and CCL20 release

To better understand the consequences of immune cell infiltration in muscle tissue of IIM patients (4,5), a model of co-culture between myoblasts and PBMC was used. CCL20 and IL-6 were quantified in co-culture supernatants after 48 hrs. PBMC from healthy donors were used. No contribution of alloreactivity has been detected in the same short-term co-culture model (19,20). The PBMC-myoblast interaction induced a strong CCL20 and IL-6 production by comparison to myoblasts alone or PBMC alone ($P<0.01$) (Figure 3A and B). PBMC activation with PHA was not required for the increase of CCL20 and IL-6 secretion in co-cultures (Figure 3A to F).

To determine the role of PBMC-myoblast contact in the inflammatory process, transwell cell culture inserts allowing the circulation of soluble factors but not direct cell-cell contact were used. Myoblasts were first added to the bottom of the well and PBMC to the insert. The use of these inserts reduced strongly CCL20 secretion by 86% in resting co-cultures ($p<0.01$) and by 77% in PHA-stimulated co-cultures ($p<0.01$) (Figure 3C). In contrast, the IL-6 release in co-cultures stimulated or not with PHA was not affected (Figure 3D). Therefore, the induction of IL-6 in co-cultures was mainly mediated through soluble mediators between PBMCs and myoblasts.

To determine the contribution of IL-17 and TNFα produced by PBMCs on the CCL20 and IL-6 release in co-cultures, PBMCs activated with PHA for 24 hours were exposed to specific inhibitors of IL-17 and/or TNFα and then added to myoblast cultures. This PBMC pre-incubation step was used to better mimic the *in vivo* conditions in chronic inflammatory state. As shown in figures 3E and 3F, neutralization of IL-17 did not reduce the CCL20 and IL-6 secretion in our co-culture system. By contrast, the anti-TNFα antibody inhibited the CCL20 and IL-6 production both in unstimulated condition (36% and 42% of inhibition respectively, $p<0.05$) and PHA condition (34% and 35% of inhibition respectively, $p<0.05$). Moreover, the use of both anti-IL-17 and anti-TNFα decreased also significantly the CCL20 and IL-6 secretion without additive or synergistic inhibitory effects (Figures 3E and F). Therefore, TNFα contributed to the induction of CCL20 and IL-6 release in PHA-activated co-cultures.

IL-17 and TNFα increase store operated calcium entry (SOCE)

Inflammation increases intracellular Ca^{2+} concentrations in several inflammatory muscle disorders. The routes of calcium entry include calcium leak channels, stretch-activated channels, receptor-operated channels, and store-operated calcium channels. Ca^{2+} influx is sufficient to induce muscular dystrophy through a TRPC-dependent mechanism (21). The pro-inflammatory cytokine TNFα has been shown to enhance SOCE in human airway smooth muscle cells (22). The effect of IL-17 and TNFα on the calcium homeostasis has not been investigated in the context of IIM pathogenesis.

Orai1 and STIM1 mRNA levels were first measured in human myoblasts after 6 and 12 hrs of IL-17 and/or TNFα exposure. No significant effect in mRNA levels of ORAI1 and STIM1 was detected at 6 hrs (data not shown) and 12 hrs. (Figure 4A and B). Orai1 and STIM1 protein levels were next investigated by western blot. IL-17/TNFα increased significantly STIM1 expression compared to control (Figure 4E and F). STIM1 puncta formation analysis revealed no significant

difference in the puncta density, but an increase of STIM1-puncta surface in IL17/TNFα conditions (Figure 4G and H).

To confirm the effect of cytokines on SOCE, a fluorescence-based measurement of SOCE in human myoblasts was performed after cytokine treatment. IL-17 and TNFα single treatment modified the slope of SOCE by 1.4-fold and the IL-17/TNFα combination by 2-fold compared to control condition ($p<0.01$) (Figure 4I and J).

Inhibition of SOCE reduces IL-6 production induced by the IL-17/TNFα combination

To assess the SOCE contribution to the myoblast inflammatory response, myoblasts were stimulated with IL-17 and/or TNFα in presence or not of 2-APB or BTP2 SOCE inhibitors. IL-6 release by myoblasts was quantified at 48 hrs. The 2-ABP and BTP2 inhibitors inhibited the induction of IL-6 production by IL-17 and/or TNFα in a dose-dependent manner. The induction of IL-6 secretion by the IL-17 and TNFα combination was reduced by 42% ($p<0.05$) with 2-ABP at 50 μM, and by 19% ($p<0.05$) and 33% ($p<0.01$) with BTP-2 at 10 and 20 μM, respectively (Figure 5A and B). Therefore, SOCE interacts with immune mechanisms to further increase the myoblast inflammatory response.

Discussion

Both immune and non-immune mechanisms contribute to IIM pathogenesis. The interplay between these two mechanisms was studied in human myoblasts stimulated with the pro-inflammatory cytokines IL-17 and TNFα. Immature muscle precursors are immunologically active cells, playing an important role in disease progression and probably in muscle regeneration defects observed in IIM patients (15,23,24). The results indicate that in addition of the inflammatory response induced by IL-17 and TNFα, IL-17 and TNFα promoted also ER and mitochondrial stress and calcium dysregulation in myoblasts, leading to muscle cell dysfunction.

The immune cell infiltrate contributes to the pathogenesis of IIM through cell-cell interactions and the release of cytokines. Myoblasts may contribute to mononuclear cell attraction by secreting chemokines in response to local inflammation (23). CCL20 expression was found in dermatomyositis and polymyositis muscle samples and was associated with dendritic and Th17 cell homing (10). In this study, IL-17 and TNFα had a cooperative synergistic effect on CCL20 secretion by isolated myoblasts. These results are in line with our previous reports indicating that IL-17 increases TLR-3 agonist and IL-1β-induced CCL20 production by myoblasts (9,15). Therefore, IL-17 can exacerbate the weak effects of low concentrations of TNFα and IL-1β on CCL20 release. Because CCL20 induces Th17 and dendritic cell recruitment, this local increase of CCL20 could contribute to the induction and perpetuation of the IIM local inflammation.

IL-6 is a pleiotropic inflammatory cytokine produced by myoblasts in response to inflammation. The IL-17/IL-1β, TNFα/IL-1β and TNFα/IFNγ combinations have been previously shown to have additive/synergistic effects on the induction of IL-6 secretion by myoblasts (9,14,23,25). Here, IL-17 and TNFα interactions increased in synergy the production of IL-6.

To mimic the *in vivo* environment characterized by immune cell infiltration in IIM, a PBMC and myoblast co-culture system was used. The interactions between PBMC and myoblasts induced a strong CCL20 and IL-6 release and PBMC activation with PHA was not required for this induction. In synoviocyte-PBMC or skin fibroblast-PBMC co-cultures, cell interactions were also sufficient to induce the IL-6 or IL-8 secretion (20,26). The increase of CCL20 production in co-cultures was mediated through direct PBMC-myoblast contacts since the use of cell culture insert reduced strongly its production in co-culture. Another study showed that the T cell – myoblast direct interactions can lead to T cell proliferation (27). By contrast, the IL-6 secretion was mainly induced through soluble factors in myoblast-PBMC co-culture whereas the direct cell contact was significantly involved in IL-6 generation in synoviocyte-PBMC or skin fibroblast-PBMC co-cultures (20,26). Neutralization of IL-17 and/or TNFα in the co-culture system with

pre-incubated PBMCs and myoblasts reduced IL-6 release, confirming the contribution of the soluble factors. Therefore, the soluble inflammatory cytokines IL-17 and TNFα could have an important role in initiating and maintaining inflammation *in vivo* in IIM through the production of IL-6, which then contributes to Th17 cell differentiation.

The ER stress pathways are activated in tissues from patients with IIM (28,29) and interplay with mitochondrial dysfunction and ROS generation (30). In myoblasts, we identified that IL-17 and/or TNFα mediated ER stress and mitochondrial ROS, suggesting that these cytokines may participate *in vivo* to these non-immune mechanisms. Moreover, ER is the main intracellular Ca^{2+} storage, and ER stress induces Ca^{2+} ER release. Changes in Ca^{2+} homeostasis can affect muscle contractibility (31). Ca^{2+} dysregulation was reported in sporadic inclusion body myositis (32). SOCE is a key component of the intracellular calcium concentration and plays an important role in muscle function and development (3,33,34). SOCE is modified during inflammation (22,35). In this study, IL-17 and/or TNFα exposure increased SOCE in myoblasts with a higher effect when IL-17 and TNFα were combined. In human airway smooth muscle cells, TNFα enhanced Orai1, STIM1, and SOCE (35). In myoblasts, TNFα alone did not increase significantly Orai1 and STIM1 expression but the concentration was 20-fold lower. However, the IL-17/TNFα combination increased significantly STIM1 protein level in myoblasts but had no significant effect on STIM1 and Orai1 mRNA expression or Orai1 protein level. TNFα and IL-13 have been shown to increase STIM1 aggregation in human airway smooth muscle cells, contributing to SOCE induction (22). Therefore, IL-17 and/or TNFα may increase SOCE by inducing Ca^{2+} release from ER and enhancing STIM1 aggregation.

To determine the role of SOCE in the inflammatory response induced by IL-17/TNFα, SOCE inhibitors were used. SOCE inhibition reduced the secretion of IL-6 following IL-17/TNFα exposure. In human bronchial epithelial cells, SOCE inhibition with BTP2 inhibited IL-6 and IL-8 production after allergen stimulation (36). Therefore, in addition to muscle cell dysfunction, SOCE may have a central role in the induction of inflammation. Its neutralization could be a promising therapeutic strategy in IIM. These results in myoblasts are summarized in Figure 6.

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Figure legends:

Figure 1: Synergistic effect of IL-17 and TNFα on the CCL20 and IL-6 production by myoblasts. Myoblasts were treated with IL-17 (50 ng/mL) and/or TNFα (1 ng/mL) for 48 hrs. IL-6 and CCL20 secretion by myoblasts was quantified by ELISA **(A, B)**. Data are the mean of 5 to 8 independent experiments ± SEM; * $p<0.05$, ** $p<0.01$ vs. control untreated condition and # $p<0.05$, ## $p<0.01$ vs. other inflammatory conditions.

Figure 2: IL-17 and TNFα increase ER stress and mitochondrial ROS in myoblasts. Myoblasts were treated with IL-17 (50 ng/mL) and/or TNFα (1 ng/mL) for 24 hrs. Expression of BiP/Grp78 protein was measured by western-blot and the band density was normalized with tubulin expression **(A, B)**. Mitochondrial oxidative stress measurements (ROS) of human myoblasts was measured with the fluorescence intensity of CellRox Dye, using 40x objective of a confocal microscope Nikon A1r, scale bar 70µm **(C, D)**. Data are the mean of 4 to 7 independent experiments ± SEM, *** $p<0.001$ and **** $p<0.0001$, vs. control untreated condition.

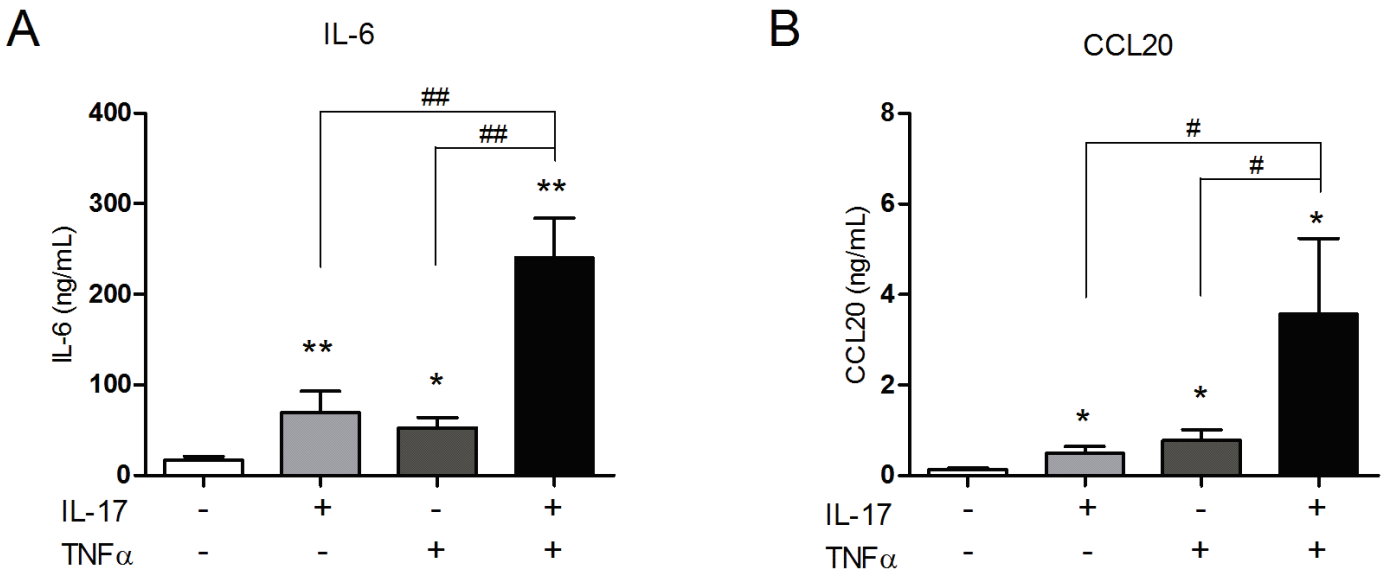
Figure 3: PBMC-myoblast interaction induces a strong production CCL20 and IL-6. PBMC and myoblasts were cultured alone or in co-culture at a ratio of 5 PBMCs for 1 myoblast for 48 hrs in the presence or not of PHA (5 µg/mL). CCL20 and IL-6 secretion by myoblasts was quantified by ELISA **(A-F)**. The contribution of direct cell-cell contact was investigated with a cell culture permeable insert **(C, D)**. PBMCs were pre-incubated for 24h in presence or not of PHA and then exposed or not to an anti-IL-17 antibody and/or an anti-TNFα antibody for 3h before being added to the myoblast cultures. Data are expressed as CCL20 and IL-6 supernatant level percentages compared to the non-activated pre-incubated PBMC – myoblast co-cultures **(E, F)**.. Data are the mean of 6 to 14 independent experiments ± SEM; * $p<0.05$, ** $p<0.01$ vs. control co-culture condition.

Figure 4: IL-17 and TNFα increase store-operated calcium entry. Myoblasts were treated with IL-17 (50 ng/mL) and/or TNFα (1 ng/mL). mRNA levels of STIM1 and ORAI1 at 12 hrs was expressed as fold changes compared to control **(A, B)**. ORAI1 and STIM1 protein was measured by western-blot and the band density was normalized with the tubulin expression. **(C-F)**. Representative image of STIM1 puncta in human myoblast treated with IL-17 (50 ng/mL) and TNFα (1 ng/mL) for 24 hours. Image Correlation Spectroscopy (ICS) analysis of STIM1 puncta (left inset) mean density of puncta (µm). (right inset) mean surface of puncta (puncta/µm). Data are the mean of 3 independent experiments with cells from 3 different donors ± SEM; * $p<0.05$ vs. control untreated condition, scale bar 3µm **(G, H)**. SOCE was measured by using Fura2-AM dye in human myoblasts. Cells were imaged on an epifluorescence microscope using a 40x objective. Trace is a representative measurement of SOCE in IL17/TNFα treated myoblasts. **(I)**. Slope analysis of the SOCE **(J)**. Data are the mean of 4-5 independent experiments ± SEM; * $p<0.05$ and **** $p<0.00001$, vs. control untreated condition.

Figure 5: Inhibition of SOCE reduces IL-6 production induced by the IL-17/TNFα combination. Myoblasts were stimulated with IL-17 and/or TNFα in presence or not of the SOCE inhibitor 2-APB (10; 25 and 50 µM) or BTP2 (10 and 20 µM) for 48 hrs. CCL20 and IL-6 secretion by myoblasts was quantified by ELISA **(A, B)**. Data are the mean of 7 independent experiments ± SEM; ** $p<0.01$ vs. control untreated condition and # $p<0.05$, ## $p<0.01$ vs. other inflammatory conditions

Figure 6: IL-17 and TNFα mediate muscle damage and weakness through immune and non-immune pathways in myoblasts. The immune cell infiltration in IIM constitutes a local

source of cytokines and promotes the cell-cell interactions. IL-17 mainly produced by Th17 cells, and TNFα act in synergy on myoblasts to increase IL-6 and CCL20 secretion. Because IL-6 is involved in the Th17 cell differentiation and CCL20 in dendritic and Th17 cell recruitment, IL-6 and CCL20 mediate a positive feedback loop promoting local IL-17 production. IL-17 and TNFα induce also non-immune pathways with ROS production, ER stress and SOCE activation. The IL-17/TNFα effect of mitochondrial dysfunction, ER stress and SOCE activation are probably closely linked. SOCE and calcium dysregulation contribute to IL-6 release induced by IL-17/TNFα.
ER: endoplasmic reticulum; ROS: reactive oxygen species; chemokine (C-C motif) ligand 20: CCL20; DC: dendritic cells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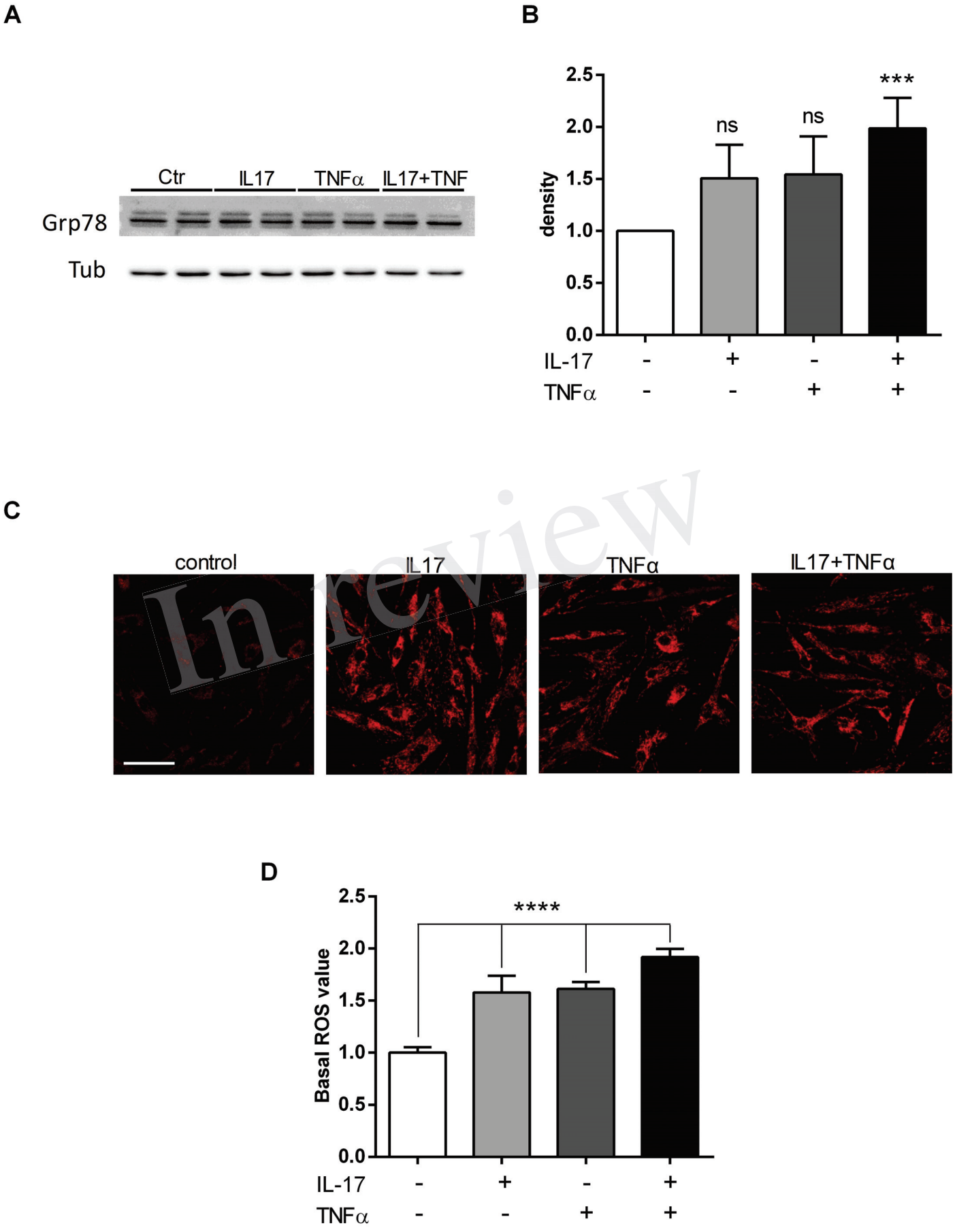

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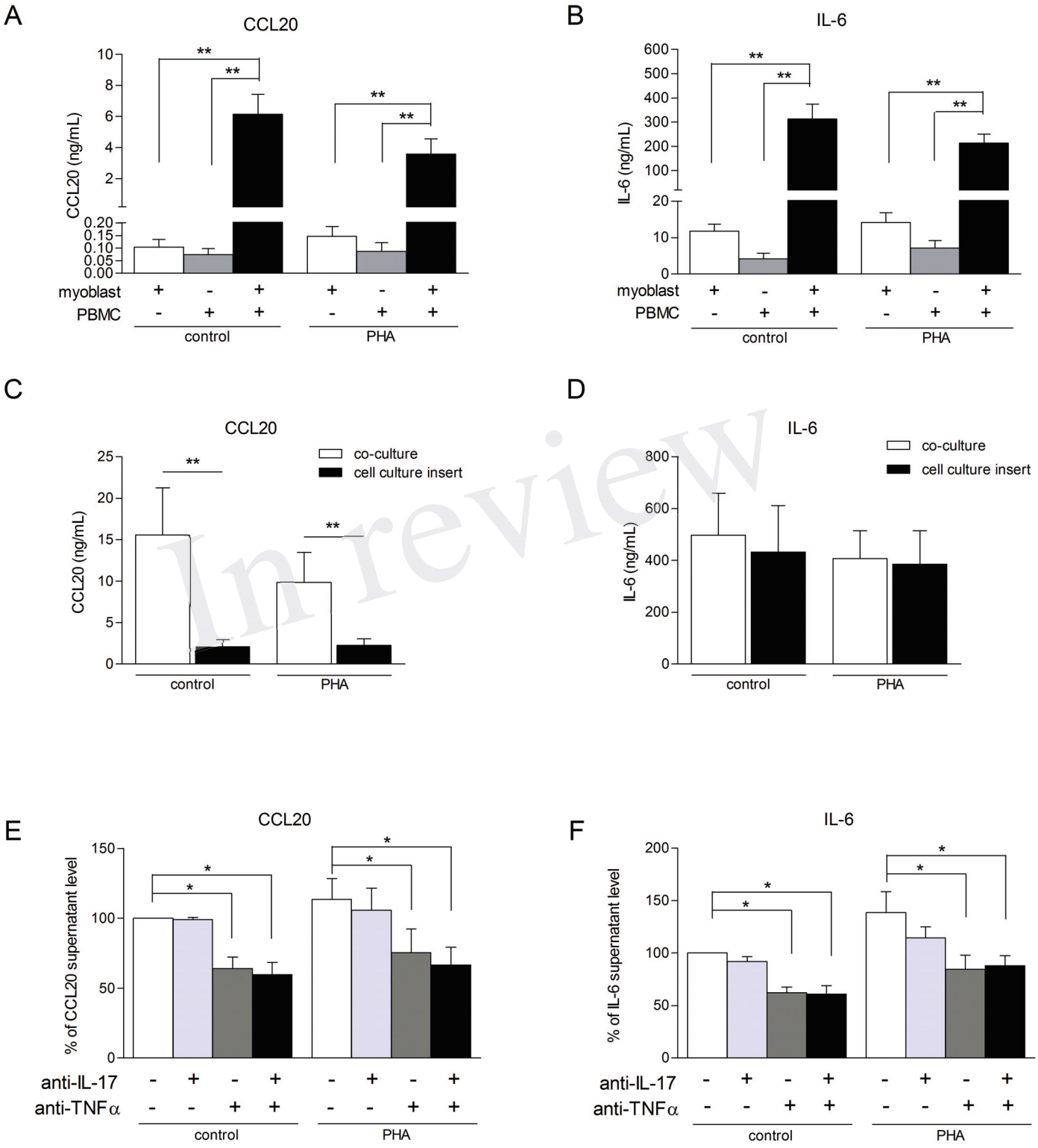

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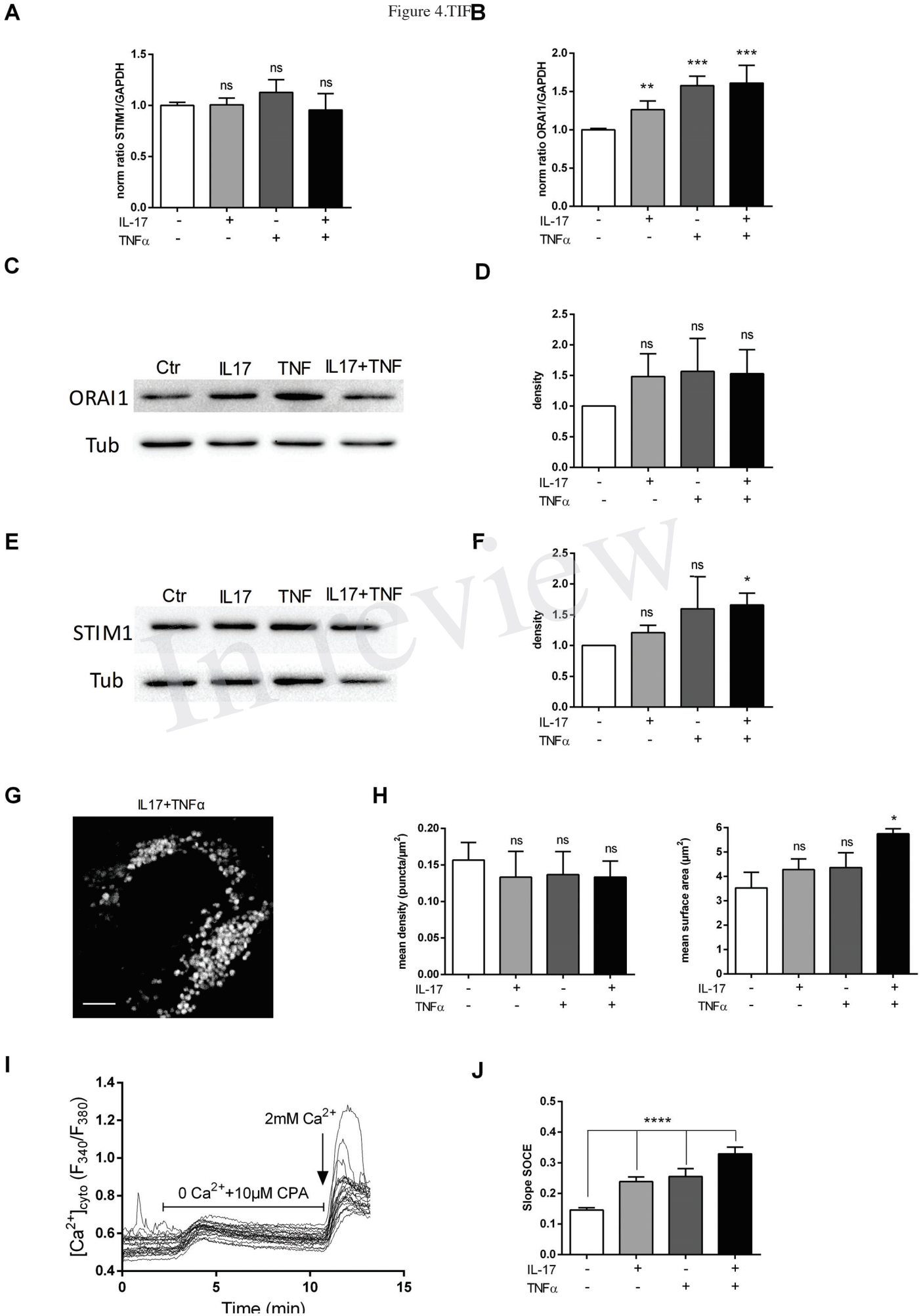

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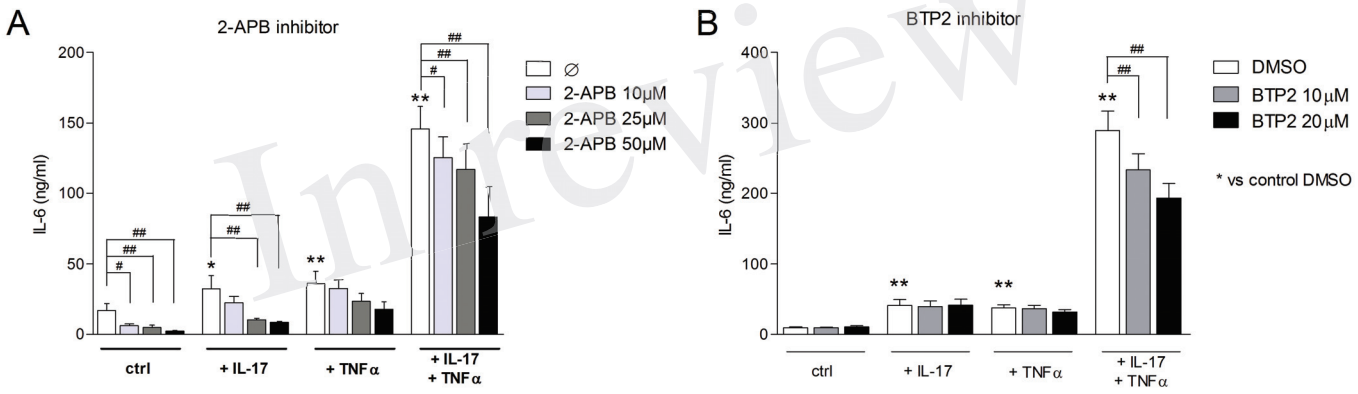

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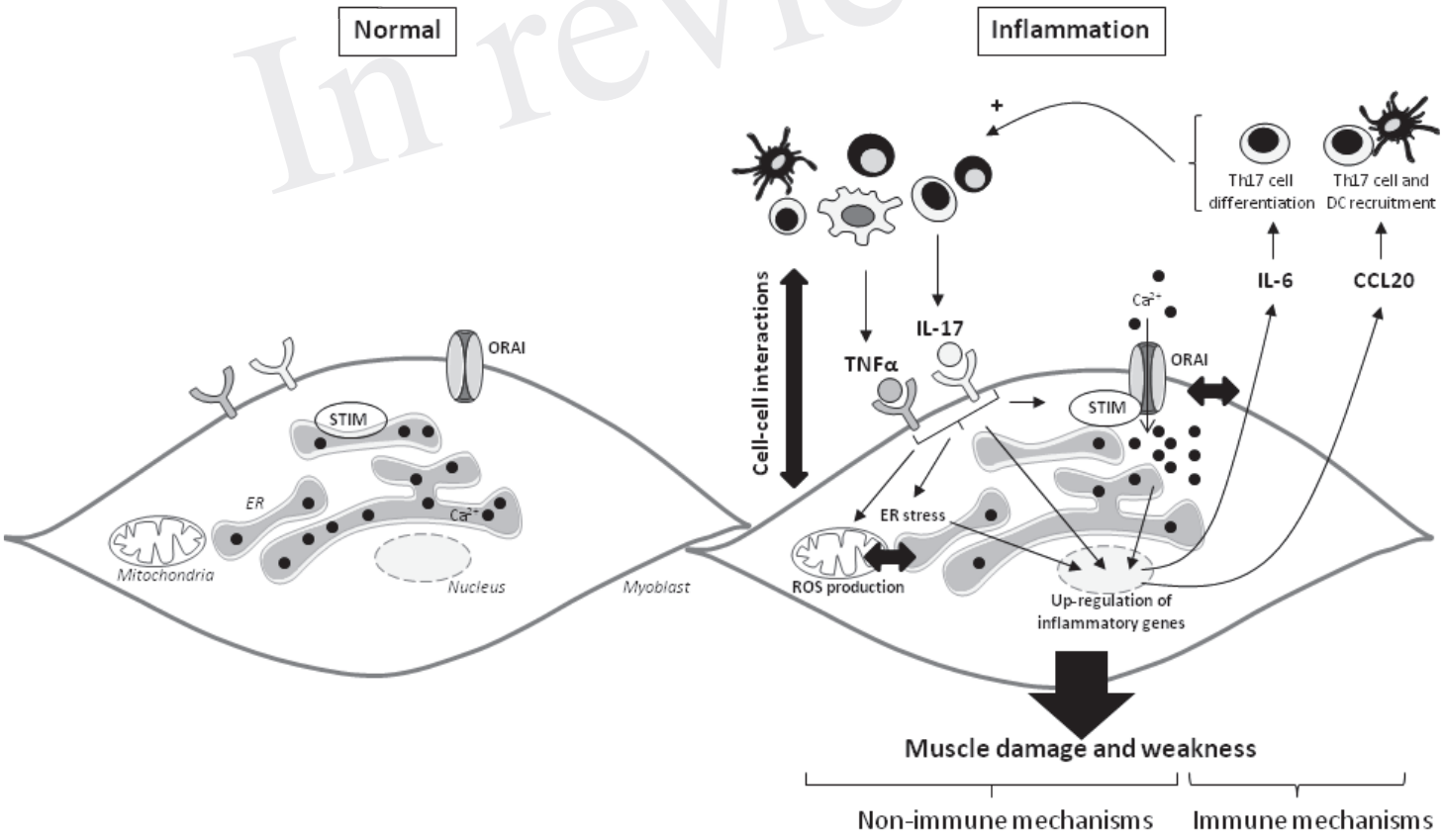

DISCUSSION

This study highlights the cooperative effects of IL-17 and TNFα on the induction of the inflammatory response in hepatocytes, HSCs and myoblasts by using human cell cultures. In addition, the IL-17 and TNFα association can also mediate non-immune adverse effects in the liver and the muscles that could impair liver and muscle functions. These findings confirm the systemic and pleiotropic activities of IL-17 and TNFα. Moreover, IL-17 and TNFα contribute partially to the increase of pro-inflammatory mediators induced by the interactions between PBMCs and hepatocytes, HSCs or myoblasts. In this way, IL-17 and TNFα are pro-inflammatory mediators in the bidirectional crosstalk between the PBMCs and the resident tissue cells, which can drive to chronic inflammation.

1 The use of human cells for the *in vitro* cellular models

1.1 Hepatocyte cultures

In order to keep the human- and the organ-specific properties, *in vitro* models with human cells from liver were used in this study. Because PHHs are from native livers, they are considered to be a gold standard approach to reflect the specific functionality and mediators of the human organ. However, liver biopsies are rare and they can have important individual differences due to several factors such as genetic factors, environmental factors or pathological conditions (e.g. infections, steatosis, cancer). For these reasons, human hepatoma cell lines are useful alternative tools. Here, three immortal cell lines (Huh7.5, HepG2 and HepaRG cells) derived from liver tissue of patients with hepatocellular carcinoma were used. Interestingly, the HepaRG cells evolve from a bipotent progenitor cells toward both hepatocyte-like and primitive biliary epithelial-like cells (Marion et al., 2010). To choose the most relevant cell line, Huh7.5, HepG2 and HepaRG cells were exposed to IL-17 and TNFα. The IL-17 and TNFα combination increases in synergy the IL-6 and IL-8 secretion in HepaRG cell and PHH cultures but not in Huh7.5 and HepG2 cell cultures. The low TNFR2 mRNA levels in Huh7.5 and HepG2 cells compared to HepaRG cells may contribute to the

different levels of IL-6 and IL-8 secretion between the cell lines following IL-17 and TNFα exposure. Based on these results, the HepaRG cell line was selected for the experiments of this study.

1.2 Hepatic stellate cell cultures

To study the role of IL-17 and TNFα as well as the effects of cell interactions on the HSCs, the LX-2 human HSCs were used. The LX-2 cells were generated by spontaneous immortalization in low serum conditions. They are now well characterized and share key features with human HSCs including cytokine signaling or fibrogenesis (Xu et al., 2005). Indeed, the LX-2 cells express and respond to IL-17, TNFα, IL-6 as well as TGFβ (Fabre et al., 2014; Meng et al., 2012; Robert et al., 2016; Schoenherr et al., 2010; Sun et al., 2012). Nevertheless, it would be interesting to use primary human HSCs to confirm our results obtained with the LX-2 cells.

1.3 Myoblast cultures

Immature myoblast precursors are present in normal muscle tissue. After muscle damage, these cells proliferate and participate in the regeneration process. The immature myoblast precursors appear to be implicated in the pathogenesis of inflammatory myopathies. Indeed, in contrast to normal muscle tissue, myositis tissues are characterized by the overexpression of HLA class I antigens and myositis-associated autoantigens, as well as activation of TLR pathway and production of type I interferon, all observed in immature myoblast precursors (Casciola-Rosen et al., 2005; Tournadre et al., 2010, 2012). In addition, damage of these cells accomplishing repair may amplify tissue damage and could explain the defective repair and the presence of necrotic cells in inflammatory muscles leading to muscular atrophy (Tournadre and Miossec, 2013). For these reasons, human myoblasts instead of mature myotubes were used in this study.

2 The IL-17 and TNFα cooperation enhances the hepatic and muscle inflammatory response

2.1 IL-17 and TNFα cooperate to enhance the expression and/or secretion of pro-inflammatory mediators

IL-17 alone is often poorly active. However, IL-17 can synergize with other cytokines such as TNFα, IL-1β or IFNγ, leading to increased production of pro-inflammatory mediators such as IL-6 and IL-8 by various cell types including synoviocytes or endothelial cells (Beringer et al., 2016; Chabaud et al., 1998; Hot et al., 2012). Nevertheless, the effects of the IL-17 and TNFα cooperation have not been investigated on liver and muscle cells whereas these two cytokines are involved in several liver and muscle disorders or systemic inflammatory diseases with hepatic and muscle complications (Beringer and Miossec, 2018; De Bleecker et al., 1999; Kuru et al., 2000; Schwabe and Brenner, 2006; Tournadre and Miossec, 2012). In this study, IL-17 and TNFα cooperate to promote systemic inflammation by increasing IL-6 secretion, which in turn, enhances the CRP expression in hepatocytes. IL-17 amplifies also the TNFα effect on the induction of IL-8, CCL20 and MCP-1 chemokine expression and/or secretion mainly through an IL-6 independent pathway in hepatocyte and LX-2 HSC cultures. By promoting the neutrophil-attracting chemokine IL-8, the IL-17 and TNFα combination may have a crucial role in the initiation of liver inflammation. These *in vitro* results are consistent with the *in vivo* murine models of liver injury showing that IL-17 deletion or neutralization reduces neutrophil accumulation as well as the levels of inflammatory cytokines in liver (Furuya et al., 2015; He et al., 2013; Tan et al., 2013). It would be therefore interesting to investigate whether blocking both IL-17 and TNFα induce a further decrease of the immune cell recruitment and the release of pro-inflammatory mediators. In myoblast cultures, IL-17 and TNFα enhance also in synergy the IL-6 and CCL20 release. Since CCL20 attracts Th17 cells and IL-6 is involved in Th17 cell differentiation, the IL-17 and TNFα synergistic cooperation may contribute to the local Th17 cell induction in the liver and the muscles by increasing the IL-6 and CCL20 production.

2.2 Mechanisms of the IL-17 and TNFα synergistic effects

Although the IL-17 and TNFα synergistic effects have been well described in several cell types, the mechanisms of this interaction on the IL-6 and IL-8 induction are not well established. Here, in HepaRG cells, first exposure to IL-17, but not to TNFα, is crucial for the initiation of the IL-17 and TNFα synergistic cooperation. Various mechanisms may act at several levels: at a receptor level, at a promoter level and at a post-transcriptional level. In synoviocytes, IL-17 up-regulated the TNFR2 and the blockade of its receptor reduced the IL-17 and TNFα synergistic cooperation on the CCL20 production (Zrioual et al., 2009). In HepaRG cells, IL-17 and TNFα together but not IL-17 alone induces the TNFR2 mRNA expression. The regulation of the TNFR2 appears therefore different between synoviocytes and HepaRG cells. Moreover, the TNFR2 mRNA lower in HepG2 and Huh7.5 cells than in HepaRG cells could explain the lack of response of the HepG2 and Huh7.5 cells to the IL-17 and TNFα synergistic effect on the IL-6 and IL-8 secretion. This data suggest that the TNFR2 could have a key role in the HepaRG cell inflammatory response induced by IL-17 and TNFα although the TNFR2 is preferentially activated by the transmembrane form of the TNFα (as seen in part 1.3.2). The use of a specific blocking anti-TNFR2 antibody could be useful to confirm its involvement in the IL-17 and TNFα cooperation.

Part of the IL-17 and TNFα cooperation may also occur at a gene transcription level. Transcription factor-binding sites for NF-κB and C/EBP (CCAAT/enhancer-binding protein) in the IL-6 promoter were both involved in the IL-17 and TNFα synergistic effect on IL-6 in an osteoblastic cell line (Ruddy et al., 2004). The up-regulation of C/EBPδ by the IL-17 and TNFα stimulation and the increase of C/EBPδ recruitment to the promoter by TNFα may participate to the IL-17 and TNFα cooperative effect on the IL-6 induction (Gaffen et al., 2014; Ruddy et al., 2004; Zimmermann et al., 2015). In addition, by enhancing IκBζ mRNA expression in synergy, IL-17 and TNFα may induce a further increase of NF-κB activation since IκBζ acts as a NF-κB co-activator (Karlsen et al., 2010; Sparna et al., 2010; Zimmermann et al., 2015).

Post-transcriptional regulation is important to control cellular transcript abundance and, in turn, the levels of secreted proteins. IL-17 can increase the stability of mRNAs induced by the TNFα stimulation such as IL-6 and IL-8 mRNA (Hartupee et al., 2007; Henness et al., 2004, 2006). In this study, IL-17 enhances the IL-6 mRNA stability in HepaRG cells. As the 3'-untranslated region of the IL-6 mRNA contains adenylate and uridylate (AU)-rich elements, IL-17 may promote the binding of stabilizing AU-binding proteins over that of destabilizing AU-binding proteins, prolonging IL-6 mRNA half-life (Chowdhury et al., 2013). This IL-17 effect on the mRNA stabilization could therefore contribute to the IL-17 and TNFα synergistic effect on the IL-6 production.

Multiple mechanisms at different levels appear therefore involved in the IL-17 and TNFα synergistic effect. The contribution of these different mechanisms differs from one cell type to another as seen with the TNFR2. Moreover, in the same cell type, the mechanism involved varies between the gene or mRNA targets of IL-17 and TNFα. Indeed, NF-κB pathways participate in the secretion of IL-6 induced by the IL-17 and TNFα cooperation but not in the IL-8 secretion in HepaRG cells. In this way, the IL-17 and TNFα synergistic effect on the IκBζ up-regulation, which intensifies NF-κB activation, cannot be involved in the IL-17/TNFα-induced IL-8 production in HepaRG cells.

2.3 Potential contribution of the IL-17 and TNFα synergistic cooperation in systemic inflammatory diseases

This study shows that the effects of TNFα at low concentration can be potentiate by IL-17 in the liver and the muscles. These results strengthen the potential important systemic role of IL-17 and TNFα in autoimmune and inflammatory diseases. However, the concentrations of IL-17 and TNFα used in our *in vitro* systems may no reflect the *in vivo* local concentrations in organs or tissues. Moreover, the range of cytokines is limited in our monocultures, which reduces the number and the diversity of the interactions between cytokines. Indeed, because cytokines act in networks, IL-17 and TNFα can interact with other cytokines to mediate additive, antagonistic or synergistic effects. For example, IL-17 can synergize with other cytokines such as IL-1β, IFNγ, IL-22 or GM-CSF, in addition to TNFα, to promote

inflammation (Beringer et al., 2016). For this reason, a cell-based assay has been developed at the laboratory to detect circulating bioactive IL-17 instead of circulating IL-17 levels to select the patients more susceptible to respond to an anti-IL-17 therapy (Ndongo-Thiam and Miossec, 2015).

3 The IL-17 and TNFα inflammatory reponse may impair the liver and muscle function

3.1 IL-17 and TNFα induce cell damage in liver

Since liver inflammation may induce liver damage, the IL-17 and TNFα association could also mediate non-immune adverse effects in the liver. Here, the IL-17 and TNFα stimulation increases the levels of the intracellular ASAT transaminase in supernatants of hepatocyte cell cultures through the induction of IL-6. The IL-17 and TNFα combination can thus mediate indirectly liver damage. This hepatotoxic effect of IL-17 has been demonstrated *in vivo* in several models of liver injury in which the IL-17 inhibition reduced the necrotic lesions (Furuya et al., 2015; He et al., 2013; Tan et al., 2013). In addition, TNFα was also associated with liver injury (Park et al., 2012; Yang and Seki, 2015).

By acting on HSCs, IL-17 and TNFα could participate to the fibrosis process. In a mice model of liver fibrosis, the plasma IL-17 level is increased and inhibition of the IL-17 pathway attenuates fibrosis whereas the *in vivo* role of TNFα in liver fibrosis is still controversial (Qin et al., 2016; Tan et al., 2013; Yang and Seki, 2015). However, IL-17 and TNFα have no direct effect on the pro-fibrotic gene expression in our HSC cultures. Nevertheless, IL-17 can enhance the secretion of the main pro-fibrotic cytokine TGFβ by the activated Kupffer cells (Hara et al., 2013). In addition, IL-17 and TNFα increase slightly the TGFβ mRNA expression in LX-2 cells. Therefore, IL-17 and TNFα could have indirect pro-fibrotic effects via the induction of TGFβ, which can be amplify by the IL-17 and TGFβ synergistic cooperation (Fabre et al., 2014). In this way, it could be interesting to study the role of IL-17, TNFα and TGFβ cytokine interplays in the fibrosis process at several time points of the cultures. Indeed, it has been suggest that the matrix breakdown mediated by

TNFα may be essential at the early stage of liver injury for the recruitment of inflammatory immune cells and, later, TGFβ could decrease the initial TNFα-mediated MMP induction activity leading to the development of liver fibrosis (Knittel et al., 1999).

3.2 IL-17 and TNFα induce endoplasmic reticulum stress and calcium dysregulation

Muscle weakness and early sarcopenia are observed in inflammatory muscle disorders. Here, in human myoblasts, IL-17 and/or TNFα stimulation increase the ER stress and the mitochondrial ROS production contributing to cell damage. In addition, the IL-17 and TNFα exposure enhances SOCE in myoblasts. This effect on SOCE could impair Ca^{2+} homeostasis that is crucial in the regulation of muscle contraction. Interestingly, the inhibition of SOCE reduces the IL-6 release by IL-17 and TNFα. Therefore, immune and non-immune mechanisms interplay in the muscles. Controlling inflammation in the muscles could prevent muscle damages and dysfunctions, and in turn, the chronicity of the inflammation since the non-immune adverse mechanisms also participate in the inflammatory state (Henriques-Pons and Nagaraju, 2009).

4 Cell interactions have an important role in the initiation and the outcome of the immune response

4.1 The co-culture system to study the cell-cell interactions

During liver or muscle injury, inflammatory immune cells infiltrate the liver or the muscles allowing interactions between the infiltrated immune cells and the liver and muscle resident cells (Quintin et al., 2010; Tournadre et al., 2009; Warren et al., 2006). These cell interplays have certainly a key role in the initiation and the outcome of the inflammatory response. This study focuses on the cell interactions between PBMCs, hepatocytes, HSCs and myoblasts as hepatocytes are the most abundant cell population in liver; the HSC activation is crucial in liver fibrosis and the myoblast precursors have a critical role in myositis. Cells were co-cultured at a ratio of 25 PBMCs : 5 HepaRG cells : 1 LX-2 cell or 5 PBMCs : 1 myoblast. These ratios were based on data from the literature (Barbero-Becerra et al., 2015; Doumba et

al., 2013). The objective of this study is to get closer to the *in vivo* conditions in chronic inflammation. For this reason, neutrophils, which are mainly involved in the acute-phase response, were not added in our co-culture model. However, to keep the overall response of the mononuclear cells, PBMCs instead of purified monocytes or PBLs were used. As PBMCs are activated in inflammatory conditions, PBMCs were stimulated with PHA. In addition, non-activated PBMCs were also used to determine whether cell interactions could modulate the immune response when the immune cells are "quiescent". Because the cells can interact through direct cell-cell contacts and paracrine interactions, cell culture inserts were used to assess the contribution of each.

However, these co-culture systems could be improved. Only one or two cell types from the muscles or the liver are studied whereas the cell composition is much varied *in vivo* in the muscles and especially in the liver. Indeed, the liver is characterized by a large hepatic cell repertoire including LSECs or Kupffer cells and it is also a rich source of NKT cells and γδ T cells (Racanelli and Rehermann, 2006). It would be interesting to develop a human multi-cell liver culture model mimicking the cellular arrangement within the liver to better understand the overall effect of the cell interplays in the liver. Such a culture system has been proposed with primary cells from rat livers (Bale et al., 2016).

4.2 Interactions between PBMCs, hepatocytes, HSCs or myoblasts increase the secretion of pro-inflammatory cytokines and chemokines

By using our co-culture model, the effects of the cell interactions on the pro-inflammatory cytokines and chemokines were studied. PBMC-HepaRG cell interactions enhance the expression and/or secretion of IL-6 as well as IL-8, CCL20 and MCP-1 chemokines partially through direct cell-cell interactions. PHA activation induces a higher IL-6, IL-8 and CCL20 release in PBMC-HepaRG cell co-cultures but the contribution of the PHA stimulation is weak for the CCL20 production. In contrast, the PHA activation has no effect on the induction of the IL-6 and CCL20 release by the PBMC-myoblast interactions. Moreover, the direct cell-cell contacts are crucial for the increase of CCL20 levels whereas the induction of the IL-6 secretion is mainly mediated by the paracrine interactions in PBMC-myoblast co-cultures.

By inducing IL-6 and several chemokines, these cell interactions can therefore promote immune cell recruitment and inflammation within the liver and the muscles, which are important in the onset and the persistence of the inflammatory state. The effects of the cell interplays on the IL-6 and chemokine levels are mediated through different mechanisms depending to the cell type. For example, direct PBMC-myoblast contacts are crucial for the induction of CCL20 secretion but not the direct contacts between PBMCs and hepatocytes. These observations could help in the identification and targeting of key pro-inflammatory molecules involved in the direct cell-cell contacts. The use of *in vivo* inflammatory models will then be needed to validate the potential targets.

4.3 IL-17 and TNFα contribute to the induction of IL-6 and IL-8 secretion by the cell interactions

Because IL-17 and TNFα cooperate to induce a pro-inflammatory response in hepatocyte, HSC and myoblast cultures, these two cytokines can contribute to the induction of IL-6, IL-8 and/or CCL20 observed in co-cultures. Neutralization of IL-17 and/or TNFα reduces the IL-6 and IL-8 secretion but not the CCL20 production induced by the interactions between PBMCs, hepatocytes and/or HSCs. TNFα also contributes to the enhanced IL-6 and CCL20 release in PBMC-myoblast co-cultures. However, the blockade of both IL-17 and TNFα does not induce a further inhibition of the IL-6, IL-8 and/ CCL20 levels in PBMC-hepatocyte and PBMC-myoblast co-cultures. It could be interesting to repeat these assays with PBMCs from patients with active systemic inflammatory diseases instead of PBMCs from healthy donors. Indeed, the phenotype and the reactivity of the PBMCs are different between these two donor populations and may affect the IL-17 and TNFα contribution in co-cultures.

4.4 Hepatocytes participate in the maintenance of immune tolerance in liver

The hepatocytes are the main parenchymal tissue of the liver, they are traditionally known for their metabolic activities. However, these cells produce acute-phase proteins and express MHC I and II molecules in inflammatory conditions. Hepatocytes can therefore participate in the immune cell response (Franco et al., 1988; Herkel et al., 2003). As described above, PBMC-HepaRG cell interactions may contribute to immune cell recruitment and

inflammation by increasing IL-6 and chemokine expression and/or synthesis through both direct cell contacts and paracrine interactions. In addition, the presence of HepaRG cells modulates T-cell polarization by increasing T-bet mRNA expression and by decreasing the $CD3^+$ $CD4^+$ IL-17^+ cell frequency in PHA-activated PBMCs. Surprising, the PBMC-HepaRG cell interactions increase the mRNA levels of some pro-inflammatory cytokines but not their secretion. Lastly, the HepaRG cells inhibit the MHC II expression on PBMCs activated with PHA as well as the PHA-induced PBMC proliferation. In contrast, the skin fibroblasts have no effect on the induction of PBMC proliferation by PHA. Therefore, the hepatocytes seem to mediate immunosuppressive signals on activated PBMCs, which certainly contribute to the maintenance of immune tolerance in liver and the low levels of graft rejection in liver transplantations. However, the HepaRG cell viability decreases in presence of PHA-activated PBMCs. Long-term exposure to PHA-activated PBMCs could lead to a massive HepaRG cell death and, consequently, a loss of tolerogenic HepaRG cell activities. This situation probably occurs in chronic liver diseases that are characterized by hepatocyte death, sustained inflammation and the development of fibrosis driving to liver cirrhosis. In this way, identification of the tolerogenic pathways that appear specific to the liver could lead to new therapeutic approaches to prevent the establishment and the progression of chronic inflammation.

CONCLUSION

Inflammation is a dynamic process in which cell interactions play an important role in the initiation, progression, persistence or resolution of the inflammatory state. The direct cell-cell contacts as well as the exchanges of soluble factors, such as IL-17 and TNFα, contribute to the immune response. By increasing in synergy the expression and the secretion of pro-inflammatory cytokines and chemokines by hepatocytes, HSCs and myoblasts, IL-17 and TNFα can promote immune cell recruitment and the production of systemic inflammatory mediators. In addition, the IL-17 and TNFα association can also mediate non-immune adverse effects in the liver and the muscles that could impair the hepatic and muscular functions. All these effects participate in the chronicity of the inflammatory response and can, in turn, enhance the IL-17 and TNFα synthesis leading to a pro-inflammatory feedback loop. Neutralization of IL-17 and/or TNFα at the onset of the inflammatory process can be a promising therapeutic strategy to control liver and muscle inflammation and therefore prevent tissue injury and dysfunction. IL-17 and TNFα inhibitors are currently available on the drug market for the treatment of several autoimmune diseases including psoriasis or ankylosing spondylitis. Their use could be extended to other systemic inflammatory disorders.

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