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

Audrey Beringer

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**IL-17 et Réponse Inflammatoire  
Systémique : Focus sur le Foie et le Muscle**

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# RESUME

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L'interleukine (IL)-17 et le TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  augmentent en synergie la sécrétion de la cytokine pro-inflammatoire IL-6 et de plusieurs chimiokines. Dans les myoblastes, l'IL-17 et le TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  contribuaient partiellement à ces effets. Les effets systémiques de l'IL-17 et du TNF $\alpha$  en font donc des cibles thérapeutiques attrayantes pour le traitement des nombreuses maladies inflammatoires systémiques.

**Mots clés :** interleukine-17, tumor necrosis factor- $\alpha$ , inflammation, interactions cellulaires, hépatocytes, cellules stellaires hépatiques, myoblastes

# RESUME SUBSTANTIEL

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L'interleukine (IL)-17 et le TNF $\alpha$  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 TNF $\alpha$  sont actuellement disponibles pour le traitement de certaines de ces pathologies. L'IL-17 peut coopérer avec le TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  dans les mécanismes pathogéniques immuns et non-immuns des myocytes 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 TNF $\alpha$  dans ces interactions

## **La combinaison IL-17 et TNF $\alpha$ amplifie la réponse inflammatoire hépatique**

Les effets de l'IL-17 et du TNF $\alpha$  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/TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$ , était crucial pour l'initiation de l'effet synergique IL-17/TNF $\alpha$  sur la sécrétion de l'IL-6 et de l'IL-8 par les hépatocytes. Le blocage de l'IL-17 et/ou du TNF $\alpha$  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 TNF $\alpha$ 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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$ . 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 TNF $\alpha$  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 TNF $\alpha$  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 phytohématagglutinine. 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 phytohématagglutinine 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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  menant à une boucle de rétroaction pro-inflammatoire. Les effets systémiques et pro-inflammatoires de l'IL-17 et du TNF $\alpha$ , 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

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Interleukin-17A (IL-17) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) 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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  stimulation induces endoplasmic reticulum stress and calcium dysregulation showing that immune and non-immune pathogenic mechanisms interplay. In hepatocytes, IL-17 and TNF $\alpha$  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 TNF $\alpha$  contribute partially to these inductions. The systemic effects of IL-17 and/or TNF $\alpha$  make them attractive therapeutic targets for the treatment of various systemic inflammatory disorders.

**Key words:** interleukin-17, tumor necrosis factor- $\alpha$ , inflammation, cell interactions, hepatocytes, hepatic stellate cells, myoblasts



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# ABBREVIATIONS

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$\alpha$ -SMA	Alpha Smooth Muscle Actin
APC	Antigen Presenting Cell
ASAT	Aspartate Amino-Transferase
Ca <sup>2+</sup>	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-3 $\alpha$	Macrophage Inflammatory Protein-3 $\alpha$
MMP	Matrix Metalloproteinase
NF $\kappa$ B	Nuclear Factor $\kappa$ B
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	Phosphatidylinositol-4,5-bisphosphate 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
TGF $\beta$	Transforming Growth Factor $\beta$
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll Like Receptors
TNF $\alpha$	Tumor Necrosis Factor $\alpha$
TNFR	TNF $\alpha$ receptor
TRADD	TNFR-Associated Death Domain

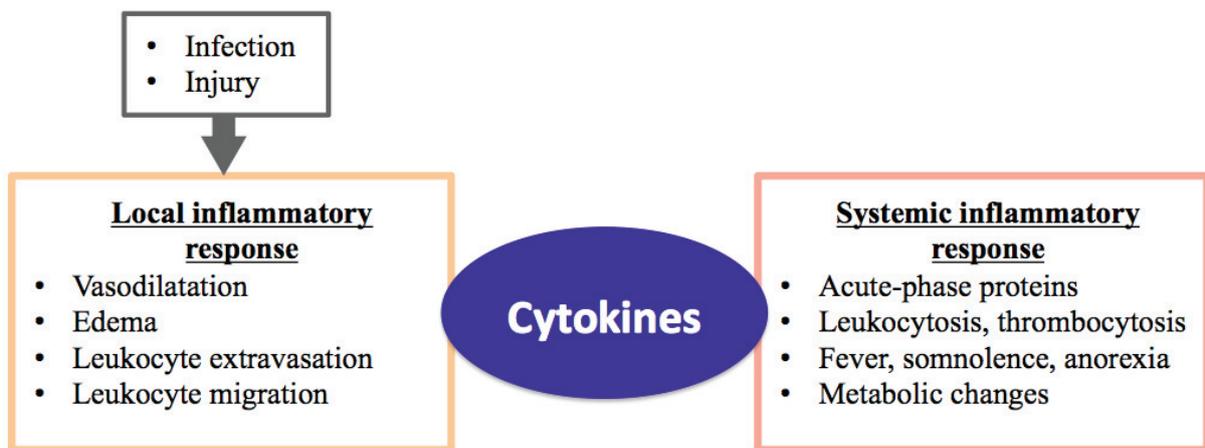


# GENERAL INTRODUCTION

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## 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 first 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, prostaglandins, 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-1 $\beta$ , tumor necrosis factor (TNF) $\alpha$  or interferon (IFN) $\gamma$ , 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 known, 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-1 $\beta$ , TNF $\alpha$  and IFN $\gamma$ . IL-6 is the major regulator of the hepatic acute-phase proteins. IL-1 $\beta$  and TNF- $\alpha$  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, TNF $\alpha$  or IL-1 $\beta$ ) or anti-inflammatory (e.g. IL-10 or transforming growth factor (TGF) $\beta$ ) cytokines.

IL-6, TNF $\alpha$  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, TNF $\alpha$  or IL-17 pathways are now available on the drug market. For this reason, this study focuses particularly on these three cytokines.

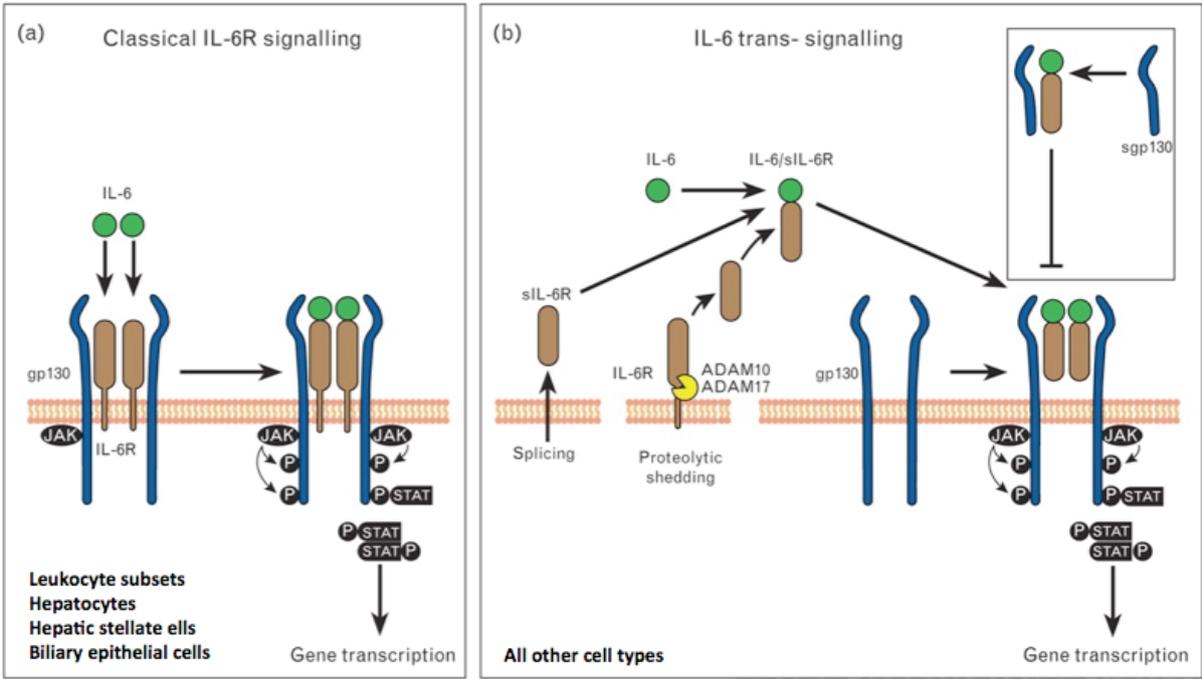
### ***1.3.1 Interleukin-6***

IL-6 is a four-helix protein of 184 amino acids with pleiotropic activities. IL-6 is synthesized 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 phosphatidylinositol-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 found 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

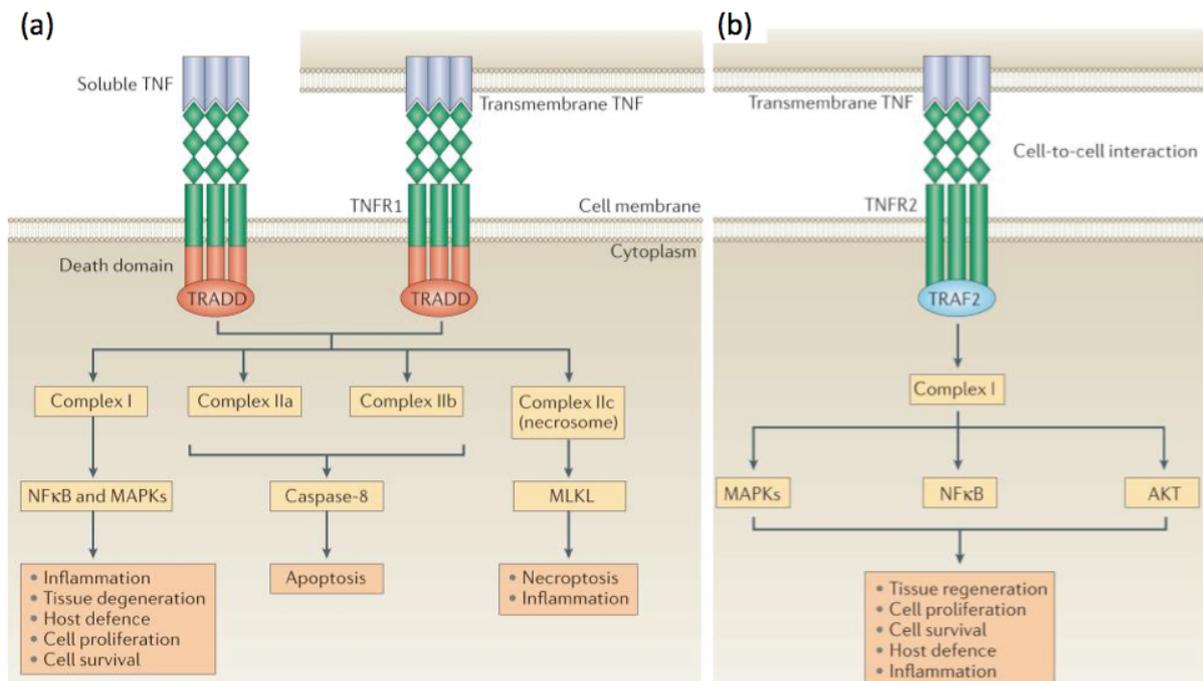
IL-6 is a pleiotropic cytokine with multiple functions in the body. It contributes to host defense against pathogens but plays also an important role in various autoimmune and inflammatory diseases such as rheumatoid arthritis (Tanaka et al., 2012). By acting on a wide variety of cells, IL-6 exerts multiple 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 $\alpha$*

TNF $\alpha$  is a pleiotropic cytokine that participates in a variety of inflammatory, infectious and malignant conditions. Activated monocytes and macrophages are the main sources of TNF $\alpha$  but a wide range of cells can also produce TNF $\alpha$  including mast cells, T cells, natural killer (NK) cells and non-immune cells such as endothelial cells (Sedger and McDermott, 2014). TNF $\alpha$  is synthesized as a 26 kDa membrane bound protein which can be cleaved into a soluble 17 kDa form by the TNF $\alpha$ -converting enzyme (TACE also known as ADAM17). Both membrane-associated and soluble TNF $\alpha$  are active and mediate their downstream signal by binding to TNF $\alpha$  receptor 1 (TNFR1) and TNFR2 (Bradley, 2008). TNFR1 (also known as CD120a) is expressed ubiquitously and activated by both transmembrane and soluble TNF $\alpha$ . 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 TNF $\alpha$  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 TNF $\alpha$  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  $\kappa$ B (NF $\kappa$ B) 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 TNF $\alpha$ -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: TNF $\alpha$  signaling pathways via TNF $\alpha$  receptor 1 and 2**

(a) Both soluble and transmembrane TNF $\alpha$  can activate TNF $\alpha$  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  $\kappa$ B (NF $\kappa$ B) driving to inflammation, tissue degeneration, host defence 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 TNF $\alpha$ . Ligation of TNFR2 leads to the recruitment of TNFR-associated factor 2 (TRAF2), which triggers assembly of complex I, and activation of MAPK, NF $\kappa$ B 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

TNF $\alpha$  can therefore trigger multiple signaling pathways involved in inflammation, host defense, proliferation and cell death. One of the major biological functions of TNF $\alpha$  is in the immune response to bacterial, viral and parasitic infections. TNF $\alpha$  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). TNF $\alpha$  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-3 $\alpha$  (MIP-3 $\alpha$ ) 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.**

## Review

IL-17 in Chronic Inflammation:  
From Discovery to TargetingAudrey Beringer,<sup>1</sup> Melissa Noack,<sup>1</sup> and Pierre Miossec<sup>1,\*</sup>

**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.

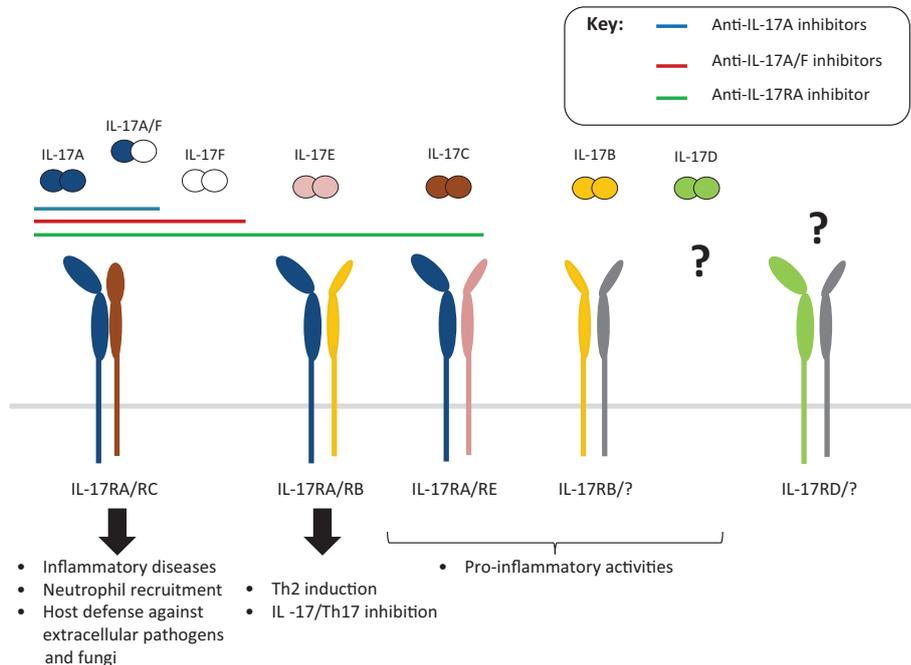
The first antibody against IL-17 was approved by the FDA and EMA in 2015 for the treatment of psoriasis.

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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## Trends in Molecular Medicine

**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- $\kappa$ B) 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 (IFN $\gamma$ ), 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- $\beta$ ) and IL-21 or IL-6, which induce the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (ROR $\gamma$ t) (human counterpart RORC). Then amplification occurs with IL-1 $\beta$  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<sup>+</sup> 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<sup>+</sup> T cells,  $\gamma\delta$  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 [TNF $\alpha$ , IL-1 $\beta$ , 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- $\kappa$ B 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 TNF $\alpha$ , IL-1 $\beta$ , IL-22, IFN $\gamma$ , 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 TNF $\alpha$ , leading to increased and prolonged protein production [42]. In addition, IL-17 induces TNF $\alpha$  receptor II expression, which increases the response to TNF $\alpha$  [41]. As a consequence, a combination of IL-17 and TNF $\alpha$  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 TNF $\alpha$  that lead to increased inflammation are the main basis for targeting both IL-17 and TNF $\alpha$ , either with a single bispecific molecule or with two inhibitors. This approach could be of interest in patients with an inadequate response to TNF $\alpha$  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<sup>+</sup> IL-17<sup>+</sup> 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
<i>IL-17A Inhibitors</i>						
Secukinumab (AIN457), Cosentyx™	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)
<i>IL-17A and IL-17F Inhibitors</i>						
Bimekizumab (UCB-4940)	UCB	Phase I	Phase I		Phase II	
ALX-0761 (MSB 0010841)	Merck Serono Ablynx	Phase I				
<i>IL-17A and TNF<math>\alpha</math> Inhibitors</i>						
ABT-122	AbbVie		Phase II		Phase II	
COVA322	Janssen/Covagen	Phase I/II	Preclinical	Preclinical	Preclinical	
<i>IL-17RA Inhibitors</i>						
Brodalumab (AMG 827)	Valeant Pharmaceuticals	Phase III		Phase III	Phase II	

Clinical status is based on data from Clinicaltrials.gov.

(AIN457, Cosentyx™), a fully human IgG1 $\kappa$  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.

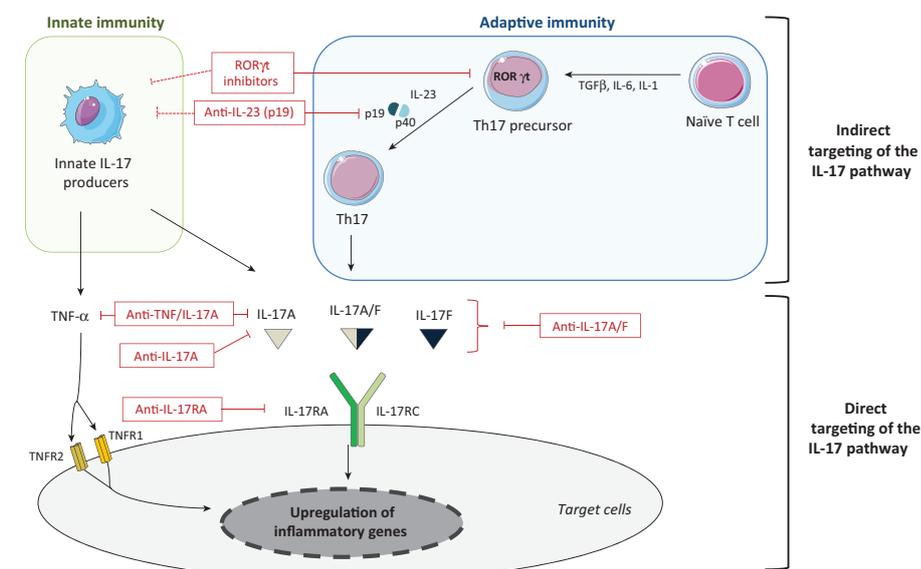
Based on the contribution of IL-17F to inflammation in addition to IL-17A, biomolecules targeting common motifs shared by IL-17A and IL-17F are now in clinical development, including the nanobody ALX-0761 and the mAb bimekizumab [61].

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 TNF $\alpha$  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 TNF $\alpha$  and the other IL-17A [62,63]. COVA322 is a fusion molecule comprising the fully human anti-TNF $\alpha$  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 TNF $\alpha$ and IL-17A (Anti-TNF $\alpha$ /IL-17A)	Antibody against IL-17 receptor type A (Anti-IL-17RA)
Secukinumab (Novartis) Ixekizumab (Lilly) CNTO 6785 (Janssen) CJM112 (Novartis) BCD 085 (BIOCAD)	Bimekizumab (UCB) ALX-122 (Merck Serono/Ablynx)	ABT-122 (AbbVie) COVA322 (Janssen/Covagen)	Brodalumab (Valeant)

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**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 (TNF $\alpha$ ) 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 (ROR $\gamma$ t).

## 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.

ROR $\gamma$ t 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 ROR $\gamma$ t 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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  and IL-17A, which may be of interest in cases of observed lack or loss of response to anti-TNF $\alpha$  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.

### References

- Miossec, P. *et al.* (2009) Interleukin-17 and type 17 helper T cells. *N. Engl. J. Med.* 361, 888–898
- Cypowyj, S. *et al.* (2012) Immunity to infection in IL-17-deficient mice and humans. *Eur. J. Immunol.* 42, 2246–2254
- Miossec, P. and Kolls, J.K. (2012) Targeting IL-17 and Th17 cells in chronic inflammation. *Nat. Rev. Drug Discov.* 11, 763–776
- Rouvier, E. *et al.* (1993) CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. *J. Immunol.* 150, 5445–5456
- Fossiez, F. *et al.* (1996) T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J. Exp. Med.* 183, 2593–2603
- Onishi, R.M. and Gaffen, S.L. (2010) Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology* 129, 311–321
- Reynolds, J.M. *et al.* (2010) IL-17 family member cytokines: regulation, and function in innate immunity. *Cytokine Growth Factor Rev.* 21, 413–423
- Gaffen, S.L. (2009) Structure and signalling in the IL-17 receptor family. *Nat. Rev. Immunol.* 9, 556–567
- Kleinschek, M.A. *et al.* (2007) IL-25 regulates Th17 function in autoimmune inflammation. *J. Exp. Med.* 204, 161–170
- Yao, Z. *et al.* (1995) Herpesvirus saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 3, 811–821
- Gaffen, S.L. (2011) Recent advances in the IL-17 cytokine family. *Curr. Opin. Immunol.* 23, 613–619
- Liu, S. *et al.* (2013) Crystal structures of interleukin 17A and its complex with IL-17 receptor A. *Nat. Commun.* 4, 1888
- Gaffen, S.L. *et al.* (2014) The IL-23–IL-17 immune axis: from mechanisms to therapeutic testing. *Nat. Rev. Immunol.* 14, 585–600
- Zrioual, S. *et al.* (2008) IL-17RA and IL-17RC receptors are essential for IL-17A-induced ELR<sup>+</sup> CX<sub>2</sub>C chemokine expression in synovial cells and are overexpressed in rheumatoid blood. *J. Immunol.* 180, 655–663
- Aarvak, T. *et al.* (1999) IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells. *J. Immunol.* 162, 1246–1251
- Yao, Z. *et al.* (1995) Human IL-17: a novel cytokine derived from T cells. *J. Immunol.* 155, 5483–5486
- Infante-Duarte, C. *et al.* (2000) Microbial lipopeptides induce the production of IL-17 in Th cells. *J. Immunol.* 165, 6107–6115
- Bettelli, E. *et al.* (2007) Th17: the third member of the effector T cell trilogy. *Curr. Opin. Immunol.* 19, 652–657
- Cua, D.J. *et al.* (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421, 744–748
- Korn, T. *et al.* (2009) IL-17 and Th17 Cells. *Annu. Rev. Immunol.* 27, 485–517
- Noack, M. and Miossec, P. (2014) Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun. Rev.* 13, 668–677
- Bettelli, E. *et al.* (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441, 235–238
- Cua, D.J. and Tato, C.M. (2010) Innate IL-17-producing cells: the sentinels of the immune system. *Nat. Rev. Immunol.* 10, 479–489
- Kenna, T.J. *et al.* (2012) Enrichment of circulating interleukin-17-secreting interleukin-23 receptor-positive  $\gamma\delta$  T cells in patients with active ankylosing spondylitis. *Arthritis Rheum.* 64, 1420–1429
- Noordenbos, T. *et al.* (2012) Interleukin-17-positive mast cells contribute to synovial inflammation in spondylarthritis. *Arthritis Rheum.* 64, 99–109
- Chabaud, M. *et al.* (2001) Enhancing effect of IL-1, IL-17, and TNF- $\alpha$  on macrophage inflammatory protein-3 $\alpha$  production in rheumatoid arthritis: regulation by soluble receptors and Th2 cytokines. *J. Immunol.* 167, 6015–6020
- Ye, P. *et al.* (2001) Interleukin-17 and lung host defense against *Klebsiella pneumoniae* infection. *Am. J. Respir. Cell Mol. Biol.* 25, 335–340
- Ishigame, H. *et al.* (2009) Differential roles of interleukin-17A and -17F in host defense against mucocutaneous bacterial infection and allergic responses. *Immunity* 30, 108–119
- Cho, J.S. *et al.* (2010) IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J. Clin. Invest.* 120, 1762–1773
- Conti, H.R. *et al.* (2009) Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J. Exp. Med.* 206, 299–311

### 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-TNF $\alpha$ /IL-17 antibodies are promising biotherapies because of the common synergistic interactions between these two cytokines. Will blocking both TNF $\alpha$  and IL-17 exacerbate the risk of infection? As the bispecific anti-TNF $\alpha$ /IL-17 antibodies are already in clinical trials, is there a benefit to combining an anti-TNF $\alpha$  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?

31. Kagami, S. *et al.* (2010) IL-23 and IL-17A, but not IL-12 and IL-22, are required for optimal skin host defense against *Candida albicans*. *J. Immunol.* 185, 5453–5462
32. de Beaucoudrey, L. *et al.* (2008) Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. *J. Exp. Med.* 205, 1543–1550
33. Puel, A. *et al.* (2011) Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science* 332, 65–68
34. Okada, S. *et al.* (2015) Immunodeficiencies. Impairment of immunity to *Candida* and *Mycobacterium* in humans with bi-allelic RORC mutations. *Science* 349, 606–613
35. Chabaud, M. *et al.* (1999) Human interleukin-17: a T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum.* 42, 963–970
36. Shahrara, S. *et al.* (2010) IL-17-mediated monocyte migration occurs partially through CC chemokine ligand 2/monocyte chemoattractant protein-1 induction. *J. Immunol.* 184, 4479–4487
37. Chabaud, M. *et al.* (1998) Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J. Immunol.* 161, 409–414
38. Toh, M.L. *et al.* (2010) Role of interleukin 17 in arthritis chronicity through survival of synoviocytes via regulation of synovial expression. *PLoS ONE* 5, e13416
39. Koenders, M.I. *et al.* (2005) Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinases 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall-induced arthritis. *Arthritis Rheum.* 52, 3239–3247
40. Chabaud, M. and Miossec, P. (2001) The combination of tumor necrosis factor alpha blockade with interleukin-1 and interleukin-17 blockade is more effective for controlling synovial inflammation and bone resorption in an *ex vivo* model. *Arthritis Rheum.* 44, 1293–1303
41. Zrioual, S. *et al.* (2009) Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. *J. Immunol.* 182, 3112–3120
42. Hartupée, J. *et al.* (2007) IL-17 enhances chemokine gene expression through mRNA stabilization. *J. Immunol.* 179, 4135–4141
43. Fischer, J.A.A. *et al.* (2015) Combined inhibition of tumor necrosis factor  $\alpha$  and interleukin-17 as a therapeutic opportunity in rheumatoid arthritis: development and characterization of a novel bispecific antibody. *Arthritis Rheumatol.* 67, 51–62
44. Miossec, P. and Kolls, J.K. (2012) Targeting IL-17 and TH17 cells in chronic inflammation. *Nat. Rev. Drug Discov.* 11, 763–776
45. Johansen, C. *et al.* (2009) Characterization of the interleukin-17 isoforms and receptors in lesional psoriatic skin. *Br. J. Dermatol.* 160, 319–324
46. Menon, B. *et al.* (2014) Interleukin-17<sup>+</sup>CD8<sup>+</sup> T cells are enriched in the joints of patients with psoriatic arthritis and correlate with disease activity and joint damage progression. *Arthritis Rheumatol.* 66, 1272–1281
47. Raychaudhuri, S.P. *et al.* (2012) IL-17 receptor and its functional significance in psoriatic arthritis. *Mol. Cell. Biochem.* 359, 419–429
48. Metavi, S.A. *et al.* (2011) Serum and synovial fluid levels of interleukin-17 in correlation with disease activity in patients with RA. *Clin. Rheumatol.* 30, 1201–1207
49. Lubberts, E. *et al.* (2004) Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. *Arthritis Rheum.* 50, 650–659
50. Mei, Y. *et al.* (2011) Increased serum IL-17 and IL-23 in the patient with ankylosing spondylitis. *Clin. Rheumatol.* 30, 269–273
51. Jandus, C. *et al.* (2008) Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. *Arthritis Rheum.* 58, 2307–2317
52. Appel, H. *et al.* (2011) Analysis of IL-17<sup>+</sup> cells in facet joints of patients with spondyloarthritis suggests that the innate immune pathway might be of greater relevance than the Th17-mediated adaptive immune response. *Arthritis Res. Ther.* 13, R95
53. Ciccia, F. *et al.* (2015) Type 3 innate lymphoid cells producing IL-17 and IL-22 are expanded in the gut, in the peripheral blood, synovial fluid and bone marrow of patients with ankylosing spondylitis. *Ann. Rheum. Dis.* 74, 1739–1747
54. Durelli, L. *et al.* (2009) T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon- $\beta$ . *Ann. Neurol.* 65, 499–509
55. Tzartos, J.S. *et al.* (2008) Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am. J. Pathol.* 172, 146–155
56. Yen, D. *et al.* (2006) IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J. Clin. Invest.* 116, 1310
57. Awasthi, A. and Kuchroo, V.K. (2009) IL-17A directly inhibits Th1 cells and thereby suppresses development of intestinal inflammation. *Nat. Immunol.* 10, 568–570
58. Jiang, W. *et al.* (2014) Elevated levels of Th17 cells and Th17-related cytokines are associated with disease activity in patients with inflammatory bowel disease. *Inflamm. Res.* 63, 943–950
59. Sahin, A. *et al.* (2014) Serum interleukin 17 levels in patients with Crohn's disease: real life data. *Dis. Markers* 2014, 690853
60. Ueno, A. *et al.* (2013) Increased prevalence of circulating novel IL-17 secreting Foxp3 expressing CD4<sup>+</sup> T cells and defective suppressive function of circulating Foxp3<sup>+</sup> regulatory cells support plasticity between Th17 and regulatory T cells in inflammatory bowel disease patients. *Inflamm. Bowel Dis.* 19, 2522–2534
61. Bartlett, H.S. and Million, R.P. (2015) Targeting the IL-17-Th17 pathway. *Nat. Rev. Drug Discov.* 14, 11–12
62. Hsieh, C.M. *et al.* (2014) FRI0303 discovery and characterization of Abt-122, an anti-TNF/IL-17 DVD-Ig molecule as a potential therapeutic candidate for rheumatoid arthritis. *Ann. Rheum. Dis.* 73, 495
63. Ahmed, G.F. *et al.* (2015) FRI0156 pharmacokinetics of ABT-122, a dual TNF- and IL-17A-targeted DVD-Ig<sup>TM</sup>, after single dosing in healthy volunteers and multiple dosing in subjects with rheumatoid arthritis. *Ann. Rheum. Dis.* 74, 479
64. Silacci, M. *et al.* (2015) Discovery and characterization of COVA322, a clinical-stage bispecific TNF/IL-17A inhibitor for the treatment of inflammatory diseases. *MAbs* 8, 141–149
65. Isono, F. *et al.* (2014) Inhibiting ROR $\gamma$ /Th17 axis for autoimmune disorders. *Drug Discov. Today* 19, 1205–1211
66. Solt, L.A. *et al.* (2011) Suppression of Th17 differentiation and autoimmunity by a synthetic ROR ligand. *Nature* 472, 491–494
67. Xiao, S. *et al.* (2014) Small-molecule ROR $\gamma$  antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms. *Immunity* 40, 477–489
68. Hueber, W. *et al.* (2010) Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. *Sci. Transl. Med.* 2, 52ra72
69. Langley, R.G. *et al.* (2014) Secukinumab in plaque psoriasis – results of two Phase 3 trials. *N. Engl. J. Med.* 371, 326–338
70. Thaci, D. *et al.* (2015) Secukinumab is superior to ustekinumab in clearing skin of subjects with moderate to severe plaque psoriasis: CLEAR, a randomized controlled trial. *J. Am. Acad. Dermatol.* 73, 400–409
71. Leonardi, C. *et al.* (2012) Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. *N. Engl. J. Med.* 366, 1190–1199
72. Griffiths, C.E.M. *et al.* (2015) Comparison of ixekizumab with etanercept or placebo in moderate-to-severe psoriasis (UNCOVER-2 and UNCOVER-3): results from two Phase 3 randomised trials. *Lancet* 386, 541–551
73. Papp, K.A. *et al.* (2012) Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. *N. Engl. J. Med.* 366, 1181–1189
74. Papp, K. *et al.* (2014) Safety and efficacy of brodalumab for psoriasis after 120 weeks of treatment. *J. Am. Acad. Dermatol.* 71, 1183–1190
75. Lebwohl, M. *et al.* (2015) Phase 3 studies comparing brodalumab with ustekinumab in psoriasis. *N. Engl. J. Med.* 373, 1318–1328

76. Sofen, H. *et al.* (2014) Guselkumab (an IL-23-specific mAb) demonstrates clinical and molecular response in patients with moderate-to-severe psoriasis. *J. Allergy Clin. Immunol.* 133, 1032–1040
77. Gordon, K.B. *et al.* (2015) A Phase 2 trial of guselkumab versus adalimumab for plaque psoriasis. *N. Engl. J. Med.* 373, 136–144
78. Kopp, T. *et al.* (2015) Clinical improvement in psoriasis with specific targeting of interleukin-23. *Nature* 521, 222–226
79. Papp, K. *et al.* (2015) Tildrakizumab (MK-3222), an anti-interleukin-23p19 monoclonal antibody, improves psoriasis in a Phase IIb randomized placebo-controlled trial. *Br. J. Dermatol.* 173, 930–939
80. McInnes, I.B. *et al.* (2014) Efficacy and safety of secukinumab, a fully human anti-interleukin-17A monoclonal antibody, in patients with moderate-to-severe psoriatic arthritis: a 24-week, randomised, double-blind, placebo-controlled, Phase II proof-of-concept trial. *Ann. Rheum. Dis.* 73, 349–356
81. McInnes, I.B. *et al.* FUTURE 2 Study Group (2015) Secukinumab, a human anti-interleukin-17A monoclonal antibody, in patients with psoriatic arthritis (FUTURE 2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 386, 1137–1146
82. Mease, P.J. *et al.* (2015) Secukinumab inhibition of interleukin-17A in patients with psoriatic arthritis. *N. Engl. J. Med.* 373, 1329–1339
83. Mease, P.J. *et al.* (2014) Brodalumab, an anti-IL17RA monoclonal antibody, in psoriatic arthritis. *N. Engl. J. Med.* 370, 2295–2306
84. Baeten, D. *et al.* (2013) Anti-interleukin-17A monoclonal antibody secukinumab in treatment of ankylosing spondylitis: a randomised, double-blind, placebo-controlled trial. *Lancet* 382, 1705–1713
85. Baraliakos, X. *et al.* (2015) Long-term effects of secukinumab on MRI findings in relation to clinical efficacy in subjects with active ankylosing spondylitis: an observational study. *Ann. Rheum. Dis.* Published online August 6, 2015. <http://dx.doi.org/10.1136/annrheumdis-2015-207544>
86. Genovese, M.C. *et al.* (2013) Efficacy and safety of secukinumab in patients with rheumatoid arthritis: a Phase II, dose-finding, double-blind, randomised, placebo controlled study. *Ann. Rheum. Dis.* 72, 863–869
87. Genovese, M.C. *et al.* (2014) One-year efficacy and safety results of secukinumab in patients with rheumatoid arthritis: Phase II, dose-finding, double-blind, randomized, placebo-controlled study. *J. Rheumatol.* 41, 414–421
88. Strand, V. *et al.* (2014) Secukinumab treatment in rheumatoid arthritis is associated with incremental benefit in the clinical outcomes and HRQoL improvements that exceed minimally important thresholds. *Health Qual. Life Outcomes* 12, 31
89. Burmester, G.R. *et al.* (2015) Association of HLA-DRB1 alleles with clinical responses to the anti-interleukin-17A monoclonal antibody secukinumab in active rheumatoid arthritis. *Rheumatology (Oxford)* 55, 49–55
90. Genovese, M.C. *et al.* (2010) LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: a Phase I randomized, double-blind, placebo-controlled, proof-of-concept study. *Arthritis Rheum.* 62, 929–939
91. Genovese, M.C. *et al.* (2014) A Phase II randomized study of subcutaneous ixekizumab, an anti-interleukin-17 monoclonal antibody, in rheumatoid arthritis patients who were naive to biologic agents or had an inadequate response to tumor necrosis factor inhibitors. *Arthritis Rheum.* 66, 1693–1704
92. Pavelka, K. *et al.* (2015) A study to evaluate the safety, tolerability, and efficacy of brodalumab in subjects with rheumatoid arthritis and an inadequate response to methotrexate. *J. Rheumatol.* 42, 912–919
93. Martin, D.A. *et al.* (2013) A Phase Ib multiple ascending dose study evaluating safety, pharmacokinetics, and early clinical response of brodalumab, a human anti-IL-17R antibody, in methotrexate-resistant rheumatoid arthritis. *Arthritis Res. Ther.* 15, R164
94. van Oosten, B.W. *et al.* (1996) Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. *Neurology* 47, 1531–1534
95. Mardiguan, S. *et al.* (2013) Anti-IL-17A treatment reduces clinical score and VCAM-1 expression detected by *in vivo* magnetic resonance imaging in chronic relapsing EAE ABH mice. *Am. J. Pathol.* 182, 2071–2081
96. Knier, B. *et al.* (2014) Novel monoclonal antibodies for therapy of multiple sclerosis. *Expert Opin. Biol. Ther.* 14, 503–513
97. Luchtman, D.W. *et al.* (2014) IL-17 and related cytokines involved in the pathology and immunotherapy of multiple sclerosis: current and future developments. *Cytokine Growth Factor Rev.* 25, 403–413
98. Hueber, W. *et al.* (2012) Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* 61, 1693–1700
99. Patel, D.D. *et al.* (2013) Effect of IL-17A blockade with secukinumab in autoimmune diseases. *Ann. Rheum. Dis.* 72 (Suppl. 2), ii116–ii123
100. Raine, T. and Kaser, A. (2012) Seventeen in Crohn's disease: less prime than we thought? *Gut* 61, 1653–1654
101. Maxwell, J.R. *et al.* (2015) Differential roles for interleukin-23 and interleukin-17 in intestinal immunoregulation. *Immunity* 43, 739–750
102. Busse, W.W. *et al.* (2013) Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. *Am. J. Respir. Crit. Care Med.* 188, 1294–1302
103. Grosskreutz, C.L. *et al.* (2015) Dry eye signs and symptoms persist during systemic neutralization of IL-1 $\beta$  by canakinumab or IL-17A by secukinumab. *Cornea* 34, 1551–1555
104. Danesh, M.J. and Kimball, A.B. (2016) Brodalumab and suicidal ideation in the context of a recent economic crisis in the United States. *J. Am. Acad. Dermatol.* 74, 190–192
105. Ndongo-Thiam, N. and Miossec, P. (2015) A cell-based bioassay for circulating bioactive IL-17: application to destruction in rheumatoid arthritis. *Ann. Rheum. Dis.* 74, 1629–1631

### **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 Rehmann, 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.**



## 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;  $\alpha$ GalCer,  $\alpha$ -galactosylceramide;  $\alpha$ -SMA,  $\alpha$ -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- $\beta$ , requires transforming growth factor- $\beta$

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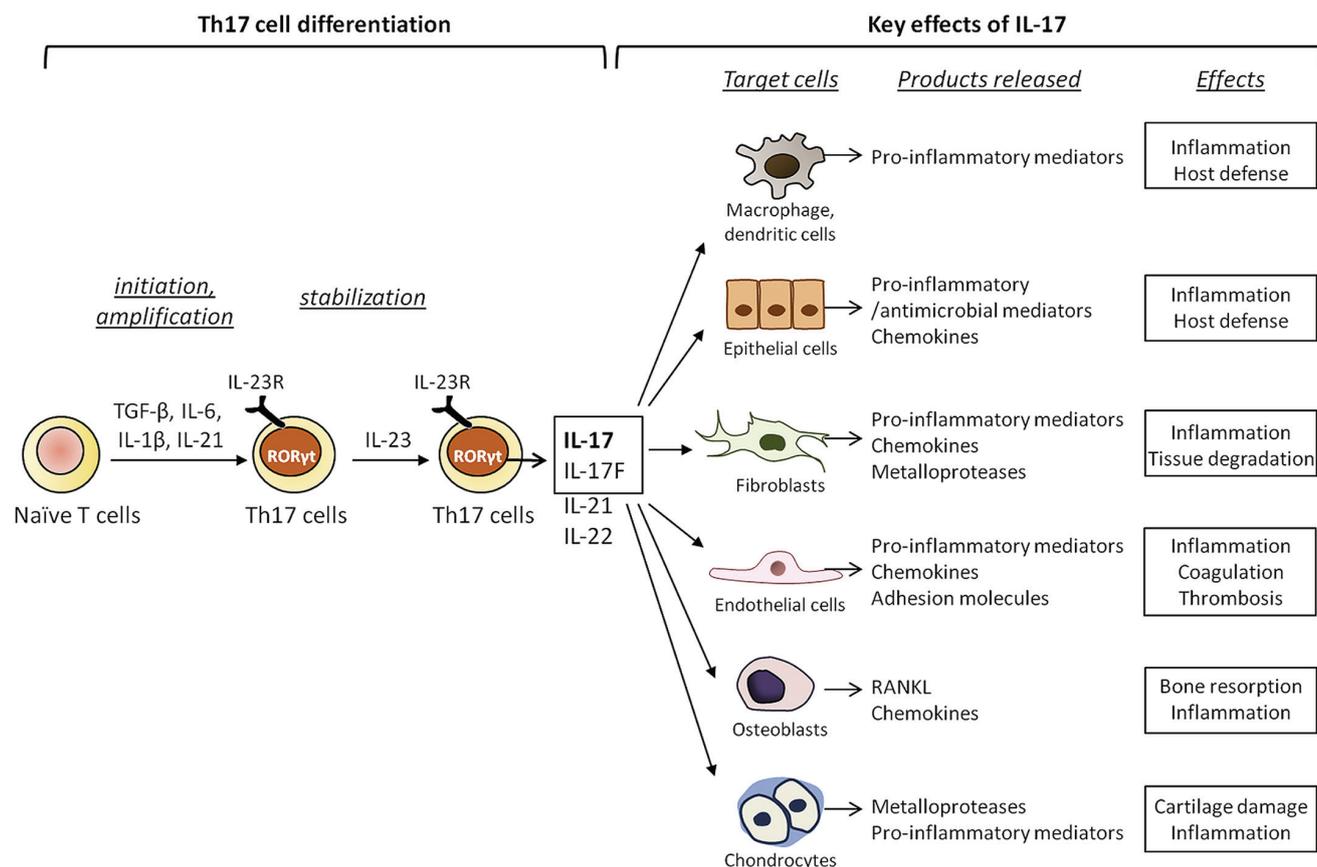


Fig. 1. Differentiation of Th17 cells and key biological effects of IL-17.

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 metalloprotease and RANKL by IL-17 leads to tissue damage as observed in joint inflammation. By acting on endothelial cells, IL-17 promotes coagulation and thrombosis. IL-23R, IL-23 receptor; RANKL, receptor activator of nuclear factor-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<sup>+</sup> Th17 cells were the first identified IL-17 producing cells. The differentiation of Th17 from naïve CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells following PMA/ionomycin activation [13]. Th17 cells and IL-17<sup>+</sup> CD8<sup>+</sup> 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<sup>+</sup> 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, TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  response [28]. In addition, IL-17 increases mRNA stability of cytokines induced by TNF $\alpha$  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 TNF $\alpha$  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-3 $\alpha$ ) are key chemokines up-regulated by the IL-17 and TNF $\alpha$  synergistic interaction, but independently of IL-6 [25]. By acting on IL-8, IL-17 and TNF $\alpha$  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 TNF $\alpha$  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- $\gamma$  and TNF $\alpha$  increases Th17 cell and T CD8<sup>+</sup> IL-17<sup>+</sup> 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-1 $\beta$  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-1 $\beta$  and TNF $\alpha$ ) and profibrotic mediators (TGF- $\beta$  and  $\alpha$ -smooth muscle actin ( $\alpha$ -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-1 $\beta$ , 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- $\alpha$  (GRO $\alpha$ ) and IL-8, which then induces the recruitment of neutrophils [44,45]. Moreover,  $\alpha$ -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- $\beta$  in the development of fibrosis. IL-17 up-regulates TGF- $\beta$ 1 and TGF- $\beta$ -RII expression and induces TGF- $\beta$ -RII signaling in LX-2 cells, which enhances HSCs response to TGF- $\beta$  and activates fibrosis [7,46]. Therefore, IL-17 induces HSCs activation and amplifies the TGF- $\beta$ -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-

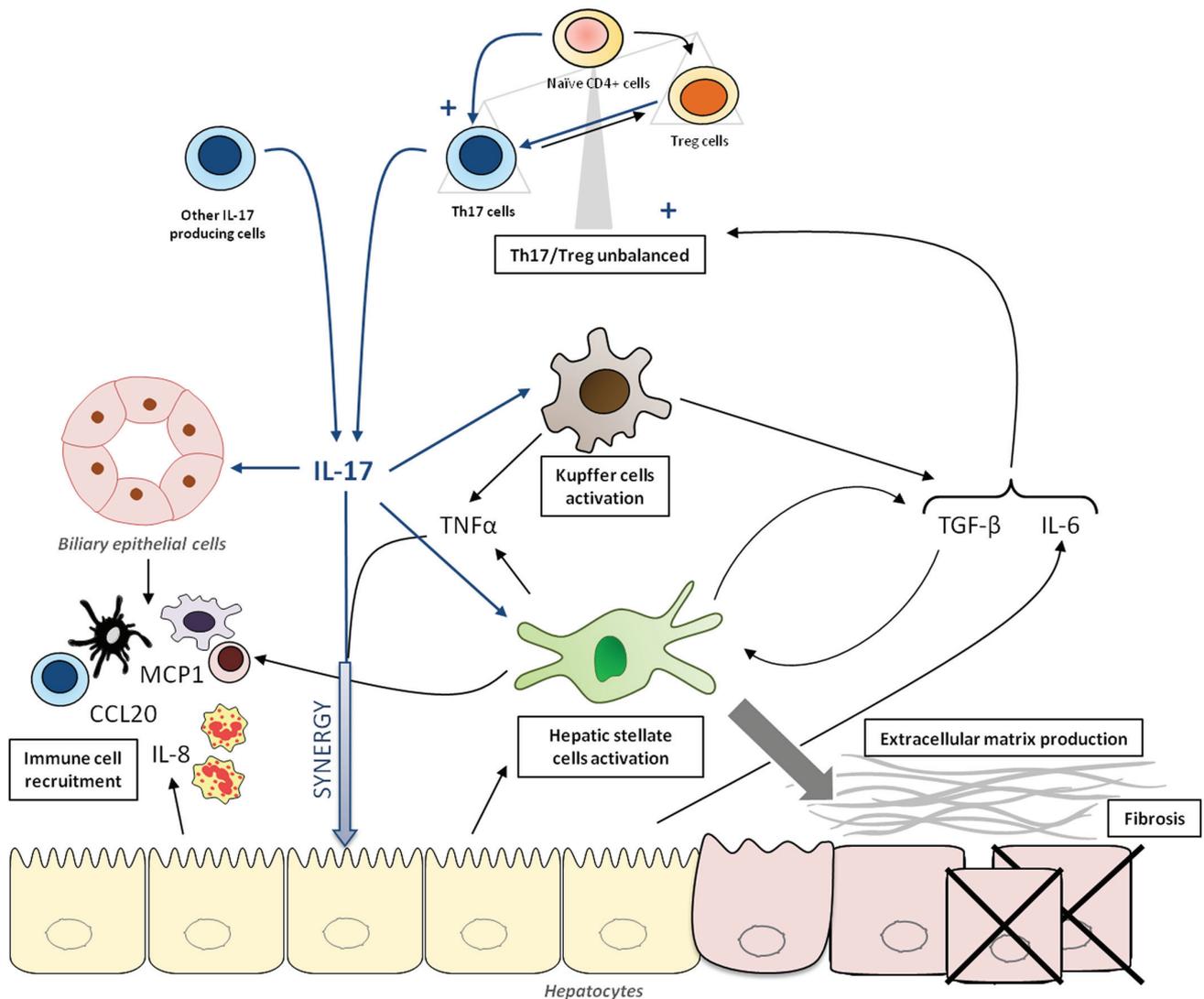


Fig. 2. Main effects of IL-17 on liver resident cells.

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 producing cells (e.g.  $\gamma\delta$  T cells, NK cells or group 3 innate lymphoid cells) induces TNF $\alpha$ , IL-6 and TGF- $\beta$  release by Kupffer cells (KC) and hepatic stellate cells (HSC). The IL-17 and TNF $\alpha$  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- $\beta$  by enhancing TGF- $\beta$  and TGF- $\beta$ -receptor expression in a positive feedback loop. HSC activation by IL-17, TGF- $\beta$  and periostin leads to collagen production and liver fibrosis. In addition, IL-6 and TGF- $\beta$  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.

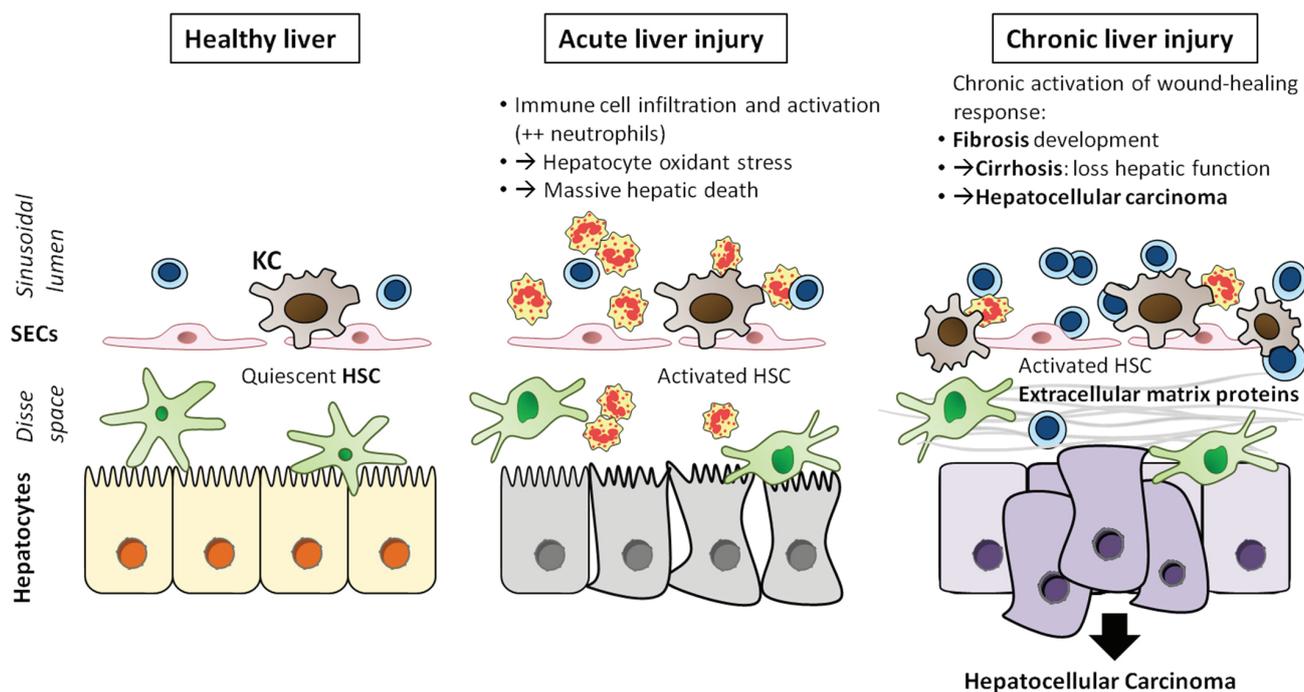
inflammatory cytokines (IL-6, IL-1 $\beta$ , TNF $\alpha$ ) and the profibrotic cytokine TGF- $\beta$  [7,47]. KCs from a mice model of hepatitis induce Th17 differentiation from naïve CD4<sup>+</sup> T cells in co-cultures [48]. KC and HSC co-cultures from wild type (WT) or IL-17RA<sup>-/-</sup> mice show that the percentage of collagen- $\alpha$ 1 is lower in WT KC - IL-17RA<sup>-/-</sup> HSC or IL-17RA<sup>-/-</sup> KC - WT HSC co-cultures than WT KC - WT HSC co-cultures, and this reduction is even higher in IL-17RA<sup>-/-</sup> KC - IL-17RA<sup>-/-</sup> HSC co-cultures [7]. IL-17 may therefore contribute to collagen production by acting both on KCs and HSCs. The increase of TGF- $\beta$  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 endothelial 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  $\alpha$ -galactosylceramide ( $\alpha$ GalCer)-induced hepatitis model. This surprising effect could be linked to the model itself [56].  $\alpha$ GalCer-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<sup>+</sup> 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-17<sup>+</sup> 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-17<sup>+</sup> cells represent around 2–3% of the CD3<sup>+</sup> T cell infiltrate, with CD4<sup>+</sup> Th17 cells present at slightly higher frequency than T CD8<sup>+</sup> IL-17<sup>+</sup> cells [16]. Because BECs may attract Th17 cells through CCL20 chemokine up-regulation in inflammatory conditions, IL-17<sup>+</sup> 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.  $\alpha$ GalCer,  $\alpha$ -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.
<i>Chemical compounds-induced hepatitis models</i>		
Carbon tetrachloride (CCl <sub>4</sub> )-induced liver fibrosis mice	Increase of plasma IL-17 level and splenic Th17 cell frequency following CCl <sub>4</sub> 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 <sup>-/-</sup> 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 $\gamma\delta$ 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]
$\alpha$ -galactosylceramide ( $\alpha$ GalCer)-induced hepatitis mice	$\alpha$ GalCer injection in mice induces IL-17 production by NKT cells. Neutralization of IL-17 before $\alpha$ GalCer injection exacerbates hepatitis and IL-17 administration ameliorates $\alpha$ GalCer-induced hepatitis.	[56]
<i>Viral hepatitis models</i>		
Adenovirus	Adenovirus increases hepatic IL-17A and IL-17F production, predominantly by $\gamma\delta$ 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- $\gamma$ regulates negatively virus induced-Th17 cell response by increasing Th17 apoptosis.	[106]
<i>Drug-induced liver injury models</i>		
Diclofenac overdose	Up-regulation of the hepatic expression of the Th17 cell-related factors ROR $\gamma$ t 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]
<i>Alcoholic liver disease models</i>		
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]
<i>Non-alcoholic fatty liver disease (NAFLD) models</i>		
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 <sup>-/-</sup> mice on MCDD are protected from increased pro-inflammatory cytokine expression, immune cell infiltration and hepatocellular damage.	[112,113]

lymphopoietin is associated to CD4<sup>+</sup> 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]
Alcoholic liver disease (ALD)	Increase of IL-17 plasma level and liver IL-17 <sup>+</sup> cell infiltrates in ALD patients. The IL-17-secreting cell infiltrates are associated with fibrosis score and the Th17/Treg imbalance with poor prognosis.	[32,71,72] [45,114]
Non-alcoholic fatty liver (NAFL) disease	Increase of IL-17 <sup>+</sup> 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 <sup>+</sup> 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-producing CD8 <sup>+</sup> 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 CXCL 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<sup>+</sup> and RORC<sup>+</sup> cells in the newly generated Treg (nTreg CD25<sup>-</sup>) 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 anti-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-1 $\beta$  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-producing 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 TNF $\alpha$  often work in synergy, bispecific inhibitors blocking both IL-17 and TNF $\alpha$  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-1 $\beta$  are involved in the Th17 cell differentiation, the inhibition of the IL-6 pathway (by anti-IL-6R or JAK inhibitors) or the IL-1 $\beta$  pathway (by anti-IL-1 $\beta$  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

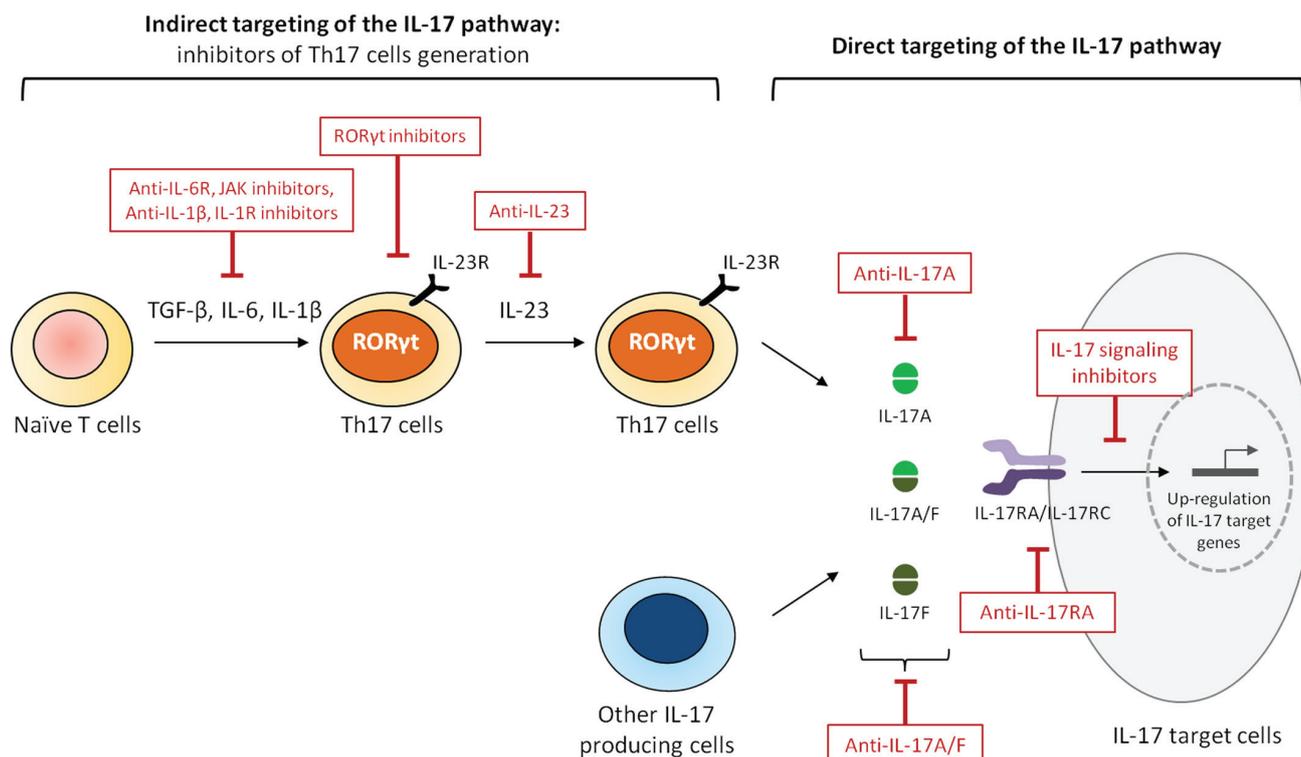


Fig. 4. Therapeutic strategies to target IL-17 pathway and Th17 cell generation.

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 inhibitors) or IL-1 pathway (IL-1R antagonist or anti-IL-1 $\beta$ ) may inhibit the initiation of Th17 cell generation and anti-IL-23 the stabilization of the Th17 cell phenotype. ROR $\gamma$ t inhibitors reduce the Th17 cell differentiation. IL-6R, IL-6 receptor; IL-1R, IL-1 receptor; JAK, janus kinase; ROR $\gamma$ t, retinoic acid receptor-related orphan nuclear receptor gamma

Th17 cell phenotype [90]. Different pathway inhibitors of IL-6 (tocilizumab or the anti-JAK tofacitinib), IL-1 $\beta$  (anakinra or canakinumab) and IL-23 (tildrakizumab or guselkumab) are now tested. Targeting the Th17 cell transcription factor ROR $\gamma$ t/ 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 diseases. 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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### References

- [1] Heymann F, Tacke F. Immunology in the liver—from homeostasis to disease. *Nat Rev Gastroenterol Hepatol* 2016;13:88–110.
- [2] Racanelli V, Rehermann B. The liver as an immunological organ. *Hepatology* 2006;43:S54–62.
- [3] Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *N Engl J Med* 2009;361:888–98.
- [4] Lavocat F, Ndongo-Thiam N, Miossec P. Interleukin-25 Produced by Synovialocytes Has Anti-inflammatory Effects by Acting As a Receptor Antagonist for Interleukin-17A Function. *Front Immunol* 2017;8:647.
- [5] Gaffen SL, Jain R, Garg AV, Cua DJ. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nat Rev Immunol* 2014;14:585–600.
- [6] Lafdil F, Miller AM, Ki SH, Gao B. Th17 cells and their associated cytokines in liver diseases. *Cell Mol Immunol* 2010;7:250–4.
- [7] Meng F, Wang K, Aoyama T, Grivnenikov SI, Paik Y, Scholten D, et al. Interleukin-17 signaling in inflammatory. Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice. *Gastroenterology* 2012;143(765–76):e1–3.

- [8] Noack M, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev* 2014;13:668–77.
- [9] Roh YS, Park S, Lim CW, Kim B. Depletion of Foxp3+ Regulatory T Cells Promotes Profibrogenic Milieu of Cholestasis-Induced Liver Injury. *Dig Dis Sci* 2015;60:2009–18.
- [10] Sun XF, Gu L, Deng WS, Xu Q. Impaired balance of T helper 17/T regulatory cells in carbon tetrachloride-induced liver fibrosis in mice. *World J Gastroenterol* 2014;20:2062–70.
- [11] Ju JH, Heo YJ, Cho ML, Jhun JY, Park JS, Lee SY, et al. Modulation of STAT-3 in rheumatoid synovial T cells suppresses Th17 differentiation and increases the proportion of Treg cells. *Arthritis Rheum* 2012;64:3543–52.
- [12] Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* 2010;10:479–89.
- [13] Tang XZ, Jo J, Tan AT, Sandalova E, Chia A, Tan KC, et al. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J Immunol* 2013;190:3142–52.
- [14] Hammerich L, Heymann F, Tacke F. Role of IL-17 and Th17 cells in liver diseases. *Clin Dev Immunol* 2011;2011:345803.
- [15] Grafmueller S, Billerbeck E, Blum HE, Neumann-Haefelin C, Thimme R. Differential antigen specificity of hepatitis C virus-specific interleukin 17- and interferon gamma-producing CD8(+) T cells during chronic infection. *J Infect Dis* 2012;205:1142–6.
- [16] Oo YH, Banz V, Kavanagh D, Liaskou E, Withers DR, Humphreys E, et al. CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver. *J Hepatol* 2012;57:1044–51.
- [17] Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, Monroe HR, et al. IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J Clin Invest* 2010;120:1762–73.
- [18] Kagami S, Rizzo HL, Kurtz SE, Miller LS, Blauvelt A. IL-23 and IL-17A, but not IL-12 and IL-22, are required for optimal skin host defense against *Candida albicans*. *J Immunol* 2010;185:5453–62.
- [19] Okada S, Markle JG, Deenick EK, Mele F, Averbuch D, Lagos M, et al. Impairment of immunity to *Candida* and *Mycobacterium* in humans with bi-allelic RORC mutations. *Science* 2015;349:606–13.
- [20] de Beaucoudrey L, Puel A, Filipe-Santos O, Cobat A, Ghandil P, Chrabieh M, et al. Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. *J Exp Med* 2008;205:1543–50.
- [21] Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science* 2011;332:65–8.
- [22] Hot A, Lenief V, Miossec P. Combination of IL-17 and TNFalpha induces a pro-inflammatory, pro-coagulant and pro-thrombotic phenotype in human endothelial cells. *Ann Rheum Dis* 2012;71:768–76.
- [23] Katz Y, Nadviv O, Beer Y. Interleukin-17 enhances tumor necrosis factor alpha-induced synthesis of interleukins 1,6, and 8 in skin and synovial fibroblasts: a possible role as a "fine-tuning cytokine" in inflammation processes. *Arthritis Rheum* 2001;44:2176–84.
- [24] Osta B, Roux JP, Lavocat F, Pierre M, Ndongo-Thiam N, Boivin G, et al. Differential Effects of IL-17A and TNF-alpha on Osteoblastic Differentiation of Isolated Synovial Cells and on Bone Explants from Arthritis Patients. *Front Immunol* 2015;6:151.
- [25] A. Beringer, N. Ndongo, J. Molle, B. Bartosch and P. Miossec, Synergistic effect of interleukin-17 and tumor necrosis factor- $\alpha$  on inflammatory response in hepatocytes through interleukin-6 dependent and independent pathways, *Clin Exp Immunol* (in press).
- [26] Ruddy MJ, Wong GC, Liu XK, Yamamoto H, Kasayama S, Kirkwood KL, et al. Functional cooperation between interleukin-17 and tumor necrosis factor-alpha is mediated by CCAAT/enhancer-binding protein family members. *J Biol Chem* 2004;279:2559–67.
- [27] Sparna T, Retej J, Schlich K, Albrecht U, Naumann K, Gretz N, et al. Genome-wide comparison between IL-17 and combined TNF-alpha/IL-17 induced genes in primary murine hepatocytes. *BMC Genomics* 2010;11:226.
- [28] Zrioual S, Ecochard R, Tournadre A, Lenief V, Cazalis MA, Miossec P. Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synovial cells. *J Immunol* 2009;182:3112–20.
- [29] Hartupee J, Liu C, Novotny M, Li X, Hamilton T. IL-17 enhances chemokine gene expression through mRNA stabilization. *J Immunol* 2007;179:4135–41.
- [30] Gu FM, Li QL, Gao Q, Jiang JH, Zhu K, Huang XY, et al. IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma. *Mol Cancer* 2011;10:150.
- [31] Patel DN, King CA, Bailey SR, Holt JW, Venkatachalam K, Agrawal A, et al. Interleukin-17 stimulates C-reactive protein expression in hepatocytes and smooth muscle cells via p38 MAPK and ERK1/2-dependent NF-kappaB and C/EBPbeta activation. *J Biol Chem* 2007;282:27229–38.
- [32] Zhao L, Tang Y, You Z, Wang Q, Liang S, Han X, et al. Interleukin-17 contributes to the pathogenesis of autoimmune hepatitis through inducing hepatic interleukin-6 expression. *PLoS One* 2011;6:e18909.
- [33] Tang Y, Bian Z, Zhao L, Liu Y, Liang S, Wang Q, et al. Interleukin-17 exacerbates hepatic steatosis and inflammation in non-alcoholic fatty liver disease. *Clin Exp Immunol* 2011;166:281–90.
- [34] Amara S, Lopez K, Banan B, Brown SK, Whalen M, Myles E, et al. Synergistic effect of pro-inflammatory TNFalpha and IL-17 in peristoin mediated collagen deposition: potential role in liver fibrosis. *Mol Immunol* 2015;64:26–35.
- [35] O'Brien KM, Allen KM, Rockwell CE, Towery K, Luyendyk JP, Coppole BL. IL-17A synergistically enhances bile acid-induced inflammation during obstructive cholestasis. *Am J Pathol* 2013;183:1498–507.
- [36] Fabbri E, Cella M, McCartney SA, Fuchs A, Abumrad NA, Pietka TA, et al. Association between specific adipose tissue CD4+ T-cell populations and insulin resistance in obese individuals. *Gastroenterology* 2013;145(366–74):e1–3.
- [37] Carambia A, Frenzel C, Bruns OT, Schwinge D, Reimer R, Hohenberg H, et al. Inhibition of inflammatory CD4 T cell activity by murine liver sinusoidal endothelial cells. *J Hepatol* 2013;58:112–8.
- [38] Harada K, Shimoda S, Sato Y, Isse K, Ikeda H, Nakanuma Y. Periductal interleukin-17 production in association with biliary innate immunity contributes to the pathogenesis of cholangiopathy in primary biliary cirrhosis. *Clin Exp Immunol* 2009;157:261–70.
- [39] Huang Q, Chu S, Yin X, Yu X, Kang C, Li X, et al. Interleukin-17A-Induced Epithelial-Mesenchymal Transition of Human Intrahepatic Biliary Epithelial Cells: Implications for Primary Biliary Cirrhosis. *Tohoku J Exp Med* 2016;240:269–75.
- [40] Tan Z, Qian X, Jiang R, Liu Q, Wang Y, Chen C, et al. IL-17A plays a critical role in the pathogenesis of liver fibrosis through hepatic stellate cell activation. *J Immunol* 2013;191:1835–44.
- [41] Sun HQ, Zhang JY, Zhang H, Zou ZS, Wang FS, Jia JH. Increased Th17 cells contribute to disease progression in patients with HBV-associated liver cirrhosis. *J Viral Hepat* 2012;19:396–403.
- [42] Hara M, Kono H, Furuya S, Hirayama K, Tsuchiya M, Fujii H. Interleukin-17A plays a pivotal role in cholestatic liver fibrosis in mice. *J Surg Res* 2013;183:574–82.
- [43] Botcher K, Rombouts K, Saffioti F, Roccarina D, Rosselli M, Hall A, et al. MAIT cells are chronically activated in patients with autoimmune liver disease and promote pro-fibrogenic hepatic stellate cell activation. *Hepatology* (Baltimore, Md.) 2018 July;68(no. 1):172–86. <https://doi.org/10.1002/hep.29782>.
- [44] Shi T, Zhang T, Zhang L, Yang Y, Zhang H, Zhang F. The Distribution and the Fibrotic Role of Elevated Inflammatory Th17 Cells in Patients With Primary Biliary Cirrhosis. *Medicine* (Baltimore) 2015;94:e1888.
- [45] Lemmers A, Moreno C, Gustot T, Marechal R, Degre D, Demetter P, et al. The interleukin-17 pathway is involved in human alcoholic liver disease. *Hepatology* 2009;49:646–57.
- [46] Fabre T, Kared H, Friedman SL, Shoukry NH. IL-17A enhances the expression of profibrotic genes through upregulation of the TGF-beta receptor on hepatic stellate cells in a JNK-dependent manner. *J Immunol* 2014;193:3925–33.
- [47] Lafdil F, Wang H, Park O, Zhang W, Moritoki Y, Yin S, et al. Myeloid STAT3 inhibits T cell-mediated hepatitis by regulating T helper 1 cytokine and interleukin-17 production. *Gastroenterology* 2009;137(2125–35):e1–2.
- [48] Zhang Y, Wang X, Zhong M, Zhang M, Suo Q, Lv K. MicroRNA let-7a ameliorates con A-induced hepatitis by inhibiting IL-6-dependent Th17 cell differentiation. *J Clin Immunol* 2013;33:630–9.
- [49] Jaeschke H, Hasegawa T. Role of neutrophils in acute inflammatory liver injury. *Liver Int* 2006;26:912–9.
- [50] Lefkowitz JH. The Pathology of Acute Liver Failure. *Adv Anat Pathol* 2016;23:144–58.
- [51] McDowell Torres D, Stevens RD, Gurakar A. Acute liver failure: a management challenge for the practicing gastroenterologist. *Gastroenterol Hepatol (N Y)* 2010;6:444–50.
- [52] Xu M, Morishima N, Mizoguchi I, Chiba Y, Fujita K, Kuroda M, et al. Regulation of the development of acute hepatitis by IL-23 through IL-22 and IL-17 production. *Eur J Immunol* 2011;41:2828–39.
- [53] Yan S, Wang L, Liu N, Wang Y, Chu Y. Critical role of interleukin-17/interleukin-17 receptor axis in mediating Con A-induced hepatitis. *Immunol Cell Biol* 2012;90:421–8.
- [54] He J, Lang G, Ding S, Li L. Pathological role of interleukin-17 in poly I:C-induced hepatitis. *PLoS One* 2013;8:e73909.
- [55] Furuya S, Kono H, Hara M, Hirayama K, Sun C, Fujii H. Interleukin 17A plays a role in lipopolysaccharide/D-galactosamine-induced fulminant hepatic injury in mice. *J Surg Res* 2015;199:487–93.
- [56] Wondimu Z, Santodomingo-Garzon T, Le T, Swain MG. Protective role of interleukin-17 in murine NKT cell-driven acute experimental hepatitis. *Am J Pathol* 2010;177:2334–46.
- [57] Kobayashi E, Kobayashi M, Tsuneyama K, Fukami T, Nakajima M, Yokoi T. Halothane-induced liver injury is mediated by interleukin-17 in mice. *Toxicol Sci* 2009;111:302–10.
- [58] Wang X, Jiang Z, Xing M, Fu J, Su Y, Sun L, et al. Interleukin-17 mediates tripolite-induced liver injury in mice. *Food Chem Toxicol* 2014;71:33–41.
- [59] Wang X, Sun R, Wei H, Tian Z. High-mobility group box 1 (HMGB1)-Toll-like receptor (TLR)4-interleukin (IL)-23-IL-17A axis in drug-induced damage-associated lethal hepatitis: Interaction of gammadelta T cells with macrophages. *Hepatology* 2013;57:373–84.
- [60] Zhu X, Uetrecht J. A novel T(H)17-type cell is rapidly increased in the liver in response to acetaminophen-induced liver injury: T(H)17 cells and the innate immune response. *J Immunotoxicol* 2013;10:287–91.
- [61] Yasumi Y, Takikawa Y, Endo R, Suzuki K. Interleukin-17 as a new marker of severity of acute hepatic injury. *Hepatology Res* 2007;37:248–54.
- [62] Lai R, Xiang X, Mo R, Bao R, Wang P, Guo S, et al. Protective effect of Th22 cells and intrahepatic IL-22 in drug induced hepatocellular injury. *J Hepatol* 2015;63:148–55.
- [63] Sarra M, Cupi ML, Bernardini R, Ronchetti G, Monteleone I, Ranalli M, et al. IL-25 prevents and cures fulminant hepatitis in mice through a myeloid-derived suppressor cell-dependent mechanism. *Hepatology* 2013;58:1436–50.
- [64] Zhang F, Yao S, Yuan J, Zhang M, He Q, Yang G, et al. Elevated IL-6 receptor expression on CD4+ T cells contributes to the increased Th17 responses in patients with chronic hepatitis B. *Virology* 2011;8:270.
- [65] Wang Q, Zhou J, Zhang B, Tian Z, Tang J, Zheng Y, et al. Hepatitis B virus induces IL-23 production in antigen presenting cells and causes liver damage via the IL-23/IL-17 axis. *PLoS Pathog* 2013;9:e1003410.
- [66] Li J, Wang FP, She WM, Yang CQ, Li L, Tu CT, et al. Enhanced high-mobility group box 1 (HMGB1) modulates regulatory T cells (Treg)/T helper 17 (Th17) balance via toll-like receptor (TLR)-4-interleukin (IL)-6 pathway in patients with chronic hepatitis B. *J Viral Hepat* 2014;21:129–40.
- [67] Jhun J, Lee S, Kim H, Her YM, Byun JK, Kim EK, et al. HMGB1/RAGE induces IL-17 expression to exaggerate inflammation in peripheral blood cells of hepatitis B patients. *J Transl Med* 2015;13:310.

- [68] Lee HC, Sung SS, Krueger PD, Jo YA, Rosen HR, Ziegler SF, et al. Hepatitis C virus promotes T-helper (Th)17 responses through thymic stromal lymphopoietin production by infected hepatocytes. *Hepatology* 2013;57:1314–24.
- [69] Feng TT, Zou T, Wang X, Zhao WF, Qin AL. Clinical significance of changes in the Th17/Treg ratio in autoimmune liver disease. *World J Gastroenterol* 2017;23:3832–8.
- [70] Yu H, Huang J, Liu Y, Ai G, Yan W, Wang X, et al. IL-17 contributes to autoimmune hepatitis. *J Huazhong Univ Sci Technol Med Sci* 2010;30:443–6.
- [71] Grant CR, Liberal R, Holder BS, Cardone J, Ma Y, Robson SC, et al. Dysfunctional CD39(POS) regulatory T cells and aberrant control of T-helper type 17 cells in autoimmune hepatitis. *Hepatology* 2014;59:1007–15.
- [72] Longhi MS, Liberal R, Holder B, Robson SC, Ma Y, Mieli-Vergani G, et al. Inhibition of interleukin-17 promotes differentiation of CD25(–) cells into stable T regulatory cells in patients with autoimmune hepatitis. *Gastroenterology* 2012;142:1526–35. [e6].
- [73] Qian C, Jiang T, Zhang W, Ren C, Wang Q, Qin Q, et al. Increased IL-23 and IL-17 expression by peripheral blood cells of patients with primary biliary cirrhosis. *Cytokine* 2013;64:172–80.
- [74] Rong G, Zhou Y, Xiong Y, Zhou L, Geng H, Jiang T, et al. Imbalance between T helper type 17 and T regulatory cells in patients with primary biliary cirrhosis: the serum cytokine profile and peripheral cell population. *Clin Exp Immunol* 2009;156:217–25.
- [75] Lan RY, Salunga TL, Tsuneyama K, Lian ZX, Yang GX, Hsu W, et al. Hepatic IL-17 responses in human and murine primary biliary cirrhosis. *J Autoimmun* 2009;32:43–51.
- [76] Yang CY, Ma X, Tsuneyama K, Huang S, Takahashi T, Chalasani NP, et al. IL-12/Th1 and IL-23/Th17 biliary microenvironment in primary biliary cirrhosis: implications for therapy. *Hepatology* 2014;59:1944–53.
- [77] Kawata K, Tsuda M, Yang GX, Zhang W, Tanaka H, Tsuneyama K, et al. Identification of potential cytokine pathways for therapeutic intervention in murine primary biliary cirrhosis. *PLoS One* 2013;8:e74225.
- [78] Katt J, Schwinge D, Schoknecht T, Quaaas A, Sobotka I, Burandt E, et al. Increased T helper type 17 response to pathogen stimulation in patients with primary sclerosing cholangitis. *Hepatology* 2013;58:1084–93.
- [79] Pollheimer MJ, Trauner M, Fickert P. Will we ever model PSC? - "it's hard to be a PSC model!" *Clin Res Hepatol Gastroenterol* 2011;35:792–804.
- [80] Sarin SK, Choudhury A. Acute-on-chronic liver failure: terminology, mechanisms and management. *Nat Rev Gastroenterol Hepatol* 2016;13:131–49.
- [81] Yang B, Wang Y, Zhao C, Yan W, Che H, Shen C, et al. Increased Th17 cells and interleukin-17 contribute to immune activation and disease aggravation in patients with chronic hepatitis B virus infection. *Immunol Lett* 2013;149:41–9.
- [82] Kim HY, Jhun JY, Cho ML, Choi JY, Byun JK, Kim EK, et al. Interleukin-6 upregulates Th17 response via mTOR/STAT3 pathway in acute-on-chronic hepatitis B liver failure. *J Gastroenterol* 2014;49:1264–73.
- [83] Niu Y, Liu H, Yin D, Yi R, Chen T, Xue H, et al. The balance between intrahepatic IL-17(+) T cells and Foxp3(+) regulatory T cells plays an important role in HBV-related end-stage liver disease. *BMC Immunol* 2011;12:47.
- [84] Qi ZX, Wang LY, Fan YC, Zhang JJ, Li T, Wang K. Increased peripheral RORalpha and RORgammat mRNA expression is associated with acute-on-chronic hepatitis B liver failure. *J Viral Hepatol* 2012;19:811–22.
- [85] Wang LY, Meng QH, Zou ZQ, Fan YC, Han J, Qi ZX, et al. Increased frequency of circulating Th17 cells in acute-on-chronic hepatitis B liver failure. *Dig Dis Sci* 2012;57:667–74.
- [86] Zhang GL, Xie DY, Lin BL, Xie C, Ye YN, Peng L, et al. Imbalance of interleukin-17-producing CD4 T cells/regulatory T cells axis occurs in remission stage of patients with hepatitis B virus-related acute-on-chronic liver failure. *J Gastroenterol Hepatol* 2013;28:513–21.
- [87] Zhang JY, Zhang Z, Lin F, Zou ZS, Xu RN, Jin L, et al. Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. *Hepatology* 2010;51:81–91.
- [88] Zhai S, Zhang L, Dang S, Yu Y, Zhao Z, Zhao W, et al. The ratio of Th-17 to Treg cells is associated with survival of patients with acute-on-chronic hepatitis B liver failure. *Viral Immunol* 2011;24:303–10.
- [89] Xue-Song L, Cheng-Zhong L, Ying Z, Mo-Bin W. Changes of Treg and Th17 cells balance in the development of acute and chronic hepatitis B virus infection. *BMC Gastroenterol* 2012;12:43.
- [90] Beringer A, Noack M, Miossec P. IL-17 in Chronic Inflammation: From Discovery to Targeting. *Trends Mol Med* 2016;22:230–41.
- [91] Silacci M, Lembke W, Woods R, Attinger-Toller I, Baenziger-Tobler N, Batey S, et al. Discovery and characterization of COVA322, a clinical-stage bispecific TNF/IL-17A inhibitor for the treatment of inflammatory diseases. *MAbs* 2016;8:141–9.
- [92] Glatt S, Baeten D, Baker T, Griffiths M, Ionescu L, Lawson ADG, et al. Dual IL-17A and IL-17F neutralisation by bimekizumab in psoriatic arthritis: evidence from preclinical experiments and a randomised placebo-controlled clinical trial that IL-17F contributes to human chronic tissue inflammation. *Ann Rheum Dis* 2018;77:523–32.
- [93] Samson M, Audia S, Janikashvili N, Ciudad M, Trad M, Fraszczak J, et al. Brief report: inhibition of interleukin-6 function corrects Th17/Treg cell imbalance in patients with rheumatoid arthritis. *Arthritis Rheum* 2012;64:2499–503.
- [94] Ghoreschi K, Jesson MI, Li X, Lee JL, Ghosh S, Alsop JW, et al. Modulation of innate and adaptive immune responses by tofacitinib [CP-690,550]. *J Immunol* 2011;186:4234–43.
- [95] Zong M, Dorph C, Dastmalchi M, Alexanderson H, Pieper J, Amoudruz P, et al. Anakinra treatment in patients with refractory inflammatory myopathies and possible predictive response biomarkers: a mechanistic study with 12 months follow-up. *Ann Rheum Dis* 2014;73:913–20.
- [96] Solt LA, Kumar N, Nuhant P, Wang Y, Lauer JL, Liu J, et al. Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand. *Nature* 2011;472:491–4.
- [97] Isono F, Fujita-Sato S, Ito S. Inhibiting RORgammat/Th17 axis for autoimmune disorders. *Drug Discov Today* 2014;19:1205–11.
- [98] Hamada S, Umemura M, Shiono T, Tanaka K, Yahagi A, Begum MD, et al. IL-17A produced by gammadelta T cells plays a critical role in innate immunity against listeria monocytogenes infection in the liver. *J Immunol* 2008;181:3456–63.
- [99] Schulz SM, Kohler G, Holscher C, Iwakura Y, Alber G. IL-17A is produced by Th17, gammadelta T cells and other CD4+ lymphocytes during infection with Salmonella enterica serovar Enteritidis and has a mild effect in bacterial clearance. *Int Immunol* 2008;20:1129–38.
- [100] Miossec P. Update on interleukin-17: a role in the pathogenesis of inflammatory arthritis and implication for clinical practice. *RMD Open* 2017;3:e000284.
- [101] Gerard R, Sendid B, Colombel JF, Poulain D, Jouault T. An immunological link between *Candida albicans* colonization and Crohn's disease. *Crit Rev Microbiol* 2015;41:135–9.
- [102] Conti HR, Gaffen SL. IL-17-Mediated Immunity to the Opportunistic Fungal Pathogen *Candida albicans*. *J Immunol* 2015;195:780–8.
- [103] Qin SY, Lu DH, Guo XY, Luo W, Hu BL, Huang XL, et al. A deleterious role for Th9/IL-9 in hepatic fibrogenesis. *Sci Rep* 2016;6:18694.
- [104] Hou L, Jie Z, Desai M, Liang Y, Soong L, Wang T, et al. Early IL-17 production by intrahepatic T cells is important for adaptive immune responses in viral hepatitis. *J Immunol* 2013;190:621–9.
- [105] Jie Z, Liang Y, Hou L, Dong C, Iwakura Y, Soong L, et al. Intrahepatic innate lymphoid cells secrete IL-17A and IL-17F that are crucial for T cell priming in viral infection. *J Immunol* 2014;192:3289–300.
- [106] Yang W, Ding X, Deng J, Lu Y, Matsuda Z, Thiel A, et al. Interferon-gamma negatively regulates Th17-mediated immunopathology during mouse hepatitis virus infection. *J Mol Med (Berl)* 2011;89:399–409.
- [107] Yano A, Higuchi S, Tsuneyama K, Fukami T, Nakajima M, Yokoi T. Involvement of immune-related factors in diclofenac-induced acute liver injury in mice. *Toxicology* 2012;293:107–14.
- [108] Chen RC, Xu LM, Du SJ, Huang SS, Wu H, Dong JJ, et al. *Lactobacillus rhamnosus* GG supernatant promotes intestinal barrier function, balances Treg and TH17 cells and ameliorates hepatic injury in a mouse model of chronic-binge alcohol feeding. *Toxicol Lett* 2016;241:103–10.
- [109] Shi W, Zhu Q, Gu J, Liu X, Lu L, Qian X, et al. Anti-IL-17 antibody improves hepatic steatosis by suppressing interleukin-17-related fatty acid synthesis and metabolism. *Clin Dev Immunol* 2013;2013:253046.
- [110] Harley IT, Stankiewicz TE, Giles DA, Softic S, Flick LM, Cappelletti M, et al. IL-17 signaling accelerates the progression of nonalcoholic fatty liver disease in mice. *Hepatology* 2014;59:1830–9.
- [111] Gomes AL, Teixeira A, Buren S, Tummala KS, Yilmaz M, Waisman A, et al. Metabolic Inflammation-Associated IL-17A Causes Non-alcoholic Steatohepatitis and Hepatocellular Carcinoma. *Cancer Cell* 2016;30:161–75.
- [112] Giles DA, Moreno-Fernandez ME, Stankiewicz TE, Cappelletti M, Huppert SS, Iwakura Y, et al. Regulation of Inflammation by IL-17A and IL-17F Modulates Non-Alcoholic Fatty Liver Disease Pathogenesis. *PLoS One* 2016;11:e0149783.
- [113] Rolla S, Alchera E, Imarisio C, Bardina V, Valente G, Cappello P, et al. The balance between IL-17 and IL-22 produced by liver-infiltrating T-helper cells critically controls NASH development in mice. *Clin Sci (Lond)* 2016;130:193–203.
- [114] Kasztelan-Szczerbinska B, Surdacka A, Celinski K, Rolinski J, Zwolak A, Miacz S, et al. Prognostic Significance of the Systemic Inflammatory and Immune Balance in Alcoholic Liver Disease with a Focus on Gender-Related Differences. *PLoS One* 2015;10:e0128347.
- [115] Rau M, Schilling AK, Meertens J, Hering I, Weiss J, Jurowicz C, et al. Progression from Nonalcoholic Fatty Liver to Nonalcoholic Steatohepatitis Is Marked by a Higher Frequency of Th17 Cells in the Liver and an Increased Th17/Resting Regulatory T Cell Ratio in Peripheral Blood and in the Liver. *J Immunol* 2016;196:97–105.
- [116] Ye Y, Xie X, Yu J, Zhou L, Xie H, Jiang G, et al. Involvement of Th17 and Th1 effector responses in patients with Hepatitis B. *J Clin Immunol* 2010;30:546–55.
- [117] Liu B, Gao W, Zhang L, Wang J, Chen M, Peng M, et al. Th17/Treg imbalance and increased interleukin-21 are associated with liver injury in patients with chronic severe hepatitis B. *Int Immunopharmacol* 2017;46:48–55.
- [118] Paquiss FC. Immunity and Fibrogenesis: The Role of Th17/IL-17 Axis in HBV and HCV-Induced Chronic Hepatitis and Progression to Cirrhosis. *Front Immunol* 2017;8:1195.
- [119] Chang Q, Wang YK, Zhao Q, Wang CZ, Hu YZ, Wu BY. Th17 cells are increased with severity of liver inflammation in patients with chronic hepatitis C. *J Gastroenterol Hepatol* 2012;27:273–8.
- [120] Foster RG, Golden-Mason L, Rutebemberwa A, Rosen HR. Interleukin (IL)-17/IL-22-producing T cells enriched within the liver of patients with chronic hepatitis C viral (HCV) infection. *Dig Dis Sci* 2012;57:381–9.
- [121] Bowen DG. Finding a role for Th17 cells in hepatitis C pathogenesis. *J Gastroenterol Hepatol* 2012;27:188–91.
- [122] Balanescu P, Ladaru A, Voiosu T, Nicolau A, Ene M, Balanescu E. Th17 and IL-17 immunity in chronic hepatitis C infection. *Rom J Intern Med* 2012;50:13–8.
- [123] Kuang DM, Zhao Q, Wu Y, Peng C, Wang J, Xu Z, et al. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. *J Hepatol* 2011;54:948–55.
- [124] Yan J, Liu XL, Xiao G, Li NL, Deng YN, Han LZ, et al. Prevalence and clinical relevance of T-helper cells, Th17 and Th1, in hepatitis B virus-related hepatocellular carcinoma. *PLoS One* 2014;9:e96080.

### **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 TNF $\alpha$  and TGF $\beta$  were not affected by the presence of HSCs (Wang et al., 2014). Genome-wide expression profiling was 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-1 $\beta$  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- $\alpha$ 1 and TGF $\beta$  whereas the key HSC activator marker  $\alpha$ -smooth muscle actin ( $\alpha$ -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  $\alpha$ -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<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells and hepatocytes from murine liver increased the IL-10 secretion by CD4<sup>+</sup> 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 concanavalin A (Con A). The CD4<sup>+</sup> T cells primed by hepatocytes were able to suppress proliferation of responder T cells upon TCR stimulation. Interestingly, most of the CD4<sup>+</sup> IL-10<sup>+</sup> cells co-express IFN $\gamma$  but not Foxp3 (Burghardt et al., 2013). However, the same research team shows that murine hepatocytes in the presence of TGF $\beta$  promoted Foxp3 expression within CD4<sup>+</sup> 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<sup>+</sup> T cells, the CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cell co-cultures (Dangi et al., 2012). In addition, tumor-specific HSCs but not quiescent HSCs promoted T-cell hyporesponsiveness 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<sup>+</sup> T cell population, they can induce at the same time CD4<sup>+</sup> 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, TNF $\alpha$  and IL-1 $\beta$ ) by HSCs whereas the production of the anti-

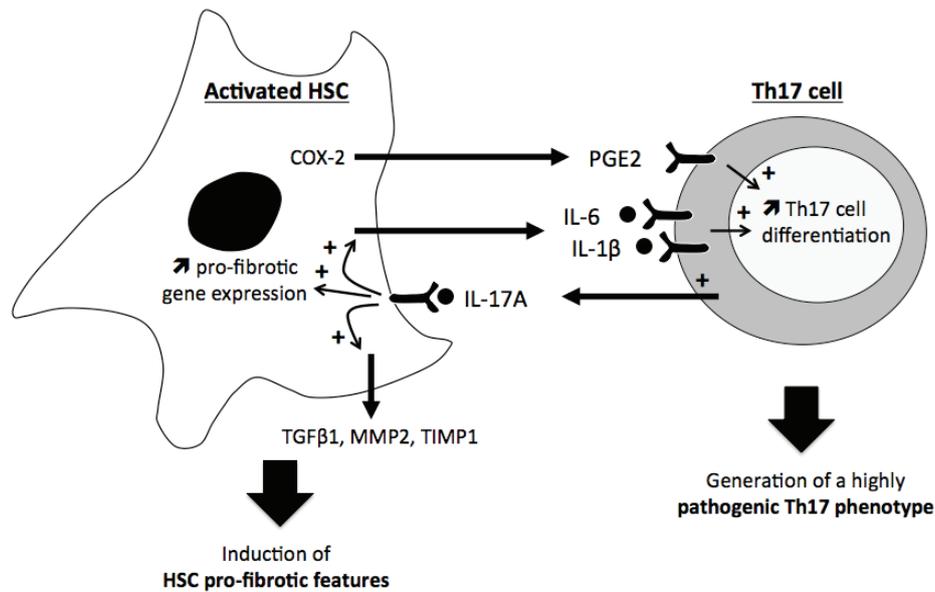
inflammatory cytokine IL-10 was increased by the presence of Treg cells in LPS-stimulated HSC cultures (Dangi et al., 2012). By producing IL-8 and TGF $\beta$ 1, Treg cells may also down-regulate pivotal ligands for activating NK cell receptors on HSCs and therefore reduce the anti-fibrotic activity of NK cells (Langhans et al., 2015).

The effect of HSCs on T cell response was also investigated in other CD4<sup>+</sup> T cell subtypes. Interactions between activated HSCs from rats and rat CD4<sup>+</sup> T cells reduced the Th1/Th2 ratio *in vitro* by promoting apoptosis and by inhibiting IFN $\gamma$  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<sup>+</sup> T cells from patients with chronic hepatitis B (CHB). The interactions between CD4<sup>+</sup> T cells from CHB patients and activated HSCs enhanced the proliferation of CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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  $\alpha$ -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  $\alpha$ -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 TGF $\beta$ 1 as well as the release of IL-6 and IL-1 $\beta$  when Th17 cells were purified from patients with CHB. Gene expressions of Collagen-III, MMP2, TIMP1 and the pro-fibrotic cytokines TGF $\beta$ 1, 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 oncogenes, 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-1 $\beta$ , 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-1 $\beta$  inhibited significantly the frequency of IL-17A-producing CD4<sup>+</sup> T cells and the secretion of IL-17 in activated T CD4<sup>+</sup> 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<sup>+</sup> 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-1 $\beta$  and TGF- $\beta$ 1) 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<sup>+</sup> 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<sup>+</sup> 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-1 $\beta$  pathways. In turn, IL-17 promotes the activation of HSCs by inducing the expression of pro-fibrotic genes ( $\alpha$ -SMA, Collagen-III, TGF $\beta$ 1, CTGF, EGF and PEGF-BB) and the secretion of IL-6, IL-1 $\beta$ , TGF $\beta$ 1, MMP2 and TIMP1.

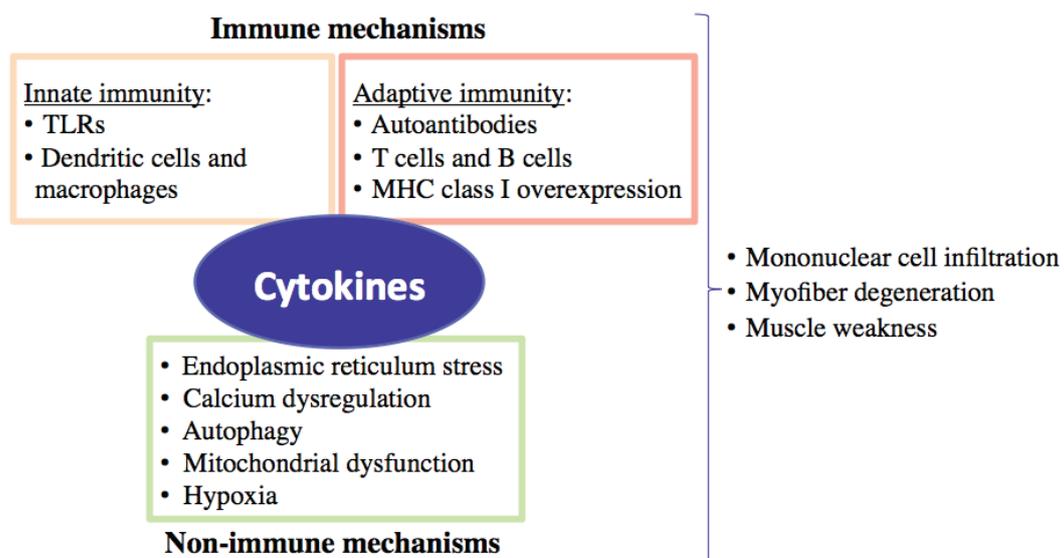
The immunoregulatory role of activated HSCs on immune cells is complex and appears to depend on the *in vitro* cell model used and the cell stimulation. In chronically inflamed liver, HSCs are activated and trans-differentiate into pro-fibrogenic myofibroblasts. By inducing immunosuppressive functions 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). TGF $\beta$  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<sup>+</sup> 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<sup>+</sup> 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<sup>2+</sup>) 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  $\text{Ca}^{2+}$  regulation. The sarcoplasmic reticulum (SR) is a specialized form of ER found in muscle cells. SR is a  $\text{Ca}^{2+}$  storage depot and regulates  $\text{Ca}^{2+}$  release during muscle contraction. The ER and SR can rapidly refill  $\text{Ca}^{2+}$  store by inducing  $\text{Ca}^{2+}$  entry from the extracellular environment via the  $\text{Ca}^{2+}$  sensors stromal interaction molecule (STIM1) and the  $\text{Ca}^{2+}$  channel Orai (Henriques-Pons and Nagaraju, 2009). Abnormalities of this  $\text{Ca}^{2+}$  pathway, known as store-operated  $\text{Ca}^{2+}$  entry (SOCE), can impair  $\text{Ca}^{2+}$  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-1 $\alpha$  and IL-1 $\beta$  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- $\kappa$ B 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 IFN $\gamma$ -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<sup>+</sup> CD4<sup>+</sup> IL-17<sup>+</sup> cells was not higher in IBM patients than healthy controls (Allenbach et al., 2014). In contrast, the frequency of CD3<sup>+</sup> CD4<sup>+</sup> IL-17<sup>+</sup> 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-1 $\beta$  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-1 $\beta$  were combined

(Chevrel et al., 2003). IL-17, but not IFN $\gamma$ , 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

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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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  in immune and non-immune processes in myoblasts

# **1 Synergistic effect of IL-17 and TNF $\alpha$ 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- $\alpha$  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 TNF $\alpha$ , IL-1 $\beta$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  in the hepatic inflammatory response and the contribution of IL-6 in the effects triggered by IL-17 and TNF $\alpha$ .

## **1.2 Results**

**The IL-17 and TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  effect on the CRP and ASAT levels in PHH and HepaRG cell cultures.

**The IL-17 and TNF $\alpha$  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/TNF $\alpha$  exposure, the IL-17 and TNF $\alpha$  effect on IL-8, MCP-1 and CCL20 is mainly independent of the IL-6 pathway.

**IL-17 initiates the IL-17 and TNF $\alpha$  synergistic effect on the IL-6 and IL-8 production.**

Indeed, pre-incubation first with IL-17 followed by the addition of TNF $\alpha$  induces two-fold higher IL-6 and IL-8 release than the pre-incubation first with TNF $\alpha$ , 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 TNF $\alpha$  synergistic effect on IL-6 in hepatocytes.

**IL-17 and TNF- $\alpha$  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- $\kappa$ B transcription factor.** ERK inhibition reduces both IL-6 and IL-8 production induced by IL-17 and TNF $\alpha$  whereas the activation of PI3K/Akt and NF- $\kappa$ B pathways are only involved in the synthesis of IL-6 but not IL-8.

### ***1.3 Conclusion***

IL-17 and TNF $\alpha$  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 TNF $\alpha$  induce in synergy the expression and/or production of IL-8, MCP-1 and CCL20 chemokines. Blocking IL-17 and/or TNF $\alpha$  could be a promising therapeutic strategy to control systemic inflammation but also the local cell recruitment and associated liver cell injury.

# Synergistic effect of interleukin-17 and tumour necrosis factor- $\alpha$ 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)- $\alpha$  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- $\alpha$ . To determine the contribution of the IL-6 pathway in the IL-17/TNF- $\alpha$ -mediated effect, an anti-IL-6 receptor antibody was used. IL-17 and TNF- $\alpha$  increased in synergy IL-6 secretion by HepaRG cells and PHH but not by Huh7.5 and HepG2 cells. This IL-17/TNF- $\alpha$  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- $\alpha$  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- $\alpha$ , was crucial for the initiation of the IL-17/TNF- $\alpha$  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- $\alpha$  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- $\kappa$ B and/or protein kinase B (Akt)-phosphatidylinositol 3-kinase signalling pathways. Therefore, the IL-17/TNF- $\alpha$  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- $\alpha$  combination may also induce immune cell recruitment by chemokine up-regulation. IL-17 and/or TNF- $\alpha$  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- $\alpha$

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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- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$  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- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  1 ng/ml (R&D systems). To block the IL-6, IL-17 or TNF- $\alpha$  pathways, tocilizumab (Roche, Welwyn, UK), anti-IL-17A (R&D Systems) and infliximab (MSD, Courbevoie, France) were used at 10  $\mu$ g/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- $\kappa$ B) 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  $\mu$ M 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- $\alpha$  for 12 h. Cells were then washed and incubated with 5  $\mu$ g/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<sup>®</sup> Plus Mini kit (Quiagen, Hilden, Germany). cDNA was synthesized using the iScript<sup>™</sup> kit (Bio-Rad, Hercules, CA, USA). Polymerase chain reaction (PCR) amplification was performed using the CFX96<sup>™</sup> real-time system instrument (Bio-Rad) with the iTaq<sup>™</sup> universal SYBR<sup>®</sup> green supermix (Bio-Rad) and the Qiagen QuantiTect<sup>®</sup> 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- $\alpha$  alone (10 ng/ml) or a combination of IL-17 (50 ng/ml) and TNF- $\alpha$  (1 ng/ml) for 30 min. Cells were lysed using the Halt<sup>™</sup> 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  $\mu$ g) 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- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ) (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<sup>TM</sup> XR+ Gel system (Bio-Rad) and Image Lab 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<sup>®</sup> (Siemens, 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  $\pm$  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- $\alpha$ combination increases IL-6 expression and production synergistically

IL-17 and TNF- $\alpha$  have been shown previously to induce a massive IL-6 production by various cell types in synergy [16,17]. Here, the IL-17/TNF- $\alpha$  effect on IL-6 was studied in hepatic cell lines and PHH. In HepaRG cells and PHH, the IL-17/TNF- $\alpha$  combination exposure increased the IL-6 secretion synergistically compared to IL-17 or TNF- $\alpha$  alone. However, the IL-6 production by Huh 7.5 and HepG2 cell lines was not affected by IL-17/TNF- $\alpha$  exposure (Fig. 1a). Based on these results, the HepaRG cell line was used for the following experiments. The IL-17/TNF- $\alpha$  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- $\alpha$  receptors may contribute to this IL-17/TNF- $\alpha$  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- $\alpha$  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- $\alpha$

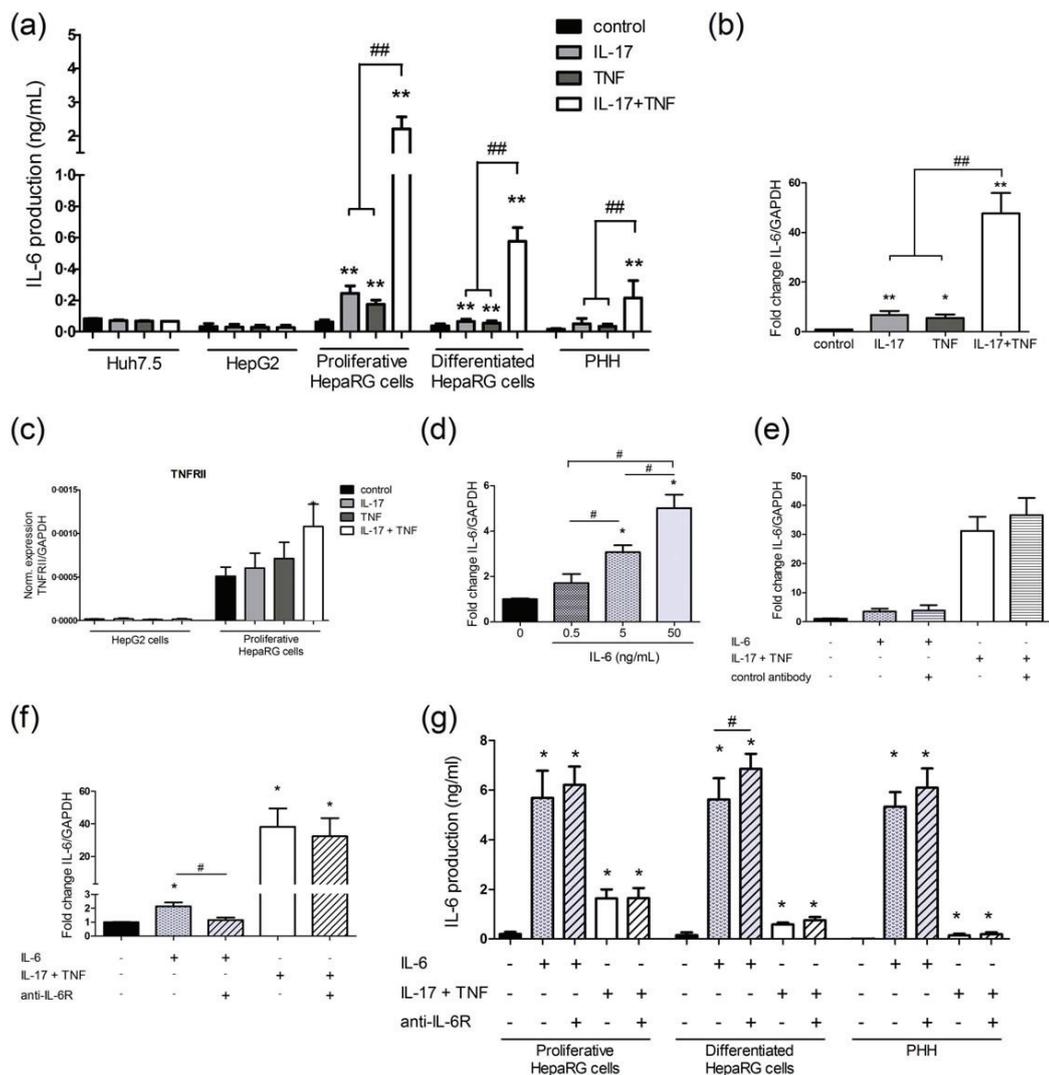
stimulation (Fig. 1c and data not shown). IL-17R and TNF-R expressions were also compared between the HepG2 and Huh7.5 cells, which did not produce IL-6 after the IL-17/TNF- $\alpha$  stimulation, and the proliferative HepaRG cells, which released IL-6 following the IL-17/TNF- $\alpha$  stimulation (Fig. 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- $\alpha$ .

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- $\alpha$  (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- $\alpha$  significantly (Fig. 1f). The contribution of the IL-6-positive feedback loop in the IL-6 induction by IL-17/TNF- $\alpha$  was therefore very weak compared to the IL-17/TNF- $\alpha$  direct effect on IL-6. The IL-6 supernatant levels in hepatocyte cultures stimulated with IL-6 or IL-17/TNF- $\alpha$  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- $\alpha$ 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- $\alpha$  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- $\alpha$  (Fig. 2a,b). Moreover, CRP production by PHH from different donors following IL-17/TNF- $\alpha$  stimulation correlated strongly with the PHH ability to produce IL-6 (Fig. 2c,d). Therefore, CRP up-regulation by IL-17 and/or TNF- $\alpha$  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- $\alpha$  stimulation. These



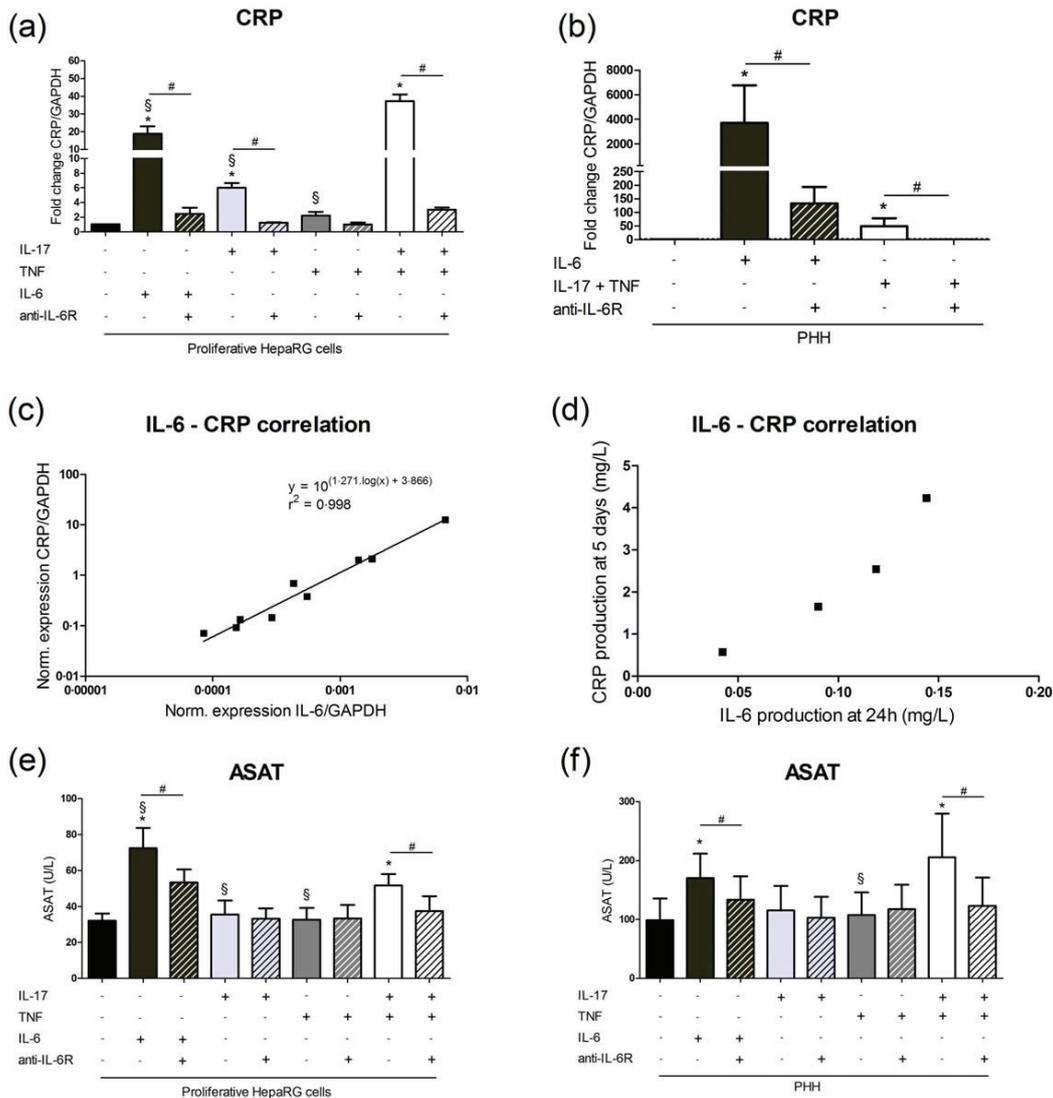
**Fig. 1.** Interleukin (IL)-17 and tumour necrosis factor (TNF)- $\alpha$  combination increases IL-6 expression and production synergistically. Hepatocytes were exposed to IL-17 and/or TNF- $\alpha$  or IL-6 with/without the anti-IL-6R. (a,g) IL-6 production by hepatocytes was quantified by enzyme-linked 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- $\alpha$  stimulation in HepaRG cells (e, data not shown). Data are the mean of three to 18 independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control; # $P$  < 0.05 and ## $P$  < 0.01 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- $\alpha$  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- $\alpha$  co-operation enhanced IL-8 production by HepaRG cells and PHH versus IL-17 alone, TNF- $\alpha$  alone and control (Fig. 3a). IL-8 mRNA expression in proliferative HepaRG cells was up-regulated by IL-17 alone (5-fold) and TNF- $\alpha$  alone (5-fold), with a synergistic effect of both (24-fold) (Fig. 3b). The IL-17/TNF- $\alpha$  synergistic interactions also increased IL-8 mRNA levels by up to 14-fold in PHH (Fig. 3b).

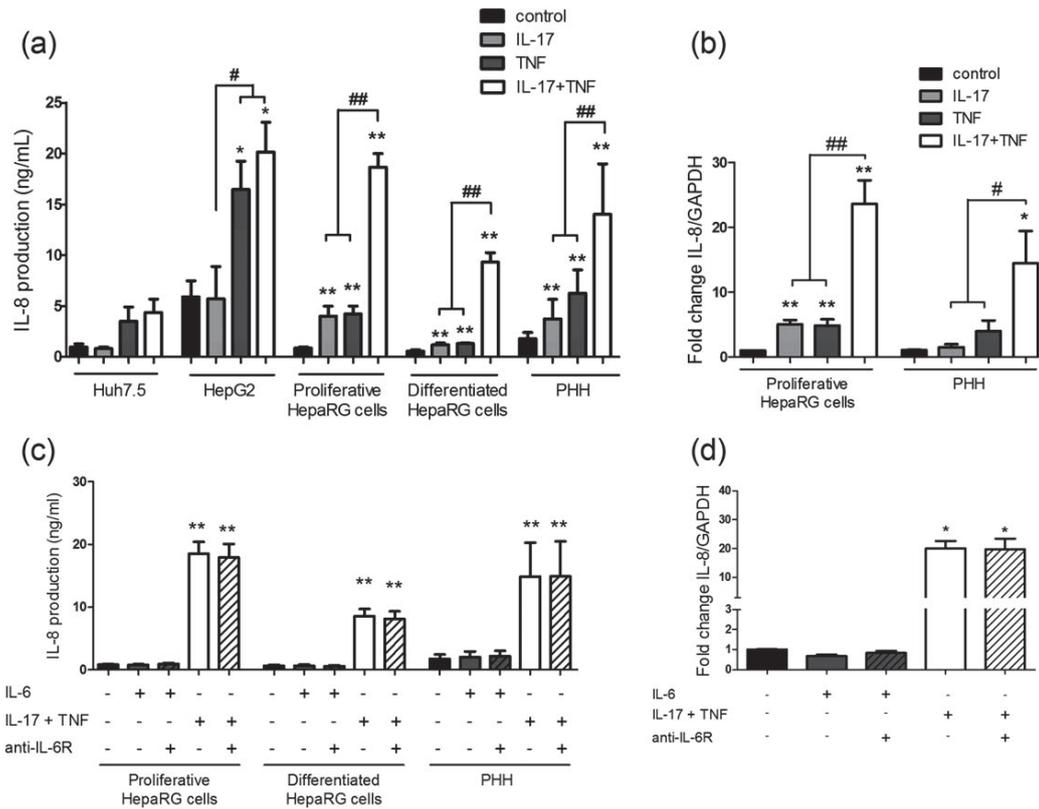


**Fig. 2.** Induction of the interleukin (IL)-6-dependent C-reactive protein (CRP) expression and aspartate aminotransferase (ASAT) activity level following IL-17 and/or tumour necrosis factor (TNF)- $\alpha$  stimulation. Hepatocytes were treated with IL-17 and/or TNF- $\alpha$  or IL-6 with/without 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- $\alpha$  stimulation in HepaRG cells (data not shown). (c) Correlation between IL-6 and CRP mRNA levels in PHH stimulated with IL-17/TNF- $\alpha$  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- $\alpha$ . (e,f) ASAT supernatant levels were quantified at 120 h. Data are the mean of four to six independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P < 0.05$  versus control, \$ $P < 0.05$  versus IL-17/TNF- $\alpha$  condition, # $P < 0.05$  versus other cytokine conditions.

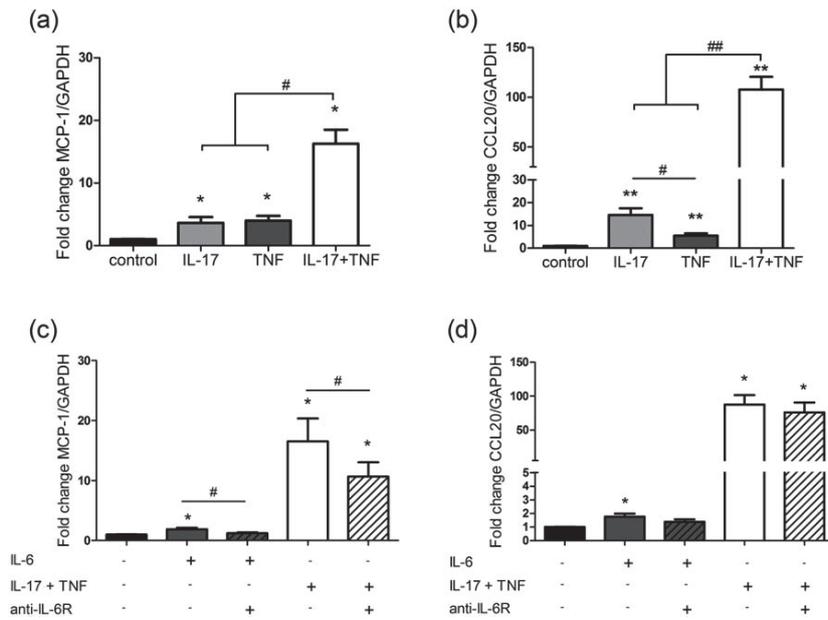
However, the IL-8 mRNA and protein level was unchanged after IL-6 exposure. Moreover, the IL-6 pathway inhibition had no effect on the induction of IL-8 expression and production by IL-17/TNF- $\alpha$  (Fig. 3c,d). Therefore, IL-8 induction by IL-17 and TNF- $\alpha$  was not mediated through IL-6. The IL-17 and TNF- $\alpha$  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- $\alpha$  increase in synergy MCP-1 and CCL20 expression mainly through an IL-6-independent pathway**

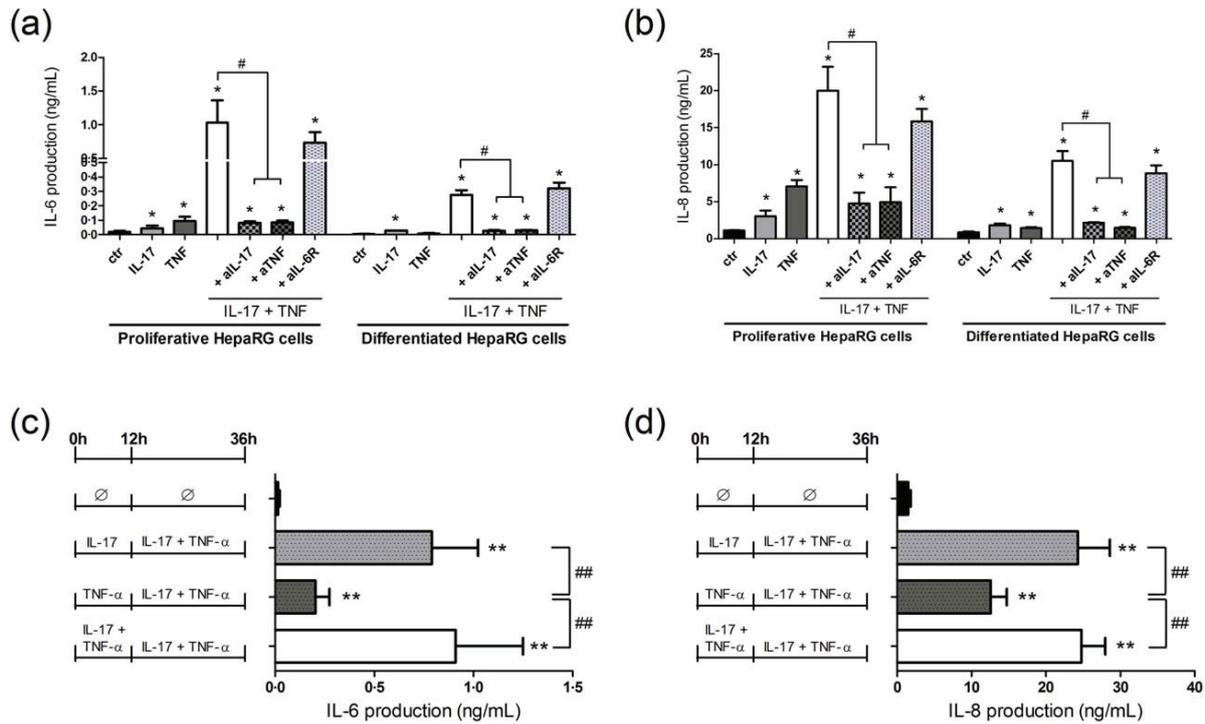
MCP-1 and CCL20 are two chemokines acting on mononuclear cells involved in the chronicity of the inflammatory response [27–29]. MCP-1 and CCL20 mRNA levels increased in the presence of IL-17 or TNF- $\alpha$ , with a clear



**Fig. 3.** Interleukin (IL)-17 and tumour necrosis factor (TNF)- $\alpha$  combination increases IL-8 expression and production synergistically independently of the IL-6 pathway. Hepatocytes were exposed to IL-17 and/or TNF- $\alpha$  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- $\alpha$  stimulation in human HepaRG cells (data not shown). Data are the mean of three to 18 independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control; # $P$  < 0.05 and ## $P$  < 0.01 versus other cytokine conditions.



**Fig. 4.** Interleukin (IL)-17 and tumour necrosis factor (TNF)- $\alpha$  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- $\alpha$  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- $\alpha$  stimulation (data not shown). Data are the mean of seven to eight independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control; # $P$  < 0.05 and ## $P$  < 0.01 versus other cytokine conditions.



**Fig. 5.** Interleukin (IL)-17 initiates the IL-17 and tumour necrosis factor (TNF)- $\alpha$  synergistic effect on IL-6 and IL-8 production. (a,b) To evaluate the contribution of IL-17, TNF- $\alpha$  and IL-6 pathways in the IL-17/TNF- $\alpha$  synergistic effect, human hepatoma cells (HepaRG) cells were treated with IL-17 and/or TNF- $\alpha$  with/without anti-IL-17A (aIL-17), anti-TNF- $\alpha$  (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- $\alpha$  stimulation (data not shown). (c,d) Proliferative HepaRG cells were pre-exposed to IL-17 and/or TNF- $\alpha$  overnight (for 12 h) before IL-17 and/or TNF- $\alpha$  addition to have both cytokines in the culture medium (except for the control ( $\emptyset$ )). 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  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control; # $P$  < 0.05 and ## $P$  < 0.01 versus other cytokine conditions.

synergistic effect of both cytokines (16- and 108-fold, respectively,  $P$  < 0.01) versus control (Fig. 4a,b).

To determine whether the IL-17/TNF- $\alpha$  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- $\alpha$  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- $\alpha$  combination in proliferative HepaRG cells (Fig. 4c,d). Therefore, IL-17 and TNF- $\alpha$  can act independently of the IL-6 signalling pathway on the induction of MCP-1 and CCL20 expression. IL-17/TNF- $\alpha$  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- $\alpha$ synergistic effect on IL-6 and IL-8 production

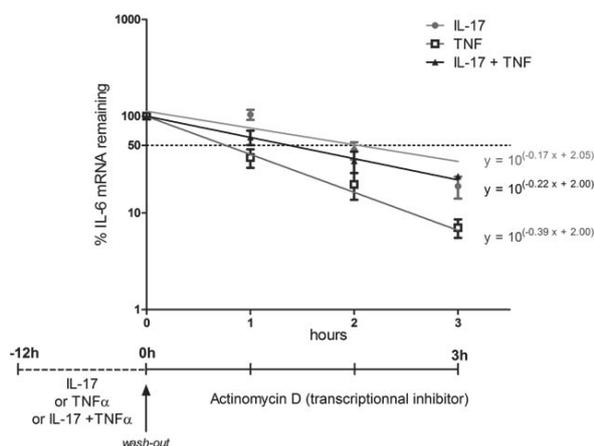
To understand more clearly the contribution of each cytokine on the IL-17 and TNF- $\alpha$  synergistic effect, antibodies

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

#### IL-17 enhanced the stability of IL-6 mRNA

Post-transcriptional regulations could contribute to the IL-17 and TNF- $\alpha$  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)- $\alpha$  (empty squares) or the IL-17/TNF- $\alpha$  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  $\pm$  standard error of the mean (s.e.m.).

and TNF- $\alpha$  effect on mRNA stabilization, HepaRG cells were treated with IL-17 and/or TNF- $\alpha$  for 12 h and then transcription was inhibited. TNF- $\alpha$ -induced IL-6 transcripts had a half-life of 46 min, whereas IL-17 and the IL-17/TNF- $\alpha$  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- $\alpha$ increase IL-6 and IL-8 production in synergy via the activation of ERK and/or PI3K/Akt signalling pathways and/or NF- $\kappa$ B transcription factor

The effect of the TNF- $\alpha$ /IL-17 combination on the downstream signalling pathways was then investigated, focusing on the MAPKs (JNK, p38, ERK), NF- $\kappa$ B 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- $\alpha$  association induced phosphorylation of I $\kappa$ B $\alpha$  (leading to NF- $\kappa$ B release and activation) and ERK. The IL-17/TNF- $\alpha$  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- $\alpha$  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  $\mu$ M, respectively) and IL-8 (31 and 69% of inhibition for 1 and 10  $\mu$ M, respectively) in a dose-dependent manner

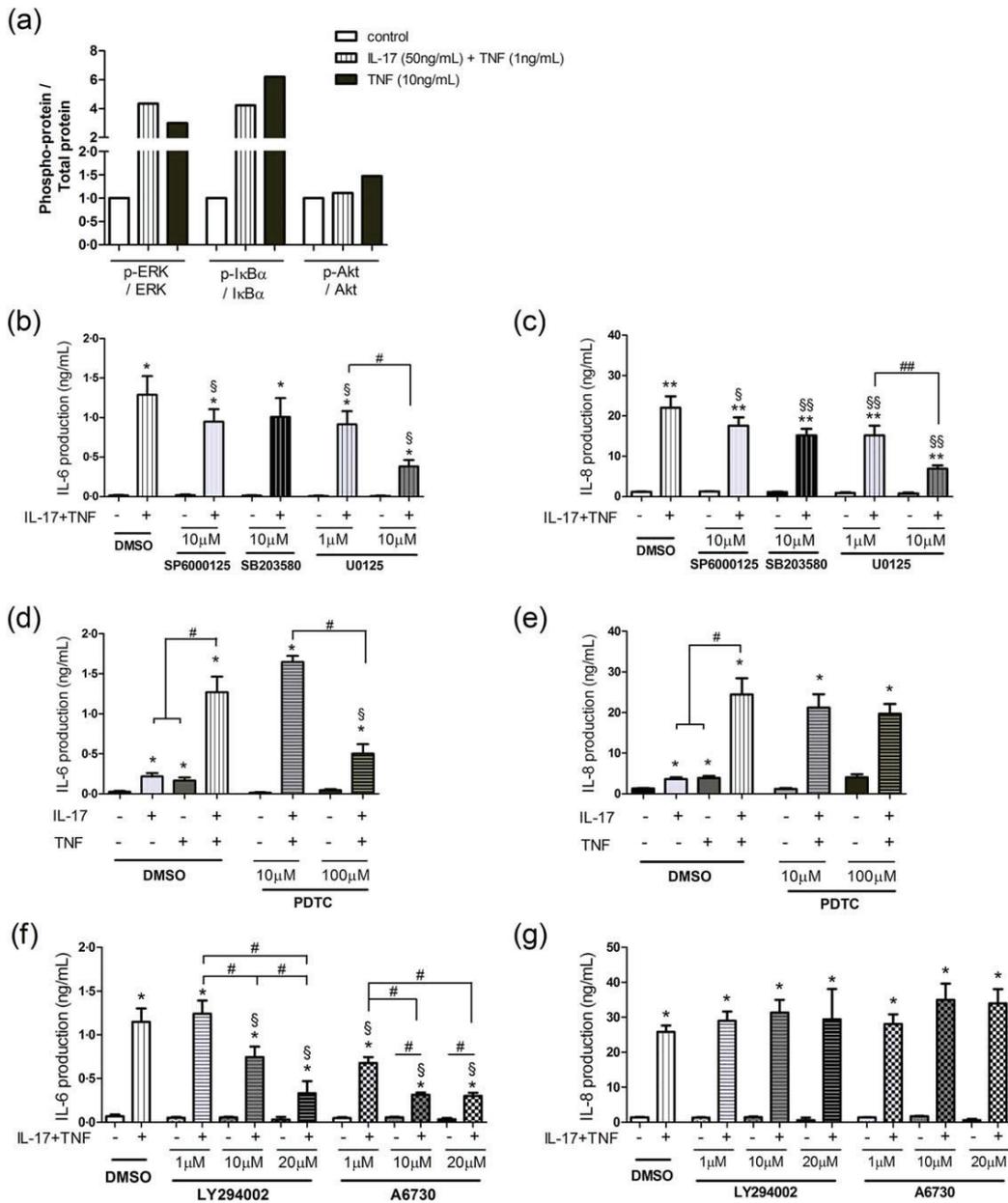
induced by the IL-17/TNF- $\alpha$  combination (Fig. 7b,c). IL-6 production induced by the IL-17/TNF- $\alpha$  combination was also inhibited in the presence of the NF- $\kappa$ B inhibitor (60% of inhibition), the Akt inhibitor (more than 70% of inhibition for 10 and 20  $\mu$ M) and the PI3K inhibitor (40 and 65% of inhibition for 1 and 10  $\mu$ M, respectively), whereas IL-8 production was not impacted significantly (Fig. 7d-g). Activation of ERK, NF- $\kappa$ B and/or PI3K/Akt signalling pathways was therefore involved in the IL-17/TNF- $\alpha$  synergistic effect on IL-6 and/or IL-8 production.

## Discussion

This study shows how the IL-17/TNF- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  synergistic interaction was very weak at 12 h (Fig. 1g) and 24 h (data not shown) compared to the direct IL-17/TNF- $\alpha$  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- $\alpha$  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- $\alpha$ . In PHH treated with the IL-17/TNF- $\alpha$  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- $\alpha$  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)- $\alpha$  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 phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signalling pathways and nuclear factor kappa B (NF- $\kappa$ B) transcription factor. (a) Proliferative human HepaRG cells were incubated with IL-17 and/or TNF- $\alpha$  for 30min. The total and phosphorylated forms of ERK, NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ) 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- $\kappa$ B, Akt and PI3K pathway inhibitors for 1 h followed by the IL-17 and/or TNF- $\alpha$  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- $\kappa$ B inhibitor; LY294002: PI3K inhibitor and A6730: Akt inhibitor. Data are the mean of at least four to eight independent experiments  $\pm$  standard error of the mean (s.e.m.); \* $P$  < 0.05, \*\* $P$  < 0.01 versus control in dimethylsulphoxide (DMSO) condition, § $P$  < 0.05 and §§ $P$  < 0.01 versus IL-17/TNF- $\alpha$  condition in DMSO medium; # $P$  < 0.05 and ## $P$  < 0.01 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- $\alpha$  combination increased the ASAT activity level through the IL-6 pathway. These results appear consistent with our *in-vivo* studies, which showed that TNF- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  can increase the accumulation of a Th17-driven response and lead to a chronic inflammatory state.

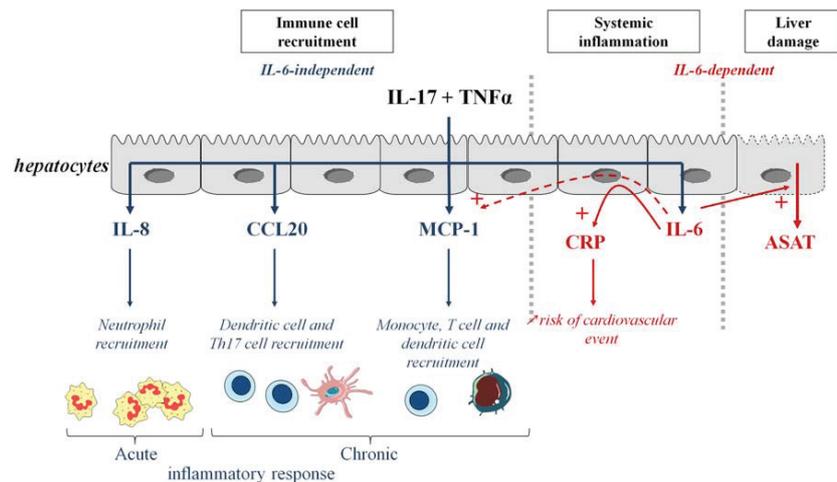
Although the IL-17/TNF- $\alpha$  synergistic effect has been well described in several cell types [16–19,45,46], the mechanism of this effect on IL-6 and IL-8 production has not yet been studied in hepatocytes. Various mechanisms may act at several levels: at a receptor level, at a post-transcriptional level and at a promoter level. In synoviocytes, the TNF receptor II (TNF-RII) contributed to the IL-17 and TNF- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  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 uridylylate (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- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  (Fig. 1f) in HepaRG cells. Therefore, the IL-17/TNF- $\alpha$  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- $\kappa$ B and C/EBP (CCAAT/enhancer-binding protein) in the IL-6 promoter were both involved in the IL-17 and TNF- $\alpha$  synergistic effect on IL-6 in an osteoblastic cell line [56]. Part of the IL-17/TNF- $\alpha$  synergistic effect on IL-6 may occur at the gene transcription level through the up-regulation of C/EBP $\delta$  by IL-17/TNF- $\alpha$  and the increase of C/EBP $\delta$  recruitment to the promoter by TNF- $\alpha$  [31,56,57]. In primary murine hepatocytes, NF- $\kappa$ B contributed to the activation of many IL-17 target genes related to inflammation [58]. Here, in HepaRG cells, the NF- $\kappa$ B pathway appears to be involved in IL-6 release induced by the IL-17/TNF- $\alpha$  combination (Fig. 7d). Both IL-17 and TNF- $\alpha$  signalling



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

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

In conclusion, targeting IL-17 and/or TNF- $\alpha$  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.

### References

- Zhou Z, Xu MJ, Gao B. Hepatocytes: a key cell type for innate immunity. *Cell Mol Immunol* 2016; **13**:301–15.

- Yoshizaki K. Pathogenic role of IL-6 combined with TNF-alpha or IL-1 in the induction of acute phase proteins SAA and CRP in chronic inflammatory diseases. *Adv Exp Med Biol* 2011; **691**: 141–50.
- Patel DN, King CA, Bailey SR *et al.* Interleukin-17 stimulates C-reactive protein expression in hepatocytes and smooth muscle cells via p38 MAPK and ERK1/2-dependent NF-kappaB and C/EBPbeta activation. *J Biol Chem* 2007; **282**:27229–38.
- Kramer E, Torzewski J, Kamenz J *et al.* Interleukin-1beta stimulates acute phase response and C-reactive protein synthesis by inducing an NFkappaB- and C/EBPbeta-dependent autocrine interleukin-6 loop. *Mol Immunol* 2008; **45**:2678–89.
- Choy E, Ganeshalingam K, Semb AG *et al.* Cardiovascular risk in rheumatoid arthritis: recent advances in the understanding of the pivotal role of inflammation, risk predictors and the impact of treatment. *Rheumatology (Oxf)* 2014; **53**:2143–54.
- Nishimoto N, Miyasaka N, Yamamoto K *et al.* Long-term safety and efficacy of tocilizumab, an anti-IL-6 receptor monoclonal antibody, in monotherapy, in patients with rheumatoid arthritis (the STREAM study): evidence of safety and efficacy in a 5-year extension study. *Ann Rheum Dis* 2009; **68**:1580–4.
- Calabrese LH, Rose-John S. IL-6 biology: implications for clinical targeting in rheumatic disease. *Nat Rev Rheumatol* 2014; **10**: 720–7.
- Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol* 2014; **5**:491.
- Matthews VB, Allen TL, Risis S *et al.* Interleukin-6-deficient mice develop hepatic inflammation and systemic insulin resistance. *Diabetologia* 2010; **53**:2431–41.
- Taniguchi K, Wu LW, Grivennikov SI *et al.* A gp130-Src-YAP module links inflammation to epithelial regeneration. *Nature* 2015; **519**:57–62.
- Genovese MC, Kremer JM, van Vollenhoven RF *et al.* Transaminase levels and hepatic events during tocilizumab treatment: pooled analysis of long-term clinical trial safety data in rheumatoid arthritis. *Arthritis Rheumatol* 2017; **69**:1751–61.
- Strang AC, Bisioendial RJ, Kootte RS *et al.* Pro-atherogenic lipid changes and decreased hepatic LDL receptor expression by tocilizumab in rheumatoid arthritis. *Atherosclerosis* 2013; **229**:174–81.

- 13 McInnes IB, Sieper J, Braun J *et al.* Efficacy and safety of secukinumab, a fully human anti-interleukin-17A monoclonal antibody, in patients with moderate-to-severe psoriatic arthritis: a 24-week, randomised, double-blind, placebo-controlled, phase II proof-of-concept trial. *Ann Rheum Dis* 2014; **73**:349–56.
- 14 Zimmermann HW, Tacke F. Modification of chemokine pathways and immune cell infiltration as a novel therapeutic approach in liver inflammation and fibrosis. *Inflamm Allergy Drug Targets* 2011; **10**:509–36.
- 15 Lemmers A, Moreno C, Gustot T *et al.* The interleukin-17 pathway is involved in human alcoholic liver disease. *Hepatology* 2009; **49**:646–57.
- 16 Hot A, Lenief V, Miossec P. Combination of IL-17 and TNF- $\alpha$  induces a pro-inflammatory, pro-coagulant and pro-thrombotic phenotype in human endothelial cells. *Ann Rheum Dis* 2012; **71**:768–76.
- 17 Katz Y, Nadviv O, Beer Y. Interleukin-17 enhances tumor necrosis factor  $\alpha$ -induced synthesis of interleukins 1, 6, and 8 in skin and synovial fibroblasts: a possible role as a 'fine-tuning cytokine' in inflammation processes. *Arthritis Rheum* 2001; **44**: 2176–84.
- 18 Osta B, Roux JP, Lavocat F *et al.* Differential effects of IL-17A and TNF- $\alpha$  on osteoblastic differentiation of isolated synovial cells and on bone explants from arthritis patients. *Front Immunol* 2015; **6**:151.
- 19 Chiricozzi A, Guttman-Yassky E, Suarez-Farinas M *et al.* Integrative responses to IL-17 and TNF- $\alpha$  in human keratinocytes account for key inflammatory pathogenic circuits in psoriasis. *J Invest Dermatol* 2011; **131**:677–87.
- 20 Hammerich L, Heymann F, Tacke F. Role of IL-17 and Th17 cells in liver diseases. *Clin Dev Immunol* 2011; **2011**:345803.
- 21 Zhang JY, Zhang Z, Lin F *et al.* Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. *Hepatology* 2010; **51**:81–91.
- 22 Schwabe RF, Brenner DA. Mechanisms of Liver Injury. I. TNF- $\alpha$ -induced liver injury: role of IKK, JNK, and ROS pathways. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G583–9.
- 23 Meng F, Wang K, Aoyama T *et al.* Interleukin-17 signaling in inflammatory, Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice. *Gastroenterology* 2012; **143**:765–76.e1–3.
- 24 Grigorov B, Reungoat E, Gentil Dit Maurin A *et al.* Hepatitis C virus infection propagates through interactions between Syndecan-1 and CD81 and impacts the hepatocyte glycocalyx. *Cell Microbiol* 2017; **19**. doi: 10.1111/cmi.12711.
- 25 Lucifora J, Durantel D, Testoni B *et al.* Control of hepatitis B virus replication by innate response of HepaRG cells. *Hepatology* 2010; **51**:63–72.
- 26 Quintin E, Scaozec JY, Marotte H *et al.* Rare incidence of methotrexate-specific lesions in liver biopsy of patients with arthritis and elevated liver enzymes. *Arthritis Res Ther* 2010; **12**: R143.
- 27 Miao H, Zhang Y, Lu Z *et al.* FOXO1 increases CCL20 to promote NF- $\kappa$ B-dependent lymphocyte chemotaxis. *Mol Endocrinol* 2012; **26**:423–37.
- 28 Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *N Engl J Med* 2009; **361**:888–98.
- 29 Melgarejo E, Medina MA, Sanchez-Jimenez F *et al.* Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. *Int J Biochem Cell Biol* 2009; **41**:998–1001.
- 30 Beringer A, Noack M, Miossec P. IL-17 in Chronic inflammation: from discovery to targeting. *Trends Mol Med* 2016; **22**: 230–41.
- 31 Gaffen SL, Jain R, Garg AV *et al.* The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nat Rev Immunol* 2014; **14**:585–600.
- 32 Zhao L, Tang Y, You Z *et al.* Interleukin-17 contributes to the pathogenesis of autoimmune hepatitis through inducing hepatic interleukin-6 expression. *PLOS ONE* 2011; **6**:e18909.
- 33 Gu FM, Li QL, Gao Q *et al.* IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma. *Mol Cancer* 2011; **10**:150.
- 34 Kaptoge S, Di Angelantonio E, Pennells L *et al.* C-reactive protein, fibrinogen, and cardiovascular disease prediction. *N Engl J Med* 2012; **367**:1310–20.
- 35 Toulemonde G, Scaozec JY, Miossec P. Treatment with etanercept of autoimmune hepatitis associated with rheumatoid arthritis: an open label proof of concept study. *Ann Rheum Dis* 2012; **71**:1423–4.
- 36 Iwamoto S, Kido M, Aoki N *et al.* TNF- $\alpha$  is essential in the induction of fatal autoimmune hepatitis in mice through upregulation of hepatic CCL20 expression. *Clin Immunol* 2013; **146**: 15–25.
- 37 Stout-Delgado HW, Du W, Shirali AC *et al.* Aging promotes neutrophil-induced mortality by augmenting IL-17 production during viral infection. *Cell Host Microbe* 2009; **6**:446–56.
- 38 Nagata T, McKinley L, Peschon JJ *et al.* Requirement of IL-17RA in Con A induced hepatitis and negative regulation of IL-17 production in mouse T cells. *J Immunol* 2008; **181**:7473–9.
- 39 Bonder CS, Ajuebor MN, Zbytniuk LD *et al.* Essential role for neutrophil recruitment to the liver in concanavalin A-induced hepatitis. *J Immunol* 2004; **172**:45–53.
- 40 Gujral JS, Farhood A, Bajt ML *et al.* Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. *Hepatology* 2003; **38**:355–63.
- 41 O'Brien KM, Allen KM, Rockwell CE *et al.* IL-17A synergistically enhances bile acid-induced inflammation during obstructive cholestasis. *Am J Pathol* 2013; **183**:1498–507.
- 42 Ziraldo C, Vodovotz Y, Namas RA *et al.* Central role for MCP-1/CCL2 in injury-induced inflammation revealed by *in vitro*, *in silico*, and clinical studies. *PLOS ONE* 2013; **8**:e79804.
- 43 Mandrekar P, Ambade A, Lim A *et al.* An essential role for monocyte chemoattractant protein-1 in alcoholic liver injury: regulation of proinflammatory cytokines and hepatic steatosis in mice. *Hepatology* 2011; **54**:2185–97.
- 44 Sarma NJ, Tiriveedhi V, Crippin JS *et al.* Hepatitis C virus-induced changes in microRNA 107 (miRNA-107) and miRNA-449a modulate CCL2 by targeting the interleukin-6 receptor complex in hepatitis. *J Virol* 2014; **88**:3733–43.
- 45 Hartupej J, Liu C, Novotny M *et al.* IL-17 enhances chemokine gene expression through mRNA stabilization. *J Immunol* 2007; **179**:4135–41.
- 46 Hennes S, Johnson CK, Ge Q *et al.* IL-17A augments TNF- $\alpha$ -induced IL-6 expression in airway smooth muscle by enhancing mRNA stability. *J Allergy Clin Immunol* 2004; **114**: 958–64.
- 47 Zrioual S, Ecochard R, Tournadre A *et al.* Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synovial cells. *J Immunol* 2009; **182**: 3112–20.

- 48 Hennes S, van Thoor E, Ge Q *et al.* IL-17A acts via p38 MAPK to increase stability of TNF- $\alpha$ -induced IL-8 mRNA in human ASM. *Am J Physiol Lung Cell Mol Physiol* 2006; **290**: L1283–90.
- 49 Shimada M, Andoh A, Hata K *et al.* IL-6 secretion by human pancreatic periacinar myofibroblasts in response to inflammatory mediators. *J Immunol* 2002; **168**:861–8.
- 50 Witowski J, Pawlaczyk K, Breborowicz A *et al.* IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO  $\alpha$  chemokine from mesothelial cells. *J Immunol* 2000; **165**:5814–21.
- 51 Chowdhury S, Dijkhuis A, Steiert S *et al.* IL-17 attenuates degradation of ARE-mRNAs by changing the cooperation between AU-binding proteins and microRNA16. *PLOS Genet* 2013; **9**: e1003747.
- 52 Andoh A, Shimada M, Bamba S *et al.* Extracellular signal-regulated kinases 1 and 2 participate in interleukin-17 plus tumor necrosis factor- $\alpha$ -induced stabilization of interleukin-6 mRNA in human pancreatic myofibroblasts. *Biochim Biophys Acta* 2002; **1591**:69–74.
- 53 Chen Y, Kijlstra A, Yang P. IL-17A stimulates the production of inflammatory mediators via Erk1/2, p38 MAPK, PI3K/Akt, and NF- $\kappa$ B pathways in ARPE-19 cells. *Mol Vis* 2011; **17**: 3072–7.
- 54 Shahrara S, Pickens SR, Mandelin AM 2nd *et al.* IL-17-mediated monocyte migration occurs partially through CC chemokine ligand 2/monocyte chemoattractant protein-1 induction. *J Immunol* 2010; **184**:4479–87.
- 55 Huang F, Kao CY, Wachi S *et al.* Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF- $\kappa$ B activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. *J Immunol* 2007; **179**:6504–13.
- 56 Ruddy MJ, Wong GC, Liu XK *et al.* Functional cooperation between interleukin-17 and tumor necrosis factor- $\alpha$  is mediated by CCAAT/enhancer-binding protein family members. *J Biol Chem* 2004; **279**:2559–67.
- 57 Zimmermann M, Aguilera FB, Castellucci M *et al.* Chromatin remodelling and autocrine TNF $\alpha$  are required for optimal interleukin-6 expression in activated human neutrophils. *Nat Commun* 2015; **6**:6061.
- 58 Sparna T, Retey J, Schmich K *et al.* Genome-wide comparison between IL-17 and combined TNF- $\alpha$ /IL-17 induced genes in primary murine hepatocytes. *BMC Genomics* 2010; **11**:226.
- 59 Karlsen JR, Borregaard N, Cowland JB. Induction of neutrophil gelatinase-associated lipocalin expression by co-stimulation with interleukin-17 and tumor necrosis factor- $\alpha$  is controlled by I $\kappa$ B-zeta but neither by C/EBP- $\beta$  nor C/EBP- $\delta$ . *J Biol Chem* 2010; **285**:14088–100.

## **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 these 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 TNF $\alpha$  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<sup>+</sup> CD4<sup>+</sup> IL-17<sup>+</sup> 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). These interactions are facilitated 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<sup>®</sup> 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<sup>®</sup> Plus Mini kit (Quiagen, Hilden, Germany). cDNA was synthesized using the iScript<sup>™</sup> kit (Bio-Rad, Hercules, CA, USA). PCR amplification was performed using the CFX96<sup>™</sup> Real time system instrument (Bio-Rad) with the iTaq<sup>™</sup> universal SYBR<sup>®</sup> green supermix (Bio-Rad) and the Qiagen QuantiTect<sup>®</sup> 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, TNF $\alpha$ , IFN $\gamma$ , IL-1 $\beta$  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<sup>+</sup> CD4<sup>+</sup> IL-17<sup>+</sup> 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<sup>6</sup> cells/mL) were labelled with 1  $\mu$ M 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 software (version 1.2, Beckman Coulter).

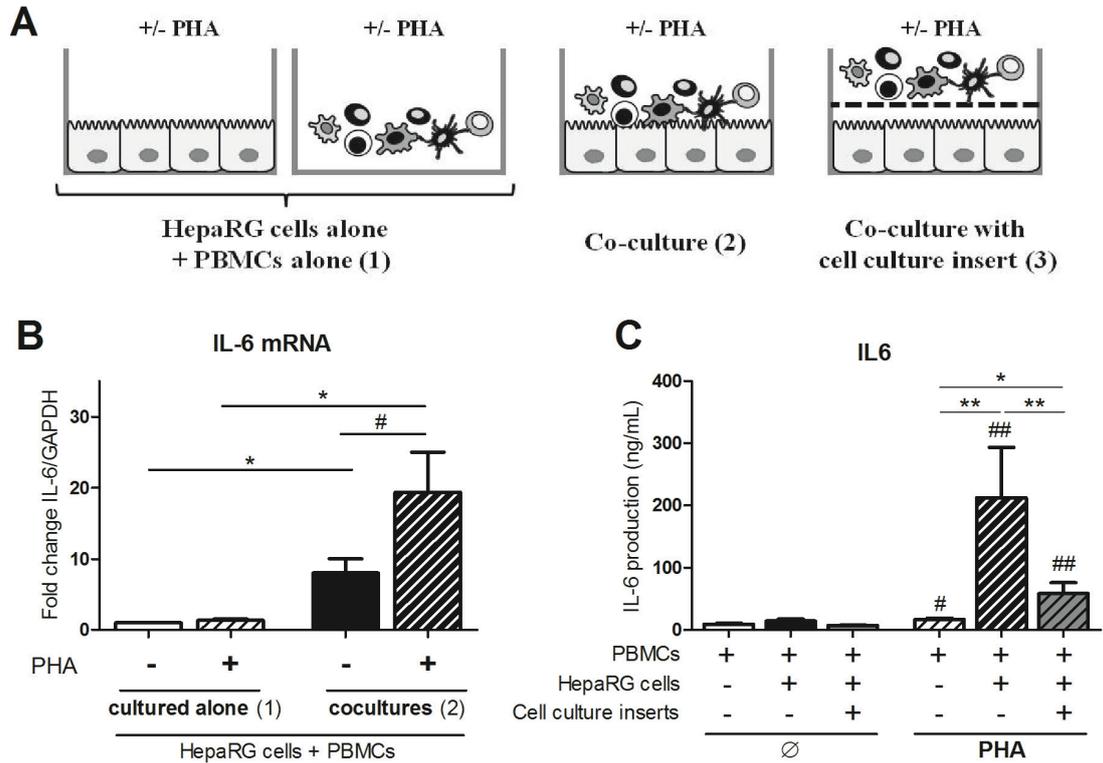
## **Statistical analysis**

Calculations were performed with GraphPad Prism version 5.01 software. Data are the mean of at least 3 independent experiments  $\pm$  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.

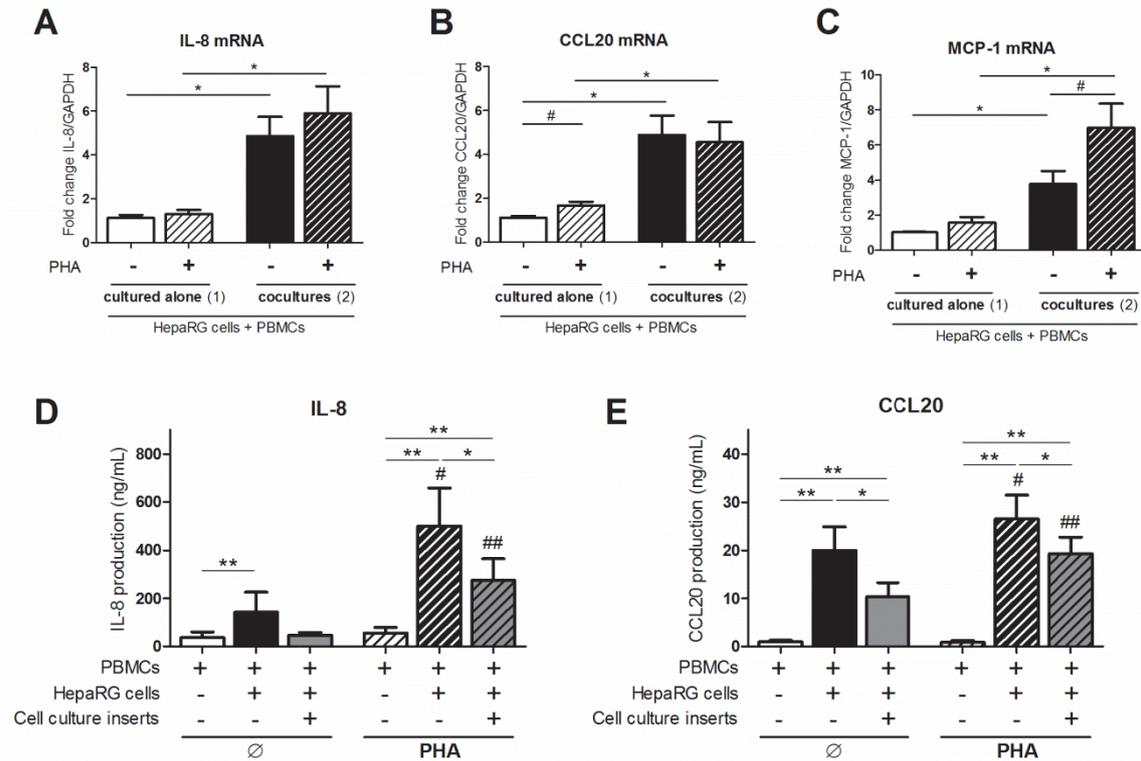


**FIGURE 6: PBMC-HepaRG cell interactions increase the expression and/or secretion of IL-6 in activated condition mainly through direct cell-cell contacts**

(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  $\pm$  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.



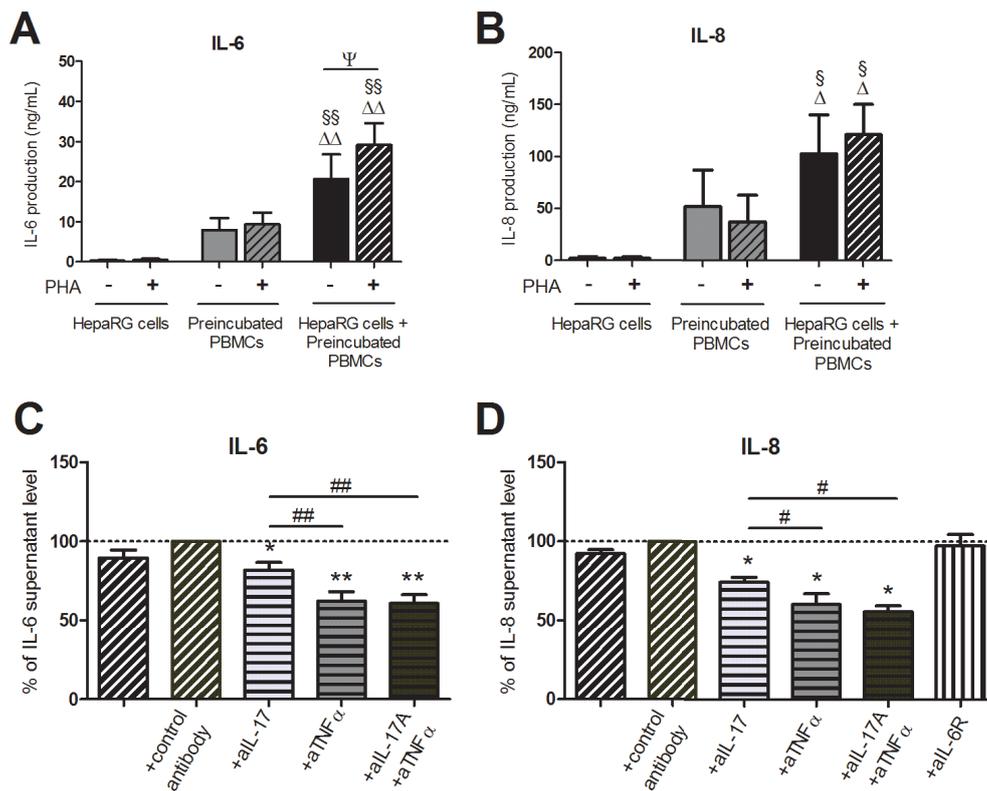
**FIGURE 7: PBMC-HepaRG cell interactions increase the expression and/or secretion of IL-8, CCL20 and MCP-1 chemokines**

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  $\pm$  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 TNF $\alpha$ contribute to the induction of the IL-6 and IL-8 secretion by the PBMC-HepaRG cell interactions**

IL-17 and TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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, TNF $\alpha$  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-TNF $\alpha$  alone or the combination of anti-IL-17 and anti-TNF $\alpha$  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-TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  inhibitors had no additive effects on the IL-6 and IL-8 inhibition.



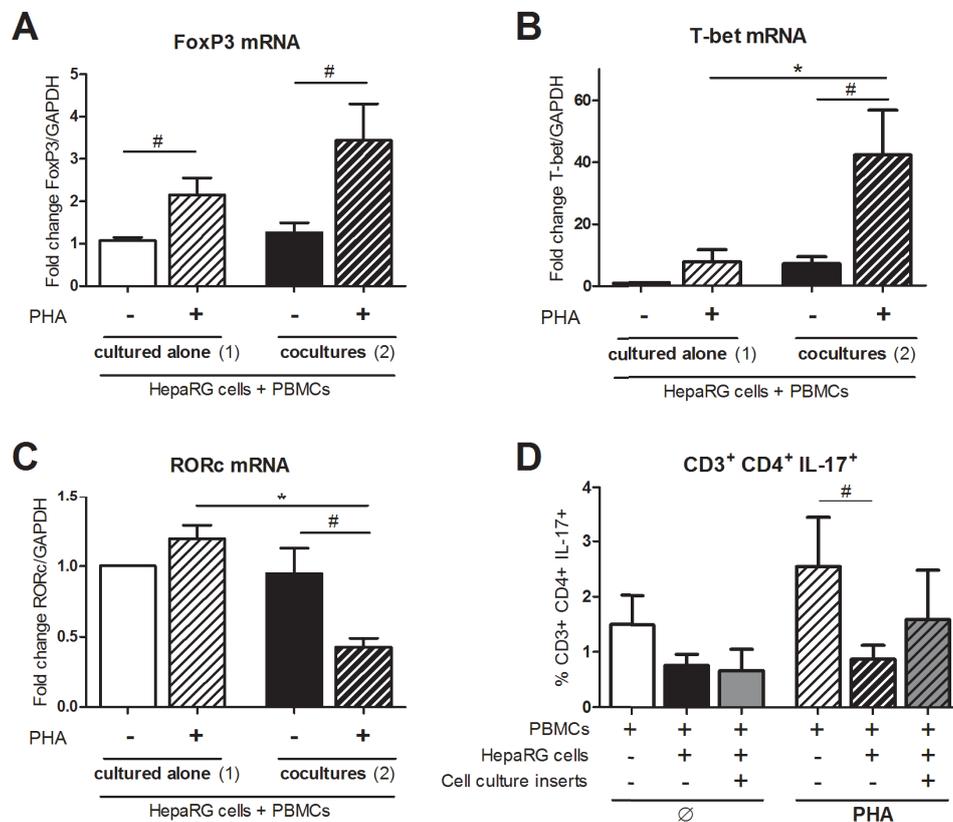
**FIGURE 8: IL-17 and TNF $\alpha$  contribute to the induction of IL-6 and IL-8 secretion by the PBMC-HepaRG cell interactions**

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-TNF $\alpha$  (aTNF $\alpha$ ) 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  $\pm$  SEM; Mann Whitney test,  $\Delta p < 0.05$  and  $\Delta\Delta p < 0.01$  versus HepaRG cells alone;  $\$ p < 0.05$  and  $\$\$ p < 0.01$  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-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, Tbet 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  $CD3^+ CD4^+ IL-17^+$  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  $CD3^+ CD4^+ IL-17^+$  cell frequency.

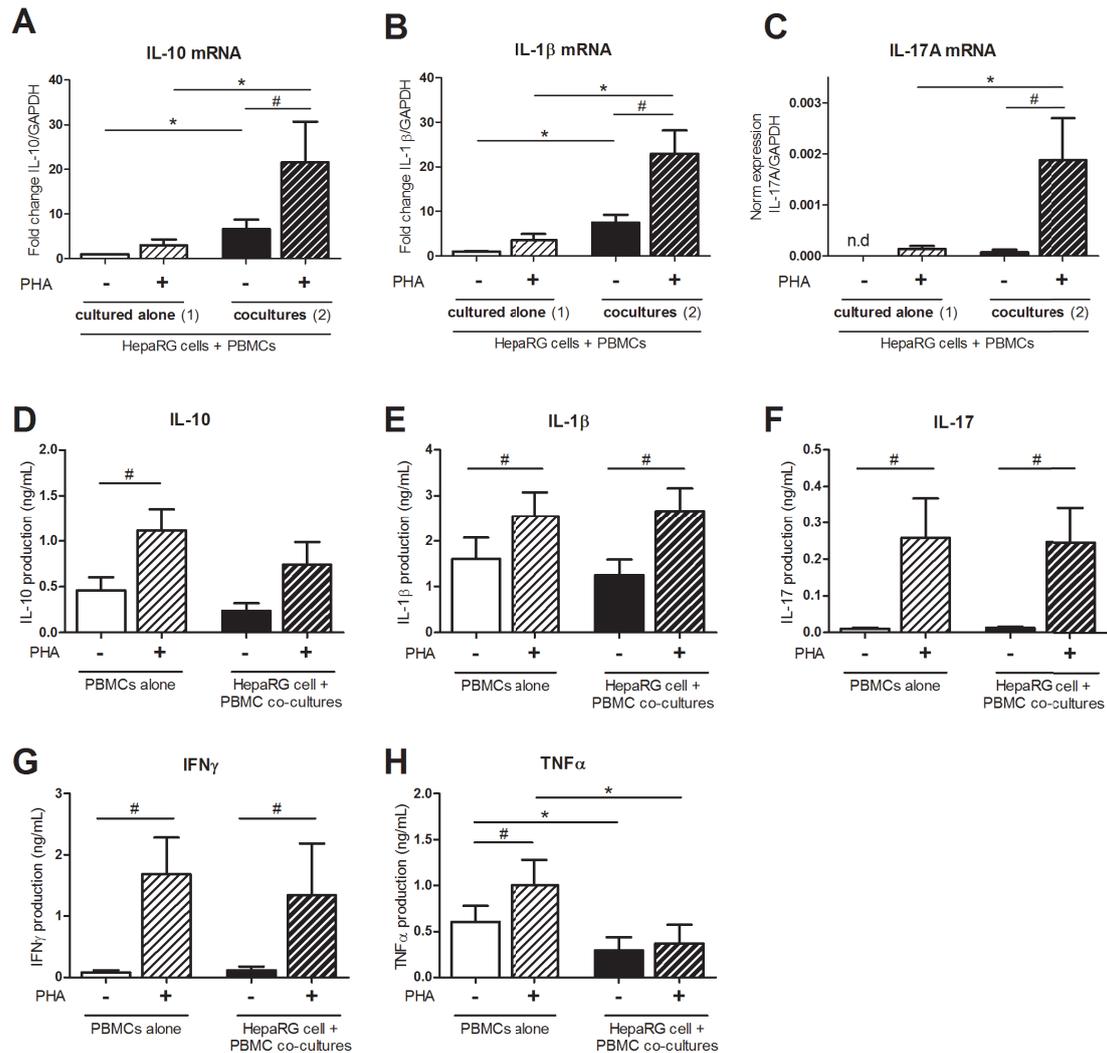


**FIGURE 9: PBMC-HepaRG cell interactions up-regulate Tbet expression whereas RORc expression and  $IL-17^+ CD4^+ CD3^+$  cell frequency decreased in PHA-activated conditions**

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, Tbet 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  $\pm$  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 TNF $\alpha$ but not the IL-10, IL-1 $\beta$ , IL-17 and IFN $\gamma$ release**

Because hepatocytes can act on T cell polarization, mRNA and/or culture supernatant levels of the pro-inflammatory cytokines IL-1 $\beta$ , IL-17, IFN $\gamma$  and TNF $\alpha$  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-1 $\beta$  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-1 $\beta$ , IL-17 and IFN $\gamma$  were higher in PHA-conditions ( $p < 0.05$ ) but similar between PBMCs alone and in co-cultures (Figures 10D, E, F, G). In contrast, TNF $\alpha$  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-1 $\beta$  and IL-17 but not their secretion in culture supernatants in PHA-activated conditions.



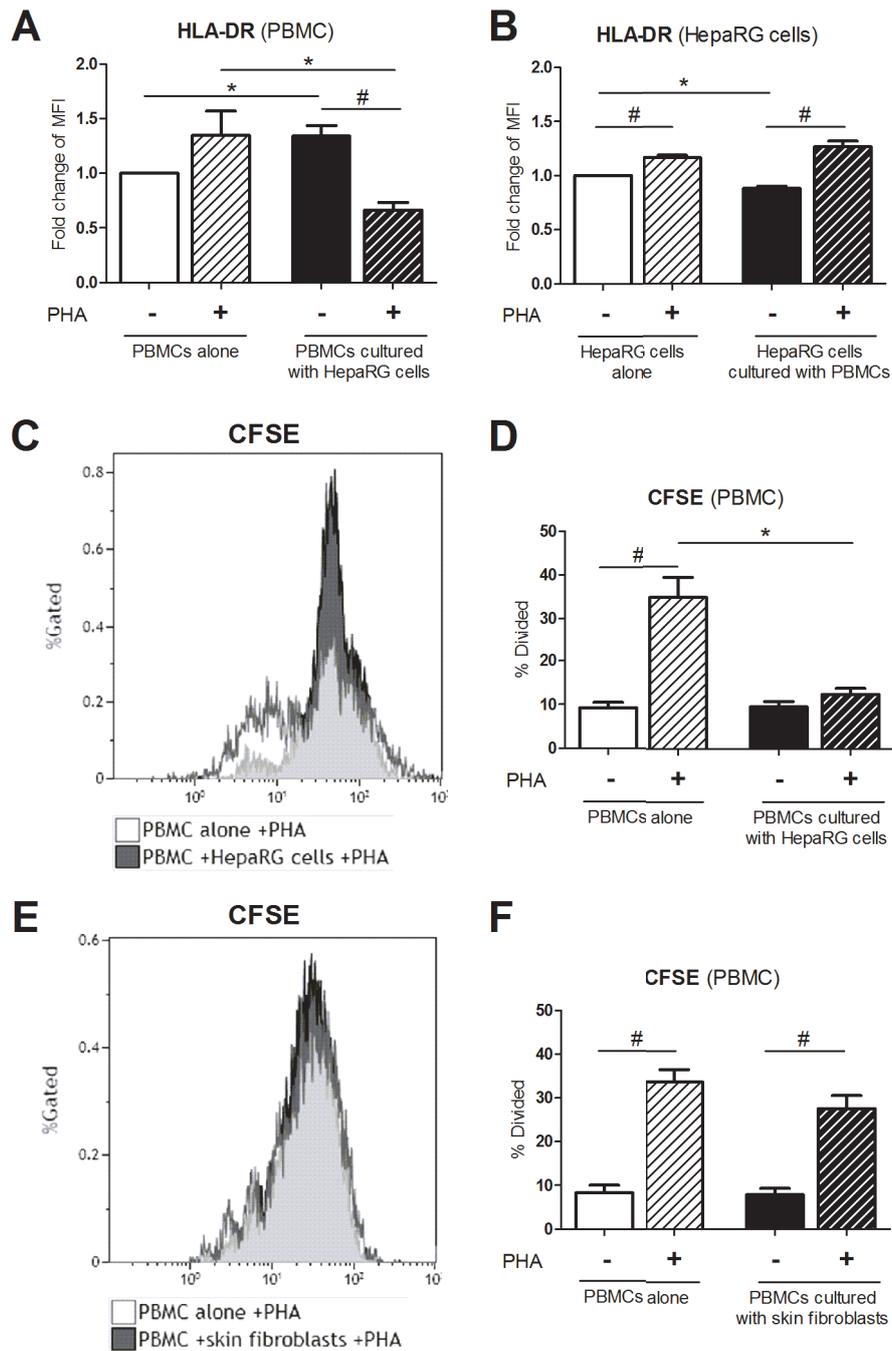
**FIGURE 10: PBMC-HepaRG cell interactions decrease the secretion of TNF $\alpha$  and had no effect on the IL-10, IL-1 $\beta$ , IL-17 and IFN $\gamma$  release in PHA-activated condition**

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-1 $\beta$  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-1 $\beta$ , IL-17, IFN $\gamma$  and TNF $\alpha$  supernatant levels were quantified by ELISA after 48h of culture. Data are the mean of 6 to 11 independent experiments  $\pm$  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**

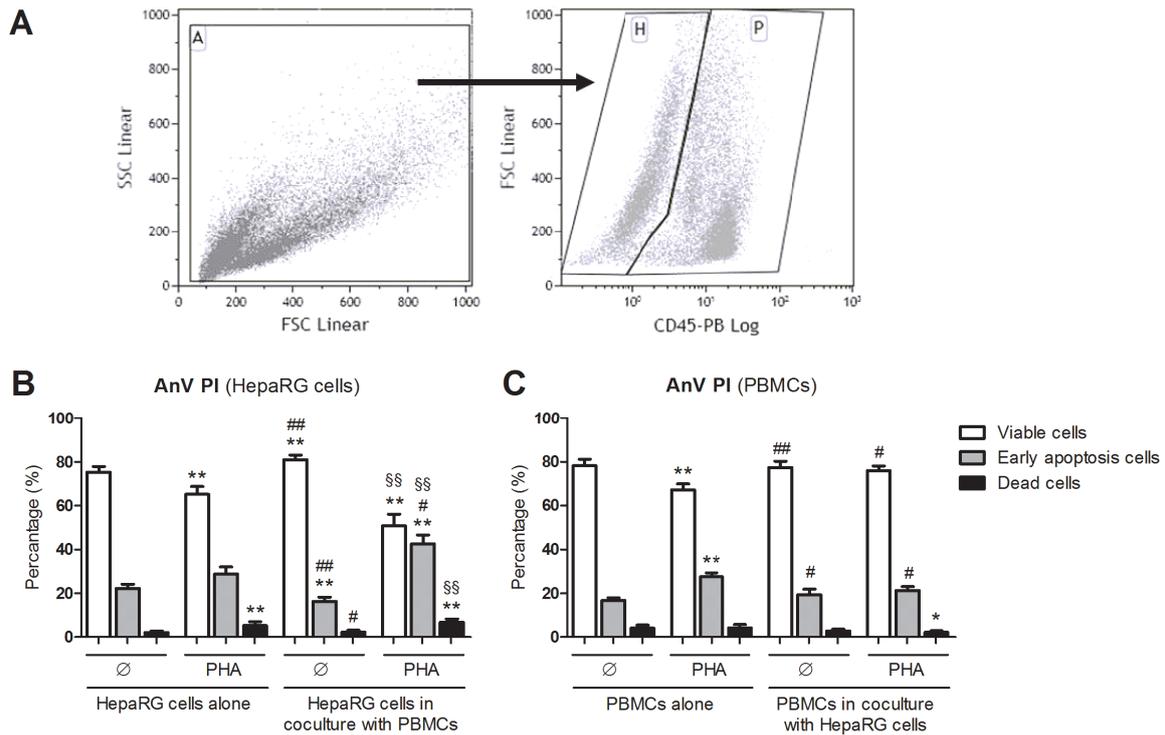
The effect of PBMC-HepaRG cell interactions on the antigen presenting cell capacity to CD4<sup>+</sup> T cells was next determined by looking at the human leukocyte antigens (HLA)-DR

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  $\pm$  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<sup>+</sup>) and the HepaRG cell population (CD45<sup>-</sup>). (C, D) Cells were stained with Annexin V (AnV)-FITC and propidium iodide (PI) to quantify the percentage of viable (AnV<sup>-</sup> PI<sup>-</sup>), early apoptosis (AnV<sup>+</sup> PI<sup>-</sup>) and dead (AnV<sup>+</sup> PI<sup>+</sup>) 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 not 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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  inhibited the IL-6 and IL-8 production in co-cultures without an additional inhibitory effect of the blockade of both IL-17 and TNF $\alpha$ . 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 TNF $\alpha$  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-1 $\beta$ , TNF $\alpha$  and IL-10 compared to THP-1 cultured alone (Honda and Inagawa, 2016). In addition, the interactions between CD4<sup>+</sup> T cells and hepatocytes from murine livers increased the IL-10 secretion by CD4<sup>+</sup> 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<sup>+</sup> FoxP3<sup>+</sup> Treg cells in presence of TGF $\beta$  through Notch signaling (Burghardt et al., 2014). The induction of IL-10 and FoxP3<sup>+</sup> Treg cells was even more pronounced when CD4<sup>+</sup> T cells were co-cultured with hepatocytes from regenerated livers of mice pretreated with the lectin concanavalin A (Burghardt et al., 2013, 2014). The CD4<sup>+</sup> 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 IFN $\gamma$ , known as a signature cytokine of Th1 cells, was similar between these two conditions. Surprisingly, the PBMC-HepaRG cell interactions decreased the RORc expression and the frequency of CD3<sup>+</sup> CD4<sup>+</sup> IL-17<sup>+</sup> 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 TNF $\alpha$  release was lower in co-cultures. In addition, gene expressions of IL-10, IL-1 $\beta$  and IL-17 were up-regulated in PHA-activated co-cultures but not the IL-10, IL-1 $\beta$  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<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 were 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 TNF $\alpha$ cooperation enhances the hepatic stellate cell pro-inflammatory response**

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

**Keywords:** hepatic stellate cells, interleukin-17, tumor necrosis factor- $\alpha$ , 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 TNF $\alpha$  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 TNF $\alpha$ . IL-17 and TNF $\alpha$  contribution was determined in a co-culture model including non- or PHA-activated PBMCs, LX-2 cells and/or HepaRG cells. IL-17 enhanced TNF $\alpha$  effects on the induction of IL-6 and IL-1 $\beta$  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 TNF $\alpha$  had no direct pro-fibrotic effects in collagen I  $\alpha$ 1, TIMP and MMP2 gene expression whereas MMP3 mRNA levels are up-regulated in LX-2 cells. Nevertheless, IL-17 and TNF $\alpha$  could act indirectly on fibrosis at later time points through the induction of TGF $\beta$ . Lastly, IL-17 and TNF $\alpha$  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 TNF $\alpha$  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 TGF $\beta$ , the master profibrotic cytokine, to induce the human HSC activation (Fabre et al., 2014). Since IL-17 enhanced the TNF $\alpha$  production by Kupffer cells and the TNF $\alpha$  mRNA expression in HSCs, TNF $\alpha$  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 TNF $\alpha$  to up-regulate in synergy some pro-inflammatory genes (Beringer et al., 2016, 2018; Katz et al., 2001; Zrioual et al., 2009). TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  effects. Finally, the IL-17 and TNF $\alpha$  contribution was determined in a co-culture model including PBMCs, LX-2 cells and/or HepaRG cells. The IL-17 exposure enhanced the TNF $\alpha$  effects on the induction of pro-inflammatory cytokines and chemokines. Moreover, IL-17 and TNF $\alpha$  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  $\mu\text{g/ml}$  phytohemagglutinin (PHA) (Sigma-Aldrich) and exposed to the anti-IL-17 secukinumab (Novartis, Basel, Switzerland) and the anti-TNF $\alpha$  infliximab (Merck, Kenilworth, USA) or the control antibody at 10  $\mu\text{g/mL}$  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 synthesized 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**

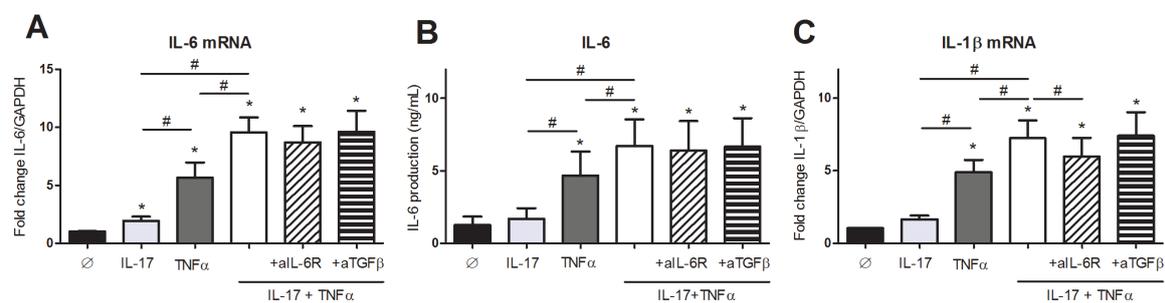
Calculations were performed with GraphPad Prism version 5.01 software. Data are the mean of at least 3 independent experiments  $\pm$  SEM. Statistical differences were analyzed using the Mann Whitney test. P values less than to 0.05 were considered significant.

## ***3.3 Results***

### **IL-17 amplified the TNF $\alpha$ effect on the induction IL-6 and IL-1 $\beta$ in the LX-2 cells**

IL-17 can cooperate with TNF $\alpha$  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 TNF $\alpha$  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-1 $\beta$  as well as IL-6 secretion compared to control condition. In contrast, the LX-2 cells stimulation with TNF $\alpha$  alone enhanced strongly the IL-6 and IL-1 $\beta$  mRNA

levels and the IL-6 supernatant level ( $p < 0.05$ ). Interestingly, addition of IL-17 to the TNF $\alpha$  induced a further increase of the IL-6 and IL-1 $\beta$  mRNA expression and the IL-6 production versus TNF $\alpha$  alone ( $p < 0.05$ ) (Figures 13A-C). Therefore, IL-17 amplified the TNF $\alpha$  effect on the IL-6 and IL-1 $\beta$  induction in the LX-2 cell cultures. Because the LX-2 cells have functional IL-6 and TGF $\beta$  receptors (Fabre et al., 2014; Schmidt-Arras and Rose-John, 2016; Schoenherr et al., 2010), a part of the IL-17 and TNF $\alpha$  effects could be mediated indirectly through the HSC autocrine IL-6 and TGF $\beta$  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 TGF $\beta$  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 TGF $\beta$  pathway, reduced by 18% the IL-1 $\beta$  up-regulation induced by IL-17 and TNF $\alpha$  (Figures 13A-C). The induction of IL-6 and IL-1 $\beta$  following the IL-17 and TNF $\alpha$  stimulation is therefore mainly independent of the IL-6 and TGF $\beta$  pathway activation.

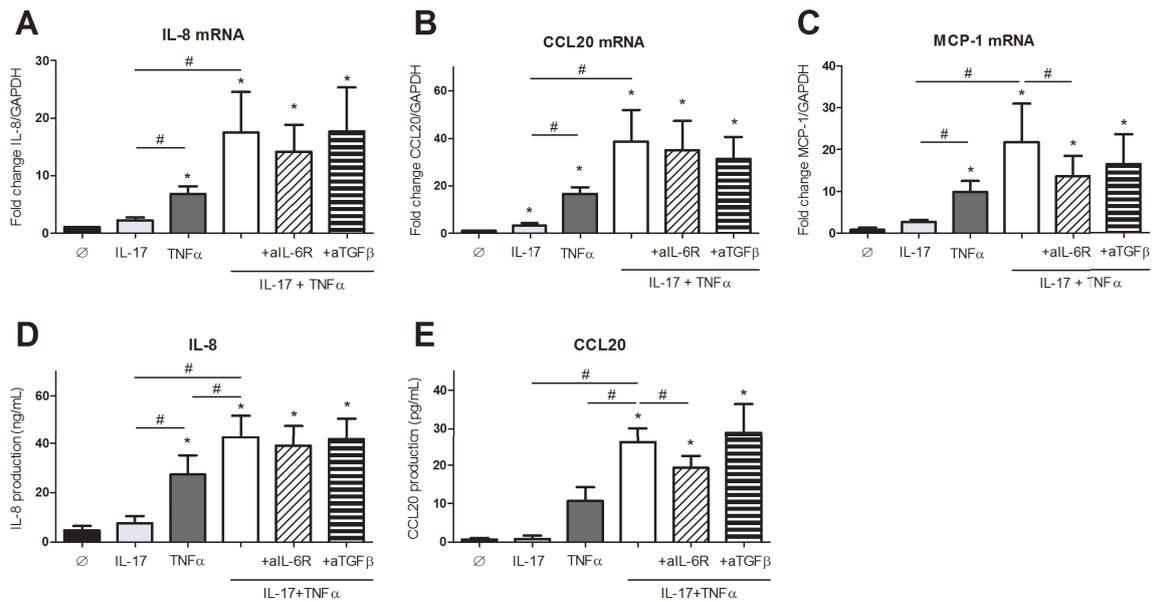


**FIGURE 13: IL-17 amplifies the TNF $\alpha$  effect on the induction of IL-6 and IL-1 $\beta$  in the LX-2 cells mainly independently of IL-6 and TGF $\beta$  pathways**

LX-2 cells were exposed to IL-17 and/or TNF $\alpha$  with/without anti-IL-6R (aIL-6R) or anti-TGF $\beta$  (aTGF $\beta$ ). (A, C) IL-6 and IL-1 $\beta$  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 TNF $\alpha$  stimulation (data not shown). Data are the mean of 6 independent experiments  $\pm$  SEM; Mann Whitney test, \* $p < 0.05$  versus control, # $p < 0.05$  versus other cytokine conditions.

### **IL-17 amplifies the TNF $\alpha$ 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 TNF $\alpha$  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. TNF $\alpha$  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-1 $\beta$ , IL-17 amplified the TNF $\alpha$  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 TNF $\alpha$  stimulation compared to TNF $\alpha$  alone ( $p < 0.05$ ) confirmed that IL-17 potentiated the TNF $\alpha$  effects on the increase of chemokine expression and/or production (Figures 14D, E). In addition, IL-8 induction following IL-17/TNF $\alpha$  was independent of IL-6 and TGF $\beta$  pathways since specific inhibitors of IL-6 and TGF $\beta$  pathways had no effect on the IL-8 levels induced by IL-17/TNF $\alpha$  (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 TGF $\beta$  pathway blockade had no effect (Figure 14B, C, E). This IL-17 and TNF $\alpha$  cooperation on the chemokine induction can intensify the local immune cell recruitment mainly through pathways independent of IL-6 and TGF $\beta$ .



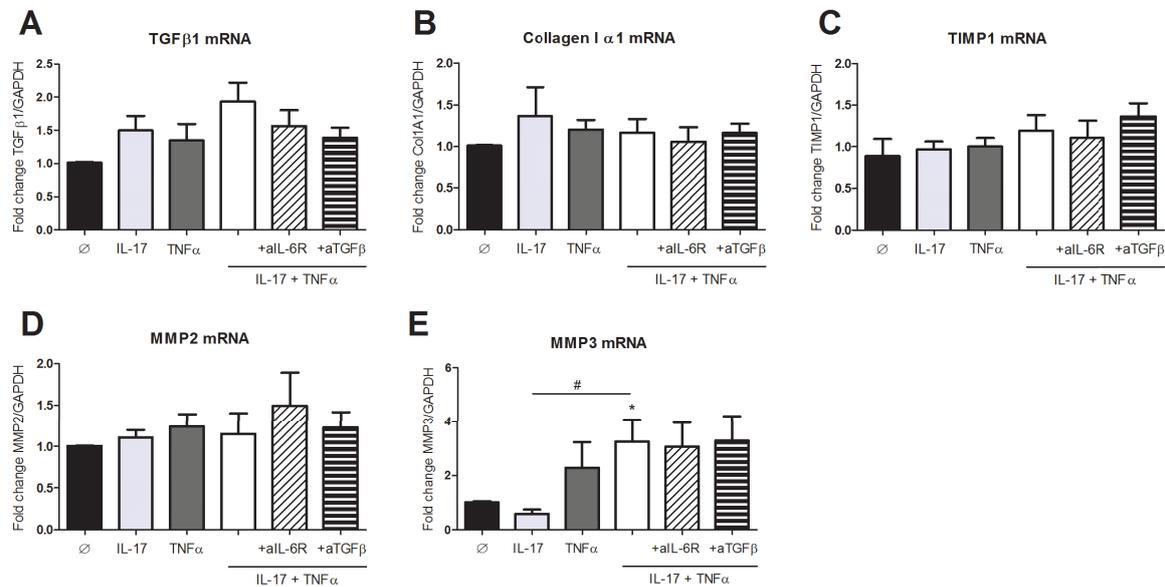
**FIGURE 14: IL-17 amplifies the TNF $\alpha$  effect on the increase of the IL-8, CCL20 and MCP-1 chemokine expression and/or secretion**

LX-2 cells were exposed to IL-17 and/or TNF $\alpha$  with/without anti-IL-6R (aIL-6R) or anti-TGF $\beta$  (aTGF $\beta$ ). (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 TNF $\alpha$  stimulation (data not shown). Data are the mean of 6 independent experiments  $\pm$  SEM; Mann Whitney test, \* $p$ <0.05 versus control, # $p$ <0.05 versus other cytokine conditions.

### IL-17 and TNF $\alpha$ 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 TNF $\alpha$  stimulation increased mRNA expression of the master pro-fibrotic cytokine TGF $\beta$ 1 by 1.9-fold (non-significant) in LX-2 cells (Figure 15A). Moreover, IL-17 and/or TNF $\alpha$  stimulation had no effect on mRNA levels of collagen I  $\alpha$ 1, TIMP1 and MMP2 (Figures 15B-D). In contrast, MMP3 mRNA expression was enhanced in presence of the IL-17 and TNF $\alpha$  combination ( $p$ <0.05) (Figure 15E). IL-6 and TGF $\beta$  pathways were not involved in the effects mediated by IL-17 and TNF $\alpha$  since the inhibition of IL-6 or TGF $\beta$  pathway had no effect on the gene expression levels in IL-17 and/or TNF $\alpha$  conditions. Therefore, IL-17 and TNF $\alpha$  have no direct pro-fibrotic effects on LX-2 cells.

However, by enhancing MMP3, IL-17 and TNF $\alpha$  could reduce or/and prevent extracellular matrix deposition.



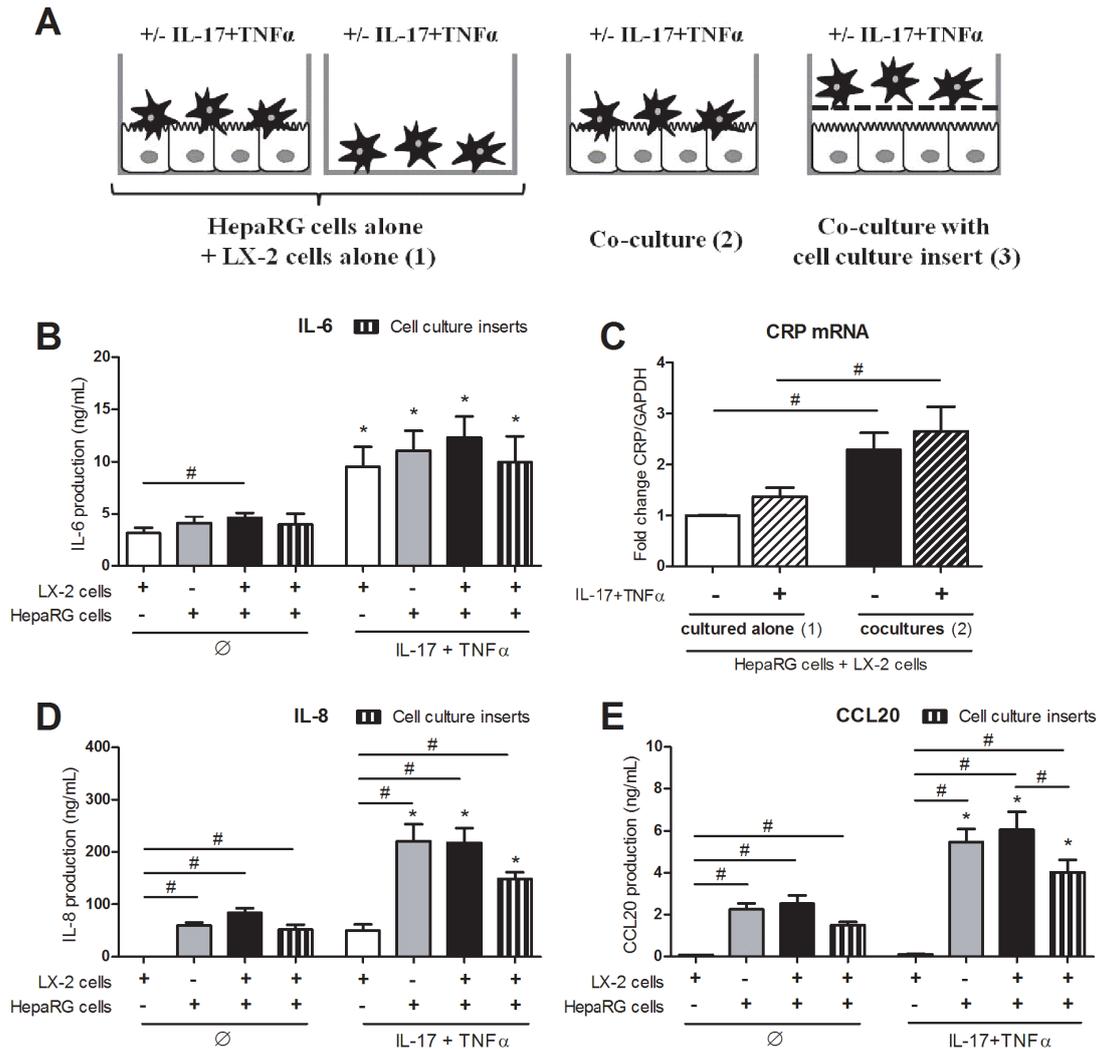
**FIGURE 15: IL-17 and TNF $\alpha$  have no direct effects on profibrotic genes**

LX-2 cells were exposed to IL-17 and/or TNF $\alpha$  with/without anti-IL-6R or anti-TGF $\beta$ . (A-E) TGF $\beta$ 1 mRNA expression at 10h and collagen I  $\alpha$ 1, TIMP1, MMP2 and MMP3 mRNA expression at 48h were expressed as fold changes compared to control. Data are the mean of 6 independent experiments  $\pm$  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/TNF $\alpha$ -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 TNF $\alpha$  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/TNF $\alpha$ -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/TNF $\alpha$ -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/TNF $\alpha$ .



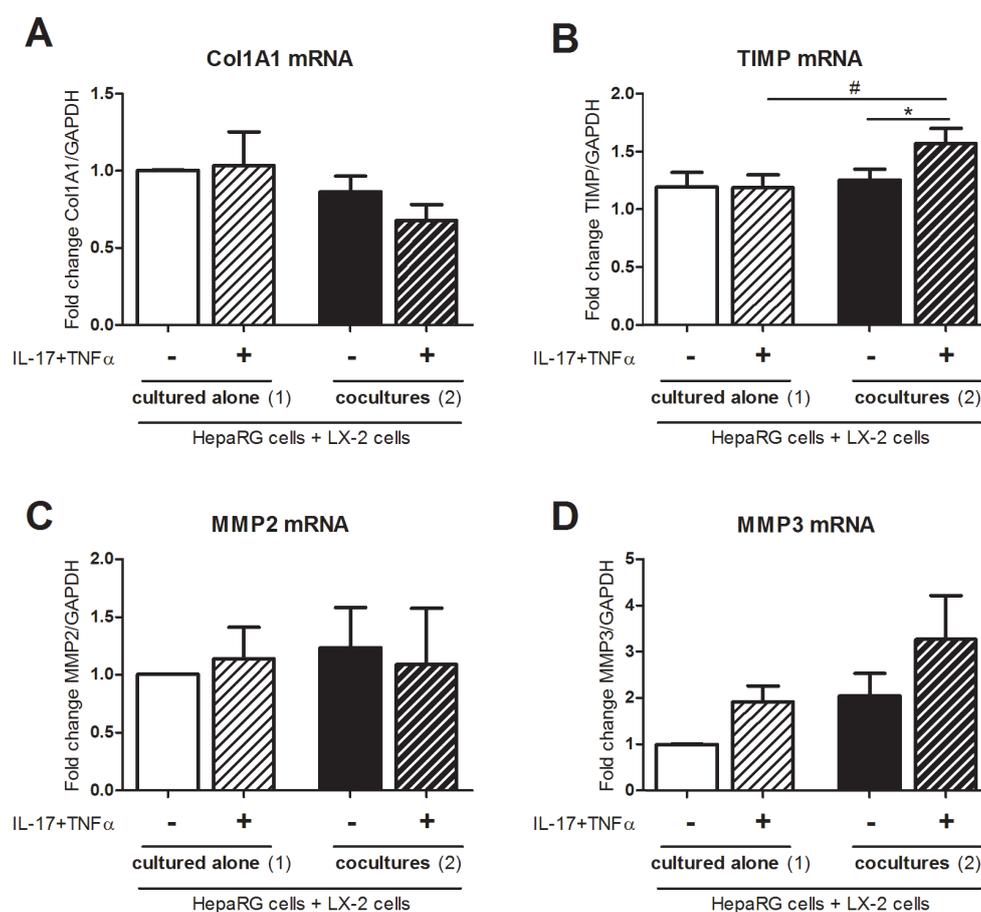
**FIGURE 16: LX-2 cell-HepaRG cell interactions enhance CRP expression but not the IL-6, IL-8 and CCL20 secretion by HepaRG cells**

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 TNF $\alpha$  (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  $\pm$  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 TNF $\alpha$ activated conditions

The effects of LX-2 cell-HepaRG cell interactions on fibrotic genes were next assessed with or without IL-17 and TNF $\alpha$  stimulation. Compared to LX-2 cells and HepaRG cells cultured separately, collagen type I  $\alpha 1$  mRNA 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/TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  on the fibrotic genes.

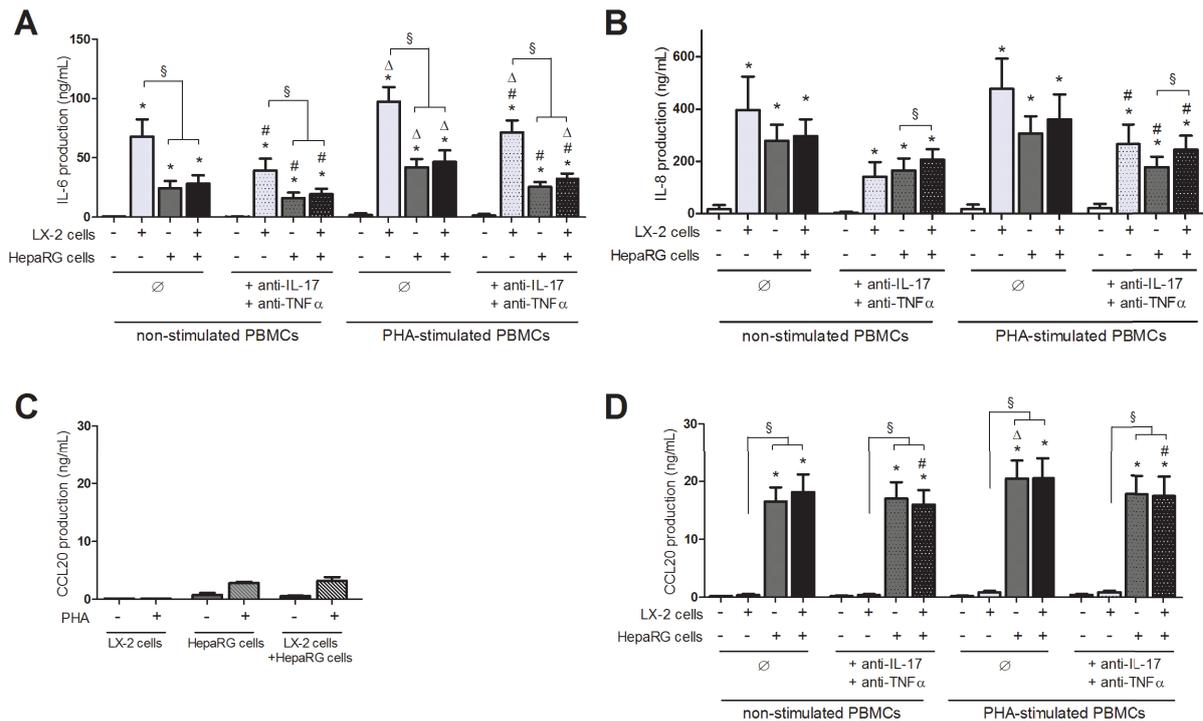


**FIGURE 17: LX-2 cell-HepaRG cell interactions modulated Collagen I  $\alpha$ 1, TIMP and MMP3 expression in IL-17 and TNF $\alpha$  activated conditions**

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 TNF $\alpha$ . (A-C) Collagen I  $\alpha$ 1, TIMP1 and MMP3 mRNA expression at 48h were expressed as fold changes compared to control. Data are the mean of 6 independent experiments  $\pm$  SEM; Mann Whitney test, \* $p < 0.05$  versus conditions without cytokines, # $p < 0.05$  versus co-cultures.

### **IL-17 and TNF $\alpha$ 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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  by up to 32% in PHA-activated co-cultures ( $p < 0.05$ ) (Figure 18B). IL-17 and TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$ 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-TNF $\alpha$  (aTNF $\alpha$ ) 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 not 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  $\pm$  SEM; Mann Whitney test, \* $p$ <0.05 versus PBMC alone,  $\S$  $p$ <0.05 versus LX-2 cell-PBMC co-cultures, # $p$ <0.05 versus without anti-IL-17 and anti-TNF $\alpha$ ,  $\Delta$  $p$ <0.05 versus non-activated conditions.

### 3.4 Discussion

This study shows that IL-17 amplified the TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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-1 $\beta$  as well as IL-8 and growth-related oncogene- $\alpha$  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-1 $\beta$  and IL-8 mRNA expression and/or secretion in LX-2 cell cultures compared to the TNF $\alpha$  stimulation. However, IL-17 potentiated the TNF $\alpha$  effects on the IL-6, IL-1 $\beta$  and IL-8 induction. This IL-17 and TNF $\alpha$  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 TNF $\alpha$  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 TGF $\beta$  expression in HSCs (Meng et al., 2012; Sun et al., 2012) and HSCs have functional IL-6 and TGF $\beta$  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 TNF $\alpha$ -mediated effects was assessed. The IL-6 and TGF $\beta$  pathways were not involved in the IL-17 and TNF $\alpha$  effects of the pro-inflammatory mediators studied with the exception of the IL-1 $\beta$  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 TNF $\alpha$  on the induction of the pro-inflammatory mediators studied here. As IL-17 may enhance the local TNF $\alpha$  production in liver (Hara et al., 2013; Meng et al., 2012), TNF $\alpha$  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 TGF $\beta$ 1 as well as collagen type I  $\alpha$ 1 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  $\alpha$ 1 and TIMP1 expression was very weak or

absent compared to TGF $\beta$  stimulation. However, IL-17 enhanced the TGF $\beta$  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 TGF $\beta$  pathway activation. TNF $\alpha$  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 TNF $\alpha$  stimulation whereas the collagen type I  $\alpha$ 1, TIMP1 and MMP2 expressions were unchanged (Robert et al., 2016; Tarrats et al., 2011). This matrix breakdown mediated by TNF $\alpha$  could be essential at the early stage of liver injury for the recruitment of inflammatory immune cells and later, TGF $\beta$  could decrease the initial TNF $\alpha$ -mediated MMP induction activity leading to the development of liver fibrosis (Knittel et al., 1999). Here, IL-17 and/or TNF $\alpha$  had no effect on the collagen I  $\alpha$ 1, TIMP1 and MMP2 mRNA expressions in LX-2 cells. However, as expected, the presence of TNF $\alpha$  enhanced MMP3 expression, this could prevent the extracellular matrix accumulation. IL-17 and/or TNF $\alpha$  have thereby no direct pro-fibrotic activities on HSCs. Nevertheless, by inducing the TGF $\beta$ 1 secretion by Kupffer cells (Hara et al., 2013), IL-17 may have indirect pro-fibrotic effects which can be amplified by the IL-17 and TGF $\beta$  cooperation described by Fabre *et al.* (Fabre et al., 2014). The potential indirect effects mediated by TGF $\beta$  may occur at later time points and therefore they were not 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-1 $\beta$ , 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/TNF $\alpha$ -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/TNF $\alpha$  activated conditions. Indeed, by inducing the release of soluble factors such as periostin by hepatocytes, IL-17 and TNF $\alpha$  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 TNF $\alpha$  in LX-2 cell-HepaRG cell co-cultures and could therefore counterbalance partially the IL-17/TNF $\alpha$  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 TNF $\alpha$  can participate to the cell-to-cell communication in liver. Indeed, HSCs exposed to conditioned medium from activated PBMCs increased the growth-related oncogene- $\alpha$  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-1 $\beta$ , 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). TNF $\alpha$ -released from macrophages was also a key factor of HSC activation since the blockage of TNF $\alpha$  decreased mRNA levels of fibrosis markers in HSCs co-cultured with CCR9<sup>+</sup> macrophages (Chu et al., 2013). In addition, TNF $\alpha$  contributed to the increase of HSC survival induced by the HSC–hepatic macrophage interactions via the activation of NF- $\kappa$ B 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 TNF $\alpha$  were involved in the induction of IL-6 and IL-8 release

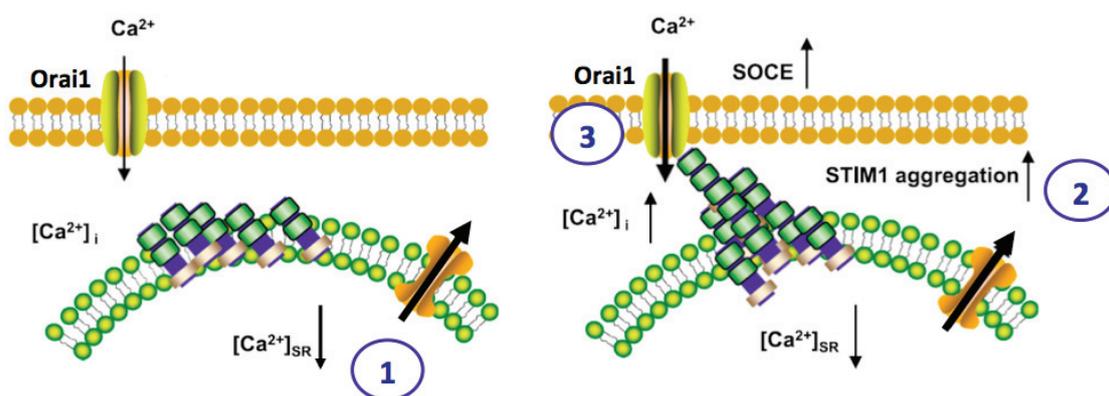
in non- and/or activated co-cultures, IL-17 and TNF $\alpha$  contribute most probably to the perpetuation of the chronic inflammatory state in liver. Since sustained liver inflammation drive to liver fibrosis, neutralization of IL-17 and/or TNF $\alpha$  at the early phase of the liver injury can be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

## 4 IL-17 and TNF $\alpha$ 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 TNF $\alpha$  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<sup>2+</sup> 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<sup>2+</sup> and its overexpression was associated with muscular dystrophy (Edwards et al., 2010). SOCE is activated by Ca<sup>2+</sup> depletion from the ER that triggers STIM1 translocation to Orai leading to store-operated Ca<sup>2+</sup> channel opening (Figure 19).



**FIGURE 19: Store-operated calcium entry process**

The increase of endoplasmic reticulum Ca<sup>2+</sup> store depletion (1) enhances STIM1 aggregation (2) leading to store-operated channels Ca<sup>2+</sup> opening (3) and Ca<sup>2+</sup> 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 TNF $\alpha$  on the inflammatory response and SOCE in human myoblasts.

## 4.2 Results

**IL-17 and TNF $\alpha$  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 TNF $\alpha$  can contribute to the local Th17 cell induction.

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

**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 TNF $\alpha$  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 TNF $\alpha$  contribute to this induction.

**IL-17 and TNF $\alpha$  increase SOCE.** IL-17 and TNF $\alpha$  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 TNF $\alpha$  exposure increase significantly SOCE.

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

## 4.3 Conclusion

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

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

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2  
3 **BLOCKADE OF STORE-OPERATED CALCIUM ENTRY REDUCES**  
4 **IL-17/TNF CYTOKINE-INDUCED INFLAMMATORY RESPONSE**  
5 **IN HUMAN MYOBLASTS**  
6

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23 **Word count:** 2,969

24 **Number of figures:** 6  
25

26       **Abstract**

27       Muscle inflammation as in idiopathic inflammatory myopathies (IIM) leads to muscle  
28 weakness, mononuclear cell infiltration and myofiber dysfunction affecting calcium channels. The  
29 effects of interleukin-17A (IL-17) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) on inflammation and  
30 calcium changes were investigated in human myoblasts. Human myoblasts were exposed to IL-17  
31 and/or TNF $\alpha$  with/without store-operated Ca<sup>2+</sup> entry (SOCE) inhibitors (2-ABP or BTP2). For co-  
32 cultures, peripheral blood mononuclear cells (PBMC) from healthy donors activated or not with  
33 phytohemagglutinin (PHA) were added to myoblasts at a 5:1 ratio.  
34 IL-17 and TNF $\alpha$  induced in synergy CCL20 and IL-6 production by myoblasts (>14-fold).  
35 PBMC-myoblast co-cultures enhanced CCL20 and IL-6 production in the presence or not of PHA  
36 compared to PBMC or myoblast monocultures. Anti-IL-17 and/or anti-TNF $\alpha$  decreased the  
37 production of IL-6 in co-cultures (p<0.05). Transwell system that prevents direct cell-cell contact  
38 reduced CCL20 (p<0.01) but not IL-6 secretion. IL-17 and/or TNF $\alpha$  increased the level of the ER  
39 stress marker Grp78, mitochondrial ROS and promoted SOCE activation by 2-fold (p<0.01) in  
40 isolated myoblasts. SOCE inhibitors reduced the IL-6 production induced by IL-17/TNF $\alpha$ .  
41 Therefore, muscle inflammation induced by IL-17 and/or TNF $\alpha$  may increase muscle cell  
42 dysfunction, which, in turn, increased inflammation. Such close interplay between immune and  
43 non-immune mechanisms may drive and increase muscle inflammation and weakness.

44  
45       **Keywords:** inflammatory myopathies, interleukin-17, tumor necrosis factor- $\alpha$ , store-operated  
46 calcium entry, myoblasts

In review

47 **Introduction**

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

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

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

70  
71 **Materials and methods**

72  
73 **Isolation and culture of muscle cells.** Muscle samples were obtained from subjects  
74 undergoing orthopedic surgery. Biopsies were performed on m. vastus lateralis (femoral  
75 quadriceps) at distance of the joint. Written informed consent was obtained before surgery  
76 according to the policies of the local ethical committee and the Ministry of Research, which  
77 approved the study (reference number: AC-2010-1164). After surgery, muscle samples were  
78 immediately placed in sterile PBS with antibiotics (penicillin and streptomycin, Eurobio,  
79 Courtaboeuf, France) and washed. The fat and fibrous tissues were removed. Muscle samples  
80 were cut into fragments ( $1\text{-}2\text{mm}^3$ ) and incubated at  $37^\circ\text{C}$  for 30 minutes with  $1\text{mg/mL}$  collagenase  
81 (Sigma-Aldrich, St Louis, MO, USA). After washing and filtration, a first selection was done to  
82 remove fibroblasts by incubating the supernatants in petri dishes at  $37^\circ\text{C}$  for 1 hour. Unattached  
83 myoblasts were then transferred and cultured at  $37^\circ\text{C}/5\% \text{CO}_2$  in Ham's-F10 medium (Eurobio)  
84 supplemented with 20% fetal bovine serum (Life Technologies, Carlsbad, USA), 2% Penicillin-  
85 Streptomycin (Eurobio), 1% L-glutamine (Eurobio) and 1% Amphotericin B (Eurobio). After 10  
86 days, adherent cells were detached with trypsin (Eurobio), and myoblasts were purified by positive  
87 selection with CD56 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to  
88 the instructions of the manufacturer. Myoblasts were used between passages 2 and 8.

89  
90 **Myoblast culture exposures.** Myoblasts were seeded at a density of  $50,000 \text{ cells/cm}^2$ . After  
91 adhesion, cells were stimulated with  $50\text{ng/mL}$  IL-17A (Dendritics, Lyon, France) or  $1\text{ng/mL}$   
92  $\text{TNF}\alpha$  (R&D Systems, Minneapolis, USA) alone or in combination. To inhibit SOCE, BTP2 (or  
93 YM58483) and 2-aminoethyl diphenylborinate (2-APB) inhibitors (Sigma-Aldrich) were used  
94 between 10 to  $50\mu\text{M}$ .

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

109  
110 **Enzyme-linked immunosorbent assay (ELISA).** After 48 hours of treatment, supernatants  
111 were harvested and the IL-6 and chemokine (C-C motif) ligand 20 (CCL20) productions were  
112 quantified with commercially available ELISA kits (R&D Systems) according to the  
113 manufacturer's instructions.

114  
115 **Quantitative real time-PCR.** Total RNA was purified using an RNeasy® Plus Mini kit  
116 (Qiagen, Hilden, Germany). cDNA was synthesized using the iScript™ kit (Bio-Rad, Hercules,  
117 CA, USA). PCR amplification was performed using the CFX96™ Real time system instrument  
118 (Bio-Rad) with the iTaq™ universal SYBR® green supermix (Bio-Rad) and the Qiagen  
119 QuantiTect® primers (QT00083538 for STIM1 and QT01870043 for ORAI1). The expression of  
120 the genes of interest was normalized to the expression of the housekeeping GAPDH gene.

121  
122 **Cell lysis and western blotting.** Cell lysates were obtained by lysing cells with RIPA buffer  
123 supplemented with 1mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 20mM NAF, 5mM EDTA and a cocktail of  
124 proteases inhibitor. Total protein concentration was determined using Bicinchoninic acid method  
125 (BCA, Interchim) and 25µg of protein of each sample was loaded on 12% sodium dodecyl sulfate  
126 polyacrylamide gel (SDS-PAGE). Migration was performed during 15 minutes at 90V followed  
127 by 60 minutes at 130V. Proteins were then blotted on a polyvinylidene difluoride (PVDF)  
128 membrane by electro transfer (Trans-Blot Turbo Transfer, Bio-Rad). PVDF membrane was  
129 incubated at room temperature for 1 hour with 5% milk in PBS for blocking and then incubates  
130 overnight at 4°C in the same buffer with the primary antibody (Grp78, sc-376768; STIM1,  
131 ab108994; ORAI1, sc68895; Tubulin, sc-5286). Secondary Horse radish peroxidase (HRP)  
132 coupled antibodies and ECL (entry-level peroxidase substrate for enhanced chemiluminescence)  
133 plus kit and Western Blotting detection system from GE Healthcare were used to reveal the  
134 proteins. The protein amount was determined using ImageLab software (Bio-Rad).

135  
136 **Wide-field microscopy for Ca<sup>2+</sup> live cell imaging.** Cells were imaged on an epifluorescence  
137 microscope Leica DMI6000B using 40x objective equipped with Orca-Flash4.0 digital camera  
138 (Hamamatsu). Myoblasts were double excited at 340 and 380nm and emission was collected at  
139 510nm with identical acquisition parameters. Medium was replaced by a Calcium Containing  
140 Buffer (CCB) (in mmol/L: 140NaCl, 5KCl, 10HEPES, 1MgCl<sub>2</sub>, 2CaCl<sub>2</sub>, 10glucose, adjusted to  
141 pH7.4) containing 3µmol/L of fura2-AM during 30min at room temperature. Cells were washed  
142 twice with calcium free buffer in which 0.1mmol/L EGTA was added and placed under the  
143 microscope. For depletion of Ca<sup>2+</sup> stores, cyclopiazonic acid (CPA) (10µM) was used and then  
144 2mM calcium solution (CCB) was added to trigger the SOCE. Fluorescence ratios were calculated

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

147

148 **Confocal microscopy for oxidative stress detection.** Cells were imaged on a confocal  
149 microscope Nikon A1r using 40x objective. Myoblasts were excited at 640nm and emission was  
150 collected at 665nm. Medium was replaced by a Calcium Containing Buffer (CCB) (in mmol/L:  
151 140NaCl, 5KCl, 10HEPES, 1MgCl<sub>2</sub>, 2CaCl<sub>2</sub>, 10glucose, adjusted to pH7.4) containing  
152 2.5μmol/L of CellROX™ Deep Red Reagent during 30min at 37°C. Fluorescence intensity was  
153 analyzed in ImageJ Fiji (<https://fiji.sc/#>, NIH). A threshold at the third quartile of the pixel  
154 intensity distribution was applied before the analysis.

155

#### 156 **Confocal microscopy for STIM1 puncta analysis**

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

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

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

172

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

176

## 177 **Results**

178

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

180 IL-6 is a pro-inflammatory cytokine involved in the differentiation of Th17 cells, the main IL-  
181 17-producing cells. By attracting Th17 cells and dendritic cells, CCL20 plays an important role in  
182 the local immune cell recruitment (17). IL-17 and TNFα are involved in IL-6 and/or CCL20  
183 production by muscle cells (9,14,15). but the effect of their combination has not yet been  
184 investigated. The IL-17/TNFα effect on IL-6 and CCL20 release was studied in human myoblasts.  
185 IL-17, TNFα and the IL-17/TNFα combination increased significantly IL-6 production by 4-, 3-  
186 and 14-fold respectively compared to untreated condition (Figure 1A). CCL20 secretion by  
187 myoblasts was also induced by IL-17 alone (4-fold), TNFα alone (6-fold) and the IL-17/TNFα  
188 combination (29-fold) compared to the control condition (p<0.05) (Figure 1B). Therefore, the IL-  
189 17/TNFα cooperation increased synergistically the secretion of CCL20 and IL-6. By acting on IL-  
190 6 and CCL20 secretion, IL-17 and TNFα may contribute to the local Th17 cell induction.

191

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

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

195 sensors and to initiate the ER stress response known as the unfolded-protein response (18). As  
196 observed for the secretion of CCL20 and IL-6, IL-17 and TNF $\alpha$  cooperated to increase the  
197 expression of BiP/Grp78 protein compared to IL-17 and TNF $\alpha$  alone (Figure 2A and B). In  
198 addition to the unfolded-protein response, oxidative stress and accumulation of reactive oxygen  
199 species (ROS) initiate and contribute to the inflammatory response. Using confocal microscopy, a  
200 1.5-fold increase in mitochondrial ROS and a 2-fold increase with both cytokines ( $p < 0.0001$ ) was  
201 observed in TNF $\alpha$  and IL-17 treated myoblasts (Figure 2C and D).

202

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

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

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

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

229

### 230 **IL-17 and TNF $\alpha$ increase store operated calcium entry (SOCE)**

231 Inflammation increases intracellular Ca<sup>2+</sup> concentrations in several inflammatory muscle  
232 disorders. The routes of calcium entry include calcium leak channels, stretch-activated channels,  
233 receptor-operated channels, and store-operated calcium channels. Ca<sup>2+</sup> influx is sufficient to  
234 induce muscular dystrophy through a TRPC-dependent mechanism (21). The pro-inflammatory  
235 cytokine TNF $\alpha$  has been shown to enhance SOCE in human airway smooth muscle cells (22). The  
236 effect of IL-17 and TNF $\alpha$  on the calcium homeostasis has not been investigated in the context of  
237 IIM pathogenesis.

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

243 difference in the puncta density, but an increase of STIM1-puncta surface in IL17/TNF $\alpha$   
244 conditions (Figure 4G and H).

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

249

### 250 **Inhibition of SOCE reduces IL-6 production induced by the IL-17/TNF $\alpha$ combination**

251 To assess the SOCE contribution to the myoblast inflammatory response, myoblasts were  
252 stimulated with IL-17 and/or TNF $\alpha$  in presence or not of 2-APB or BTP2 SOCE inhibitors. IL-6  
253 release by myoblasts was quantified at 48 hrs. The 2-APB and BTP2 inhibitors inhibited the  
254 induction of IL-6 production by IL-17 and/or TNF $\alpha$  in a dose-dependent manner. The induction of  
255 IL-6 secretion by the IL-17 and TNF $\alpha$  combination was reduced by 42% ( $p < 0.05$ ) with 2-APB at  
256 50  $\mu$ M, and by 19% ( $p < 0.05$ ) and 33% ( $p < 0.01$ ) with BTP-2 at 10 and 20  $\mu$ M, respectively (Figure  
257 5A and B). Therefore, SOCE interacts with immune mechanisms to further increase the myoblast  
258 inflammatory response.

259

### 260 **Discussion**

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

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

278 IL-6 is a pleiotropic inflammatory cytokine produced by myoblasts in response to  
279 inflammation. The IL-17/IL-1 $\beta$ , TNF $\alpha$ /IL-1 $\beta$  and TNF $\alpha$ /IFN $\gamma$  combinations have been previously  
280 shown to have additive/synergistic effects on the induction of IL-6 secretion by myoblasts  
281 (9,14,23,25). Here, IL-17 and TNF $\alpha$  interactions increased in synergy the production of IL-6.

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

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

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

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

320

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323

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326

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## References

336

337

1. Pan Z, Brotto M, Ma J. Store-operated Ca<sup>2+</sup> entry in muscle physiology and diseases. *BMB Rep* (2014) **47**:69–79. doi:10.5483/BMBRep.2014.47.2.015

338

339

2. Edwards JN, Friedrich O, Cully TR, von Wegner F, Murphy RM, Launikonis BS. Upregulation of store-operated Ca<sup>2+</sup> entry in dystrophic mdx mouse muscle. *Am J Physiol-Cell Physiol* (2010) **299**:C42–C50. doi:10.1152/ajpcell.00524.2009

340

341

342

3. Kiviluoto S, Decuyper J-P, De Smedt H, Missiaen L, Parys JB, Bultynck G. STIM1 as a key regulator for Ca<sup>2+</sup> homeostasis in skeletal-muscle development and function. *Skelet Muscle* (2011) **1**:16. doi:10.1186/2044-5040-1-16

343

344

345

4. Dalakas MC, Hohlfeld R. Polymyositis and dermatomyositis. *Lancet Lond Engl* (2003) **362**:971–982. doi:10.1016/S0140-6736(03)14368-1

346

347

5. Tournadre A, Porcherot M, Chérin P, Marie I, Hachulla E, Miossec P. Th1 and Th17 balance in inflammatory myopathies: Interaction with dendritic cells and possible link with response to high-dose immunoglobulins. *Cytokine* (2009) **46**:297–301. doi:10.1016/j.cyto.2009.02.013

348

349

350

6. De Bleecker JL, Meire VI, Declercq W, Van Aken EH. Immunolocalization of tumor necrosis factor-alpha and its receptors in inflammatory myopathies. *Neuromuscul Disord* (1999) **9**:239–246. doi:10.1016/S0960-8966(98)00126-6

351

352

353

354

7. Kuru S, Inukai A, Liang Y, Doyu M, Takano A, Sobue G. Tumor necrosis factor-alpha expression in muscles of polymyositis and dermatomyositis. *Acta Neuropathol (Berl)* (2000) **99**:585–588.

355

356

357

8. Figarella-Branger D, Civatte M, Bartoli C, Pellissier J-F. Cytokines, chemokines, and cell adhesion molecules in inflammatory myopathies. *Muscle Nerve* (2003) **28**:659–682. doi:10.1002/mus.10462

358

359

360

9. Chevrel G, Page G, Granet C, Streichenberger N, Varennes A, Miossec P. Interleukin-17 increases the effects of IL-1 beta on muscle cells: arguments for the role of T cells in the pathogenesis of myositis. *J Neuroimmunol* (2003) **137**:125–133.

361

362

363

10. Page G, Chevrel G, Miossec P. Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: Interaction with chemokines and Th1 cytokine-producing cells. *Arthritis Rheum* (2004) **50**:199–208. doi:10.1002/art.11428

364

365

366

11. Szodoray P, Alex P, Knowlton N, Centola M, Dozmorov I, Csipo I, Nagy AT, Constantin T, Ponyi A, Nakken B, et al. Idiopathic inflammatory myopathies, signified by distinctive peripheral cytokines, chemokines and the TNF family members B-cell activating factor and a proliferation inducing ligand. *Rheumatol Oxf Engl* (2010) **49**:1867–1877. doi:10.1093/rheumatology/keq151

367

368

369

370

12. Higgs BW, Zhu W, Richman L, Fiorentino DF, Greenberg SA, Jallal B, Yao Y. Identification of activated cytokine pathways in the blood of systemic lupus erythematosus, myositis, rheumatoid arthritis, and scleroderma patients. *Int J Rheum Dis* (2012) **15**:25–35. doi:10.1111/j.1756-185X.2011.01654.x

371

372

373

374

13. Moran EM, Mastaglia FL. Cytokines in immune-mediated inflammatory myopathies: cellular sources, multiple actions and therapeutic implications. *Clin Exp Immunol* (2014) **178**:405–415. doi:10.1111/cei.12445

375

376

377

14. Chevrel G, Granet C, Miossec P. Contribution of tumour necrosis factor alpha and interleukin (IL) 1beta to IL6 production, NF-kappaB nuclear translocation, and class I MHC expression in muscle cells: in vitro regulation with specific cytokine inhibitors. *Ann Rheum Dis* (2005) **64**:1257–1262. doi:10.1136/ard.2004.032359

378

379

380

381

15. Tournadre A, Lenief V, Miossec P. Expression of Toll-like receptor 3 and Toll-like receptor 7 in muscle is characteristic of inflammatory myopathy and is differentially regulated by Th1 and Th17 cytokines. *Arthritis Rheum* (2010) **62**:2144–2151. doi:10.1002/art.27465

382

383

384

- 385 16. Bidaux G, Le Nézet C, Pisfil MG, Henry M, Furlan A, Bensaude O, Vandebunder B,  
386 Héliot L. FRET Image Correlation Spectroscopy Reveals RNAPII-Independent P-TEFb  
387 Recruitment on Chromatin. *Biophys J* (2018) **114**:522–533. doi:10.1016/j.bpj.2017.11.3783
- 388 17. Tournadre A, Miossec P. Interleukin-17 in inflammatory myopathies. *Curr Rheumatol*  
389 *Rep* (2012) **14**:252–256. doi:10.1007/s11926-012-0242-x
- 390 18. Afroze D, Kumar A. ER stress in skeletal muscle remodeling and myopathies. *FEBS J*  
391 (2017) doi:10.1111/febs.14358
- 392 19. Eljaafari A, Tartelin M-L, Aissaoui H, Chevrel G, Osta B, Lavocat F, Miossec P. Bone  
393 marrow-derived and synovium-derived mesenchymal cells promote Th17 cell expansion and  
394 activation through caspase 1 activation: Contribution to the chronicity of rheumatoid arthritis.  
395 *Arthritis Rheum* (2012) **64**:2147–2157. doi:10.1002/art.34391
- 396 20. Noack M, Ndongo-Thiam N, Miossec P. Interaction among activated lymphocytes and  
397 mesenchymal cells through podoplanin is critical for a high IL-17 secretion. *Arthritis Res*  
398 *Ther* (2016) **18**:148. doi:10.1186/s13075-016-1046-6
- 399 21. Millay DP, Goonasekera SA, Sargent MA, Maillet M, Aronow BJ, Molkenin JD.  
400 Calcium influx is sufficient to induce muscular dystrophy through a TRPC-dependent  
401 mechanism. *Proc Natl Acad Sci U S A* (2009) **106**:19023–19028.  
402 doi:10.1073/pnas.0906591106
- 403 22. Jia L, Delmotte P, Aravamudan B, Pabelick CM, Prakash YS, Sieck GC. Effects of the  
404 Inflammatory Cytokines TNF- $\alpha$  and IL-13 on Stromal Interaction Molecule-1 Aggregation in  
405 Human Airway Smooth Muscle Intracellular Ca<sup>2+</sup> Regulation. *Am J Respir Cell Mol Biol*  
406 (2013) **49**:601–608. doi:10.1165/rcmb.2013-0040OC
- 407 23. De Rossi M, Bernasconi P, Baggi F, de Waal Malefyt R, Mantegazza R. Cytokines  
408 and chemokines are both expressed by human myoblasts: possible relevance for the immune  
409 pathogenesis of muscle inflammation. *Int Immunol* (2000) **12**:1329–1335.
- 410 24. Tournadre A, Lenief V, Eljaafari A, Miossec P. Immature muscle precursors are a  
411 source of interferon- $\beta$  in myositis: role of Toll-like receptor 3 activation and contribution to  
412 HLA class I up-regulation. *Arthritis Rheum* (2012) **64**:533–541. doi:10.1002/art.33350
- 413 25. Bartoccioni E, Michaelis D, Hohlfeld R. Constitutive and cytokine-induced production  
414 of interleukin-6 by human myoblasts. *Immunol Lett* (1994) **42**:135–138.
- 415 26. Noack M, Ndongo-Thiam N 'd, Miossec P. Role of podoplanin in the high interleukin-  
416 17A secretion resulting from interactions between activated lymphocytes and psoriatic skin-  
417 derived mesenchymal cells. *Clin Exp Immunol* (2016) **186**:64–74. doi:10.1111/cei.12830
- 418 27. Waschbisch A, Meuth SG, Herrmann AM, Wrobel B, Schwab N, Lochmüller H,  
419 Wiendl H. Intercellular exchanges of membrane fragments (trogonocytosis) between human  
420 muscle cells and immune cells: A potential mechanism for the modulation of muscular  
421 immune responses. *J Neuroimmunol* (2009) **209**:131–138.  
422 doi:10.1016/j.jneuroim.2009.02.005
- 423 28. Nagaraju K, Casciola-Rosen L, Lundberg I, Rawat R, Cutting S, Thapliyal R, Chang J,  
424 Dwivedi S, Mitsak M, Chen Y-W, et al. Activation of the endoplasmic reticulum stress  
425 response in autoimmune myositis: potential role in muscle fiber damage and dysfunction.  
426 *Arthritis Rheum* (2005) **52**:1824–1835. doi:10.1002/art.21103
- 427 29. Vitadello M, Doria A, Tarricone E, Ghirardello A, Gorza L. Myofiber stress-response  
428 in myositis: parallel investigations on patients and experimental animal models of muscle  
429 regeneration and systemic inflammation. *Arthritis Res Ther* (2010) **12**:R52.  
430 doi:10.1186/ar2963
- 431 30. Rayavarapu S, Coley W, Nagaraju K. Endoplasmic reticulum stress in skeletal muscle  
432 homeostasis and disease. *Curr Rheumatol Rep* (2012) **14**:238–243. doi:10.1007/s11926-012-  
433 0247-5
- 434 31. Coley W, Rayavarapu S, Nagaraju K. Role of non-immune mechanisms of muscle

435 damage in idiopathic inflammatory myopathies. *Arthritis Res Ther* (2012) **14**:209.  
436 doi:10.1186/ar3791

437 32. Amici DR, Pinal-Fernandez I, Mázala DAG, Lloyd TE, Corse AM, Christopher-Stine  
438 L, Mammen AL, Chin ER. Calcium dysregulation, functional calpainopathy, and endoplasmic  
439 reticulum stress in sporadic inclusion body myositis. *Acta Neuropathol Commun* (2017) **5**:24.  
440 doi:10.1186/s40478-017-0427-7

441 33. Stiber J, Hawkins A, Zhang Z-S, Wang S, Burch J, Graham V, Ward CC, Seth M,  
442 Finch E, Malouf N, et al. STIM1 signalling controls store-operated calcium entry required for  
443 development and contractile function in skeletal muscle. *Nat Cell Biol* (2008) **10**:688–697.  
444 doi:10.1038/ncb1731

445 34. Darbellay B, Arnaudeau S, König S, Jousset H, Bader C, Demaurex N, Bernheim L.  
446 STIM1- and Orai1-dependent store-operated calcium entry regulates human myoblast  
447 differentiation. *J Biol Chem* (2009) **284**:5370–5380. doi:10.1074/jbc.M806726200

448 35. Sathish V, Abcejo AJ, Thompson MA, Sieck GC, Prakash YS, Pabelick CM.  
449 Caveolin-1 regulation of store-operated Ca<sup>2+</sup> influx in human airway smooth muscle. *Eur*  
450 *Respir J* (2012) **40**:470–478. doi:10.1183/09031936.00090511

451 36. Jairaman A, Maguire CH, Schleimer RP, Prakriya M. Allergens stimulate store-  
452 operated calcium entry and cytokine production in airway epithelial cells. *Sci Rep* (2016)  
453 **6**:32311. doi:10.1038/srep32311

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In review

456 **Figure legends:**

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**Figure 1: Synergistic effect of IL-17 and TNF $\alpha$  on the CCL20 and IL-6 production by myoblasts.** Myoblasts were treated with IL-17 (50 ng/mL) and/or TNF $\alpha$  (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  $\pm$  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 TNF $\alpha$  increase ER stress and mitochondrial ROS in myoblasts.** Myoblasts were treated with IL-17 (50 ng/mL) and/or TNF $\alpha$  (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 70 $\mu$ m (C, D). Data are the mean of 4 to 7 independent experiments  $\pm$  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  $\mu$ g/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-TNF $\alpha$  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  $\pm$  SEM; \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. control co-culture condition.

**Figure 4: IL-17 and TNF $\alpha$  increase store-operated calcium entry.** Myoblasts were treated with IL-17 (50 ng/mL) and/or TNF $\alpha$  (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 TNF $\alpha$  (1 ng/mL) for 24 hours. Image Correlation Spectroscopy (ICS) analysis of STIM1 puncta (left inset) mean density of puncta ( $\mu$ m). (right inset) mean surface of puncta (puncta/ $\mu$ m). Data are the mean of 3 independent experiments with cells from 3 different donors  $\pm$  SEM; \*  $p < 0.05$  vs. control untreated condition, scale bar 3 $\mu$ m (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/TNF $\alpha$  treated myoblasts. (I). Slope analysis of the SOCE (J). Data are the mean of 4-5 independent experiments  $\pm$  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/TNF $\alpha$  combination.** Myoblasts were stimulated with IL-17 and/or TNF $\alpha$  in presence or not of the SOCE inhibitor 2-APB (10; 25 and 50  $\mu$ M) or BTP2 (10 and 20  $\mu$ M) for 48 hrs. CCL20 and IL-6 secretion by myoblasts was quantified by ELISA (A, B). Data are the mean of 7 independent experiments  $\pm$  SEM; \*\*  $p < 0.01$  vs. control untreated condition and #  $p < 0.05$ , ##  $p < 0.01$  vs. other inflammatory conditions

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

506 source of cytokines and promotes the cell-cell interactions. IL-17 mainly produced by Th17 cells,  
507 and TNF $\alpha$  act in synergy on myoblasts to increase IL-6 and CCL20 secretion. Because IL-6 is  
508 involved in the Th17 cell differentiation and CCL20 in dendritic and Th17 cell recruitment, IL-6  
509 and CCL20 mediate a positive feedback loop promoting local IL-17 production. IL-17 and TNF $\alpha$   
510 induce also non-immune pathways with ROS production, ER stress and SOCE activation. The IL-  
511 17/TNF $\alpha$  effect of mitochondrial dysfunction, ER stress and SOCE activation are probably closely  
512 linked. SOCE and calcium dysregulation contribute to IL-6 release induced by IL-17/TNF $\alpha$ .  
513 ER: endoplasmic reticulum; ROS: reactive oxygen species; chemokine (C-C motif) ligand 20:  
514 CCL20; DC: dendritic cells

In review

In review

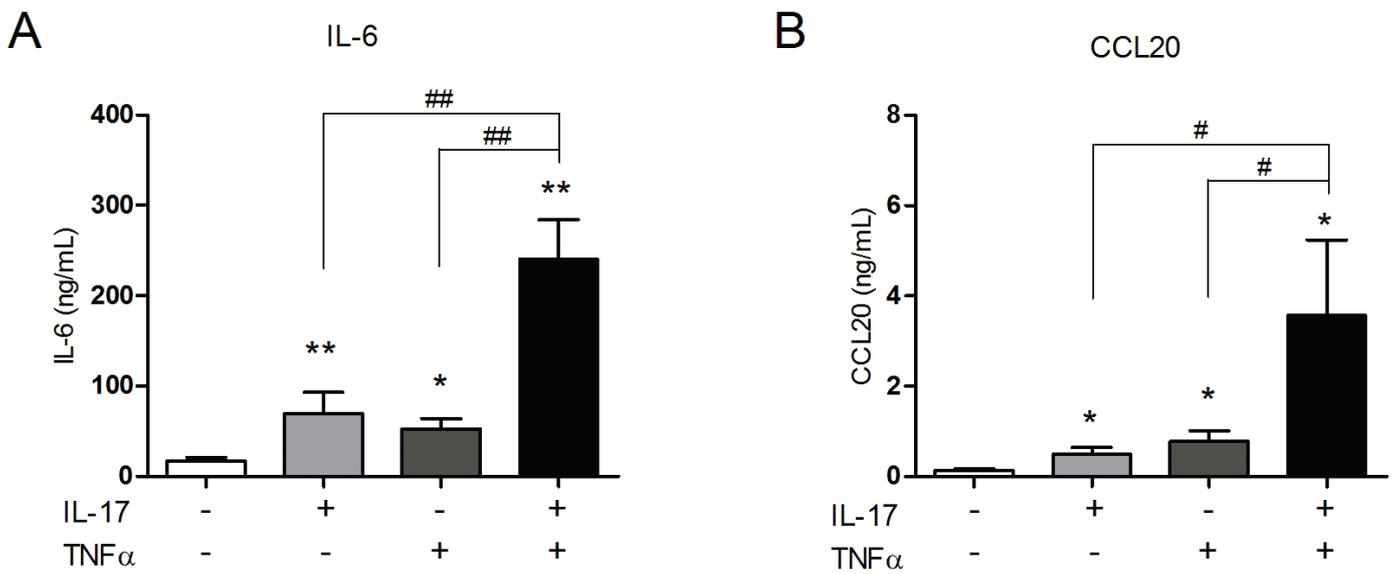


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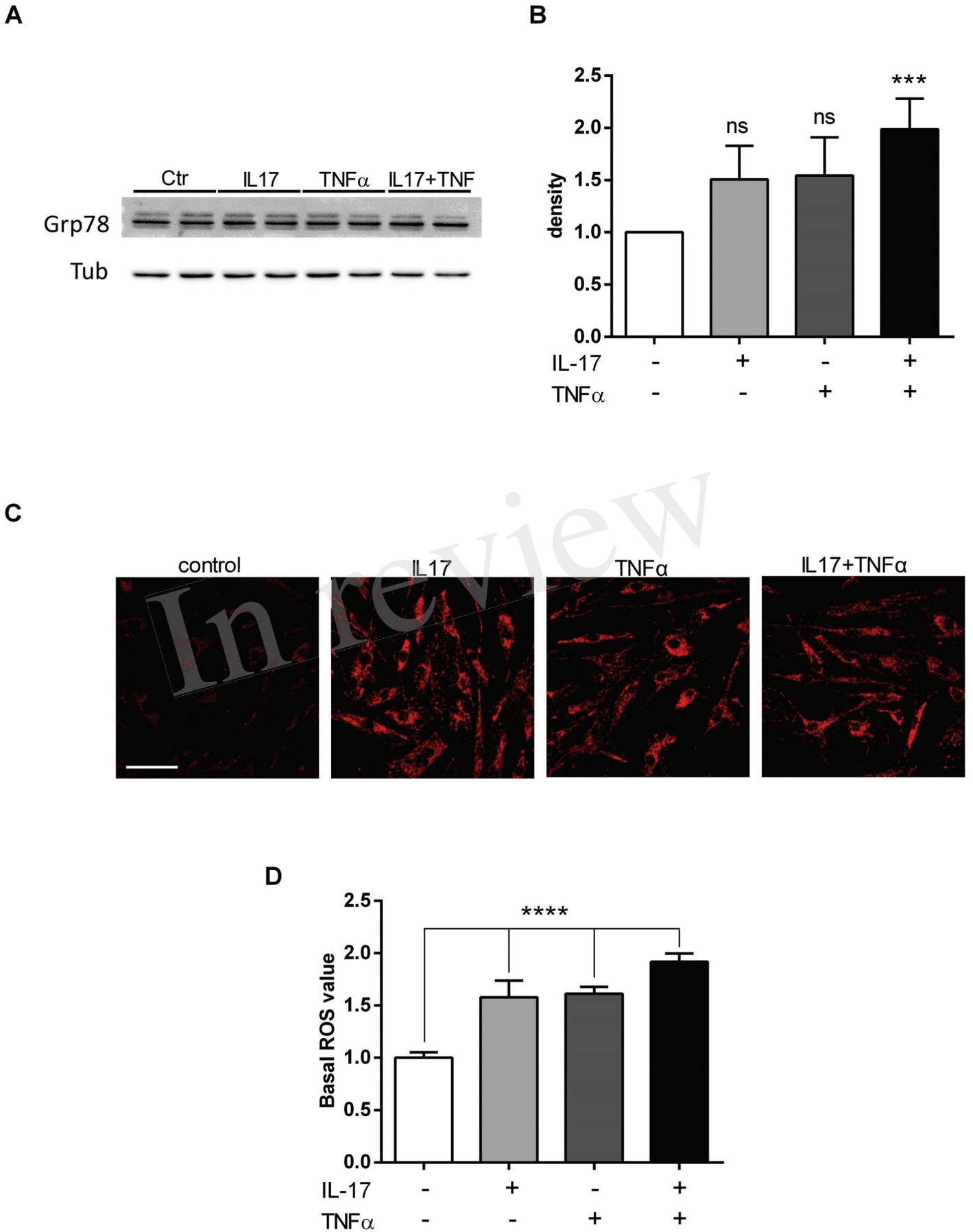


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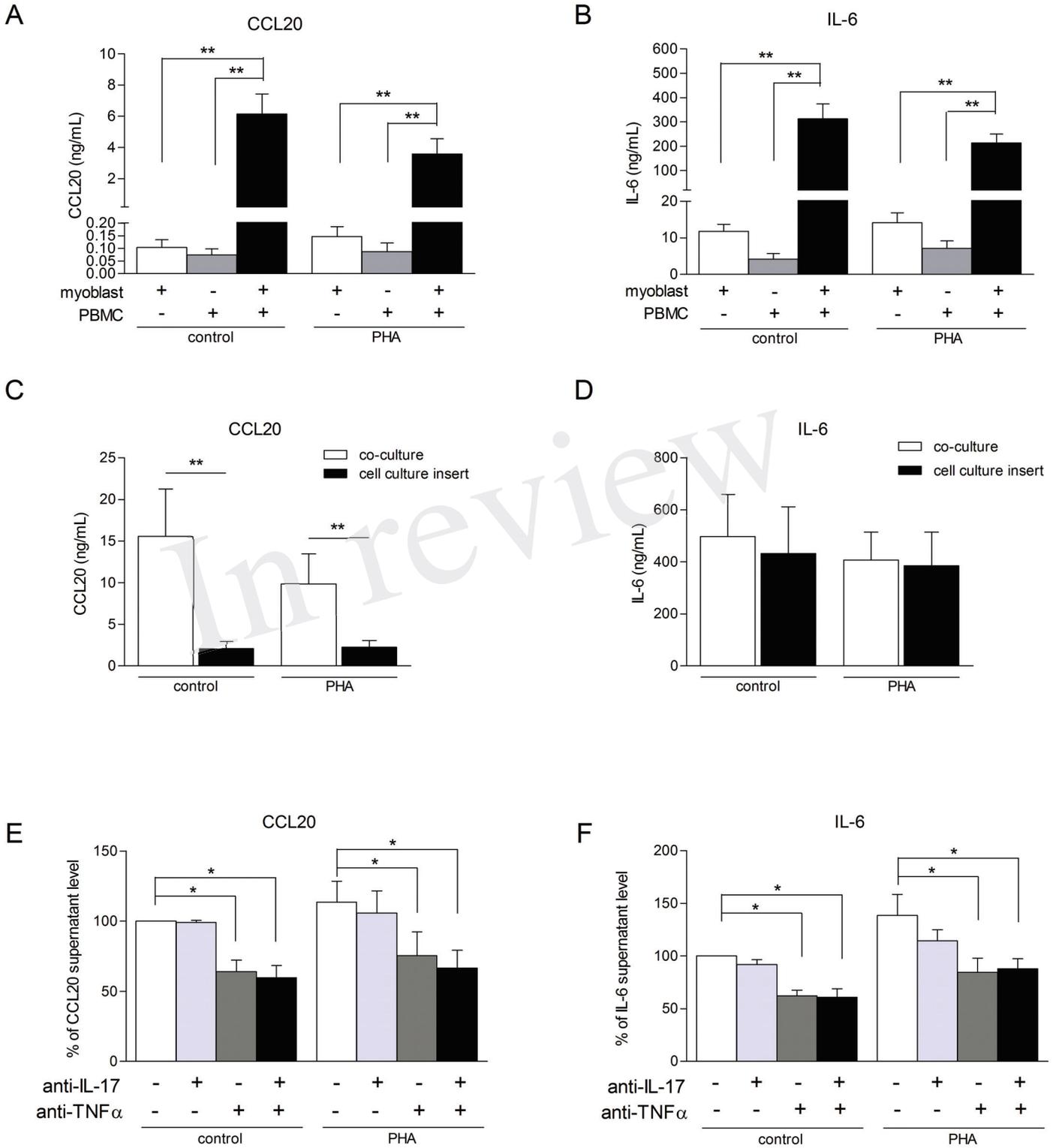
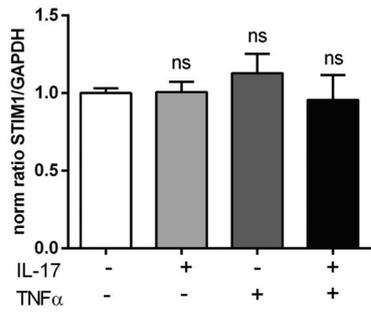
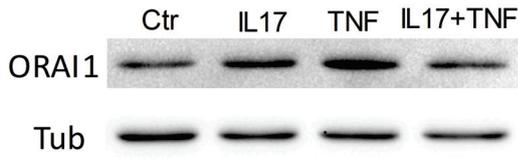


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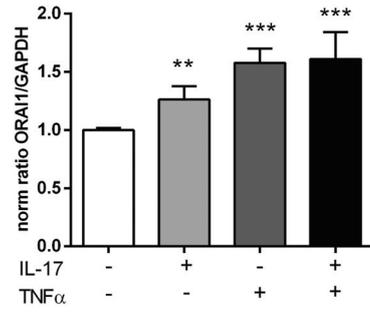
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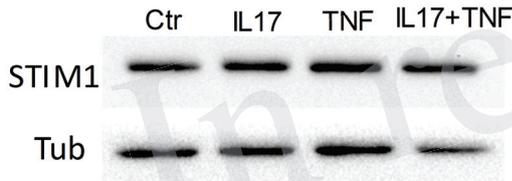
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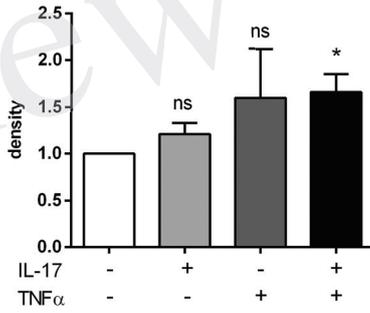
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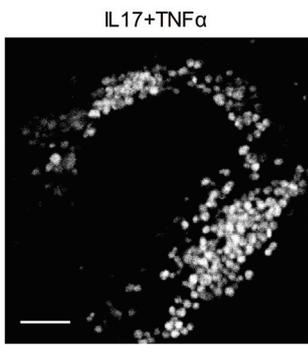
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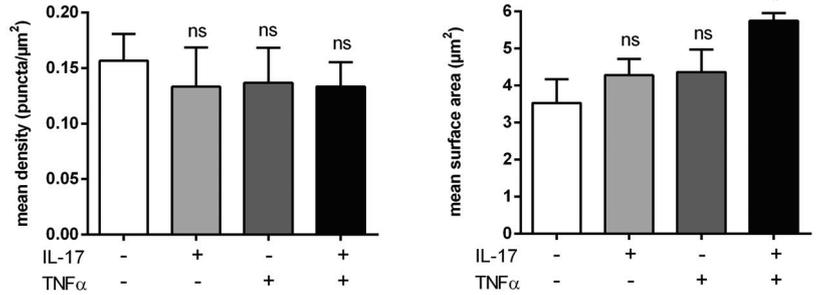
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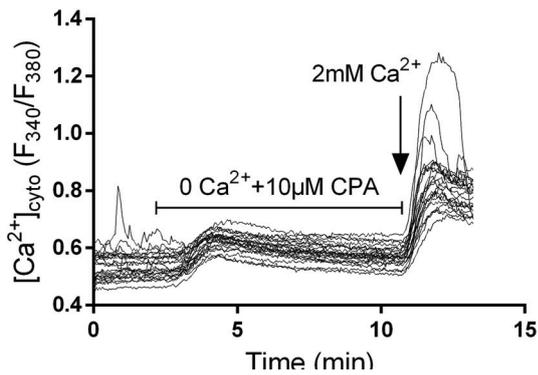
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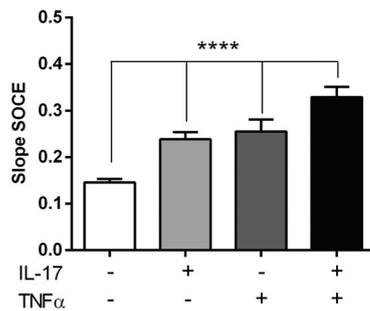


Figure 5.TIF

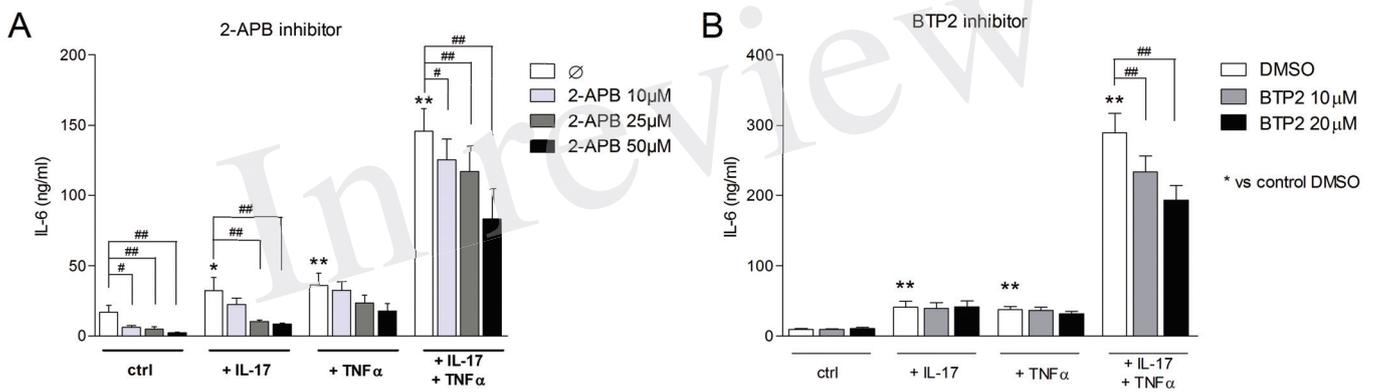
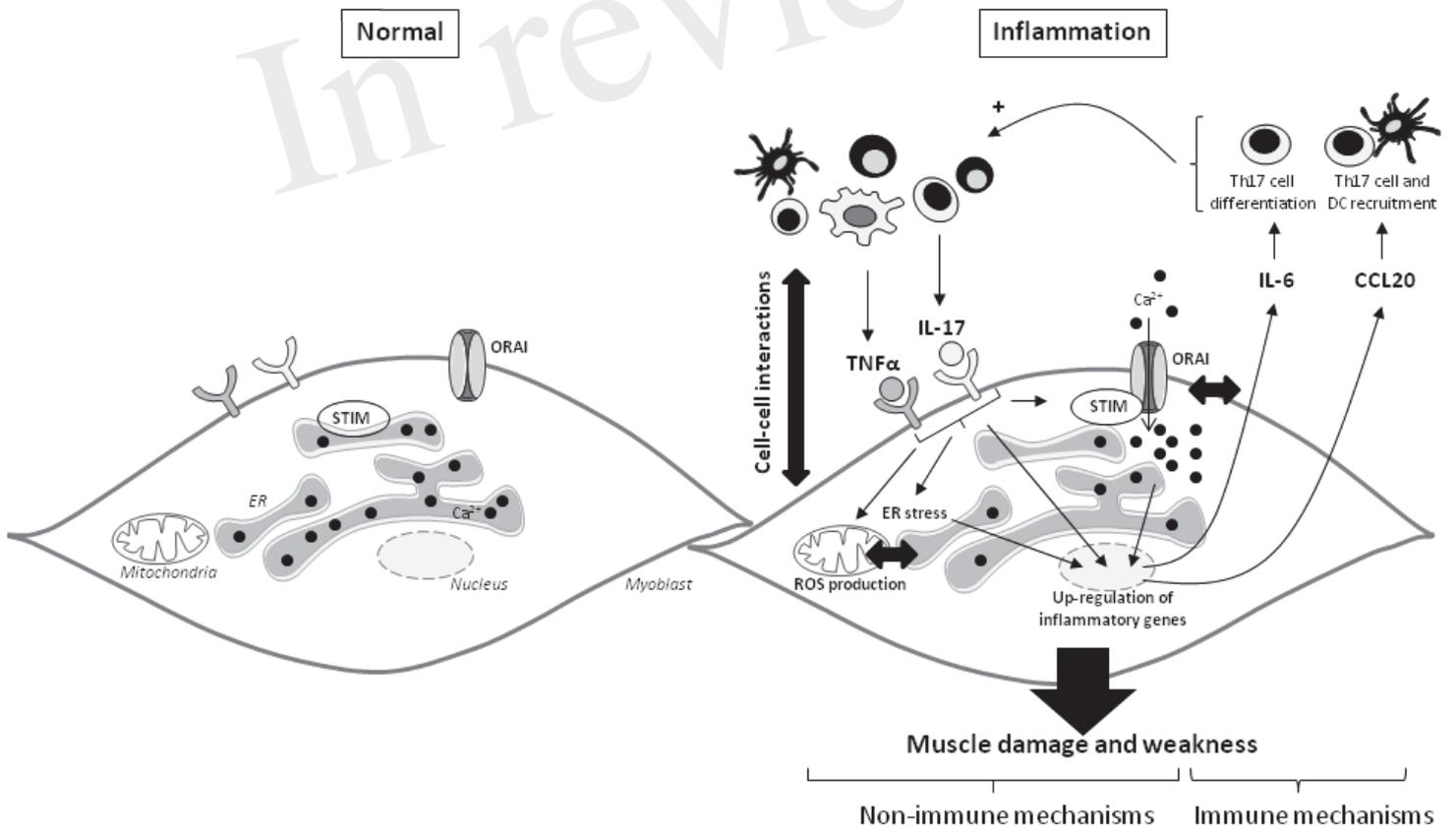


Figure 6.TIF





# DISCUSSION

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This study highlights the cooperative effects of IL-17 and TNF $\alpha$  on the induction of the inflammatory response in hepatocytes, HSCs and myoblasts by using human cell cultures. In addition, the IL-17 and TNF $\alpha$  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 TNF $\alpha$ . Moreover, IL-17 and TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$ . The IL-17 and TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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, TNF $\alpha$ , IL-6 as well as TGF $\beta$  (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 TNF $\alpha$ cooperation enhances the hepatic and muscle inflammatory response**

### **2.1 IL-17 and TNF $\alpha$ 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 TNF $\alpha$ , IL-1 $\beta$  or IFN $\gamma$ , 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 TNF $\alpha$  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 Bleeker et al., 1999; Kuru et al., 2000; Schwabe and Brenner, 2006; Tournadre and Miossec, 2012). In this study, IL-17 and TNF $\alpha$  cooperate to promote systemic inflammation by increasing IL-6 secretion, which in turn, enhances the CRP expression in hepatocytes. IL-17 amplifies also the TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  induce a further decrease of the immune cell recruitment and the release of pro-inflammatory mediators. In myoblast cultures, IL-17 and TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$ synergistic effects***

Although the IL-17 and TNF $\alpha$  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 TNF $\alpha$ , is crucial for the initiation of the IL-17 and TNF $\alpha$  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 TNF $\alpha$  synergistic cooperation on the CCL20 production (Zrioual et al., 2009). In HepaRG cells, IL-17 and TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  although the TNFR2 is preferentially activated by the transmembrane form of the TNF $\alpha$  (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 TNF $\alpha$  cooperation.

Part of the IL-17 and TNF $\alpha$  cooperation may also occur at a gene transcription level. Transcription factor-binding sites for NF- $\kappa$ B and C/EBP (CCAAT/enhancer-binding protein) in the IL-6 promoter were both involved in the IL-17 and TNF $\alpha$  synergistic effect on IL-6 in an osteoblastic cell line (Ruddy et al., 2004). The up-regulation of C/EBP $\delta$  by the IL-17 and TNF $\alpha$  stimulation and the increase of C/EBP $\delta$  recruitment to the promoter by TNF $\alpha$  may participate to the IL-17 and TNF $\alpha$  cooperative effect on the IL-6 induction (Gaffen et al., 2014; Ruddy et al., 2004; Zimmermann et al., 2015). In addition, by enhancing I $\kappa$ B $\zeta$  mRNA expression in synergy, IL-17 and TNF $\alpha$  may induce a further increase of NF- $\kappa$ B activation since I $\kappa$ B $\zeta$  acts as a NF- $\kappa$ B 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 TNF $\alpha$  stimulation such as IL-6 and IL-8 mRNA (Hartupee et al., 2007; Hennes 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 TNF $\alpha$  synergistic effect on the IL-6 production.

Multiple mechanisms at different levels appear therefore involved in the IL-17 and TNF $\alpha$  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 TNF $\alpha$ . Indeed, NF- $\kappa$ B pathways participate in the secretion of IL-6 induced by the IL-17 and TNF $\alpha$  cooperation but not in the IL-8 secretion in HepaRG cells. In this way, the IL-17 and TNF $\alpha$  synergistic effect on the I $\kappa$ B $\zeta$  up-regulation, which intensifies NF- $\kappa$ B activation, cannot be involved in the IL-17/TNF $\alpha$ -induced IL-8 production in HepaRG cells.

### **2.3 Potential contribution of the IL-17 and TNF $\alpha$ synergistic cooperation in systemic inflammatory diseases**

This study shows that the effects of TNF $\alpha$  at low concentration can be potentiated by IL-17 in the liver and the muscles. These results strengthen the potential important systemic role of IL-17 and TNF $\alpha$  in autoimmune and inflammatory diseases. However, the concentrations of IL-17 and TNF $\alpha$  used in our *in vitro* systems may not 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 TNF $\alpha$  can interact with other cytokines to mediate additive, antagonistic or synergistic effects. For example, IL-17 can synergize with other cytokines such as IL-1 $\beta$ , IFN $\gamma$ , IL-22 or GM-CSF, in addition to TNF $\alpha$ , 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 TNF $\alpha$ inflammatory response may impair the liver and muscle function**

#### **3.1 IL-17 and TNF $\alpha$ induce cell damage in liver**

Since liver inflammation may induce liver damage, the IL-17 and TNF $\alpha$  association could also mediate non-immune adverse effects in the liver. Here, the IL-17 and TNF $\alpha$  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 TNF $\alpha$  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, TNF $\alpha$  was also associated with liver injury (Park et al., 2012; Yang and Seki, 2015).

By acting on HSCs, IL-17 and TNF $\alpha$  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 TNF $\alpha$  in liver fibrosis is still controversial (Qin et al., 2016; Tan et al., 2013; Yang and Seki, 2015). However, IL-17 and TNF $\alpha$  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 TGF $\beta$  by the activated Kupffer cells (Hara et al., 2013). In addition, IL-17 and TNF $\alpha$  increase slightly the TGF $\beta$  mRNA expression in LX-2 cells. Therefore, IL-17 and TNF $\alpha$  could have indirect pro-fibrotic effects via the induction of TGF $\beta$ , which can be amplified by the IL-17 and TGF $\beta$  synergistic cooperation (Fabre et al., 2014). In this way, it could be interesting to study the role of IL-17, TNF $\alpha$  and TGF $\beta$  cytokine interplays in the fibrosis process at several time points of the cultures. Indeed, it has been suggested that the matrix breakdown mediated by

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

### **3.2 IL-17 and TNF $\alpha$ 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 TNF $\alpha$  stimulation increase the ER stress and the mitochondrial ROS production contributing to cell damage. In addition, the IL-17 and TNF $\alpha$  exposure enhances SOCE in myoblasts. This effect on SOCE could impair Ca<sup>2+</sup> homeostasis that is crucial in the regulation of muscle contraction. Interestingly, the inhibition of SOCE reduces the IL-6 release by IL-17 and TNF $\alpha$ . 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  $\gamma\delta$  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 TNF $\alpha$ contribute to the induction of IL-6 and IL-8 secretion by the cell interactions**

Because IL-17 and TNF $\alpha$  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 TNF $\alpha$  reduces the IL-6 and IL-8 secretion but not the CCL20 production induced by the interactions between PBMCs, hepatocytes and/or HSCs. TNF $\alpha$  also contributes to the enhanced IL-6 and CCL20 release in PBMC-myoblast co-cultures. However, the blockade of both IL-17 and TNF $\alpha$  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 TNF $\alpha$  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<sup>+</sup> CD4<sup>+</sup> IL-17<sup>+</sup> cell frequency in PHA-activated PBMCs. Surprisingly, 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

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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 TNF $\alpha$ , 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 TNF $\alpha$  can promote immune cell recruitment and the production of systemic inflammatory mediators. In addition, the IL-17 and TNF $\alpha$  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 TNF $\alpha$  synthesis leading to a pro-inflammatory feedback loop. Neutralization of IL-17 and/or TNF $\alpha$  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 TNF $\alpha$  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.



## REFERENCES

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- Allenbach, Y., Chaara, W., Rosenzweig, M., Six, A., Prevel, N., Mingozi, F., Wanschitz, J., Musset, L., Charuel, J.-L., Eymard, B., et al. (2014). Th1 Response and Systemic Treg Deficiency in Inclusion Body Myositis. *PLoS ONE* 9.
- Amara, S., Lopez, K., Banan, B., Brown, S.-K., Whalen, M., Myles, E., Ivy, M.T., Johnson, T., Schey, K.L., and Tiriveedhi, V. (2015). Synergistic effect of pro-inflammatory TNF $\alpha$  and IL-17 in periostin mediated collagen deposition: potential role in liver fibrosis. *Mol. Immunol.* 64, 26–35.
- Bale, S.S., Geerts, S., Jindal, R., and Yarmush, M.L. (2016). Isolation and co-culture of rat parenchymal and non-parenchymal liver cells to evaluate cellular interactions and response. *Sci. Rep.* 6, 25329.
- Barbero-Becerra, V.J., Giraudi, P.J., Chávez-Tapia, N.C., Uribe, M., Tiribelli, C., and Rosso, N. (2015). The interplay between hepatic stellate cells and hepatocytes in an in vitro model of NASH. *Toxicol. Vitro Int. J. Publ. Assoc. BIBRA* 29, 1753–1758.
- Beringer, A., and Miossec, P. (2018). IL-17 and IL-17-producing cells and liver diseases, with focus on autoimmune liver diseases. *Autoimmun. Rev.* 17, 1176–1185.
- Beringer, A., Noack, M., and Miossec, P. (2016). IL-17 in Chronic Inflammation: From Discovery to Targeting. *Trends Mol. Med.* 22, 230–241.
- Beringer, A., Thiam, N., Molle, J., Bartosch, B., and Miossec, P. (2018). Synergistic effect of interleukin-17 and tumour necrosis factor- $\alpha$  on inflammatory response in hepatocytes through interleukin-6-dependent and independent pathways. *Clin. Exp. Immunol.* 193, 221–233.
- Bertolino, P., Trescol-Biémont, M.C., and Rabourdin-Combe, C. (1998). Hepatocytes induce functional activation of naive CD8<sup>+</sup> T lymphocytes but fail to promote survival. *Eur. J. Immunol.* 28, 221–236.
- Bode, J.G., Albrecht, U., Häussinger, D., Heinrich, P.C., and Schaper, F. (2012). Hepatic acute phase proteins--regulation by IL-6- and IL-1-type cytokines involving STAT3 and its crosstalk with NF- $\kappa$ B-dependent signaling. *Eur. J. Cell Biol.* 91, 496–505.
- Bordoni, V., Alonzi, T., Agrati, C., Poccia, F., Borsellino, G., Mancino, G., Fimia, G.M., Piacentini, M., Fantoni, A., and Tripodi, M. (2004). Murine hepatocyte cell lines promote expansion and differentiation of NK cells from stem cell precursors. *Hepatology* 39, 1508–1516.
- Böttcher, K., Rombouts, K., Saffioti, F., Roccarina, D., Rosselli, M., Hall, A., Luong, T., Tsochatzis, E.A., Thorburn, D., and Pinzani, M. (2018). MAIT cells are chronically activated in patients with autoimmune liver disease and promote profibrogenic hepatic stellate cell activation. *Hepatology* 68, 172–186.
- Bradley, J.R. (2008). TNF-mediated inflammatory disease. *J. Pathol.* 214, 149–160.

- Brunn, A., Zornbach, K., Hans, V.H., Haupt, W.F., and Deckert, M. (2012). Toll-like receptors promote inflammation in idiopathic inflammatory myopathies. *J. Neuropathol. Exp. Neurol.* *71*, 855–867.
- Burghardt, S., Erhardt, A., Claass, B., Huber, S., Adler, G., Jacobs, T., Chalaris, A., Schmidt-Arras, D., Rose-John, S., Karimi, K., et al. (2013). Hepatocytes contribute to immune regulation in the liver by activation of the Notch signaling pathway in T cells. *J. Immunol. Baltim. Md 1950* *191*, 5574–5582.
- Burghardt, S., Claass, B., Erhardt, A., Karimi, K., and Tiegs, G. (2014). Hepatocytes induce Foxp3<sup>+</sup> regulatory T cells by Notch signaling. *J. Leukoc. Biol.* *96*, 571–577.
- Calabrese, L.H., and Rose-John, S. (2014). IL-6 biology: implications for clinical targeting in rheumatic disease. *Nat. Rev. Rheumatol.* *10*, 720–727.
- Calne, R.Y., Sells, R.A., Pena, J.R., Davis, D.R., Millard, P.R., Herbertson, B.M., Binns, R.M., and Davies, D.A. (1969). Induction of immunological tolerance by porcine liver allografts. *Nature* *223*, 472–476.
- Casciola-Rosen, L., Nagaraju, K., Plotz, P., Wang, K., Levine, S., Gabrielson, E., Corse, A., and Rosen, A. (2005). Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy. *J. Exp. Med.* *201*, 591–601.
- Chabaud, M., Fossiez, F., Taupin, J.L., and Miossec, P. (1998). Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J. Immunol. Baltim. Md 1950* *161*, 409–414.
- Charles, R., Chou, H.-S., Wang, L., Fung, J.J., Lu, L., and Qian, S. (2013). Human hepatic stellate cells inhibit T-cell response through B7-H1 pathway. *Transplantation* *96*, 17–24.
- Chevrel, G., Page, G., Granet, C., Streichenberger, N., Varennes, A., and Miossec, P. (2003). Interleukin-17 increases the effects of IL-1 beta on muscle cells: arguments for the role of T cells in the pathogenesis of myositis. *J. Neuroimmunol.* *137*, 125–133.
- Chevrel, G., Granet, C., and Miossec, P. (2005). Contribution of tumour necrosis factor alpha and interleukin (IL) 1beta to IL6 production, NF-kappaB nuclear translocation, and class I MHC expression in muscle cells: in vitro regulation with specific cytokine inhibitors. *Ann. Rheum. Dis.* *64*, 1257–1262.
- Chiricozzi, A., Guttman-Yassky, E., Suárez-Fariñas, M., Nograles, K.E., Tian, S., Cardinale, I., Chimenti, S., and Krueger, J.G. (2011). Integrative responses to IL-17 and TNF- $\alpha$  in human keratinocytes account for key inflammatory pathogenic circuits in psoriasis. *J. Invest. Dermatol.* *131*, 677–687.
- Chowdhury, S., Dijkhuis, A., Steiert, S., and Lutter, R. (2013). IL-17 attenuates degradation of ARE-mRNAs by changing the cooperation between AU-binding proteins and microRNA16. *PLoS Genet.* *9*, e1003747.
- Chu, P.-S., Nakamoto, N., Ebinuma, H., Usui, S., Saeki, K., Matsumoto, A., Mikami, Y., Sugiyama, K., Tomita, K., Kanai, T., et al. (2013). C-C motif chemokine receptor 9 positive

macrophages activate hepatic stellate cells and promote liver fibrosis in mice. *Hepatology*. Baltimore, Md 58, 337–350.

Coley, W., Rayavarapu, S., and Nagaraju, K. (2012). Role of non-immune mechanisms of muscle damage in idiopathic inflammatory myopathies. *Arthritis Res. Ther.* 14, 209.

Comerford, I., Bunting, M., Fenix, K., Haylock-Jacobs, S., Litchfield, W., Harata-Lee, Y., Turvey, M., Brazzatti, J., Gregor, C., Nguyen, P., et al. (2010). An immune paradox: how can the same chemokine axis regulate both immune tolerance and activation?: CCR6/CCL20: a chemokine axis balancing immunological tolerance and inflammation in autoimmune disease. *BioEssays News Rev. Mol. Cell. Dev. Biol.* 32, 1067–1076.

Correia, M.P., Cardoso, E.M., Pereira, C.F., Neves, R., Uhrberg, M., and Arosa, F.A. (2009). Hepatocytes and IL-15: a favorable microenvironment for T cell survival and CD8+ T cell differentiation. *J. Immunol. Baltimore, Md* 182, 6149–6159.

Coulouarn, C., Corlu, A., Glaise, D., Guenon, I., Thorgeirsson, S.S., and Clement, B. (2012). Hepatocyte - Stellate Cell Crosstalk in the Liver Engenders a Permissive Inflammatory Microenvironment that Drives Progression in Hepatocellular Carcinoma. *Cancer Res.* 72, 2533–2542.

Coutant, F., and Miossec, P. (2016). Altered dendritic cell functions in autoimmune diseases: distinct and overlapping profiles. *Nat. Rev. Rheumatol.* 12, 703–715.

Crispe, I.N. (2009). The liver as a lymphoid organ. *Annu. Rev. Immunol.* 27, 147–163.

Dalakas, M.C., and Hohlfield, R. (2003). Polymyositis and dermatomyositis. *Lancet Lond. Engl.* 362, 971–982.

Dangi, A., Sumpter, T.L., Kimura, S., Stolz, D.B., Murase, N., Raimondi, G., Vodovotz, Y., Huang, C., Thomson, A.W., and Gandhi, C.R. (2012). Selective expansion of allogeneic regulatory T cells by hepatic stellate cells: role of endotoxin and implications for allograft tolerance. *J. Immunol. Baltimore, Md* 188, 3667–3677.

De Bleecker, J.L., Meire, V.I., Declercq, W., and Van Aken, E.H. (1999). Immunolocalization of tumor necrosis factor-alpha and its receptors in inflammatory myopathies. *Neuromuscul. Disord.* 9, 239–246.

Doumba, P.P., Nikolopoulou, M., Gomatos, I.P., Konstadoulakis, M.M., and Koskinas, J. (2013). Co-culture of primary human tumor hepatocytes from patients with hepatocellular carcinoma with autologous peripheral blood mononuclear cells: study of their in vitro immunological interactions. *BMC Gastroenterol.* 13, 17.

Edwards, J.N., Friedrich, O., Cully, T.R., von Wegner, F., Murphy, R.M., and Launikonis, B.S. (2010). Upregulation of store-operated Ca<sup>2+</sup> entry in dystrophic mdx mouse muscle. *Am. J. Physiol.-Cell Physiol.* 299, C42–C50.

Fabre, T., Kared, H., Friedman, S.L., and Shoukry, N.H. (2014). IL-17A enhances the expression of profibrotic genes through upregulation of the TGF- $\beta$  receptor on hepatic stellate cells in a JNK-dependent manner. *J. Immunol. Baltimore, Md* 193, 3925–3933.

- Ferreira, R.C., Freitag, D.F., Cutler, A.J., Howson, J.M.M., Rainbow, D.B., Smyth, D.J., Kaptoge, S., Clarke, P., Boreham, C., Coulson, R.M., et al. (2013). Functional IL6R 358Ala allele impairs classical IL-6 receptor signaling and influences risk of diverse inflammatory diseases. *PLoS Genet.* 9, e1003444.
- Franco, A., Barnaba, V., Natali, P., Balsano, C., Musca, A., and Balsano, F. (1988). Expression of class I and class II major histocompatibility complex antigens on human hepatocytes. *Hepatology*. Baltimore, Md 8, 449–454.
- Furuya, S., Kono, H., Hara, M., Hirayama, K., Sun, C., and Fujii, H. (2015). Interleukin 17A plays a role in lipopolysaccharide/D-galactosamine-induced fulminant hepatic injury in mice. *J. Surg. Res.* 199, 487–493.
- Gabay, C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Res. Ther.* 8, S3.
- Gabay, C., and Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med.* 340, 448–454.
- Gaffen, S.L., Jain, R., Garg, A.V., and Cua, D.J. (2014). The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nat. Rev. Immunol.* 14, 585–600.
- Genovese, M.C., Kremer, J.M., van Vollenhoven, R.F., Alten, R., Scali, J.J., Kelman, A., Dimonaco, S., and Brockwell, L. (2017). Transaminase Levels and Hepatic Events During Tocilizumab Treatment: Pooled Analysis of Long-Term Clinical Trial Safety Data in Rheumatoid Arthritis. *Arthritis Rheumatol.* Hoboken NJ 69, 1751–1761.
- Hara, M., Kono, H., Furuya, S., Hirayama, K., Tsuchiya, M., and Fujii, H. (2013). Interleukin-17A plays a pivotal role in cholestatic liver fibrosis in mice. *J. Surg. Res.* 183, 574–582.
- Hartupée, J., Liu, C., Novotny, M., Li, X., and Hamilton, T. (2007). IL-17 enhances chemokine gene expression through mRNA stabilization. *J. Immunol.* Baltimore, Md 1950 179, 4135–4141.
- Hassan, W., Ding, L., Gao, R.-Y., Liu, J., and Shang, J. (2014). Interleukin-6 signal transduction and its role in hepatic lipid metabolic disorders. *Cytokine* 66, 133–142.
- He, J., Lang, G., Ding, S., and Li, L. (2013). Pathological role of interleukin-17 in poly I:C-induced hepatitis. *PLoS One* 8, e73909.
- Heinrich, P.C., Castell, J.V., and Andus, T. (1990). Interleukin-6 and the acute phase response. *Biochem. J.* 265, 621–636.
- Hennes, S., Johnson, C.K., Ge, Q., Armour, C.L., Hughes, J.M., and Ammit, A.J. (2004). IL-17A augments TNF-alpha-induced IL-6 expression in airway smooth muscle by enhancing mRNA stability. *J. Allergy Clin. Immunol.* 114, 958–964.
- Hennes, S., van Thoor, E., Ge, Q., Armour, C.L., Hughes, J.M., and Ammit, A.J. (2006). IL-17A acts via p38 MAPK to increase stability of TNF-alpha-induced IL-8 mRNA in human ASM. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 290, L1283–1290.

- Henriques-Pons, A., and Nagaraju, K. (2009). Nonimmune mechanisms of muscle damage in myositis: role of the endoplasmic reticulum stress response and autophagy in the disease pathogenesis. *Curr. Opin. Rheumatol.* *21*, 581–587.
- Herkel, J., Jagemann, B., Wiegand, C., Lazaro, J.F.G., Lueth, S., Kanzler, S., Blessing, M., Schmitt, E., and Lohse, A.W. (2003). MHC class II-expressing hepatocytes function as antigen-presenting cells and activate specific CD4 T lymphocytes. *Hepatology*. *37*, 1079–1085.
- Higgs, B.W., Zhu, W., Richman, L., Fiorentino, D.F., Greenberg, S.A., Jallal, B., and Yao, Y. (2012). Identification of activated cytokine pathways in the blood of systemic lupus erythematosus, myositis, rheumatoid arthritis, and scleroderma patients. *Int. J. Rheum. Dis.* *15*, 25–35.
- Höchst, B., Schildberg, F.A., Sauerborn, P., Gäbel, Y.A., Gevensleben, H., Goltz, D., Heukamp, L.C., Türler, A., Ballmaier, M., Gieseke, F., et al. (2013). Activated human hepatic stellate cells induce myeloid derived suppressor cells from peripheral blood monocytes in a CD44-dependent fashion. *J. Hepatology*. *59*, 528–535.
- Holt, A.P., Salmon, M., Buckley, C.D., and Adams, D.H. (2008). Immune Interactions in Hepatic Fibrosis. *Clin. Liver Dis.* *12*, 861–882.
- Honda, T., and Inagawa, H. (2016). Gene Expression in Lipopolysaccharide-treated Human Monocytes Following Interaction with Hepatic Cancer Cells. *Anticancer Res.* *36*, 3699–3704.
- Hot, A., Lenief, V., and Miossec, P. (2012). Combination of IL-17 and TNF $\alpha$  induces a pro-inflammatory, pro-coagulant and pro-thrombotic phenotype in human endothelial cells. *Ann. Rheum. Dis.* *71*, 768–776.
- Ji, J., Eggert, T., Budhu, A., Forgues, M., Takai, A., Dang, H., Ye, Q., Lee, J.-S., Kim, J.H., Greten, T.F., et al. (2015). Hepatic Stellate Cell and Monocyte Interaction Contributes To Poor Prognosis in Hepatocellular Carcinoma. *Hepatology*. *62*, 481–495.
- Kallioulas, G.D., and Ivashkiv, L.B. (2016). TNF biology, pathogenic mechanisms and emerging therapeutic strategies. *Nat. Rev. Rheumatol.* *12*, 49–62.
- Kamada, N., Davies, H.S., and Roser, B. (1981). Reversal of transplantation immunity by liver grafting. *Nature* *292*, 840–842.
- Karlsen, J.R., Borregaard, N., and Cowland, J.B. (2010). Induction of neutrophil gelatinase-associated lipocalin expression by co-stimulation with interleukin-17 and tumor necrosis factor-alpha is controlled by IkappaB-zeta but neither by C/EBP-beta nor C/EBP-delta. *J. Biol. Chem.* *285*, 14088–14100.
- Katz, Y., Nadiv, O., and Beer, Y. (2001). Interleukin-17 enhances tumor necrosis factor alpha-induced synthesis of interleukins 1,6, and 8 in skin and synovial fibroblasts: a possible role as a “fine-tuning cytokine” in inflammation processes. *Arthritis Rheum.* *44*, 2176–2184.
- Kim, G.-T., Cho, M.-L., Park, Y.-E., Yoo, W.H., Kim, J.-H., Oh, H.-J., Kim, D.-S., Baek, S.-H., Lee, S.-H., Lee, J.-H., et al. (2010). Expression of TLR2, TLR4, and TLR9 in dermatomyositis and polymyositis. *Clin. Rheumatol.* *29*, 273–279.

- Knittel, T., Mehde, M., Kobold, D., Saile, B., Dinter, C., and Ramadori, G. (1999). Expression patterns of matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF-alpha and TGF-beta1. *J. Hepatol.* *30*, 48–60.
- Kocić, J., Santibañez, J.F., Krstić, A., Mojsilović, S., Dorđević, I.O., Trivanović, D., Ilić, V., and Bugarski, D. (2012). Interleukin 17 inhibits myogenic and promotes osteogenic differentiation of C2C12 myoblasts by activating ERK1,2. *Biochim. Biophys. Acta* *1823*, 838–849.
- Kocić, J., Santibañez, J.F., Krstić, A., Mojsilović, S., Ilić, V., and Bugarski, D. (2013). Interleukin-17 modulates myoblast cell migration by inhibiting urokinase type plasminogen activator expression through p38 mitogen-activated protein kinase. *Int. J. Biochem. Cell Biol.* *45*, 464–475.
- Kondo, M., Murakawa, Y., Harashima, N., Kobayashi, S., Yamaguchi, S., and Harada, M. (2009). Roles of proinflammatory cytokines and the Fas/Fas ligand interaction in the pathogenesis of inflammatory myopathies. *Immunology* *128*, e589-599.
- Kramer, F., Torzewski, J., Kamenz, J., Veit, K., Hombach, V., Dedio, J., and Ivashchenko, Y. (2008). Interleukin-1beta stimulates acute phase response and C-reactive protein synthesis by inducing an NFkappaB- and C/EBPbeta-dependent autocrine interleukin-6 loop. *Mol. Immunol.* *45*, 2678–2689.
- Kubes, P., and Jenne, C. (2018). Immune Responses in the Liver. *Annu. Rev. Immunol.* *36*, 247–277.
- Kumar, S., Wang, J., Thomson, A.W., and Gandhi, C.R. (2017). Hepatic stellate cells increase the immunosuppressive function of natural Foxp3+ regulatory T cells via IDO-induced AhR activation. *J. Leukoc. Biol.* *101*, 429–438.
- Kuru, S., Inukai, A., Liang, Y., Doyu, M., Takano, A., and Sobue, G. (2000). Tumor necrosis factor-alpha expression in muscles of polymyositis and dermatomyositis. *Acta Neuropathol. (Berl.)* *99*, 585–588.
- Lafdil, F., Miller, A.M., Ki, S.H., and Gao, B. (2010). Th17 cells and their associated cytokines in liver diseases. *Cell. Mol. Immunol.* *7*, 250–254.
- Langhans, B., Alwan, A.W., Krämer, B., Glässner, A., Lutz, P., Strassburg, C.P., Nattermann, J., and Spengler, U. (2015). Regulatory CD4+ T cells modulate the interaction between NK cells and hepatic stellate cells by acting on either cell type. *J. Hepatol.* *62*, 398–404.
- Lemmers, A., Moreno, C., Gustot, T., Maréchal, R., Degré, D., Demetter, P., de Nadai, P., Geerts, A., Quertinmont, E., Vercruysse, V., et al. (2009). The interleukin-17 pathway is involved in human alcoholic liver disease. *Hepatol. Baltim. Md* *49*, 646–657.
- Lerut, J., and Sanchez-Fueyo, A. (2006). An appraisal of tolerance in liver transplantation. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* *6*, 1774–1780.
- Li, X., Su, Y., Hua, X., Xie, C., Liu, J., Huang, Y., Zhou, L., Zhang, M., Li, X., and Gao, Z. (2017). Levels of hepatic Th17 cells and regulatory T cells upregulated by hepatic stellate cells in advanced HBV-related liver fibrosis. *J. Transl. Med.* *15*, 75.

- Liu, Y., Li, L., Liu, J., She, W., Shi, J., Li, J., Wang, J., and Jiang, W. (2017). Activated hepatic stellate cells directly induce pathogenic Th17 cells in chronic hepatitis B virus infection. *Exp. Cell Res.* *359*, 129–137.
- Ma, T., Wang, Z., Yang, Z., and Chen, J. (2015). Cluster of differentiation 147 is a key molecule during hepatocellular carcinoma cell-hepatic stellate cell cross-talk in the rat liver. *Mol. Med. Rep.* *12*, 111–118.
- Marion, M.-J., Hantz, O., and Durantel, D. (2010). The HepaRG cell line: biological properties and relevance as a tool for cell biology, drug metabolism, and virology studies. *Methods Mol. Biol. Clifton NJ* *640*, 261–272.
- Matsuda, M., Tsurusaki, S., Miyata, N., Saijou, E., Okochi, H., Miyajima, A., and Tanaka, M. (2018). Oncostatin M causes liver fibrosis by regulating cooperation between hepatic stellate cells and macrophages in mice. *Hepatology. Baltim. Md* *67*, 296–312.
- Melgarejo, E., Medina, M.A., Sánchez-Jiménez, F., and Urdiales, J.L. (2009). Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. *Int. J. Biochem. Cell Biol.* *41*, 998–1001.
- Meng, F., Wang, K., Aoyama, T., Grivennikov, S.I., Paik, Y., Scholten, D., Cong, M., Iwaisako, K., Liu, X., Zhang, M., et al. (2012). Interleukin-17 signaling in inflammatory, Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice. *Gastroenterology* *143*, 765–776.e3.
- Moran, E.M., and Mastaglia, F.L. (2014). Cytokines in immune-mediated inflammatory myopathies: cellular sources, multiple actions and therapeutic implications. *Clin. Exp. Immunol.* *178*, 405–415.
- Ndongo-Thiam, N., and Miossec, P. (2015). A cell-based bioassay for circulating bioactive IL-17: application to destruction in rheumatoid arthritis. *Ann. Rheum. Dis.* *74*, 1629–1631.
- Noack, M., Ndongo-Thiam, N. 'd, and Miossec, P. (2016). Role of podoplanin in the high interleukin-17A secretion resulting from interactions between activated lymphocytes and psoriatic skin-derived mesenchymal cells. *Clin. Exp. Immunol.* *186*, 64–74.
- Osawa, Y., Hoshi, M., Yasuda, I., Saibara, T., Moriwaki, H., and Kozawa, O. (2013). Tumor necrosis factor- $\alpha$  promotes cholestasis-induced liver fibrosis in the mouse through tissue inhibitor of metalloproteinase-1 production in hepatic stellate cells. *PLoS One* *8*, e65251.
- Osta, B., Roux, J.-P., Lavocat, F., Pierre, M., Ndongo-Thiam, N., Boivin, G., and Miossec, P. (2015). Differential Effects of IL-17A and TNF- $\alpha$  on Osteoblastic Differentiation of Isolated Synoviocytes and on Bone Explants from Arthritis Patients. *Front. Immunol.* *6*, 151.
- Page, G., Chevrel, G., and Miossec, P. (2004). Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: Interaction with chemokines and Th1 cytokine-producing cells. *Arthritis Rheum.* *50*, 199–208.
- Park, J., Kang, W., Ryu, S.-W., Kim, W.-I., Chang, D.-Y., Lee, D.H., Park, D.Y., Choi, Y.-H., Choi, K., Shin, E.-C., et al. (2012). Hepatitis C virus infection enhances TNF $\alpha$ -induced cell death via suppression of NF- $\kappa$ B. *Hepatology. Baltim. Md* *56*, 831–840.

- Patel, D.N., King, C.A., Bailey, S.R., Holt, J.W., Venkatachalam, K., Agrawal, A., Valente, A.J., and Chandrasekar, B. (2007). Interleukin-17 stimulates C-reactive protein expression in hepatocytes and smooth muscle cells via p38 MAPK and ERK1/2-dependent NF-kappaB and C/EBPbeta activation. *J. Biol. Chem.* *282*, 27229–27238.
- Pellicoro, A., Ramachandran, P., Iredale, J.P., and Fallowfield, J.A. (2014). Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat. Rev. Immunol.* *14*, 181–194.
- Petrasek, J., Iracheta-Vellve, A., Saha, B., Satishchandran, A., Kodys, K., Fitzgerald, K.A., Kurt-Jones, E.A., and Szabo, G. (2015). Metabolic danger signals, uric acid and ATP, mediate inflammatory cross-talk between hepatocytes and immune cells in alcoholic liver disease. *J. Leukoc. Biol.* *98*, 249–256.
- Pradere, J.-P., Kluwe, J., De Minicis, S., Jiao, J.-J., Gwak, G.-Y., Dapito, D.H., Jang, M.-K., Guenther, N.D., Mederacke, I., Friedman, R., et al. (2013). Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology* *58*, 1461–1473.
- Qin, S., Lu, D., Guo, X., Luo, W., Hu, B., Huang, X., Chen, M., Wang, J., Ma, S.-J., Yang, X., et al. (2016). A deleterious role for Th9/IL-9 in hepatic fibrogenesis. *Sci. Rep.* *6*, 18694.
- Quah, B.J.C., Warren, H.S., and Parish, C.R. (2007). Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat. Protoc.* *2*, 2049–2056.
- Quintin, E., Scoazec, J.-Y., Marotte, H., and Miossec, P. (2010). Rare incidence of methotrexate-specific lesions in liver biopsy of patients with arthritis and elevated liver enzymes. *Arthritis Res. Ther.* *12*, R143.
- Racanelli, V., and Rehermann, B. (2006). The liver as an immunological organ. *Hepatology* *43*, S54–62.
- Rafiq, S., Frayling, T.M., Murray, A., Hurst, A., Stevens, K., Weedon, M.N., Henley, W., Ferrucci, L., Bandinelli, S., Corsi, A.-M., et al. (2007). A common variant of the interleukin 6 receptor (IL-6r) gene increases IL-6r and IL-6 levels, without other inflammatory effects. *Genes Immun.* *8*, 552–559.
- Rayavarapu, S., Coley, W., and Nagaraju, K. (2012). Endoplasmic reticulum stress in skeletal muscle homeostasis and disease. *Curr. Rheumatol. Rep.* *14*, 238–243.
- Rayavarapu, S., Coley, W., Kinder, T.B., and Nagaraju, K. (2013). Idiopathic inflammatory myopathies: pathogenic mechanisms of muscle weakness. *Skelet. Muscle* *3*, 13.
- Robert, S., Gicquel, T., Bodin, A., Lagente, V., and Boichot, E. (2016). Characterization of the MMP/TIMP Imbalance and Collagen Production Induced by IL-1 $\beta$  or TNF- $\alpha$  Release from Human Hepatic Stellate Cells. *PLoS One* *11*, e0153118.
- Robinson, M.W., Harmon, C., and O’Farrelly, C. (2016). Liver immunology and its role in inflammation and homeostasis. *Cell. Mol. Immunol.* *13*, 267.

- Ruddy, M.J., Wong, G.C., Liu, X.K., Yamamoto, H., Kasayama, S., Kirkwood, K.L., and Gaffen, S.L. (2004). Functional cooperation between interleukin-17 and tumor necrosis factor- $\alpha$  is mediated by CCAAT/enhancer-binding protein family members. *J. Biol. Chem.* *279*, 2559–2567.
- Russo, R.C., Garcia, C.C., Teixeira, M.M., and Amaral, F.A. (2014). The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. *Expert Rev. Clin. Immunol.* *10*, 593–619.
- Sancho-Bru, P., Juez, E., Moreno, M., Khurdayan, V., Morales-Ruiz, M., Colmenero, J., Arroyo, V., Brenner, D.A., Ginès, P., and Bataller, R. (2010). Hepatocarcinoma cells stimulate the growth, migration and expression of pro-angiogenic genes in human hepatic stellate cells. *Liver Int. Off. J. Int. Assoc. Study Liver* *30*, 31–41.
- Scheller, J., and Rose-John, S. (2012). The interleukin 6 pathway and atherosclerosis. *Lancet Lond. Engl.* *380*, 338.
- Scheller, J., Chalaris, A., Schmidt-Arras, D., and Rose-John, S. (2011). The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim. Biophys. Acta* *1813*, 878–888.
- Schmidt-Arras, D., and Rose-John, S. (2016). IL-6 pathway in the liver: From physiopathology to therapy. *J. Hepatol.* *64*, 1403–1415.
- Schoenherr, C., Weiskirchen, R., and Haan, S. (2010). Interleukin-27 acts on hepatic stellate cells and induces signal transducer and activator of transcription 1-dependent responses. *Cell Commun. Signal. CCS* *8*, 19.
- Schreiner, B., Voss, J., Wischhusen, J., Dombrowski, Y., Steinle, A., Lochmüller, H., Dalakas, M., Melms, A., and Wiendl, H. (2006). Expression of toll-like receptors by human muscle cells in vitro and in vivo: TLR3 is highly expressed in inflammatory and HIV myopathies, mediates IL-8 release and up-regulation of NKG2D-ligands. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* *20*, 118–120.
- Schwabe, R.F., and Brenner, D.A. (2006). Mechanisms of Liver Injury. I. TNF- $\alpha$ -induced liver injury: role of IKK, JNK, and ROS pathways. *Am. J. Physiol. Gastrointest. Liver Physiol.* *290*, G583-589.
- Sedger, L.M., and McDermott, M.F. (2014). TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. *Cytokine Growth Factor Rev.* *25*, 453–472.
- Shen, H., Xia, L., Lu, J., and Xiao, W. (2011). Interleukin-17 and interleukin-23 in patients with polymyositis and dermatomyositis. *Scand. J. Rheumatol.* *40*, 217–220.
- Shi, T., Zhang, T., Zhang, L., Yang, Y., Zhang, H., and Zhang, F. (2015). The Distribution and the Fibrotic Role of Elevated Inflammatory Th17 Cells in Patients With Primary Biliary Cirrhosis. *Medicine (Baltimore)* *94*, e1888.
- Simpson, N., Cho, Y.W., Cicciarelli, J.C., Selby, R.R., and Fong, T.-L. (2006). Comparison of renal allograft outcomes in combined liver-kidney transplantation versus subsequent

kidney transplantation in liver transplant recipients: Analysis of UNOS Database. *Transplantation* 82, 1298–1303.

Sparna, T., Rétey, J., Schmich, K., Albrecht, U., Naumann, K., Gretz, N., Fischer, H.-P., Bode, J.G., and Merfort, I. (2010). Genome-wide comparison between IL-17 and combined TNF-alpha/IL-17 induced genes in primary murine hepatocytes. *BMC Genomics* 11, 226.

Stiber, J., Hawkins, A., Zhang, Z.-S., Wang, S., Burch, J., Graham, V., Ward, C.C., Seth, M., Finch, E., Malouf, N., et al. (2008). STIM1 signalling controls store-operated calcium entry required for development and contractile function in skeletal muscle. *Nat. Cell Biol.* 10, 688–697.

Strang, A.C., Bisioendial, R.J., Kootte, R.S., Schulte, D.M., Dallinga-Thie, G.M., Levels, J.H.M., Kok, M., Vos, K., Tas, S.W., Tietge, U.J.F., et al. (2013). Pro-atherogenic lipid changes and decreased hepatic LDL receptor expression by tocilizumab in rheumatoid arthritis. *Atherosclerosis* 229, 174–181.

Sun, H.Q., Zhang, J.Y., Zhang, H., Zou, Z.S., Wang, F.S., and Jia, J.H. (2012). Increased Th17 cells contribute to disease progression in patients with HBV-associated liver cirrhosis. *J. Viral Hepat.* 19, 396–403.

Sun, X.-F., Gu, L., Deng, W.-S., and Xu, Q. (2014). Impaired balance of T helper 17/T regulatory cells in carbon tetrachloride-induced liver fibrosis in mice. *World J. Gastroenterol.* 20, 2062–2070.

Szodoray, P., Alex, P., Knowlton, N., Centola, M., Dozmorov, I., Csipo, I., Nagy, A.T., Constantin, T., Ponyi, A., Nakken, B., et al. (2010). Idiopathic inflammatory myopathies, signified by distinctive peripheral cytokines, chemokines and the TNF family members B-cell activating factor and a proliferation inducing ligand. *Rheumatol. Oxf. Engl.* 49, 1867–1877.

Tan, Z., Qian, X., Jiang, R., Liu, Q., Wang, Y., Chen, C., Wang, X., Ryffel, B., and Sun, B. (2013). IL-17A plays a critical role in the pathogenesis of liver fibrosis through hepatic stellate cell activation. *J. Immunol. Baltim. Md* 1950 191, 1835–1844.

Tanaka, T., Narazaki, M., and Kishimoto, T. (2012). Therapeutic targeting of the interleukin-6 receptor. *Annu. Rev. Pharmacol. Toxicol.* 52, 199–219.

Tang, X., Tian, X., Zhang, Y., Wu, W., Tian, J., Rui, K., Tong, J., Lu, L., Xu, H., and Wang, S. (2013). Correlation between the Frequency of Th17 Cell and the Expression of MicroRNA-206 in Patients with Dermatomyositis. *Clin. Dev. Immunol.* 2013, 345347.

Taniguchi, K., Wu, L.-W., Grivennikov, S.I., de Jong, P.R., Lian, I., Yu, F.-X., Wang, K., Ho, S.B., Boland, B.S., Chang, J.T., et al. (2015). A gp130-Src-YAP module links inflammation to epithelial regeneration. *Nature* 519, 57–62.

Tarrats, N., Moles, A., Morales, A., García-Ruiz, C., Fernández-Checa, J.C., and Marí, M. (2011). Critical role of tumor necrosis factor receptor 1, but not 2, in hepatic stellate cell proliferation, extracellular matrix remodeling, and liver fibrogenesis. *Hepatology* 54, 319–327.

Tilg, H., Kaser, A., and Moschen, A.R. (2006). How to modulate inflammatory cytokines in liver diseases. *Liver Int. Off. J. Int. Assoc. Study Liver* 26, 1029–1039.

- Tournadre, A., and Miossec, P. (2012). Interleukin-17 in inflammatory myopathies. *Curr. Rheumatol. Rep.* *14*, 252–256.
- Tournadre, A., and Miossec, P. (2013). A critical role for immature muscle precursors in myositis. *Nat. Rev. Rheumatol.* *9*, 438–442.
- Tournadre, A., Porcherot, M., Chérin, P., Marie, I., Hachulla, E., and Miossec, P. (2009). Th1 and Th17 balance in inflammatory myopathies: Interaction with dendritic cells and possible link with response to high-dose immunoglobulins. *Cytokine* *46*, 297–301.
- Tournadre, A., Lenief, V., and Miossec, P. (2010). Expression of Toll-like receptor 3 and Toll-like receptor 7 in muscle is characteristic of inflammatory myopathy and is differentially regulated by Th1 and Th17 cytokines. *Arthritis Rheum.* *62*, 2144–2151.
- Tournadre, A., Lenief, V., Eljaafari, A., and Miossec, P. (2012). Immature muscle precursors are a source of interferon- $\beta$  in myositis: role of Toll-like receptor 3 activation and contribution to HLA class I up-regulation. *Arthritis Rheum.* *64*, 533–541.
- Wang, C., Sun, J., Li, L., Wang, L., Dolan, P., and Sheil, A.G. (1998). Conversion of pancreas allograft rejection to acceptance by liver transplantation. *Transplantation* *65*, 188–192.
- Wang, Z.-M., Zhou, L.-Y., Liu, B.-B., Jia, Q.-A., Dong, Y.-Y., Xia, Y.-H., and Ye, S.-L. (2014). Rat hepatic stellate cells alter the gene expression profile and promote the growth, migration and invasion of hepatocellular carcinoma cells. *Mol. Med. Rep.* *10*, 1725–1733.
- Warren, A., Le Couteur, D.G., Fraser, R., Bowen, D.G., McCaughan, G.W., and Bertolino, P. (2006). T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells. *Hepatology* *44*, 1182–1190.
- Xia, Y.-H., Lu, Z., Zhao, M., Dai, W.-T., Ding, L., Hu, L.-X., and Jiang, G.-L. (2017). Tumor-specific hepatic stellate cells (tHSCs) induces DIgR2 expression in dendritic cells to inhibit T cells. *Oncotarget* *8*, 55084–55093.
- Xing, Z.-Z., Huang, L.-Y., Wu, C.-R., You, H., Ma, H., and Jia, J.-D. (2015). Activated rat hepatic stellate cells influence Th1/Th2 profile in vitro. *World J. Gastroenterol.* *21*, 7165–7171.
- Xu, L., Hui, A.Y., Albanis, E., Arthur, M.J., O’Byrne, S.M., Blaner, W.S., Mukherjee, P., Friedman, S.L., and Eng, F.J. (2005). Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* *54*, 142–151.
- Yang, Y.M., and Seki, E. (2015). TNF $\alpha$  in liver fibrosis. *Curr. Pathobiol. Rep.* *3*, 253–261.
- Yoshizaki, K. (2011). Pathogenic role of IL-6 combined with TNF- $\alpha$  or IL-1 in the induction of acute phase proteins SAA and CRP in chronic inflammatory diseases. *Adv. Exp. Med. Biol.* *691*, 141–150.
- Zhao, W., Su, W., Kuang, P., Zhang, L., Liu, J., Yin, Z., and Wang, X. (2012). The role of hepatic stellate cells in the regulation of T-cell function and the promotion of hepatocellular carcinoma. *Int. J. Oncol.* *41*, 457–464.

Zimmermann, M., Aguilera, F.B., Castellucci, M., Rossato, M., Costa, S., Lunardi, C., Ostuni, R., Girolomoni, G., Natoli, G., Bazzoni, F., et al. (2015). Chromatin remodelling and autocrine TNF $\alpha$  are required for optimal interleukin-6 expression in activated human neutrophils. *Nat. Commun.* 6, 6061.

Zrioual, S., Ecochard, R., Tournadre, A., Lenief, V., Cazalis, M.-A., and Miossec, P. (2009). Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. *J. Immunol. Baltim. Md 1950* 182, 3112–3120.