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# Zinc an Cadmium homeostasis changes in chronic inflammation and new treatment options in arthritis

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**Changement de l'homéostasie du Zinc et du Cadmium  
par l'inflammation chronique et nouvelles options  
thérapeutiques pour le traitement de l'arthrite**

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Devant le jury composé de :

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## Résumé de thèse

La polyarthrite rhumatoïde (PR) est une maladie inflammatoire chronique caractérisée par des atteintes articulaires. Les synoviocytes, cellules qui tapissent la membrane synoviale, deviennent réfractaires à l'apoptose et, en produisant la cytokine inflammatoire Interleukin 6 (IL-6), participent à la chronicité de l'inflammation qui est à l'origine de la perte osseuse dans la PR.

Le zinc (Zn) et le cadmium (Cd) partagent leurs propriétés physico-chimiques et leurs mécanismes de transport cellulaire. Le Zn régule des fonctions du système immunitaire, contrairement au Cd avec des propriétés sur le système immunitaire peu décrites dans la littérature.

Notre travail vise à identifier les mécanismes impliqués dans la modification de l'homéostasie des métaux dans les synoviocytes par l'inflammation et les conséquences de cette altération.

L'inflammation induit l'accumulation des métaux dans les synoviocytes en augmentant l'expression de l'importeur ZIP-8. En revanche, l'expression de l'exporteur ZnT1 et des metallothionéines (MTs, régulateurs de l'homéostasie des métaux) est dépendante de la présence des métaux. L'affinité Cd-MTs permet une accumulation irréversible du Cd dans les cellules qui réduit leur prolifération et la production d'IL-6.

Les effets antiprolifératif et anti-inflammatoire du Cd ont été testés dans le modèle d'arthrite chez le rat où une seule injection intra-articulaire de Cd à faible dose prévient la perte osseuse et réduit les scores cliniques d'arthrite. Cela pourrait représenter une nouvelle approche thérapeutique pour le traitement de la PR et d'autres pathologies caractérisées par une hyperplasie et une inflammation localisées.

## Résumé substantiel:

La polyarthrite rhumatoïde (PR), une maladie inflammatoire chronique, est le plus fréquent des rhumatismes inflammatoires. La PR est une maladie systémique, caractérisée par des atteintes articulaires liées à une hyper-prolifération locale des synoviocytes, cellules fibroblastiques qui tapissent la membrane synoviale. Les synoviocytes deviennent réfractaires à l'apoptose et participent au développement et à la chronicité de l'inflammation en produisant notamment de l'interleukin 6 (IL-6). La réaction inflammatoire chronique dans les articulations est à l'origine d'une importante perte osseuse entraînant une perte de mobilité des patients et, par conséquent, une baisse de la qualité de vie.

Le zinc (Zn) et le cadmium (Cd) appartiennent au groupe 12 des métaux divalents et partagent de nombreuses caractéristiques, notamment les propriétés physico-chimiques et leurs mécanismes de transport cellulaire. Le Zn est un important régulateur des fonctions du système immunitaire et l'inflammation chronique ainsi que le développement de plusieurs maladies chroniques ont été associés à une carence de Zn. Contrairement au Zn, le rôle du Cd dans la régulation du système immunitaire est peu décrit dans la littérature.

Notre travail vise à identifier les mécanismes impliqués dans la modification de l'homéostasie des métaux dans les synoviocytes en conditions inflammatoires. Les conséquences de l'altération de l'homéostasie des métaux par l'inflammation ont été également étudiées.

Les synoviocytes de patients atteints de PR, utilisés comme modèle cellulaire d'inflammation chronique, ont été d'abord exposés aux cytokines pro-inflammatoires IL-17 et TNF- $\alpha$ , afin de recréer un environnement inflammatoire *in vitro*. Puis, Zn et au Cd ont été ajoutés, seuls ou associés. Après avoir étudié les

mécanismes de régulations et les effets du Zn et du Cd sur les synoviocytes *in vitro*, nous avons testé leurs effets *in vivo* dans un modèle d'arthrite chez le rat.

Les résultats obtenus montrent que la combinaison IL-17/TNF- $\alpha$  induit l'accumulation des métaux dans les synoviocytes en augmentant l'expression de l'importeur transmembranaire ZIP-8, qui néanmoins n'est pas sensible à la concentration des métaux dans le milieu de culture. En revanche, l'expression de l'exporteur ZnT1 et des metallothionéines (MTs, régulateurs de l'homéostasie des métaux) est dépendante de la présence des métaux. Les résultats obtenus nous ont permis de développer un modèle mathématique représentant l'effet des cytokines sur l'homéostasie des métaux dans les synoviocytes, qui pourra être appliqué à d'autres types cellulaires.

Nous avons montré que le Cd induit une expression plus élevée des MTs que le Zn, dû à une plus grande affinité des MTs pour le Cd. La présence de cytokines inflammatoires renforce encore cet effet et permet l'accumulation irréversible du Cd dans la cellule. Pour cette raison, lorsque le Cd est ajouté dans le milieu de culture, la prolifération des cellules et la production de la cytokine pro-inflammatoire IL-6 est réduite. Au contraire, lorsque le Zn est ajouté, la viabilité des synoviocytes et l'inflammation qui en dépend augmente.

Les effets antiprolifératif et anti-inflammatoire du Cd ont été testés dans le modèle d'arthrite induite par adjuvant chez le rat. Dans ce cas, le Cd est utilisé comme traitement local par injection intra-articulaire afin de réduire l'inflammation et la destruction de l'articulation qui en découle. Les résultats montrent qu'une seule injection de Cd à faible dose prévient la perte osseuse dans les articulations et réduit les scores cliniques d'arthrite.

L'effet antiprolifératif et anti-inflammatoire du Cd, pourrait représenter une nouvelle approche thérapeutique locale pour le traitement de la PR mais aussi pour d'autres pathologies caractérisées par une hyperplasie et une inflammation localisées. Cette approche est d'ailleurs déjà utilisée dans certaines thérapies anti-cancéreuses, par l'utilisation de la toxicité intrinsèque de certains nanomatériaux.



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**Paola Bonaventura**

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## Thesis summary

Rheumatoid arthritis (RA) is a chronic inflammatory disease. Synoviocytes, cells forming the inner layer the synovium, become refractory to apoptosis and participate in the chronicity of inflammation through the production of IL-6. The perpetuation of inflammation causes an important induction of bone loss in joints.

Zinc (Zn) and Cadmium (Cd) share many physico-chemical properties and cell transport mechanism. Zn is known as a regulator of the normal function of the immune system, while Cd properties on the immune system are not well defined.

Our aim was to provide information on the metal homeostasis mechanisms in synoviocytes during chronic inflammation and on the consequences of metal homeostasis changes. After studying the differential effect of Zn and Cd at the cellular level, we could provide an innovative tool to control synoviocyte contribution to rheumatoid arthritis, which was tested on an *in vivo* model of arthritis.

Results show that IL-17/TNF- $\alpha$  combination drives the accumulation of metals inside synoviocytes through the enhancing of ZIP-8 importer expression and regardless of the concentration of metals in the culture medium. In contrast, the expression of the metal exporter ZnT1 and of the homeostasis regulators metallothioneins (MTs) was primarily dependent on metal levels.

Addition of Zn stimulated the inflammatory response, while addition of Cd can reduce both viability and inflammation.

The anti-proliferative and anti-inflammatory properties of Cd were used in the rat model of arthritis as intra-articular treatment to reduce local inflammation and joint destruction and it may represent a new local therapeutic approach for RA treatment.

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# Index

<b>I. INTRODUCTION .....</b>	<b>12</b>
<b>1. Inflammation .....</b>	<b>12</b>
1.1 Pro-inflammatory cytokines .....	13
1.2 Chronic inflammation .....	17
1.2.1 Chronic inflammatory autoimmune diseases .....	18
1.3 Rheumatoid arthritis (RA).....	19
1.3.1 Systemic inflammation in RA .....	21
1.3.2 Local inflammation in RA.....	22
1.3.2.1 Cell interactions and produced soluble factors .....	24
1.3.3 RA Treatment .....	27
1.3.3.1 Local treatment.....	29
<b>2. Transition metals and group 12 .....</b>	<b>32</b>
2.1 Metal quantification.....	34
2.2 Use of metal isotopes .....	36
2.3 Zinc (Zn).....	70
2.4 Cadmium (Cd) .....	70
2.4.1 Transport and effects of cadmium in cells .....	71
2.4.2 Competitive role of Zn and Cd.....	73
<b>II. AIMS OF THE WORK .....</b>	<b>76</b>
<b>III. ZINC AND ITS ROLE IN AUTOIMMUNITY AND INFLAMMATION .....</b>	<b>77</b>
<b>IV. A FEEDBACK LOOP BETWEEN INFLAMMATION AND ZINC UPTAKE .....</b>	<b>88</b>
<b>V. INFLAMMATION-INDUCED CADMIUM UPTAKE BY SYNOVIOCYTES PROTECTS JOINTS FROM ARTHRITIS DESTRUCTION.....</b>	<b>102</b>
<b>DISCUSSION .....</b>	<b>134</b>
<b>ANNEXE 1.....</b>	<b>140</b>
<b>BIBLIOGRAPHY .....</b>	<b>162</b>

## **List of the abbreviations used in the manuscript**

IL: interleukin  
 TNF- $\alpha$ : tumour necrosis factor- alpha  
 IFN- $\gamma$ : interferon-gamma  
 IL-17: interleukin-17  
 IL-1 $\beta$ : interleukin-1-beta  
 Th: T helper  
 Th17: T helper 17  
 Th1: T helper 1  
 IL-23: interleukin-23  
 IL-21: interleukin-21  
 IL-22: interleukin-22  
 IL-17RA: interleukin-17 receptor A  
 IL-17RC: interleukin-17 receptor C  
 PGE-2: prostaglandins-2  
 NOS: nitric oxide synthase  
 Pro-IL-1 $\beta$ : preform (precursor) IL-1 $\beta$   
 ICE: IL-1 $\beta$  converting enzyme  
 TNFR1: TNF-receptor-1  
 TNFR2: TNF-receptor-2  
 IL-8: interleukin-8  
 IL-6: interleukin-6  
 CCL-20 : Chemokine (C-C motif) ligand 20  
 CCR-6: Chemokine (C-C motif) receptor 6  
 IL-10 : Interleukin-10  
 CXCR1: Chemokine (C-X-C motif) receptor 1  
 CXCR2: Chemokine (C-X-C motif) receptor 2  
 IL-1RA: Interleukin receptor 1  
 IL-12: Interleukin-12  
 Gp130: Glycoprotein 130  
 IL-6R $\alpha$ : Interleukin-6 receptor-alpha  
 sIL-6R $\alpha$ : soluble interleukin-6 receptor-alpha  
 sgp130: soluble glycoprotein 130  
 ROS: Radical oxygen species  
 RA: Rheumatoid arthritis  
 SLE: Systemic lupus erythematosus  
 MS: Multiple sclerosis  
 IBD: Inflammatory bowel disease  
 RF: Rheumatoid factor  
 ACPAs: Anti-citrullinated protein antibodies  
 PADs: Peptidyl arginine deaminase  
 OA: Osteoarthritis  
 CTGF: connective tissue growth factor  
 CXCR4: Chemokine (C-X-C motif) receptor 4  
 CXCL12: Chemokine (C-X-C motif) ligand 12  
 CCRs: Chemokine (C-C motif) receptors  
 CCLs: Chemokine (C-C motif) ligands  
 DMARDs: Disease modulating anti-rheumatic drugs  
 GC: glucocorticoids  
 MTX: Metotrexate  
 FAAS: In flame atomic absorption spectrometry  
 ICP-AES: Inductively coupled plasma atomic emission spectrometry  
 ICP-MS: Inductively coupled plasma mass spectrometry  
 Zn: Zinc  
 Cd: Cadmium  
 MTF-1: Metallothionein transcription factor-1  
 MTs: Metallothioneins  
 Apo-MT: Apo-metallothionein  
 Holo-MT: Holo-metallothionein  
 ZIPs: Znt-Irt like proteins  
 ZnTs: Zinc transporters

## **I. INTRODUCTION**

### **1. Inflammation**

The word inflammation derives from Latin *inflammo* which means “*I ignite*” and describes the processes of the body tissues for self-protection to harmful stimuli, such as pathogens, damaged cells, or irritants. Inflammation is characterized by five cardinal signs: redness (*rubor*) resulting from vasodilation and the increased blood flow for cell recruitment, increased heat (*calor*) which manifests itself as swelling (*tumor*) and pain (*dolor*) with a possible loss of the function of the system (*function laesa*). Inflammation can be either *acute* or *chronic*, depending on the self-sustained character of the disease.

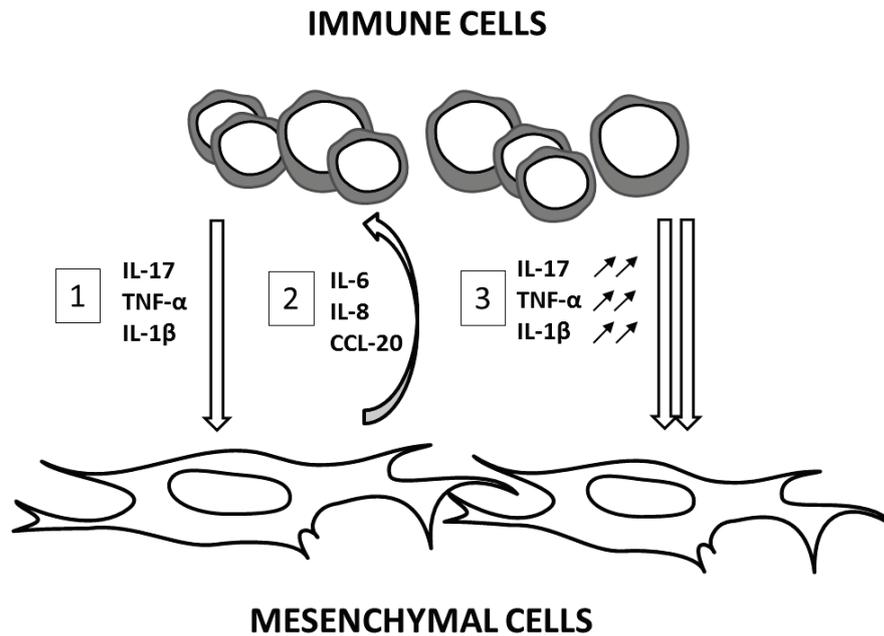
*Acute inflammation* is the initial, beneficial, short term response of the body against the harmful stimulus and it is a reversible self-limited mechanism leading to resolution at inflammatory sites. This process can also become harmful, because the components of inflammation can hurt bystander tissues. At the cellular level, acute inflammation can mainly be considered a part of the innate immunity response and it is divided into different stages of cell recruitment, from the blood into the injured tissues: during the early stage there is the activation of the innate immunity, where mainly neutrophils are recruited, first followed by monocytes-macrophages and finally by lymphocytes for the activation of the adaptive immune response. Those cells can interact with other cells at the site of inflammation and undergo activation, with the release of cellular mediators of inflammation. Pro-inflammatory cytokines are responsible, together with other chemical mediators (complement, leukotriene, prostaglandin, thromboxane, platelet activating factor), for the inflammatory clinical status.

### 1.1 Pro-inflammatory cytokines

Cytokines are small, nonstructural proteins with molecular weights ranging from 8 to 40,000 Da. Almost all nucleated cells are capable of synthesizing these proteins and, in turn, of responding to them. There is no amino acid sequence motif or three-dimensional structure that links cytokines. Even if they can be divided according to their principal biological activities into pro-inflammatory and anti-inflammatory cytokines (1), their real effect is dependent on the disease context.

Pro-inflammatory cytokines, including interleukins (IL), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and interferon  $\gamma$  (INF- $\gamma$ ) are produced predominantly by macrophages and lymphocytes but can be synthesized by other cell types. These polypeptides modulate the activity and function of other cells to coordinate and control the inflammatory response.

In this study, we focused principally on three pro-inflammatory cytokines: interleukin-17 (IL-17), interleukin-1 (IL-1), and TNF- $\alpha$ . These three cytokines are at the beginning of the inflammatory cascade as represented in figure 1 and can induce the secretion of other pro-inflammatory cytokines such as IL-6, IL-8 or CCL-20 from the cells of the tissue on which they act. This promotes the loop of inflammation, by the enhanced production of IL-17, IL-1 and TNF- $\alpha$  (**figure 1**).



**Fig. 1: Feedback loop of inflammation.** The production of IL-17, IL-1 and TNF- $\alpha$  by immune cells (1) enhances the production of IL-6, IL-8 and CCL-20 by mesenchymal cells (2) which induces a feedback loop on inflammation (3), resulting in chronicity

**IL-17**, now referred to as IL-17A (2), is part of the IL-17 family comprising six members from IL-17A to IL-17F. IL-17 is mainly produced by CD4<sup>+</sup> T lymphocytes called T-helper 17 (Th17) cells and is induced by IL-23. It acts through the two receptors IL-17 receptor A (IL-17RA) and IL-17 receptor C (IL-17RC) (3), as a potent early-mediator of inflammation by increasing chemokine production in various tissues, for the recruitment of monocytes and neutrophils to the site of inflammation. Here, IL-17 induces them to produce other mediators of inflammation, which perpetuate and amplify the inflammatory response (2). IL-17 can also induce other pro-inflammatory mediators besides cytokines and chemokines, as prostaglandins (PGE<sub>2</sub>) and nitric oxide synthase (NOS).

**IL-1 $\beta$**  is produced by activated macrophages and monocytes. It is involved in the generation of systemic and local responses to infection, injury, and other immunological challenges and it is the primary cause of chronic and acute inflammation (4). IL-1 $\beta$  is produced as an inactive cytoplasmic precursor (proIL-1 $\beta$ ) that must be cleaved (at Asp116) to generate the mature active form. The IL-1 $\beta$ -converting enzyme (ICE), better known as caspase-1 (5) is required for its cleavage.

**TNF- $\alpha$**  is mostly produced by activated macrophages but it is also expressed by neutrophils, NK-cells, endothelial cells and activated lymphocytes. The cytokine elicits biological effects by two specific receptors: TNFR1 (CD120a) – present on all kinds of cells, and TNFR2 (CD120b) – more restricted, with the highest expression on Treg lymphocytes (6).

Both IL-17 and IL-1 $\beta$  can act synergistically with TNF- $\alpha$  at the site of inflammation mobilizing and activating leukocytes, inducing B and T cell proliferation and natural killer cell cytotoxicity. The synergy between IL-17 and TNF- $\alpha$  was previously studied in our laboratory, in the context of rheumatoid arthritis (RA) and other chronic inflammatory diseases (7, 8). It was previously described that IL-17 primes the cells at the site of inflammation to respond to other cytokines, as TNF- $\alpha$ , by increasing the expression of their receptor, in this case TNFR2 (9). The synergism of IL-1 $\beta$  and TNF- $\alpha$  is also a commonly reported phenomenon, which induces the expression of endothelial adhesion molecules. These molecules are essential for the adhesion of leukocytes to endothelial surface prior to migration into the tissues (1).

These synergies between IL-17, TNF and IL-1 $\beta$  induce in loop the secretion of other cytokines, notably IL-8, IL-6 and CCL-20 from the cells at the site of

inflammation. The production of the latter is considered as a marker of response to the first three and moreover can induce in loop their production (Fig.1).

**IL-8** is produced by macrophages and other cells (as epithelial cells). It binds CXCR1 and CXCR2 receptors and functions as a chemokine, activating neutrophils to degranulate and causing tissue damage at the site of inflammation. IL-8 can also be a potent promoter of angiogenesis.

**IL-6** demonstrates contradictory activities: this cytokine is mainly recognized as a pro-inflammatory molecule, but under some circumstances, e.g. disease context, it can exhibit anti-inflammatory properties. IL-6 plays a pivotal role during the transition from innate to acquired immunity (10), leading to the switch from neutrophils to monocyte recruitment (11), by up-regulating M-CSF receptor expression and by programming Th17 differentiation through IL-21 and IL-23 pathways (12). Simultaneously, IL-6 can enhance the production of anti-inflammatory IL-1Ra and suppresses pro-inflammatory cytokines such as IL-1, TNF- $\alpha$  and IL-12 in other disease contexts (13). IL-6 receptor has a heterodimeric structure and it is composed of gp130 and IL-6R $\alpha$  subunits. Soluble form of IL-6R $\alpha$  (sIL-6R $\alpha$ ) and gp130 (sgp130) are abundantly present in body fluids. The complex effect of IL-6 may relate to the fact that the final cell response to IL-6 depends upon the ratio between the membrane-bound IL-6R $\alpha$  and gp130 on one side, and the availability of IL-6, sIL-6R $\alpha$  and sgp130 on the other (14).

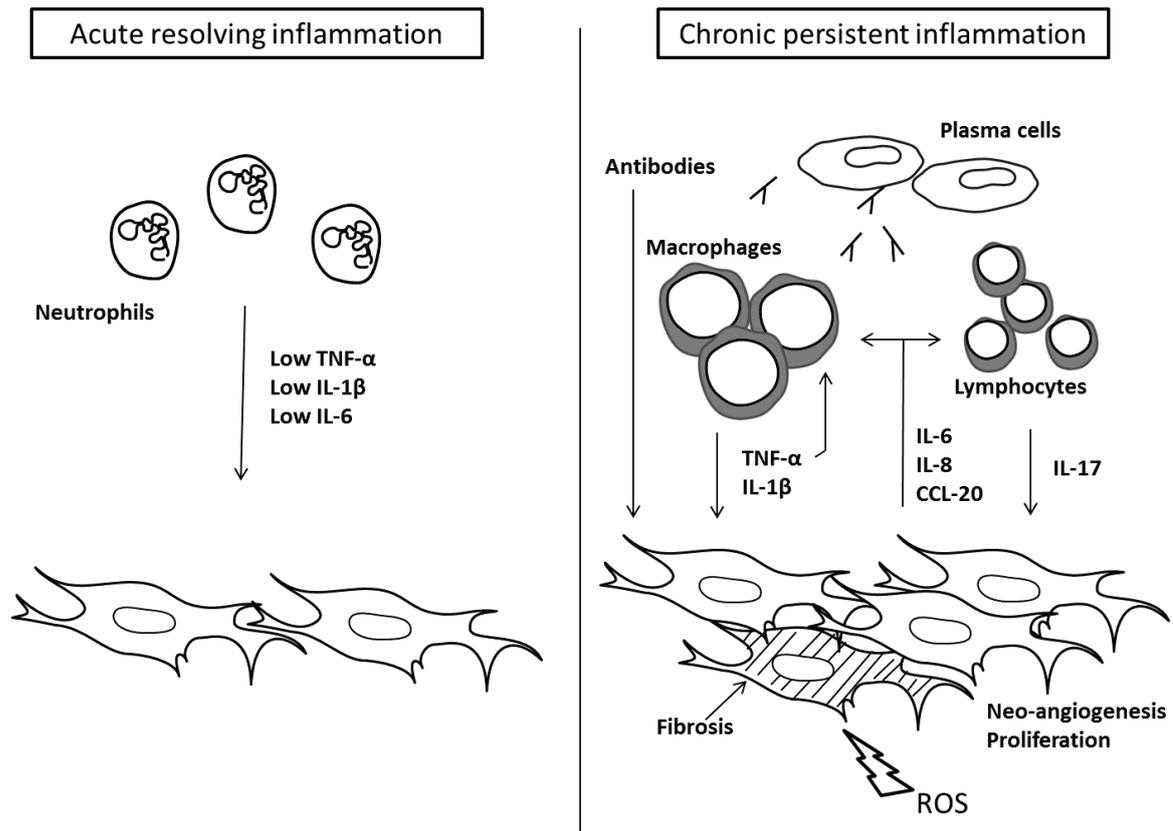
**Chemokine (C-C motif) ligand 20 (CCL-20)** is a small cytokine belonging to the CC chemokine family. It is strongly chemotactic for lymphocytes, preferentially Th17 (15, 16), and weakly attracts neutrophils through the receptor CCR6 at the site of inflammation. It is produced by immune cells but also cells at the site of

inflammation and it can be upregulated by IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$  and down-regulated by IL-10 (17).

### 1.2 Chronic inflammation

This series of biochemical events propagate and mature the inflammatory response. Prolonged, non-resolving inflammation is known as chronic inflammation and leads to a progressive shift in the type of cells present at the site of inflammation: from mainly neutrophils during acute inflammation to a lymphocyte-, plasma cell- and macrophage-enriched infiltration. The development of chronic inflammation is due to the persistent non-resolvable acute inflammation: non-degradable pathogens, viral infection, persistent foreign bodies, or autoimmune reactions can last for months or even years.

Pro-inflammatory cytokines as IL-6, IL-8, IL-1 $\beta$  and IL-17 continue to be produced in loop, causing a simultaneous destruction and healing of the tissue due to the inflammatory process, in turn leading to fibrosis, angiogenesis and eventually necrosis. Other factors involved in the persistence of inflammation can be reactive oxygen species (ROS) and some proteolytic enzymes (18). The changes involved in the process of inflammation are represented in the **figure 2**.



**Fig. 2: Acute versus chronic inflammation.** Shift of the cells at the site of inflammation induces the production of many inflammatory mediators and changes in cells

### 1.2.1 Chronic inflammatory autoimmune diseases

Inappropriate inflammatory response, when there are no foreign substances to fight off, leads to autoimmunity; an autoimmune disease is described as a self-perpetuating immune idiopathic reaction that results in tissue damage and inflammation.

Autoimmune diseases can be classified as systemic or local, depending on the extension of inflammation and on the presence or absence, respectively, of autoantibodies. In the first group are included diseases as rheumatoid arthritis (RA), dermatomyositis, scleroderma and systemic lupus erythematosus (SLE); diseases as psoriasis (dermatologic), multiple sclerosis (MS, neurologic), or inflammatory bowel

disease (IBD, gastrointestinal) are part of the second group. IL-17, alone or in association with TNF- $\alpha$  have been described as important for the pathogenesis and development of numerous of these diseases (14).

RA, being the expertise of our research group, was used as the model of chronic inflammatory disease in this work. Moreover, RA is a systemic disease, but its manifestation is primarily local: the articulation is destroyed by the perpetuation of inflammation, which causes tissue destruction with issues in self-reparation, tissue infiltration by immune system cells and neo-angiogenesis (19).

### 1.3 Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic, inflammatory, autoimmune disease of unknown etiology. The overall world prevalence of RA is approximately 1% to 2%. The physiopathology of RA is still not clear but involves a complex interplay of different causes: **genetic**, **hormonal** and **environmental**.

**Genetic** factors represent a high risk in developing RA, with an estimated heritability of 60%. There are more than 30 known polymorphisms associated with RA, notably on the gene PTPN22 (20). The most significant genetic risk factors for RA are variations in human leukocyte antigen (HLA) genes, especially the HLA-DRB1 gene. Several alleles of this gene (HLA-DRB1\*0401, \*0404, \*0101) contain a common amino acid motif (QKRAA), called shared epitope, which confer an increased susceptibility to the disease (21).

The frequency of RA is three times higher in woman, indicating an implication of sexual **hormones** in triggering the pathology, but this tendency is decreasing with age. Estrogens are implicated as enhancers of humoral immunity, while androgens

and progesterone are natural immune suppressors (22). Coherently, several studies demonstrated a relative low androgen levels in RA patients (23).

Between the **environmental factors**, infectious agents (e.g., Epstein–Barr virus, cyto-megalovirus, proteus species, and Escherichia coli) and their products (e.g., heat-shock proteins) have long been linked with rheumatoid arthritis and, although unifying mechanisms remain elusive, some form of molecular mimicry is postulated (24). The gastrointestinal microbiota has also been implicated in triggering autoantibody production, depending on the present bacteria (25). The idea that intestinal micro-organisms are associated with the development of RA comes from the evidence that the gut microbiota helps to maintain the homeostasis balance of intestinal immunity and regulates the immune response. Moreover, associations between the presence of periodontal disease and RA have been described in literature (26, 27). Most recently, *Porphyromonas gingivalis*, a periodontopathic bacterium, has been recognized as a possible link between periodontitis, peptide citrullination, autoantibody formation and joint inflammation (28, 29).

Smoking and other forms of bronchial stress (e.g., exposure to silica) increase the risk of rheumatoid arthritis among people with susceptibility HLA–DR alleles. The association between toxic metals exposure via cigarette smoking and rheumatoid arthritis incidence was also evaluated as an independent risk factor, concluding in a possible synergistic effect with other risk factors for RA development (30). Exposure to heavy metal also occurs with water and food assimilation, or occupational work and could represent an environmental risk factor for RA.

### *1.3.1 Systemic inflammation in RA*

RA is a systemic inflammatory disease characterized by heterogeneity in features and localization that can involve tissues and organs other than synovial joints. For that reason the severity and prognosis of RA can also vary significantly among individuals.

Extra-articular manifestations of RA are well described (skin, ocular, cardiovascular, pulmonary, etc.) and they are the main cause of co-morbidity and premature mortality (50-60% due to cardiovascular lesions) (31).

With the discovery of the rheumatoid factor (RF) in the '50s, it was highlighted that infections, or other factors, may trigger the induction of systemic inflammation. RF is a high-affinity auto-antibody against the Fc portion of immunoglobulin and may form with it immune complexes, which contribute to the disease. RF has long served as a diagnostic marker of RA and is present in 80% of patients and indicates a more aggressive phenotype. However, RF is not specific of RA (32).

In the late 90s, antibodies directed against citrullinated proteins (ACPAs) were discovered. In its earliest stages, even before joint inflammation occurs, RA was thought to begin with a series of events in which the immune system lost its ability to distinguish between what belonged to the body (the "self") and what is foreign to the body (the "non-self"). According to a new theory, the systemic process of inflammation begins with inadequate clearance of cells as they die. As cells reach the end of their lifespan, they undergo citrullination, a process in which enzymes called peptidylarginine deaminases (PADs) convert arginine amino acid residues of proteins within the cell into citrulline ones (33). This is a normal part of cell physiology. However, in most patients who develop RA, PADs leak from dying cells

throughout the body and citrullinate proteins in the extracellular fluid. These citrullinated proteins are selectively recognized for their acid pH as antigens by B-cells, which produce ACPAs. High levels of ACPAs are detectable in plasma or serum of RA patients and leads to an increase of the systemic inflammatory response (34).

Blood levels of different inflammatory cells and pro-inflammatory cytokines were measured in RA patients and compared to healthy or osteoarthritis (OA, non-autoimmune and less inflammatory form of arthritis) controls. Higher level of Th17, but not Th1, were found in the blood of RA patients in comparison to OA patients (35) and healthy controls (36). These results are in contrast with previous data showing no difference in Th17 in the blood of RA patients compared to controls in the early onset of arthritis (37). Moreover, the concentrations of IL-17, IL-21, and IL-22, all produced by Th17 cells, were significantly elevated in RA patients compared to healthy individuals. Furthermore IL-22 level in the serum also positively correlated with the disease activity score (36).

### *1.3.2 Local inflammation in RA*

The reason why the systemic loss of tolerance is linked to a localized onset of inflammation in the joint is still unclear, even if the biological features of auto-antigens (e.g. ACPA binding citrullinated fibrogen in joints (38)) or other biomechanical/microtrauma mechanisms can be involved.

The synovium, or synovial membrane, is the inner part of the joint, which function is to produce synovial fluid (SF), necessary to lubricate and to avoid cartilage-cartilage contact. The synovium is composed by a two-layer structure, the sub-intima and the intima; the subintima is composed by connective and adipose

tissues. The intima is a pauci-cellular structure composed by mesenchymal fibroblastic cells, named synoviocytes. In the normal condition synoviocytes are the main regulators of synovial fluid volume, immune response and leucocyte circulation as well as to the maintain of the membrane stability (39).

In the appropriate conditions, such as the presence of the specific HLA-DR alleles or perhaps cytokine-promoter polymorphisms, the inflammatory process modifies the structure of the synovium with a series of dynamic events:

Synovial membrane hyperplasia due to the acquisition of an anti-apoptotic and aggressive phenotype by synoviocytes

Synovium inflammation, with production of pro-inflammatory cytokines and chemokines to attract inflammatory cells

Immune cell infiltration in the synovial cavity, principally macrophages, B lymphocytes and CD4+ T lymphocytes and further production of cytokines

Neo-angiogenesis and proliferation of synovial cells responding to the produced cytokines

All these changes of the synovial structure are primarily due to changes at the cellular level. It is increasingly clear that the synoviocyte, rather than being simply a passive bystander, is central to the RA process and a principal actor in RA pathogenesis. Synoviocytes hyperplasia can precede the accumulation of inflammatory cells in the synovium but whether this is primarily a cause, or a consequence of the disease, remains a matter of conjecture (39).

Mechanisms for the accumulation of synoviocytes can be different inducing decreased senescence, decreased apoptosis or hyperproliferation, but always

implicating an unbalanced ratio between proliferation and apoptosis. Synoviocytes heterogeneously express a variety of pro-mitotic and anti-apoptotic mutations (as *Fas* or *p-53* abnormalities) (40), as well as several growth factors inducing their hyperproliferation. Moreover synoviocytes acquire an aggressive phenotype producing molecules increasing their invasive properties and contributing to the production of pro-inflammatory cytokines (as IL-6), metalloproteases (MMPs) and other enzymes responsible of cartilage destruction. The local production of cytokines induce the SDF1/CXCR4, HIF-1 $\alpha$  pathways for cell migration and increasing the invasive phenotype of synoviocytes (41).

Synoviocytes produce an array of chemokines that signal cells to migrate into the joint. IL-8 produced by synoviocytes is a potent neutrophil attractant that also stimulates angiogenesis. This, along with numerous other chemokines, including RANTES, IP-10, ENA-78, and MCP-1, MIP-1 $\alpha$ , and MIP-1 $\beta$  are major monocyte/macrophage recruiting proteins that are produced by synoviocytes in response to IL-1 or TNF stimulation. Release of these chemoattractants contributes to the cell mix that is subsequently recruited to the inflamed joint (42).

#### 1.3.2.1 Cell interactions and produced soluble factors

The interaction between synoviocytes and the immune cells stimulates the survival of the latter by maintaining survival signals and blocking apoptosis signals. Cell interaction also induce pro-inflammatory cytokines production from immune cells (as IL-17 and TNF- $\alpha$ ). Synoviocytes respond to the IL-17 and TNF- $\alpha$  synergistic stimulation by producing IL-6 (43). IL-6 induces chemokines (mostly monocyte attracting) as CCL2/CCL5 and further activates osteoclasts, T and B lymphocytes (44).

Among immune cells, macrophages are major producers of TNF- $\alpha$ , IL-1 $\beta$  and IL-6. TNF- $\alpha$  is a primary cytokine of the inflammatory cascade, regulating the production of chemokines and IL-6 from synoviocytes. Furthermore, TNF- $\alpha$  contributes to the neo-angiogenesis and to the activation of the inflammatory phenotype of synoviocytes (45) and consequently to the production of the connective tissue growth factor (CTGF). CTGF promotes aberrant activation of osteoclasts and disturbs the cartilage homeostasis, ultimately resulting in the destruction of the joint (46). IL-1 $\beta$  is even more potent than TNF- $\alpha$  in inducing cartilage destruction (47).

In the synovium, auto-reactive B cells can differentiate in plasma cells and produce autoantibodies such as RF and ACPA. B cells are also highly capable antigen-presenting cells and can contribute to auto-reactive T cell activation. B cells also produce several cytokines, such as IL-4 and IL-10, which promote leukocyte infiltration in the joint, angiogenesis and synovial hyperplasia (48).

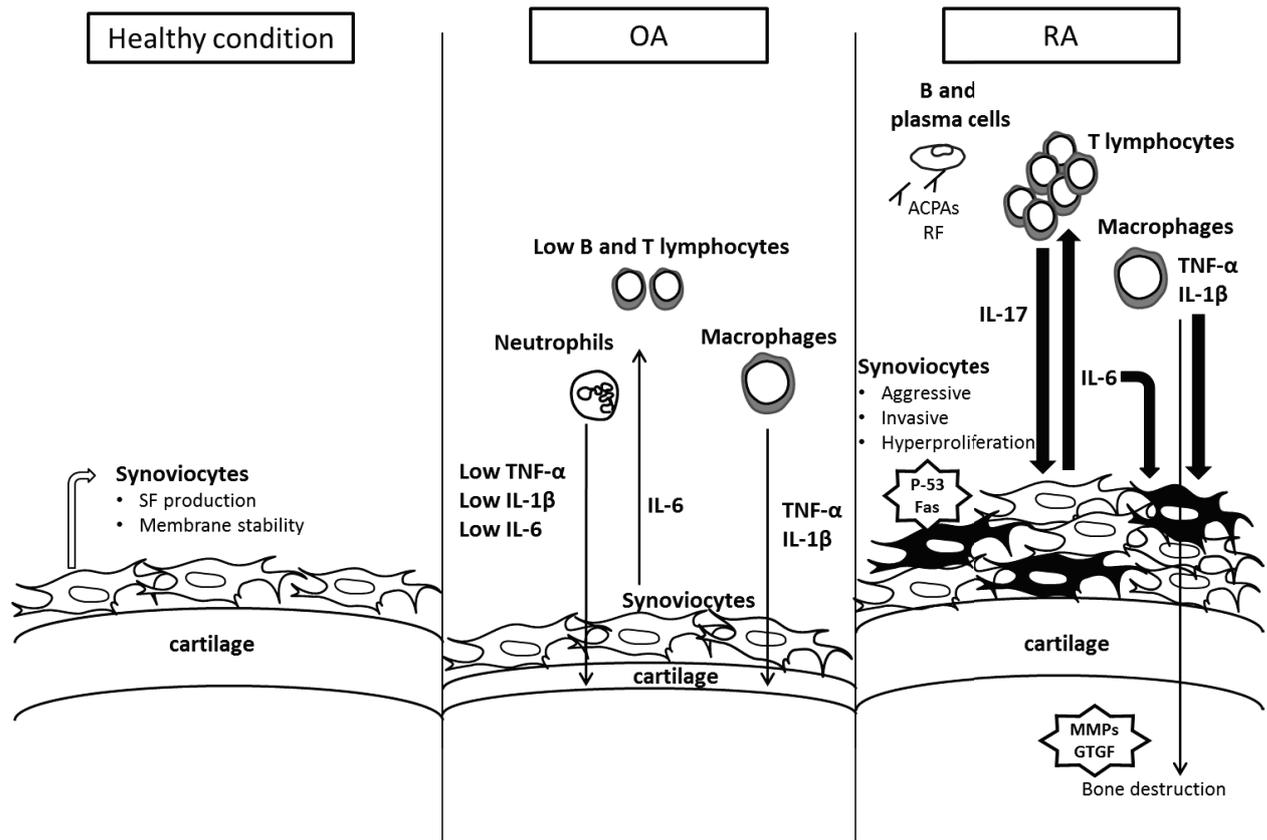
T-cells are one of the most abundant cells in the rheumatoid synovium and the majority of them are CD4<sup>+</sup> Th cells. RA has traditionally been considered associated with an 'immunological shift' from Th2 to Th1, leading to a disturbance in the healthy balance between the Th derived cytokines (INF- $\gamma$  from Th1 increased and IL-4 from Th2 decreased). Nowadays, data suggests the cardinal importance of Th17 cells in the pathogenesis of RA. The Th17 cells infiltrate in the synovium and their level is strongly higher in SF, in comparison to peripheral blood in RA patients (37). In the synovium Th17 produce IL-17 and the cytokine overexpression, which is further responsible for osteoclastic bone resorption, was previously measured in RA patient SF in comparison to OA (49, 50).

An unbalanced ratio of Th17/Tregs cells in the SF of RA patients was also described. The unbalance was due to the IL-6 dependent suppression of the TGF- $\beta$  mediated FoxP3 induction (51). Recently increased levels of IL-17-producing Tregs were identified in RA patients. This Treg subset with Th17 plasticity in peripheral blood retained suppressive functions and was associated with milder inflammatory conditions, suggesting that this Treg population works as a negative regulator in RA. Contrarily, at the synovial site, they lose their suppressive activity and their expansion is induced by IL-6 (52). Their Th17 residual activity in the synovium may be pathogenic in RA.

In contrast to IL-17 cytokine, which is mainly produced by Th17 cells, IL-17 receptors are ubiquitously expressed. Receptor expression is increased on synoviocytes of RA patients.

Moreover, CXCR4 is expressed by CD4<sup>+</sup> memory T cells in RA synovium and its unique ligand, CXCL12 (stromal cell-derived factor-1) is expressed on synoviocytes (8, 44).

Taken together these data sustain the description of RA as a systemic disease with a prevalent local manifestation. At this site, the interaction between synoviocytes and the cells of the immune system is pivotal in the perpetuation of inflammation, which in turn causes a transition of the synoviocytes from normal to “cancer-like” RA cells (represented in the **figure 3**).



**Fig. 3. Inflammatory status transition from healthy to RA condition in joints.** Cell composition changes between the healthy and RA conditions modify the status of joints and amplify inflammation

The role of the synoviocyte is thus central in RA pathogenesis and for RA perpetuation, even if it is still under-estimate in the choice of RA treatment.

### 1.3.3 RA Treatment

The denomination “disease modifying anti-rheumatic drugs” (DMARDs) indicates treatments able to impact the radiological progression of RA. DMARDs comprise a heterogeneous group of compounds with different target, biochemical and pharmacokinetic properties; nevertheless, they can be mainly divided in two large groups: synthetic DMARDs and biological DMARDs. The discovery of the first has traditionally been based on empirical data, in contrast with the second agents that were developed from bench to bedside.

The most commonly used synthetic DMARDs in clinical practice are methotrexate, and glucocorticoids (GC). Methotrexate is a purine synthesis antagonist and is considered to be the gold standard for treatment of RA (53). Since the DNA synthesis is the target of methotrexate, high rate reproducing cells, as the immune cells, are the most affected cells, even if the mechanisms of actions are still not completely clear (54). GCs are part of the group of corticosteroids and can be used for systemic or local therapy. They increase the production of anti-inflammatory cytokines and reduce pro-inflammatory ones acting at different levels (genomic and non-genomic) (55).

A deeper understanding of the pathophysiological and immunological mechanisms in RA has led to the development and introduction of biologic DMARDs, which gave relevant effect in patients with severe RA, difficult to control with conventional DMARDs. Currently, the available drugs in RA therapies are:

- Anti-TNF drugs (most commonly used)
- A IL-1 $\beta$  blocker
- A B-cell depleting agent
- An IL-6 blocker
- A T-cell co-stimulation inhibitor

The common feature of all DMARDs, independently of their synthetic or biologic nature, is the target of immune cells, leading to reduce inflammation systemically and in the joint. Even when the treatment is used locally, the principal targets are inflammatory cells, but not mesenchymal cells.

### 1.3.3.1 Local treatment

Nowadays local-treatment is mainly used in mono-articular arthritis or in rheumatoid arthritis patients where only 2-3 joints are involved. Nevertheless, there is increasing evidence, from clinical trials, that more RA patients will attain the target of remission using a combination of systemic medication and local intra-articular therapy.

Synovectomy is also a form of local treatment for rheumatoid arthritis and it's the only treatment targeting synoviocytes instead of inflammatory cells. The value of synovectomy has been demonstrated by a large number of studies, for over 30 years. The procedure can be surgical (synovectomy as surgical removal of a part of synovium) or non-surgical (synoviorthesis) which involves the intra-articular administration of an agent in order to induce fibrosis in the inflamed synovium (56); such agents may either be chemical based (osmic acid, rifampicin) or radioactive ( $^{90}\text{Y}$  isotope of yttrium or  $^{198}\text{Au}$  isotope of gold). Between those two, synoviorthesis was chosen as the first choice treatment for persistent synovitis of the joints: it is a simple procedure, which eliminates the risks associated with surgery (56).

In 1973, Menkes et al. reported their experience with the use of intra-articular osmic acid (57) but their procedure never achieved such popularity and osmic acid was never approved by the French Health Authorities and registered as a drug or a medical device for use in human being (research use only, the injections were performed under responsibility of physicians) (58). This was due mainly to the negative benefit/risk ratio according to the European legislature about pharmaceutical quality and to the occurred complications and significant pain associated with the procedure of injection (59). Furthermore, osmic acid did not have a selective effect on the synovial layer and caused a brown colouration of the

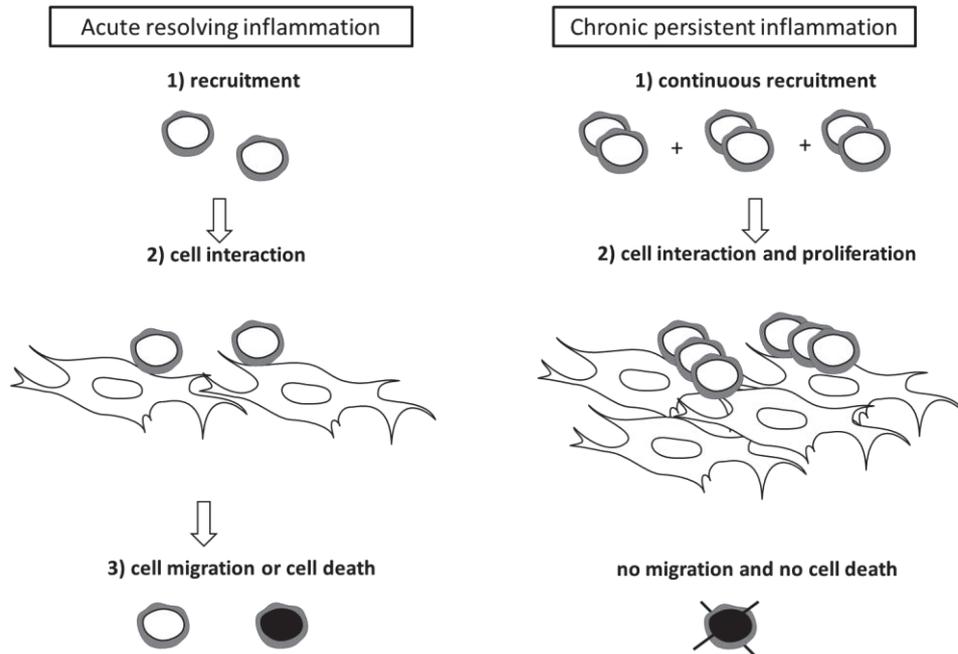
cartilage, which allows a certain amount of scepticism to be held in respect to the assurance that no actual damage to cartilage had been proven. Moreover, with osmic acid there was a wide discrepancy between the high success quota (90-95%) opposed by an improvement in only 50% after one year from injection (57, 58).

In 1980, Caruso et al. described the use of Rifampicin for the treatment of synovitis in RA but still with a high failure rate in the procedure (60).

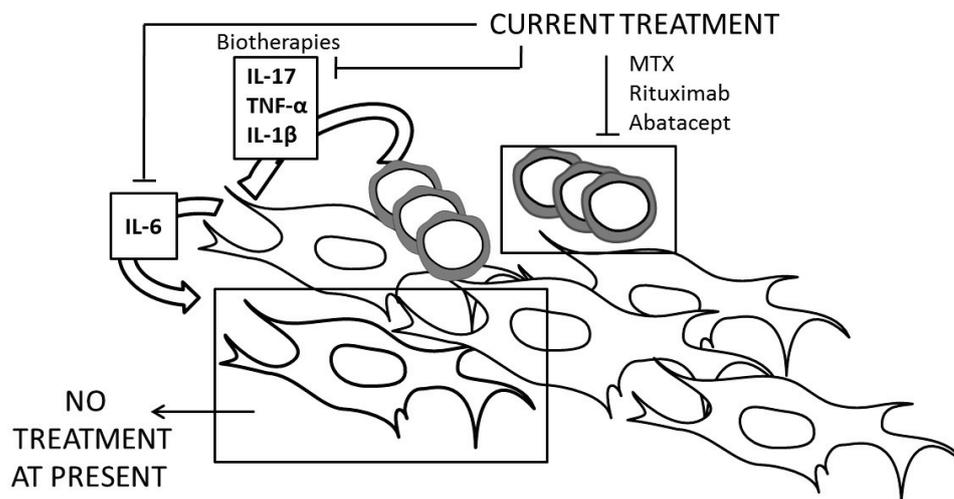
Radioactive synoviorthesis was introduced as an effective, relatively simple, virtually painless and comparatively inexpensive technique for the treatment of chronic synovitis. Global results obtained with osmic acid seem to be less favourable than those with radioisotopes except for small joints where results were comparable (61). Thus, radioactive synoviorthesis was thought to be the best choice for patients with persistent synovitis but studies on the chromosomal changes that could be attributed to the radioactive material had to be performed. After 40-years of screening, several long-term safety issues have been reported.

The central role of the synoviocyte is described in the different stages of RA pathogenesis (immune cell recruitment at the site of inflammation, inhibition of their apoptosis, perpetuation of inflammation (**Figure 4A**)) as well as the lack of a treatment which can specifically target it (**Figure 4B**). The use of non-radioactive isotopes targeting synoviocytes was evaluated in this work, with the aim of modulating hyperplasia and synovitis with an innovative local treatment.

A



B.



**Fig. 4: Cell involvement and current treatment in RA.** Stages of inflammation in RA. (A) On the left panel the acute (resolving) inflammation, on the right the chronic (non-resolving) inflammation. (B) Current treatment for chronic inflammation targeting immune system (cells and produced cytokines) but not synoviocytes

## 2. Transition metals and group 12

The biochemical principles of life are based on both organic and inorganic chemistry. Inorganic biochemical aspects include the role of essential transition metal ions in maintaining human life and how they support growth and development. Moreover, several of these transition metal ions were shown to have functions in modulating the immune system. For that reason, the effects of several metals on the inflammatory response will be analyzed in this work with particular focus on the chronic inflammatory disease RA.

The transition elements are elements having a partially filled d or f subshell in any common oxidation state. The term "transition elements" most commonly refers to the d-block transition elements and contains the group 12 of divalent metals as represented in the figure below (**Figure 5**)

Main group elements		Transitional metals										Main group elements						
H																		
Li	Be																	
Na	Mg																	
K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Al	Si	P	S	Cl	Ar	
Rb	Sr	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	Ga	Ge	As	Se	Br	Kr	
Cs	Ba	La	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	In	Sn	Sb	Te	I	Xe	
Fr	Ra	Ac	Rf	Ha	106	107	108	109					Tl	Pb	Bi	Po	At	Rn

**Fig. 5: the group 12 of transition metals.** Transition metals are represented in pink on the table of elements. The group 12 is part of transition metals and composed by Zinc, Cadmium and Mercury (Adapted from Bodner research web)

The group 12 of elements, composed by zinc (Zn), cadmium (Cd) and mercury (Hg), does not strictly meet the defining properties of transition elements, but it is usually included with this group because of their similar properties. The general properties of the transition elements are:

- They are usually high melting point metals.
- They have several oxidation states.
- They usually form colored compounds.
- They are often paramagnetic.

Transition metal ions as manganese, iron, copper, and zinc are constituents of thousands of proteins and function in enzymatic catalysis and protein structure. Transition metal ions regulate protein functions and proteins regulate their availability. For this reason the correct regulation of metal homeostasis is fundamental for cells.

Cell metal ion homeostasis requires multiple proteins for transport, sensing, chaperoning, and other functions in a network of tightly controlled interactions and with full integration into metabolism and signaling. The metal-regulatory proteins employ specific molecular mechanisms. One mechanism is the sulfur-ligand centered reactivity in zinc/thiolate coordination environments. Sulfur donors confer redox activity on the otherwise biologically redox-inert zinc ion. This coupling between zinc and redox metabolism provides a way of controlling zinc binding and protein functions (62).

Acting on the mechanisms of homeostasis regulation of transition metals, which are integrated in cellular structure and functions, we could regulate numerous cellular activities. Altering metal ions concentration could possibly have

pharmacological or toxic action, and will aid in developing strategies for preventing, diagnosing, or treating human diseases.

### 2.1 Metal quantification

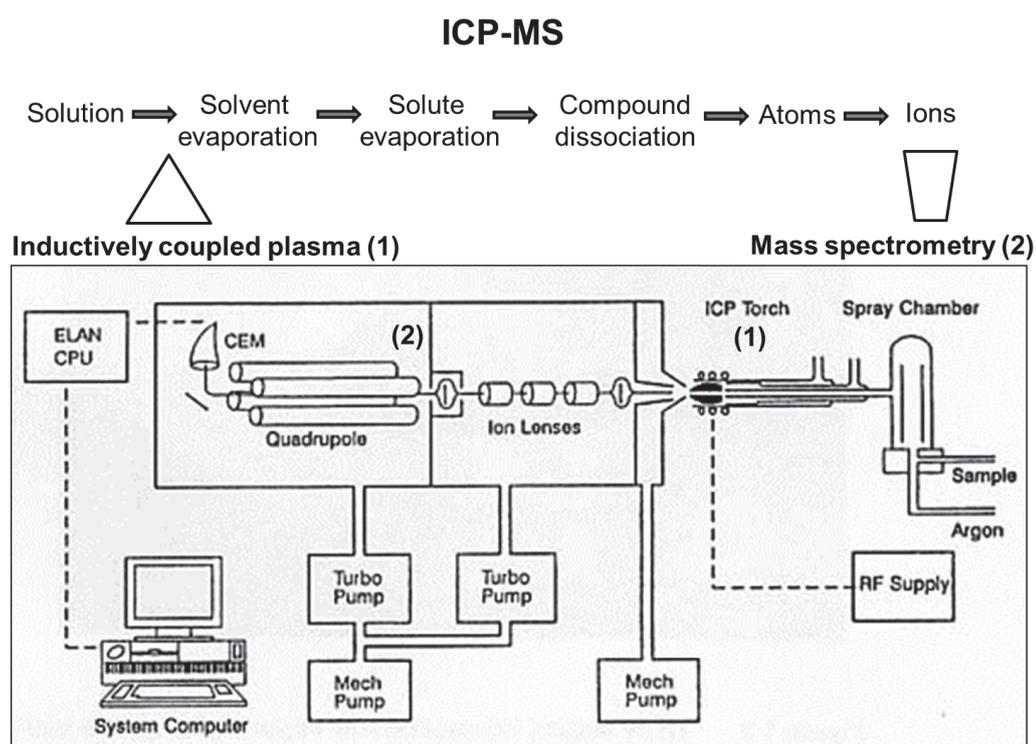
Nowadays, several techniques are available as optical methods of chemical analysis. Many techniques perform the elemental analysis on solutions, so samples need to be at the liquid status. The liquid is then transformed or introduced in the machine as a fine aerosol.

In flame atomic absorption spectrometry (FAAS), either an air/acetylene or a nitrous oxide/acetylene flame is used to evaporate the solvent and dissociate the sample into its component atoms. When light from a hollow cathode lamp passes through the cloud of atoms, the atoms of interest absorb the light from the lamp. This absorption is measured by a detector and used to calculate the concentration of that element in the original sample. The use of the flame limits the excitation temperature, which can be a problem for some elements and moreover the detection limit is in the parts-per-million (ppm) range and limited to 10 elements per analysis.

Inductively coupled plasma atomic emission spectrometry (ICP-AES), or simply ICP, is a multi-element analysis technique that uses an inductively coupled plasma source to dissociate the sample into its constituent atoms or ions, exciting them to a level where they emit light of a characteristic wavelength. A detector measures the intensity of the emitted light and calculates the concentration of that particular element in the sample.

Inductively coupled plasma mass spectrometry (ICP-MS) uses the same source of ICP. However, in this case, the ions themselves are detected, rather than the

light that they emit. The ions are extracted from the plasma and passed into the mass spectrometer, where they are separated based on their atomic mass-to-charge ratio by a quadrupole or magnetic sector analyzer. The high number of ions produced, combined with very low backgrounds, provides the best detection limits available for most elements, normally in the parts-per trillion (ppt) range. Quadrupole mass spectrometers in ICP-MS fulfill exacting requirements that demand the ultimate detectability and isotope ratio measurement, which were needed in our analysis. A schematic representation of the process in ICP-MS is represented in the **Figure 6** below:



**Fig. 6: Inductively coupled plasma mass spectrometry process.** Liquid samples are evaporated using a plasma torch inductively activated using the argon flux. Compounds of the solute are dissociated in atoms, and then ions, which can be extracted into the mass spectrometer. The quadrupole mass spectrometer divides ions on the basis of their  $m/z$  ratio and a channel electron multiplier (CEM) detects ions passed by the mass filter and send the values to the system computer for analysis.

## 2.2 Use of metal isotopes

The name isotope often reminds of the use of radioactive molecules for medical treatment, as described above in the treatment dedicated chapter. More generally, the term “isotope” can be used to describe two or more variants (isotopologues) of a molecule that differ in the isotopic identity of one or more of their constituent atoms (63). For example,  $^{64}\text{Zn}$ ,  $^{66}\text{Zn}$  and  $^{70}\text{Zn}$  are three of the naturally-occurring isotopologues of molecular Zn.

Isotopes variability between two different isotopologues of the same element is known as the “isotope effect”, a term describing the mass-dependent variations of natural isotope abundances for a particular element (63). This phenomenon is due to an increased abundance of a specific element among coexisting molecular species, or differential partitioning through reservoirs of the human body. The distribution varies as a function of the bond strength, the ionic charge and the coordination, and it also changes with kinetics. The track of the availability of isotopes, and mainly of copper (Cu), Sulfur (S) and zinc (Zn), gave the evidence of a specific fractionation of these elements in the diseased tissues (64, 65). The differences found between healthy and diseased subjects can be developed as a complement marker for cancer and/or inflammatory diseases. A supplementary descriptive work is needed to complement information, mainly about their transport, turnover and effect in human cells.

Preliminary investigations on the role of isotopes in healthy and diseased subjects are reviewed in the following review paper (Albarède F. et al, submitted). Nevertheless, isotope applications are not only related to this type of analysis (fractionation normal/ill tissue) but isotopes can be used for supplementary

investigations in the *in vitro* model, and maybe also in the *in vivo* model, as tracer, to avoid the application of radiotracers or fluorescent dyes.

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### Medical Applications of the Cu-Zn-S Isotope Effect

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#### Abstract

20 This review examines recent applications of stable copper, zinc and sulfur isotopes to  
medical cases and notably cancer. The distribution of the natural stable isotopes of a  
particular element among coexisting molecular species vary as a function of the bond  
strength, the ionic charge, and the coordination, and it also changes with kinetics. *Ab*  
25 *initio* calculations show that compounds in which metal binds to oxygen- (sulfate,  
phosphate, lactate) and nitrogen-bearing moieties (histidine) favor heavy isotopes,  
whereas bonds with sulfur (cysteine, methionine) favor the light ones. Oxidized cations  
(e.g., Cu(II)) and low coordination numbers are expected to favor heavy isotopes  
relative to their reduced counterpart (Cu(I)) and high coordination numbers.

30 Here we discuss the first observations of Cu, Zn, and S isotopic variations, three elements  
closely related along multiple biological pathways, with emphasis on serum  
samples of healthy volunteers and of cancer patients. It was found that heavy isotopes  
of Zn and to an even greater extent Cu are enriched in erythrocytes relative to serum,  
while the difference is small for sulfur. Isotopic variations related to age and sex are  
relatively small. The  $^{65}\text{Cu}/^{63}\text{Cu}$  ratio in the serum of patients with colon, breast, and  
35 liver cancer is conspicuously low relative to healthy subjects. The characteristic time  
over which Cu isotopes may change with disease progression (a few weeks) is consistent  
with both the turnover time of the element and albumin half-life. A parallel  
effect on sulfur isotopes is detected in a few un-medicated patients. Copper in liver  
tumor tissue is isotopically heavy. In contrast, isotopic shifts in the Zn of cancer patients  
are small and possibly insignificant. Possible reasons for Cu isotope variations  
40 may relate to cytosolic storage Cu lactate (Warburg effect), release of intracellular  
copper from cysteine clusters (metallothionein), or may reveal the hepatocellular and  
biosynthetic dysfunction of the liver. We suggest that Cu isotope metallomics will  
help evaluate the homeostasis of this element during patient treatment, notably by  
chelates and blockers of Cu trafficking, and understand the many biochemical pathways  
45 in which this element is essential.

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### Introduction

- For most people outside of geochemistry and physics, whenever the word 'isotope' is heard or read, it calls attention to radioactive nuclides used for dating, such as carbon 14, and for medical applications such as cobalt 60. It is also suggestive of nutrition studies in which enriched stable isotopes are added to the diet of volunteers to monitor the transit of a particular element<sup>1</sup>. Stable-isotope probing (SIP) is a related technique used in microbial ecology<sup>2</sup>. All these techniques are invasive in the sense that they interfere with the normal metabolism, even if it is usually to a trivial extent. The natural fractionation of conventional stable isotope systems involving ubiquitous elements such as C, H, O, N, and S, is only rarely used for medical purposes because they lack specificity with respect to biological processes. Metals such as alkaline-earth Ca and Mg and transition elements such as Cu and Zn, however, more promising because of their much smaller number of functional roles in biology and also because their turnover rate in the body is relatively short. Copper plays a major role in oxidizing iron and controlling electron fluxes, while Zn is a cofactor of hundreds of important enzymes. Iron is involved in a large number of biological functions and, because of the very large stores contained in the red blood cells, the muscles and the liver, its overall turnover time is of several years<sup>3</sup>. It is an essential component of heme, a cofactor made of large heterocyclic porphyrin rings. Heme is the active component of hemoglobin and myoglobin, which are metalloproteins used by the body to shuttle oxygen and carbon dioxide in blood and muscle. The purpose of the present essay is to expand the review of some appealing applications of stable metal isotopes to medicine recently published by Albarede<sup>4</sup>.
- Isotope variability is known as the *isotope effect*, a term describing the mass-dependent variations of natural isotope abundances for a particular element. The isotope effect is a consequence of the Heisenberg uncertainty principle on the distribution of energy levels of molecular vibrations. Quantum mechanics rules that the velocity and the position of a particle cannot be simultaneously known with an infinite precision. Bonds never come to rest and their lowermost energy state is referred to as the zero-point energy. This energy depends on the mass  $M$  of the bonding atoms, a character that is at the origin of isotopic variability of elements between different parts of a system such as different biological compartments.
- Otto Warburg and Adolf Krebs found in 1928<sup>5</sup> that serum copper levels increased in various chronic diseases and several types of cancers, resulting into a systemic and oncogenic<sup>6</sup> copper accumulation. Anomalously high Cu levels or Cu/Zn ratios were indeed observed in the serum of breast cancer<sup>7,8</sup> and serum ceruloplasmin was found to be significantly elevated in advanced stages of solid malignant tumors<sup>9</sup>. In itself, such observations justify that copper isotopic variability should be investigated in cancer patients. The connection between Cu and Zn through proteins such as superoxide dismutase and metallothionein involved in the control of hypoxia, and therefore of cancer in particular, also justifies interest in zinc and in the amino acids that control the coordination of both metals, notably histidine, cysteine and methionine. Hence the importance of exploring the extent of correlated sulfur isotopic variations.
- In contrast with organic biomarkers, isotope compositions can be analyzed on biological samples years after the samples have been taken. Metal isotope abundances are immune to oxidation, as they are unreactive to any chemical or biological reactions

1  
2  
3 taking place in the original sample container, even when exposed to the atmosphere.  
4 Here, we will review the variations in the abundances of stable isotopes of Cu and Zn,  
5 95 two metals tightly related in cellular and physiological activity, *naturally* present in  
6 the body of humans and other organisms. The very first Cu and Zn isotope data on  
7 blood<sup>10-21</sup> show promising relationships of isotope Cu and/or Zn compositions with  
8 age, sex, and pathologies. Iron isotopes will be left out as they have been mostly ap-  
9 plied to the iron-related disease of genetic hemochromatosis<sup>22-25</sup>. Although isotopic  
10 data on organs will also be discussed, we will focus on serum for a reason of feasibil-  
11 100 ity: it is a chemically stable liquid medium, much more available in contrast with bi-  
12 opsies and resections, even for healthy subjects, and which is commonly accessible  
13 from bio-banks. In order to assess the role of sulfur-rich amino acids and proteins and  
14 in particular the well-established connection between zinc and sulfur biochemistry  
15 through redox control<sup>26</sup>, we will also review some recent observations on the sulfur  
16 105 isotope composition of biological samples<sup>18,27</sup>.

### The isotope effect

20 Isotope fractionation is a general term referring to the variability in the isotopic abun-  
21 dances of a particular element among coexisting species (e.g., sulfide and sulfate for  
22 S) or reservoirs (e.g., S in serum and red blood cells) hosting this element. It can be  
23 110 explained in a simple way: (1) vibrational frequencies decrease approximately with  
24  $M^{1/2}$ , while bond energy  $E$  varies with vibrational frequency  $\nu$  according to  $E = (n + 1/2)$   
25  $h\nu$ , while  $h$  is the Planck constant and  $n$  a non-negative integer charac-  
26 terizing the energy 'level'. Favoring heavier isotopes in the lowermost energy levels  
27 therefore is a way of reducing the total energy of the system. High temperatures work  
28 115 to randomize the distribution of isotopes across energy levels. At ambient tempera-  
29 tures, however, the total energy is minimized when heavy isotopes concentrate into  
30 the 'stiffest' bonds, those with the lowest and therefore most stable energy levels<sup>28-32</sup>.  
31 For a given element, the strength of a particular bond is expected to be higher for the  
32 smaller ions with the higher charge and therefore developing the strongest field and  
33 120 when the overall binding energy at the site of the metal is shared among fewer part-  
34 ners. Bonds involving high oxidation states ( $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ ) and sites with small coordina-  
35 tion numbers therefore prefer heavy to light isotopes. It is worth noting at this stage  
36 that isotope variability is a very subtle phenomenon: when differences are noted be-  
37 125 tween 'light' and 'heavy' zinc or copper, a short for 'depleted' or 'enriched', respec-  
38 tively, in heavy isotopes, the effects always remain in the range of a few parts in one  
39 thousand that only modern mass spectrometry has been able to resolve.

44 In addition to the effects just described for systems at thermodynamic equilibrium, the  
45 smaller activation energy of the lighter isotopes allows them to react faster: kinetic  
46 130 effects have been advocated as a cause of biologically mediated isotope fractio-  
47 nation<sup>33</sup>, but they require either non-steady state conditions (the system grows) or the  
48 existence of competing reaction pathways (Fig. 1). What comes around goes around:  
49 the proportion of isotopes present within a system (cell, organ, body fluid) must vary  
50 if they are imported and exported at different rates. Imagine that you pick up the in-  
51 135 coming mail from your mailbox but leaving the letters coming in manila envelop be-  
52 hind, it will not take long for the content of your mailbox to take a buff color! For a  
53 period of time exceeding the mean turnover or residence in the system, input and out-  
54 put must be precisely balanced. When a pathway involves multiple outputs for a sin-  
55 gle output, the abundance of the different isotopes of a specific element may not be  
56 140 identical in each branch: this is the nature of isotope fractionation.

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3 Isotopic abundances are measured using mass spectrometers. Except for hydrogen, the  
4 isotope effect is normally very small, with variations of isotope abundances rarely ex-  
5 ceeding one part per 1000 per unit of mass difference. Measuring such small variations  
6 requires a mass spectrometer with high transmission and a magnetic mass filter (sec-  
7 145 tor). Inexpensive quadrupole mass spectrometers do not provide the precision needed  
8 for significant signal-to-noise ratios. For decades, only the isotopic abundances of el-  
9 ements that could be measured after introduction into a gas source (electron bom-  
10 bardment), such as H, C, N, O, S would be dealt with most commonly on compounds  
11 such as CO<sub>2</sub> or SO<sub>2</sub>. Mass fractionation in the mass spectrometer itself (mass bias)  
12 150 would be dealt with by swiftly alternating standard material with the samples with  
13 calibrated inlet valves. Isotopic variations are reported on a relative scale, typically  
14 the delta scale, for instance for <sup>65</sup>Cu:

$$\delta^{65}\text{Cu} = \left[ \frac{(^{65}\text{Cu}/^{63}\text{Cu})_{\text{sample}}}{(^{65}\text{Cu}/^{63}\text{Cu})_{\text{standard}}} - 1 \right]$$

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22 It is common practice to place the heavy isotope at the numerator. The gas source  
23 would, with few exceptions such as SiF<sub>4</sub>, be inefficient for metallic elements: short of  
24 155 an efficient normalization technique, the variations of metal isotope abundances has  
25 until lately remained largely unexplored. Double-spike techniques, in which the abun-  
26 dance dependence of mass fractionation is used, would relieve the constraint for ele-  
27 ments with *four* stable isotopes or more (Fe and Zn). This technique is, however, ra-  
28 ther time consuming and only found only limited applications<sup>34</sup>. In the mid 90s, Mul-  
29 160 tiple Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICP-MS) quick-  
30 ly emerged as a game changer as the technique based on very efficient ionization,  
31 high transmission, combined with sample-standard bracketing would allow unprece-  
32 dented precision (typically 0.01-0.05 parts per 1000) on metal samples as small as a  
33 few tens of nanograms of metal. The major difficulty of this technique is that the met-  
34 165 al to be analyzed represent traces in a matrix of organic material loaded with major  
35 elements such as Na, Cl, P, Mg, and Ca and the measure of its isotopic abundances  
36 cannot be determined on an unprocessed sample. The trace metal has first to be rigor-  
37 ously purified yet with a yield very close to 100 percent.

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42 170 Why take the trouble of measuring metal isotopic abundances, an excruciating and  
43 occasionally daunting task, instead of relying on the concentrations of the same metal  
44 in various parts of the body? The answer is that changes in copper or zinc concentra-  
45 tions are in general not amenable to quantitative predictions, whereas the direction  
46 and magnitude of the isotopic effect induced by bonding a metal with a chelate, typi-  
47 175 cally an amino acid such as cysteine or histidine, can be predicted by theoretical  
48 methods. In contrast with different elements, which can never truly substitute one an-  
49 other along all biochemical pathways, the isotopes of a given element behave similar-  
50 ly enough that variations in their relative abundance remain predictable. Decades ago,  
51 experimental determination of isotope fractionation of an element between coexisting  
52 180 compounds were the method of choice, but the results are now perceived as much less  
53 reliable than those obtained by the so called *ab initio* or first-principles theories. In  
54 addition, the challenge of obtaining results for the very large number of relevant or-  
55 ganic compounds is simply daunting. The most commonly used method is the Density  
56 Functional Theory or DFT<sup>35</sup>, a computational quantum mechanical modeling provid-  
57 ing the ground-state electronic structure of many-body systems. Each atom is consid-  
58 185 ered as being made of a nucleus and of orbiting electrons. Typically, each calculation

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3 is divided into two steps, one in which atoms are confined in a box and let to drift to-  
4 wards a stable molecular configuration and a subsequent step in which isotopes are  
5 substituted to infer the slight thermodynamic changes arising from the substitution.  
6 Obtaining results on compounds of biological interest is calculation intensive and re-  
7 quires special software and consistent databases.  
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10 Large proteins are so far beyond the reach of DFT but efforts to predict fractiona-  
11 tion of elements such as Fe, Cu, Zn, Ni, Ca have recently been made by a small  
12 number of groups. Isotope fractionation factors for ligand monomers, such as the  
13 most common amino acids (histidine, cysteine, methionine), glutathione, and  
14 carboxylic acids such as lactate, oxalate and citrate, have become available for Cu  
15 and Zn. As shown in [Tables 1 and 2](#), the data are tabulated as reduced partition  
16 functions  $\beta$  (usually as  $1000 \ln \beta$ ) and the order and amplitude of isotopic en-  
17 richment between two compounds 1 and 2 at equilibrium can be estimated as  
18  $\ln \beta_2 - \ln \beta_1$ . For example, the predicted  $^{65}\text{Cu}/^{63}\text{Cu}$  ratio in  $\text{Cu(II)(His)(H}_2\text{O)}_4^{2+}$  is  
19  $4.168 - 3.124 = 1.044 \text{ ‰}$  higher than in  $\text{Cu(II)(Cys)(H}_2\text{O)}_4^{2+}$ . [Table 3](#) shows some  
20 important stability constants for Cu and Zn chelates.  
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23 Some robust trends appear for Zn and Cu, two elements for which fractionation  
24 by amino acids and other organic ligands have been best studied:

- 25 (1) Isotope fractionation is less intense for Zn than for Cu  
26 (2) Cu(II) compounds are isotopically heavier than Cu(I) compounds  
27 (3) Electron donors with a strong electronegativity (N, O) and associated  
28 moieties ( $\text{NH}_2$ ,  $\text{SO}_4$ ,  $\text{PO}_4$ , OH, lactate and pyruvate, two carboxylic acids a  
29 with a side oxygen or hydroxyl) preferentially bind to heavy isotopes rela-  
30 tive to elements with smaller electronegativity, typically S and S-bearing  
31 amino acids (cysteine, methionine).  
32 (4) As demonstrated for zinc by the comparison between four- and sixfold  
33 coordination, preference for heavier isotopes decreases with increasing  
34 coordination numbers.  
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38 Understanding how these results relate to large proteins should certainly attract atten-  
39 tion in the future.  
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#### 41 [An Overview of Zinc, Copper, and Sulfur Biochemistry and Homeostasis](#)

42 We will first take an introductory tour of the biochemistry of the two important metals  
43 Zn and Cu and then summarize a few important facts about sulfur-containing amino  
44 acids and proteins. The homeostasis of each element is depicted for a 'generalized'  
45 cell in the three panels of [Fig. 2](#).  
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#### 47 *Zinc*

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49 The Zn content of the human body ranges from 1.5 to 3 g and the daily intake recom-  
50 mended for an adult is about 10 mg<sup>36</sup>. It is found in the nucleus and the cytosol of  
51 cells in all organs. About 90 percent of Zn in blood is accounted for by erythro-  
52 cytes<sup>14</sup>. Excess cytosolic Zn is bound to metallothionein, a short sulfur-rich protein  
53 and then transported to nucleus and organelles for storage. Zinc is a cofactor of car-  
54 bonic anhydrase, which interconverts carbon dioxide and bicarbonate, and thus regu-  
55 lates the acid-base balance of the cytosol. Zinc is also a cofactor of superoxide dis-  
56 mutase, which controls reactive oxygen species. Zinc regulates the glutathione metab-  
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olism and metallothionein expression<sup>37</sup>. Zinc affects signaling pathways and the activity of transcription factors with zinc finger domains.

Zinc homeostasis and its importance in various pathologies have been multiply reviewed<sup>26,38-41</sup>. Malnutrition induces cell mediated immune defects and promotes infections<sup>42</sup>. Zinc acts on the immune system by potentiating cytokines<sup>43</sup>, a mechanism that may also be controlling chronic inflammation, such as for rheumatoid arthritis<sup>44</sup>. Zinc in seminal fluid has been suggested as a biomarker of prostate cancer<sup>45</sup>. Serum albumin is the main carrier of Zn in serum. For a 'generalized' cell, the transmembrane importers consist of 14 isoforms of the ZIP family (ZIP1 to ZIP14). Different ZIP transporters are expressed specifically on different cell types<sup>41</sup>. DMT1 has a lower affinity for Zn<sup>46,47</sup>. No specific chaperone has been identified for the transfer from cytoplasm to organelles, although to some extent metallothionein may be considered one. Zn efflux from the cell and Zn stockade in organelles is controlled by ZnT protein family, which consists of 10 isoforms (ZnT1 to ZnT10). The trans-membrane ZnT1 is the only isoform to be ubiquitously expressed on the cell surface, while the expression of other ZnTs depends on the type of cell and organelles where they are localized.

*Copper*

The total copper content of the human body ranges from 50 to 150 mg and is found in all tissues and most body fluids and the daily intake recommended for an adult is about 1 mg<sup>36</sup>. About 35 percent blood copper is accounted for by erythrocytes<sup>14</sup>. Copper is a micronutrient and a catalytic and structural cofactor of many important enzymes involved in tumor development<sup>48-53</sup>. Serum ceruloplasmin is a ferroxidase enzyme synthesized in the liver, which allows iron to be transported in the blood as harmless Fe<sup>3+</sup> hydroxide. It also acts as a modulator of inflammation. A variable fraction of copper is transported by serum albumin. Cytochrome *c* oxidase is a transmembrane protein complex of the mitochondrion associated with the terminal step of electron transport and energy production. Superoxide dismutase 1 (Cu, Zn SOD1) resides mostly in the cytosol. Excess Cu may also be stored in metallothionein.

The dominant Cu importer of the cells is hCtr1 (human Cu transporter)<sup>54-56</sup>, which binds to albumin<sup>57</sup> and binds both Cu(I) and Cu(II)<sup>58</sup>. Hypoxia-induced DMT1 (divalent metal transporter, notably ferrous iron) has also been invoked in copper transport into mice intestinal cells<sup>59,60</sup>, but its relevance to other cell types is not established. Depending on the final destination, hCtr1 presents Cu<sup>+</sup> to chaperones that will deliver it to specific partners: COX17 brings copper to cytochrome *c* oxidase (CCO) in mitochondria, CCS delivers it to SOD1, while ATOX1 is the chaperone for the copper-transporting ATPases (Cu-ATPases). The latter maintain intracellular Cu(I) levels by regulating its efflux either directly or through the secretory pathway<sup>52,61</sup>. ATP7A and ATP7B differ for their pattern of tissue expression and cellular localization

*Sulfur*

The sulfur content of the human body is about 175 g<sup>36</sup>. Most body sulfur is held in two major amino acids, cysteine a thiol ending with an -SH moiety and methionine, an *S*-methyl thioether ending with a -C-S-CH<sub>3</sub> moiety. Methionine is an essential amino acid, which must be obtained from the diet, and is imported by transmembrane importers, notably the Na-independent L-type amino acid transporter 1 (LAT1)<sup>62-64</sup>. Instead, cysteine can be synthesized from methionine within the cell through the transsulfuration pathway involving methylation by *S*-adenosyl methionine (SAM).

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280 Metal binding metallothioneins are rich in cysteine, accounting for up to one third of the amino-acidic sequence<sup>65,66</sup>.

285 An essential property of cysteine is the potential of two molecules to bind into cystine by forming a covalent disulfide S-S bridge, which may open for metal chelation in a reducing environment, such as the cytosol. Disulfide bridges are very important for the structure and stability of proteins such as serum albumin, the most abundant protein of blood serum and its main sulfur carrier. The properties of the disulfide bridge are at the basis of glutathione's function, a tripeptide essential to the control of cellular redox state by easily switching between its reduced (GSH) and oxidized (GSSG) form. Glutathione is synthesized from cystine imported from the extracellular medium in exchange of glutamate by the x<sub>c</sub><sup>-</sup> 'antiporter'<sup>67</sup>.

290 Cytosolic cysteine is catabolized into pyruvate, which is used for energy production, and sulfate.

Sulfate is associated with membrane proteins known as proteoglycans, such as heparan sulfate, and is also found in heparin, an anticoagulant substance commonly used as an additive to lower the viscosity of blood samples.

#### 295 **Isotope compositions of Zn-Cu-S in the blood of healthy individuals**

Copper and zinc contents vary in the serum of control individuals in a remarkable way. Fig. 3 shows that the Cu content is high and variable among women<sup>68</sup>, whereas Zn tends to be constant. In contrast, men serum tends to have a narrow range of Cu and variable Cu/Zn. The range of overlapping values is, however, relatively large.

300 Although copper is a commonly used as biomarker to assess health status, the Zn/Cu ratio seems to have an even stronger potential<sup>19,69</sup>. Prostate cancer seems to have little effect on serum Zn levels but Cu clearly increases relative to controls. The serum of the breast cancer patients seems to plot above the reference line Zn=1200 ppm, which roughly describes the average value of women controls, whereas for colon cancer patients the value plots below this line (higher Cu and/or lower Zn).

310 How isotope compositions of metals and sulfur vary among the organs and body fluids of a mammal was essentially unknown until the first investigations by Balter et al.<sup>70,71</sup> and Moynier et al.<sup>72</sup> of sheep and mouse. For ethical reasons, access to such a variety of human material is much more restricted. The first major observation was that, in most cases, the isotope compositions of Cu, Zn, and Fe of each organ falls, for a given species, within a narrow range of values (Fig. 4). In mice, Zn is isotopically heavy in blood and bone and light in liver and brain and is not dependent on the genetic background. Copper is specifically light in kidney. This pattern reproduces for sheep except for isotopically light Zn in blood, a feature that still awaits elucidation.

315 Buechl et al.<sup>73</sup> analyzed Zn and Cu isotopes in the brain of wild type and knockout mice and demonstrated that Zn isotopes in brain tissues are sensitive to prion-related local damage.

320 Albarede et al.<sup>14</sup> conducted the first systematic analysis of Zn, Cu and Fe isotope compositions in human whole blood, serum, and erythrocytes (Table 4). They concluded that, on average, Zn and Cu are isotopically lighter in erythrocytes (red blood cells or RBC) relative to serum by ~0.3 and ~0.8 ‰, respectively. Men-woman δ<sup>66</sup>Zn and δ<sup>65</sup>Cu differences were less than 0.2 percent for both serum and RBC. The study found mean values of δ<sup>66</sup>Zn~ +0.17‰ and δ<sup>65</sup>Cu~ -0.26±0.40‰ for serum and δ<sup>66</sup>Zn~

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325  $+0.44 \pm 0.26\%$  and  $\delta^{65}\text{Cu} \sim +0.66\%$  for erythrocytes. A similar  $\delta^{65}\text{Cu}$  value  
0.29  $\pm 0.27\%$  was obtained by Costas-Rodríguez et al.<sup>20</sup> on 29 serum samples. The  
serum-RBC difference is most significant for Cu.  $\delta^{65}\text{Cu}$  is 0.2% heavier in men eryth-  
rocytes relative to women<sup>14</sup>. Total blood values depend on the erythrocyte load  
(hematocrit) and therefore may vary with sample preparation and should be consid-  
ered less reliable than serum and RBC values.

330 In a study of *whole-blood* samples on Yakut volunteers aged 18-74, Jaouen et al.<sup>16</sup>  
found that the  $^{66}\text{Zn}/^{64}\text{Zn}$  ratio increases and the  $^{65}\text{Cu}/^{63}\text{Cu}$  ratio decreases with age.  
Van Hegue et al.<sup>17</sup> observed that  $^{65}\text{Cu}/^{63}\text{Cu}$  ratio tends to increase in whole blood af-  
ter menopause but found no age or menstruation effect of Zn isotopes.

335 Comparison of their results with Albarede et al.'s<sup>14</sup> study led Jaouen et al.<sup>16</sup> to em-  
phasize the importance of the ethnic factor. On a small sample set, Van Hegue et al.<sup>74</sup>  
observed that  $\delta^{66}\text{Zn}$  in whole blood is about 0.15% higher for vegetarians relative to  
omnivorous volunteers, but the outcome for Cu isotopes was less conclusive.

340 The first substantial set of  $^{34}\text{S}/^{32}\text{S}$  values on the blood of healthy individuals were ob-  
tained by Elemental Analysis - Isotope Ratio Mass Spectrometry (EA-IRMS) (gas  
source mass spectrometry) by Balter et al.<sup>18</sup> and obtained an average value  $\delta^{34}\text{S}_{\text{V-CDT}}$   
of  $5.9 \pm 1.5\%$  on 11 serum samples and of  $5.1 \pm 1.9\%$  on 20 RBC samples. On 25 se-  
rum samples of adults, Albalat et al.<sup>27</sup> obtained a very similar mean value but within  
a reduced interval  $6.0 \pm 0.7\%$ , with the average  $\delta^{34}\text{S}_{\text{V-CDT}}$  of women being 0.2% lower  
relative to men. On the same samples, both methods agree within one permil, with  
345 serum sulfur being a fraction of permil heavier than RBC. Albalat et al.<sup>27</sup> showed that  
S in children serum is only slightly heavier but more scattered ( $6.3 \pm 1.0\%$ ) relative to  
adults.

#### Isotope compositions of Zn, Cu, and S in cancer

350 Telouk et al.<sup>19</sup> measured the  $^{65}\text{Cu}/^{63}\text{Cu}$  ratios in the serum of 20 breast and 8 colorec-  
tal cancer patients. Samples were taken at different times during the treatment, and  
amount to, respectively, 90 and 49 samples taken. Phenotypes and molecular bi-  
omarker were documented on most of the samples. When compared with the literature  
data from a control group of 50 healthy blood donors, abundances of Cu isotopes pre-  
dict mortality in the colorectal cancer group with an error probability  $p=0.018$   
355 (Fig. 5). For the breast cancer patients and the group of control women the probability  
falls even further to  $p=0.0006$ . Most patients considered in this preliminary study and  
with serum  $\delta^{65}\text{Cu}$  less than the threshold value of  $-0.35\%$  (per mil) did not survive  
beyond a few months (Figure 6). As a marker, a drop in  $\delta^{65}\text{Cu}$  precedes molecular bi-  
omarkers such as CEA (carcinoembryonic antigen) and CA15.3 (carbohydrate antigen  
360 15.3) by several months (Fig. 7), which is consistent with Cu turnover time in the  
body. The observed decrease of  $\delta^{65}\text{Cu}$  in the serum of cancer patients was assigned to  
the extensive oxidative chelation of copper by cytosolic lactate. The potential of Cu  
isotope variability as a new diagnostic tool for breast and colorectal cancer seems  
strong.

365 Lamer et al.<sup>21</sup> analyzed zinc isotope compositions of various tissues in breast cancer  
patients. Resections of breast cancers were found to have a significantly lighter Zn  
isotopic composition than the blood, serum and healthy breast tissue in both groups.  
The authors interpret the isotopically light Zn in tumors as attesting to its uptake by

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3 metallothionein in breast tissue cells, rather than in Zn-specific proteins. This reveals  
4 370 a possible mechanism of Zn delivery to Zn-sequestering vesicles by metallothionein,  
5 and is supported by a similar signature observed in the copper isotope abundances of  
6 one breast cancer patient. The number of samples used for this study remains rather  
7 small and whether and how cancer may affect the  $\delta^{66}\text{Zn}$  of the serum deserves further  
8 attention.  
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10 375 Balter et al.<sup>18</sup> found that in hepatocellular carcinomas patients, serum and erythrocyte  
11 copper and sulfur are both enriched in light isotopes relative to controls (Fig. 8, left).  
12 The magnitude of the sulfur isotope effect is similar in red blood cells and serum of  
13 hepatocellular carcinoma patients, implying that sulfur fractionation is systemic. In  
14 contrast with serum data, the  $\delta^{65}\text{Cu}$  of tumor resections is notably higher relative to  
15 healthy liver tissue (Fig. 8, right). The agreement between sulfur isotope data acquired  
16 380 on the same samples by EA-IRMS and MC-ICP-MS Albalat et al.<sup>27</sup> is reasonably  
17 good. Balter et al.<sup>18</sup> concluded that the isotopic shift of either element is not compati-  
18 ble with a dietary origin, but rather reflects the massive reallocation in the body of  
19 copper immobilized within cysteine-rich metallothionein. A related study by Costas-  
20 Rodriguez et al.<sup>20</sup> found lower  $\delta^{65}\text{Cu}$  in the serum of patients with end-stage liver  
21 385 disease, with complications such as ascites, encephalopathy, and hepatocellular carci-  
22 noma (Fig. 9). These authors pointed out that  $\delta^{65}\text{Cu}$  was positively correlated with the  
23 liver cirrhosis-related parameters, notably aspartate aminotransferase, INR (Internat-  
24 ional Normalized Ratio for prothrombin time), bilirubin and C-reactive protein, and  
25 inversely correlated with albumin and Na. They also found a negative correlation of  
26 390  $\delta^{65}\text{Cu}$  with Child-Pugh score based on albumin, bilirubin, and INR and the Mayo  
27 Clinic Model for End-stage Liver Disease score (MELD) based on creatinin, bilirubin,  
28 and INR.  
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31 Albalat et al.<sup>27</sup> analyzed sulfur isotopes in a large number of pathological samples  
32 with emphasis on serum. These serum samples departed by a much smaller S concen-  
33 395 tration from those of healthy volunteers, which echoed the negative correlation be-  
34 tween low serum albumin content<sup>75, 76</sup>. The samples, however, for which the  $\delta^{34}\text{S}$  de-  
35 parted from the range of healthy individuals were very few and corresponded to the  
36 'naive' (untreated) patients, in particular those analyzed by Balter et al.<sup>18</sup>. Cancer and  
37 400 rheumatoid arthritis conditions increase the scatter of sulfur isotope compositions by  
38 up to a factor of two, but with little effect on the mean  $\delta^{34}\text{S}$  values. It has been ob-  
39 served that medication brings  $\delta^{34}\text{S}$  back to normal values but does not change sulfur  
40 concentrations in the serum.  
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#### 45 Discussion

46 405 Before attempting a biochemical interpretation of isotopic trends in biological sam-  
47 ples, let us summarize the observations at hand. Most of the observations so far have  
48 been made on serum, on whole blood, occasionally on erythrocytes, and only excep-  
49 tionally on organ tissues and tumors. Out of the three elements, Cu and Zn seem to  
50 show a deviation of tumors from healthy tissue (heavy Cu in liver and light Zn in  
51 410 breast neoplastic tissue)<sup>18, 21</sup>. In contrast with Cu, which is definitely isotopically  
52 lighter in the serum of well over 130 cancer patients relative to a similar number of  
53 controls (colon, breast, liver)<sup>18, 19</sup>, our unpublished Zn isotope data show no discerni-  
54 ble difference between cancer patients and healthy donors of any age. Likewise, it  
55 was shown that sulfur isotope compositions in the serum of cancer patients (colon,  
56 415 breast, and liver) could not in general be distinguished from the value in control pa-  
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tients, with the exception of some hepatocellular carcinoma patients<sup>18,27</sup>, but that the spread of  $\delta^{34}\text{S}$  values is smaller for controls.

Both Telouk et al.<sup>19</sup> and Costas-Rodriguez et al.<sup>20</sup> suggested that low  $\delta^{65}\text{Cu}$  can be used for prognosis in end-stage cancer (liver, colon, breast). Copper isotopes would definitely complement other markers, such as the Child-Pugh score, albumin or transaminases. The ~6 weeks turnover time (<sup>49,77</sup>) is close enough to the 19 days of albumin<sup>78</sup> that the two parameters may have some biochemical pathways in common, one of them being that albumin is a Cu transporter. Telouk et al.<sup>19</sup> pointed out that Cu isotopes seem to be reactive over time intervals of weeks to deteriorating health conditions, whereas molecular biomarkers tend to increase, whenever they do, within months.

Among possible reasons for  $\delta^{65}\text{Cu}$  variations, some have been privileged:

1. Telouk et al.<sup>19</sup> appealed to cytosolic storage of isotopically heavy Cu chelated by lactate, which cancer cells are known to produce massively.
2. Balter et al.<sup>18</sup> suggested that the low  $\delta^{65}\text{Cu}$  value of the serum could be explained by the release of intracellular copper from cysteine clusters, with MT being the most likely source.
3. Costas-Rodriguez et al.<sup>20</sup> suggest that low  $\delta^{65}\text{Cu}$  values reveal the hepatocellular and biosynthetic dysfunction of the liver, synergistically with inflammation and water retention.

Isotope abundances add a new 'dimension' to the overall budget of each element in cells and in the organism. None of the Cu isotope studies discussed above have attempted a mass balance evaluation that would include blood components, healthy tissues, and tumor, simply because the data are not available. Liver accounts for a large fraction of bodily metals such as Cu and Fe. A legitimate concern therefore is that the Cu isotope effects observed in serum and tumors cannot be directly compared until some missing data have been collected.

Three main routes by which Cu interacts with cancer cells are cellular metabolism, angiogenesis and hypoxia. Copper is a tumor promoter and regulates oxidative phosphorylation in rapidly proliferating cancer cells inside solid tumors<sup>6</sup>. In normal cells, glycolysis, the first step of ATP production from glucose, is slow and its end product, pyruvate, is oxidized in mitochondria, where it fuels the much more efficient steps of citric acid cycle and oxidative phosphorylation. In tissues in which anaerobic condition results from reduced access to blood flow, pyruvate is reduced to L-lactate. This is lactic acid fermentation. The observation that cancer cells show enhanced glycolysis followed by lactate production in the cytosol, even in the presence of  $\text{O}_2$ , is known as the Warburg effect. Lactate levels are observed to be elevated in the serum of critically ill patients and correlate well with disease severity<sup>79,80</sup>. Lactate efflux from the cell is regulated by monocarboxylate transporters (MCT) and intracellular and extracellular lactate levels are not simply related<sup>81,82</sup>. Copper is isotopically heavy in both pyruvate and lactate. However, in healthy cells pyruvate is shuttled into mitochondria for further energy processing, whereas lactate is exported from the cell by MCT and is metabolized in the liver. To a large extent, lactate is 'available' in the cell for Cu chelation (Figure 10), whereas pyruvate is not. This is the substance of Telouk et al.'s<sup>19</sup> explanation for the accumulation of copper with high  $\delta^{65}\text{Cu}$  in the cell.

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3 A major role of copper in cancer is associated with hypoxia, a hallmark of both in-  
4 flammation and human malignancies. In order to secure delivery of oxygen and nutri-  
5 ents to tumor cells, the growth of cm-sized tumors is accompanied by pervasive neo-  
6 vascularization<sup>83</sup>. Several angiogenic factors, notably VEGF, tumor necrosis factor  
7 alpha (TNF- $\alpha$ ) and interleukin (IL1), are copper activated<sup>84</sup>. The copper-dependent  
8 Memo redox protein plays an essential role in breast-cancer metastasis<sup>85</sup>. Under hy-  
9 poxic conditions, the hypoxia-inducible factor (HIF-1 $\alpha$ ),  $\square\square\square\square$  is associated with  
10 tumor growth, vascularization, and metastasis<sup>86,87</sup>, is stabilized by ionic serum cop-  
11 per<sup>88</sup>.

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13 Copper transport and uptake are still poorly understood<sup>52</sup>, as is the mechanism of Cu  
14 reduction during uptake. It is likely that albumin is the main serum carrier presenting  
15 Cu to the cell and binds both Cu(I) and Cu(II)<sup>57,58</sup>. Both Cu<sup>+</sup> and Cu<sup>2+</sup> are captured  
16 by the terminal amino acids of Ctr1, which introduces Cu<sup>+</sup> into the cytosol. Hypoxic  
17 stimulation of the HepG2 cells (hepatocarcinoma) leads to a down-regulation of al-  
18 bumin<sup>89</sup>, which does support a connection between liver, copper, and albumin<sup>20</sup>.  
19 Transmembrane Cu uptake is the primary site where isotope fractionation takes place.  
20 Fractionation restricted to storage or efflux is unlikely, as it would lead to an open-  
21 ended shift in intracellular  $\delta^{65}\text{Cu}$ . Clearly, Cu isotopes may help understand the con-  
22 nections between tumor growth and Cu homeostasis.  
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### 25 Perspectives

26 So far, Cu has provided the strongest signal associated with a number of diseases and  
27 in particular with cancer. Zinc, iron and sulfur have not so far proved to be as in-  
28 formative as copper. The exploratory stage of Cu isotope variations in blood has been  
29 very fruitful. Now that this field is becoming mature, descriptive investigations need  
30 to be complemented. Data on organs are needed that only animal models can provide.  
31 Experiments should be run on cell cultures under hypoxic conditions. Protein expres-  
32 sion, notably those controlling metal trafficking, storage, and redox, should be evalu-  
33 ated.  
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37 490 Among the upcoming challenges, several major questions need to be addressed, nota-  
38 bly what part of the  $\delta^{65}\text{Cu}$  signal is due to cancer itself, and what is due to other fac-  
39 tors, such as age and, even more, to inflammation. Our preliminary studies of athletes  
40 and of patients with purely inflammatory diseases, such as rheumatoid arthritis, sug-  
41 gest that they can document the specific effect of inflammation.

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43 495 Reduction of the copper or ceruloplasmin level by chelates, without causing clinical  
44 copper deficiency, was proposed for therapeutic purpose. Specific copper chelators,  
45 such as tetrathiomolybdate, D-penicillamine and TPEN<sup>90-95</sup> have been shown to be a  
46 potent antiangiogenic and antimetastatic compound possibly through suppression of  
47 the NF $\kappa$ B signaling cascade. Recently, Cu-chelation therapy has been proposed as  
48 treatment of the broad spectrum of cancers containing the BRAF<sup>V600E</sup> mutation<sup>96</sup>. In-  
49 hibition of copper Atox1 trafficking has also been investigated<sup>97</sup>. The isotopic study  
50 of copper will certainly add a new dimension to the understanding of chelation path-  
51 ways and copper mass balance, at the scale of both the cell and the organism, during  
52 the treatment.  
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### Figure captions

- 790 Figure 1. Isotopes of a same element bound to a specific metalloprotein, e.g.,  $^{63}\text{Cu}$  and  $^{65}\text{Cu}$ , are depicted as blue and red spheres. Top: For transit at steady-state (no growth), the proportion of each isotope is preserved. The cell or organ is reduced to a single input and a single output. Chaperones are not expected to affect isotope compositions. Bottom: When the metal distributes itself between two coexisting channels, such as extracellular medium and cytosol, the different pathways allocate different isotope abundances to each channel. Oxidation, biosynthesis, input, storage, and output are expected to result in isotope fractionation.
- 795
- 800 Figure 2. A sketch of Zn, Cu, and S trafficking in a generalized cell. *Abbreviations Zn Panel:* Zn transporter family (ZnTx), Zn transporter ZIP family (ZIPx), metallothionein (MT), Cu,Zn-superoxide dismutase 1 (SOD1). *Zn Panel:* human copper transporter (hCtr1), cytochrome c oxidase copper chaperone (CCO), copper chaperone for superoxide dismutase (CCS), antioxidant protein 1 chaperone to the copper ATPases ATP7A and ATP7B (ATOX1).
- 805 Figure 3. Cu and Cu/Zn in the serum as indicators of cancer status. The control group shows strong correlations, reflecting the tight regulation of Zn concentrations in the body (note that  $x/y=\text{Zn}$ ). The trends for control men and women are different, with men having, on average less Cu than men. Copper remains stable in prostate cancer patients relative to control men, but increases in colon cancer patients. Zinc in the serum of breast cancer patients is decreased and Cu probably increased relative to healthy subjects.
- 810
- 815 Figure 4. Zinc and copper isotope variability among organs, bones, body fluids, and intestinal content of mice reported in delta units per mil  $^{70}$ . In orange, the range of variations for humans <sup>12-14, 22, 24, 98</sup>. Typical uncertainties are  $\pm 0.05\text{‰}$  (2-sigma error).
- 820 Figure 5. Whisker plots of serum  $\delta^{65}\text{Cu}$  values for healthy men and women compared to breast cancer and colorectal cancer patients <sup>19</sup>. Boxes represent the 75 percent middle quantiles and the whiskers 95 percent quantiles. Red lines: median; red crosses: outliers. Separation between breast cancer patients and healthy women is strong. Separation between breast cancer and colorectal cancer patients and healthy men and women seems to depend on mortality.
- 825 Figure 6. Evolution of serum  $\delta^{65}\text{Cu}$  for 20 breast cancer cases up to patient death <sup>19</sup>. Each line represents a different patient with color used for differentiation purpose. The shaded band is the 75 percent confidence limit for the serum of control women.
- 830 Figure 7. Early alarm by  $\delta^{65}\text{Cu}$  <sup>19</sup>. The plot compares the  $\delta^{65}\text{Cu}$  values (left axis, green line) and the molecular biomarkers (right axis): CEA (carcinoembryonic antigen) and CA 15.3 (carbohydrate antigens). The top bar scale shows the successive therapies given received by the patient. The copper isotope signal precedes the other markers by 2-3 months.
- 830 Figure 8. *Left:* isotopically light copper and sulfur in the serum of hepatocellular carcinoma patients relative to controls <sup>18</sup>. *Right:* isotopically heavy copper in tumor liv-

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er tissue relative to normal tissue. The opposite direction of the changes in Cu isotope abundances in serum and tumor may be explained in different ways.

835 Figure 9.  $\delta^{65}\text{Cu}$  values in the serum of liver cirrhosis patients with and without accumulation of fluid in the peritoneal cavity (ascites) relative to controls <sup>20</sup>. Ascites is often associated with cirrhosis and metastatic cancer.

840 Figure 10. Extent of copper chelation by lactates in the cytosol <sup>19</sup>. The numbers on the curves represent the  $\text{Cu}^+/\text{Cu}^{2+}$  ratio for a redox potential of 0.153 V (copper ions) and for a body potential of 0.27 V <sup>99</sup>. The vertical dashed line corresponds to a lactate concentration of 10 mMol typical of tumor cells <sup>100</sup>.

Table 1. Partition function ratios for  $^{66}\text{Zn}/^{64}\text{Zn}$  and  $^{65}\text{Cu}/^{63}\text{Cu}$  in molecular species relevant to medical studies on a 1000 ln  $\beta$  scale (reduced partition function ratios) (T=310 K). Isotopic fractionation  $\alpha$  between two coexisting species 1 and 2 can be computed as  $\alpha^{1/2} \approx \beta_1 - \beta_2$ .

Species	ln $\beta_{\text{Zn}}$	Species	ln $\beta_{\text{Cu}}$		
ZnHPO <sub>4</sub> (H <sub>2</sub> O) <sub>5</sub>	3.309	1	Cu(II)-Lact	1.725	5
ZnH <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> (H <sub>2</sub> O) <sub>4</sub> <sup>-</sup>	3.967	1	Cu(I)(H <sub>2</sub> O) <sub>2</sub> <sup>+</sup>	2.667	4
ZnH <sub>2</sub> (PO <sub>4</sub> ) <sub>2</sub> (H <sub>2</sub> O) <sub>4</sub>	4.072	1	Cu(II)H(L-ascorbate)(H <sub>2</sub> O) <sub>4</sub> <sup>+</sup>	3.087	4
<i>fourfold</i>			Cu(II)H(D-ascorbate)(H <sub>2</sub> O) <sub>4</sub> <sup>+</sup>	3.139	4
Zn(Cys)(H <sub>2</sub> O) <sub>3</sub> <sup>2+</sup>	3.072	2	Cu(II)H <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> (H <sub>2</sub> O) <sub>3</sub> <sup>-</sup>	4.176	2
Zn(Glu)(H <sub>2</sub> O) <sub>2</sub> <sup>2+</sup>	3.524	2	Cu(II)H <sub>2</sub> PO <sub>4</sub> (H <sub>2</sub> O) <sub>4</sub> <sup>+</sup>	4.355	2
Zn(H <sub>2</sub> O) <sub>4</sub> <sup>2+</sup>	3.577	2	Cu(II)H <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> (H <sub>2</sub> O) <sub>3</sub>	4.382	2
Zn(His)(H <sub>2</sub> O) <sub>3</sub> <sup>2+</sup>	3.647	2	Cu(II)Ox(H <sub>2</sub> O) <sub>2</sub>	4.931	4
Zn(Met)(H <sub>2</sub> O) <sub>3</sub> <sup>2+</sup>	3.66	2	CuH <sub>2</sub> (PO <sub>4</sub> ) <sub>2</sub> (H <sub>2</sub> O) <sub>3</sub> <sup>2-</sup>	5.024	2
Zn(His)(H <sub>2</sub> O) <sub>2</sub> <sup>2+</sup>	3.673	2			
Zn(Thr)(H <sub>2</sub> O) <sub>3</sub> <sup>2+</sup>	3.767	2	Cu(II)(Cys)(H <sub>2</sub> O) <sub>4</sub> <sup>2+</sup>	3.124	2
			Cu(II)(Met)(H <sub>2</sub> O) <sub>4</sub> <sup>2+</sup>	3.650	2
<i>sixfold</i>			Cu(II)(GS)HO	3.892	2
Zn(Cys)(H <sub>2</sub> O) <sub>5</sub> <sup>2+</sup>	2.504	2	Cu(II)(Thr)(H <sub>2</sub> O) <sub>4</sub> <sup>2+</sup>	4.110	2
Zn(Met)(H <sub>2</sub> O) <sub>5</sub> <sup>2+</sup>	2.734	2	Cu(II)(Glu)(H <sub>2</sub> O) <sub>3</sub> <sup>2+</sup>	4.117	2
Zn(His)(H <sub>2</sub> O) <sub>4</sub> <sup>2+</sup>	2.777	2	Cu(II)(His)(H <sub>2</sub> O) <sub>3</sub> <sup>2+</sup>	4.148	2
Zn(His)(H <sub>2</sub> O) <sub>5</sub> <sup>2+</sup>	2.921	2	Cu(II)(His)(H <sub>2</sub> O) <sub>4</sub> <sup>2+</sup>	4.168	2
Zn(H <sub>2</sub> O) <sub>6</sub> <sup>2+</sup>	3.026	2	Cu(II)(H <sub>2</sub> O) <sub>5</sub> <sup>2+</sup>	4.220	2
Zn(Glu)(H <sub>2</sub> O) <sub>4</sub> <sup>2+</sup>	3.053	2	Cu(II)-L-Lact(H <sub>2</sub> O) <sub>3</sub> <sup>+</sup>	4.359	2
Zn(Thr)(H <sub>2</sub> O) <sub>5</sub> <sup>2+</sup>	3.075	2	Cu(II)-L-LactH <sub>-1</sub> (H <sub>2</sub> O) <sub>2</sub>	4.969	5
			Cu(II)-L-Lact <sub>2</sub>	5.616	5
			Cu(II)-L-Lact D-Lact	5.627	6
<i>anhydrous</i>					
[Zn-Cys-H <sub>-1</sub> ] <sup>+</sup>	1.108	3			
[Zn-Cys] <sup>2+</sup>	1.211	3			
[Zn-Glu-H <sub>-1</sub> ] <sup>+</sup>	1.517	3			
[Zn-His] <sup>2+</sup>	3.336	3			
[Zn-His-H <sub>-1</sub> ] <sup>+</sup>	3.465	3			

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850 Table 2. Equilibrium  $^{34}\text{S}/^{32}\text{S}$  enrichment in ‰ of different sulfur-bearing inorganic and organic species at 298 K (Albalat et al., submitted). The calculations include the effect of one hydrate shell on  $\text{SO}_4^{2-}$ .  $^{34}\text{S}/^{32}\text{S}$  fractionation  $\alpha$  between two species may be obtained in ‰ between two coexisting species 1 and 2 can be computed as  $\alpha^{1/2} \approx \beta_1 - \beta_2$ .

$\text{HS}^-$	$\text{H}_2\text{S}$	Cysteine	Cystine	Glutathione	Methionine	Taurine	$\text{SO}_4^{2-} \cdot 6\text{H}_2\text{O}$
4.75	11.42	16.11	17.12	15.67	20.21	71.59	73.94

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860 Table 3. Stability constants for the successive chelates of Cu and Zn by relevant carboxylates.

	Species	$\log \beta_1$	$\log \beta_2$	$\log \beta_3$	
$\text{Cu}^{2+}$	pyruvate	2.2	4.9		1
	lactate	2.52	3.9	4.28	2
	ascorbate	1.57			1
$\text{Zn}^{2+}$	pyruvate	1.26	1.98		1
	lactate	1.67	2.65	2.94	2
	ascorbate	1.0			1

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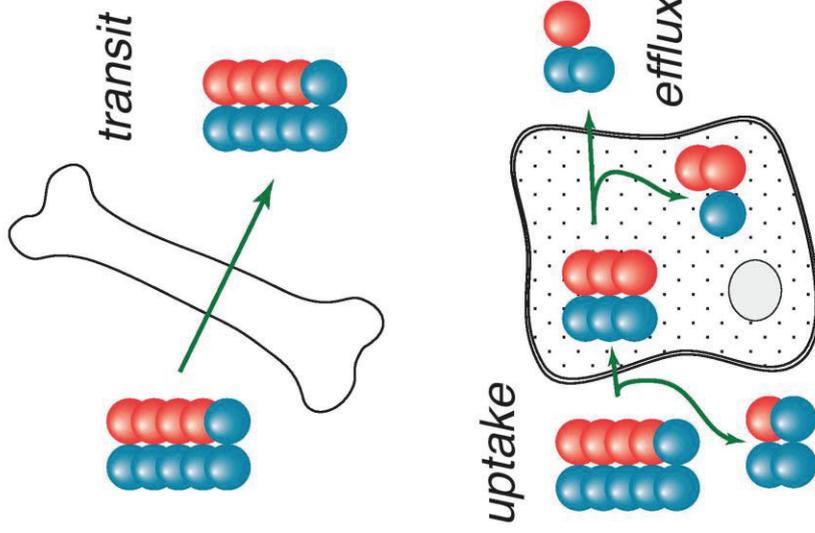
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865 Table 4. Average isotope compositions in delta units (‰) and 95% range (2s) for  
 the isotope compositions of Zn and Cu in the serum, erythrocytes, and total blood  
 of 49 blood donors <sup>4</sup>. Typical analytical uncertainties are 0.05 ‰. Men-women  
 comparison: *p* is the probability that the two sets are not identical.

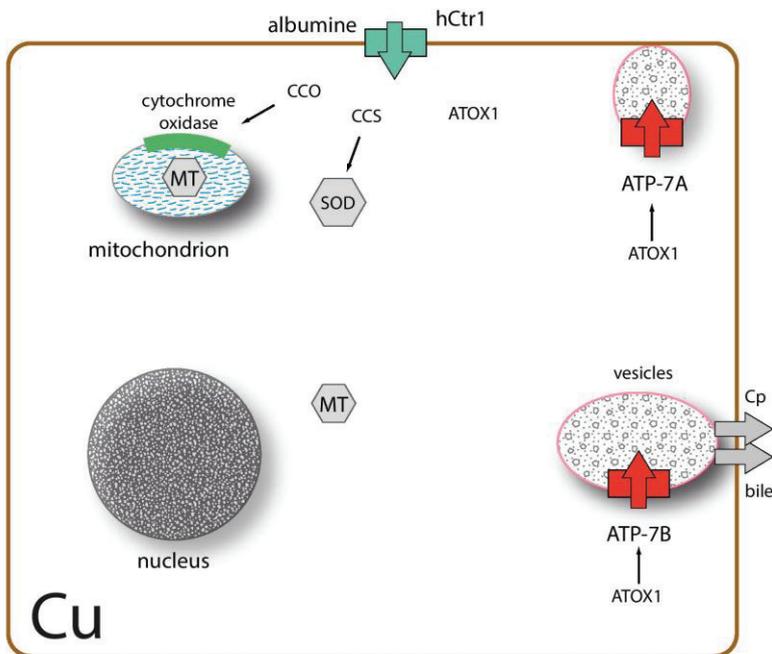
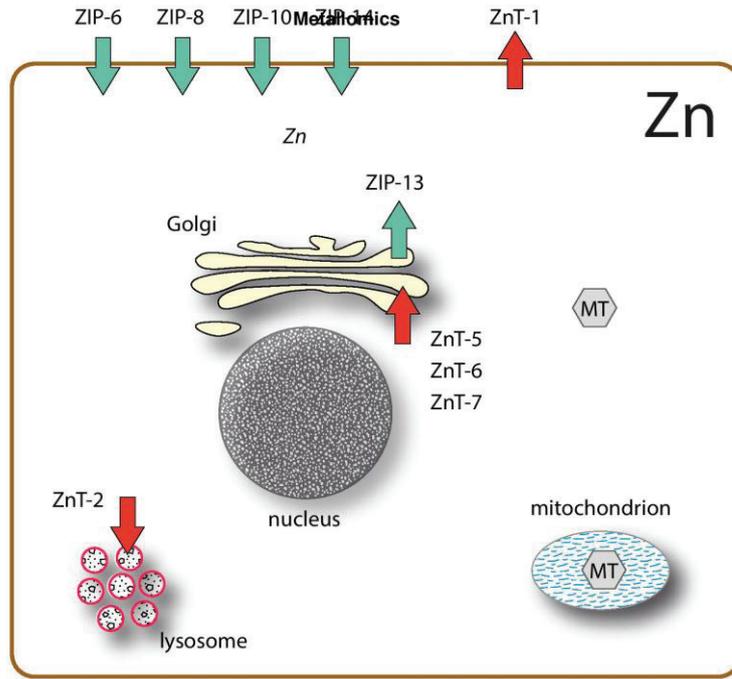
	<i>n</i>	av δ <sup>66</sup> Zn	2s	av δ <sup>65</sup> Cu	2s
<i>Serum</i>					
women	28	0.18	0.2	-0.24	0.36
men	21	0.16	0.1	-0.28	0.4
all	49	0.17	0.2	-0.26	0.4
<i>p</i> value (men/women)		0.45		0.3	
<i>Erythrocytes</i>					
women	28	0.46	0.1	0.46	0.47
men	21	0.43	0.4	0.67	0.36
all	49	0.44	0.3	0.56	0.5
<i>p</i> value (men/women)		0.39		0	
<i>Total blood</i>					
women	28	0.41	0.1	0.01	0.16
men	21	0.39	0.4	0.17	0.33
all	49	0.4	0.3	0.09	0.32
<i>p</i> value (men/women)		0.32		0.02	

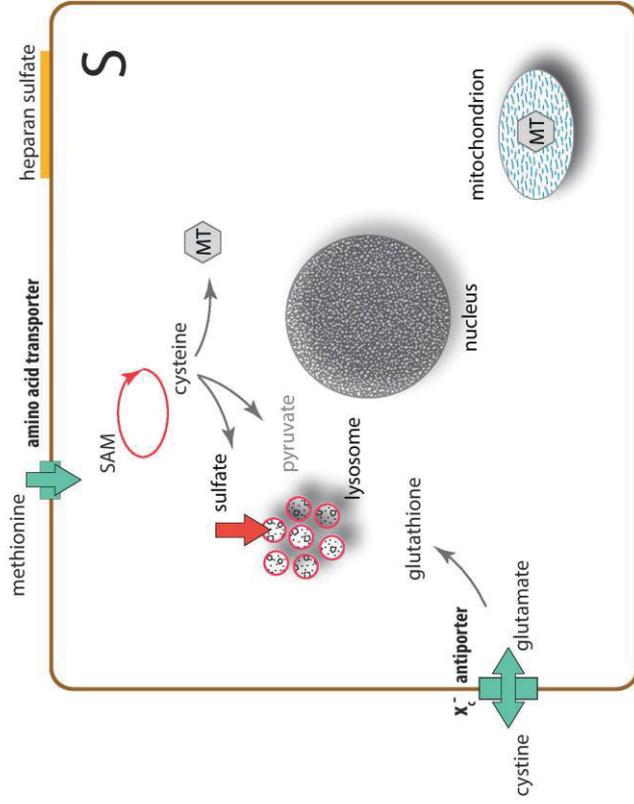
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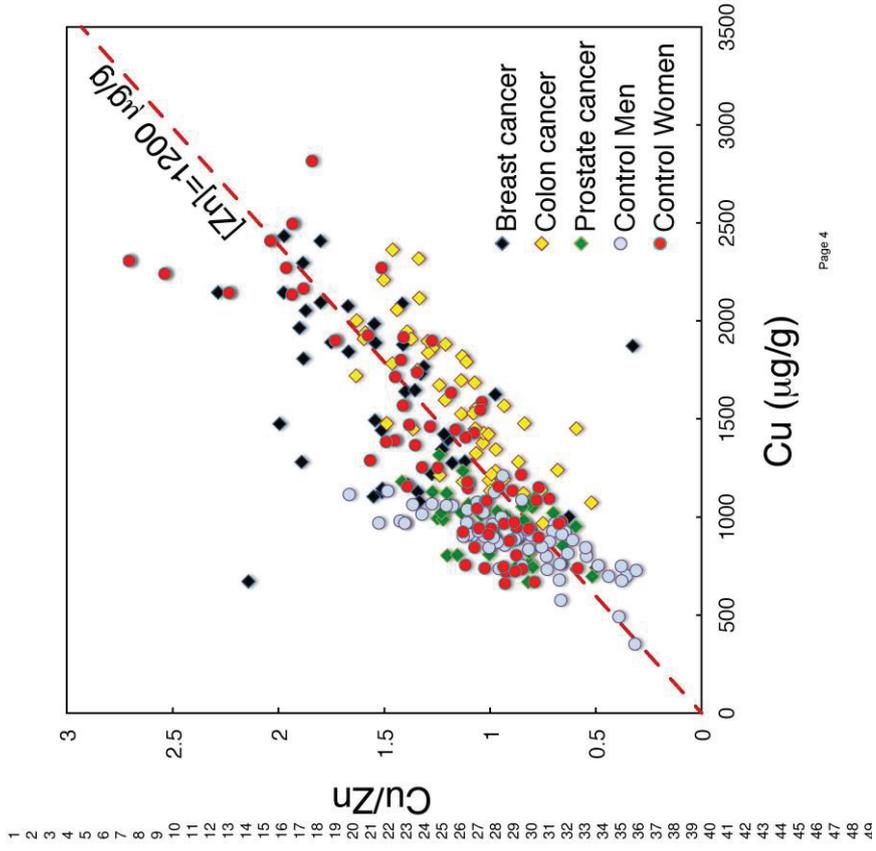
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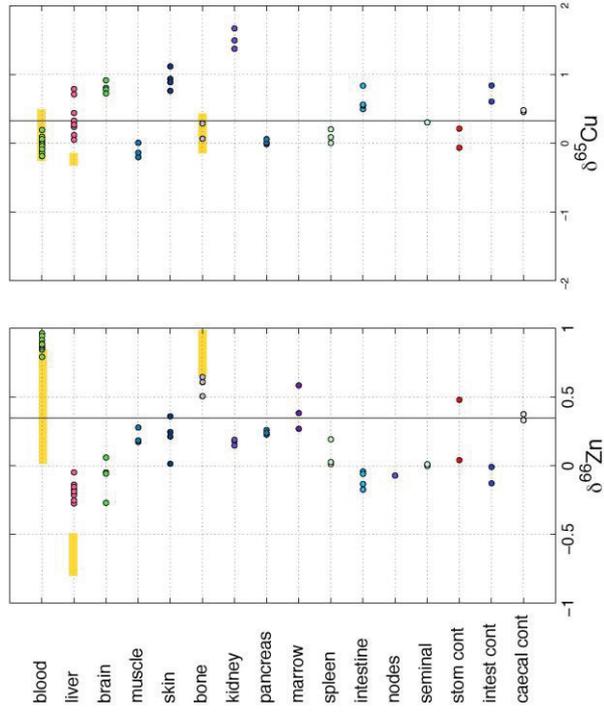


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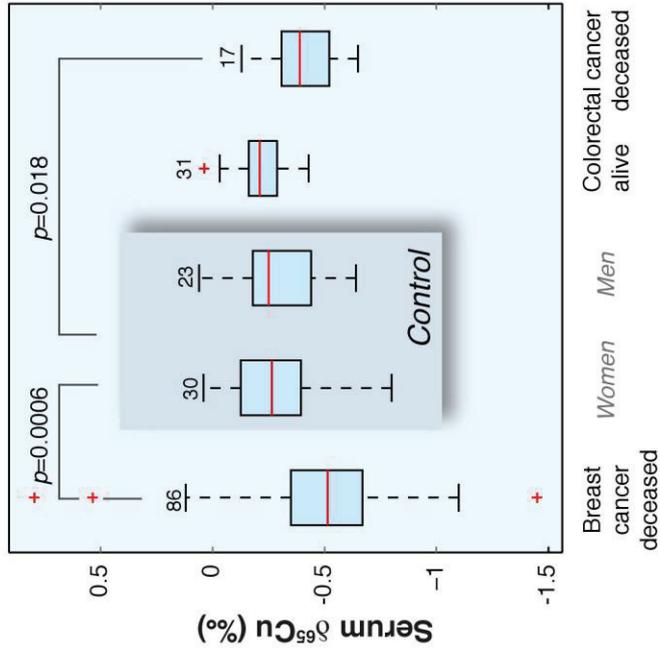






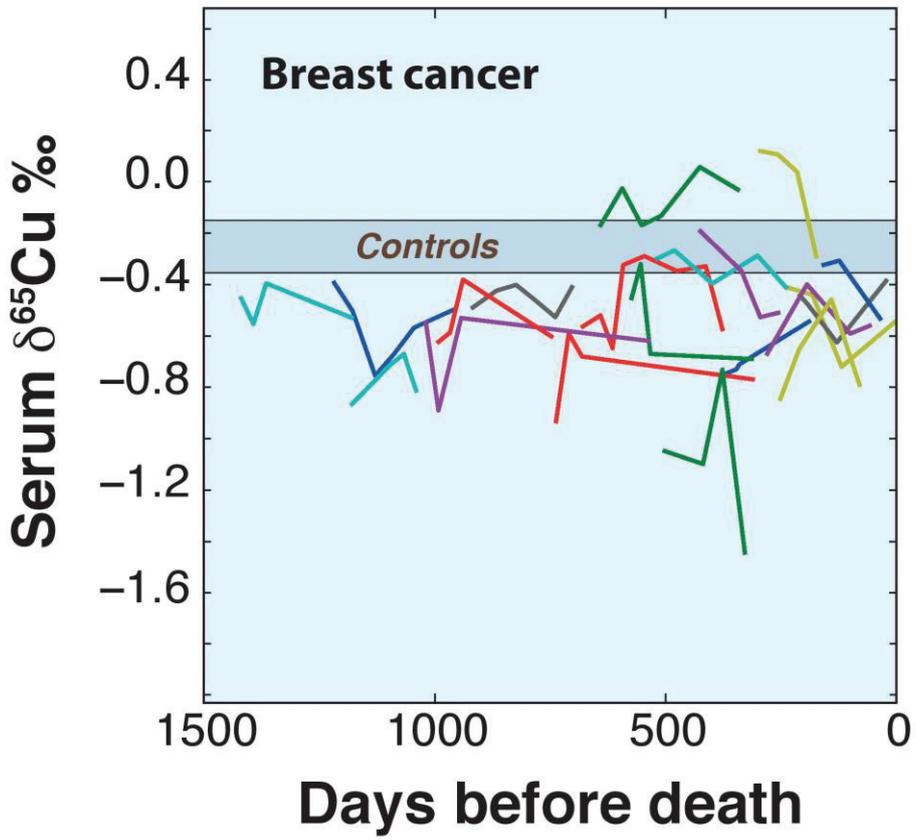


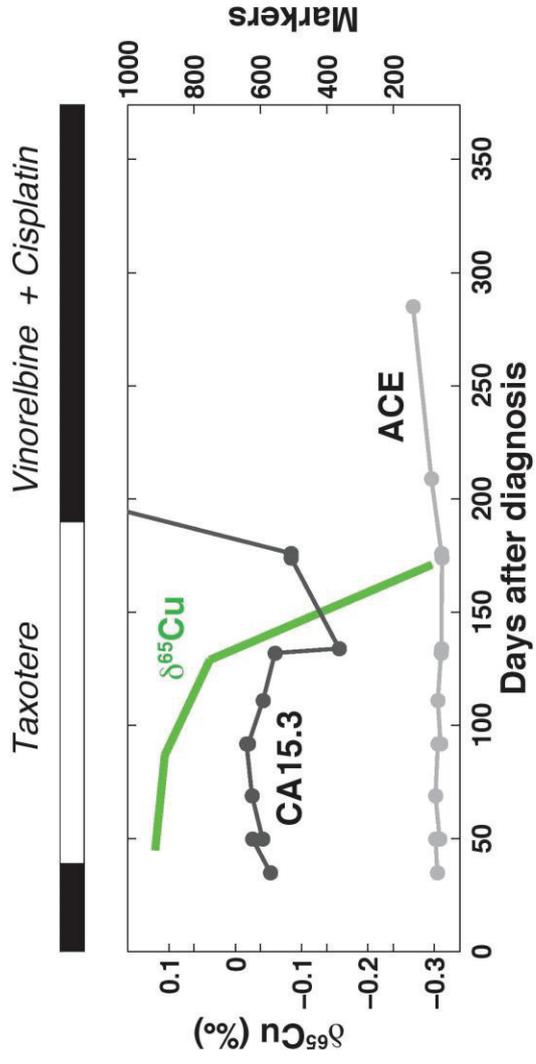
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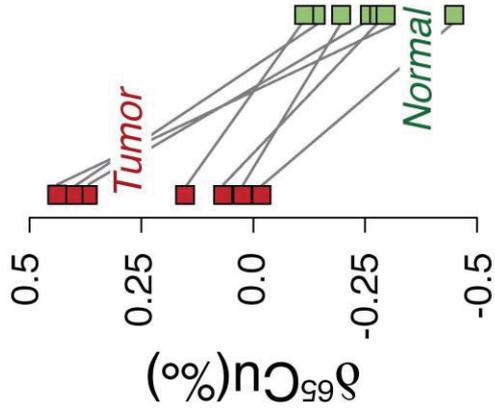
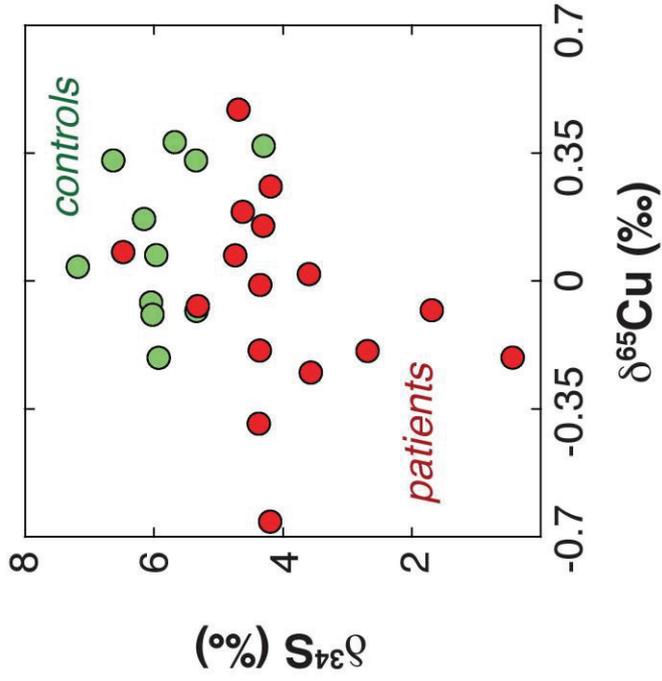
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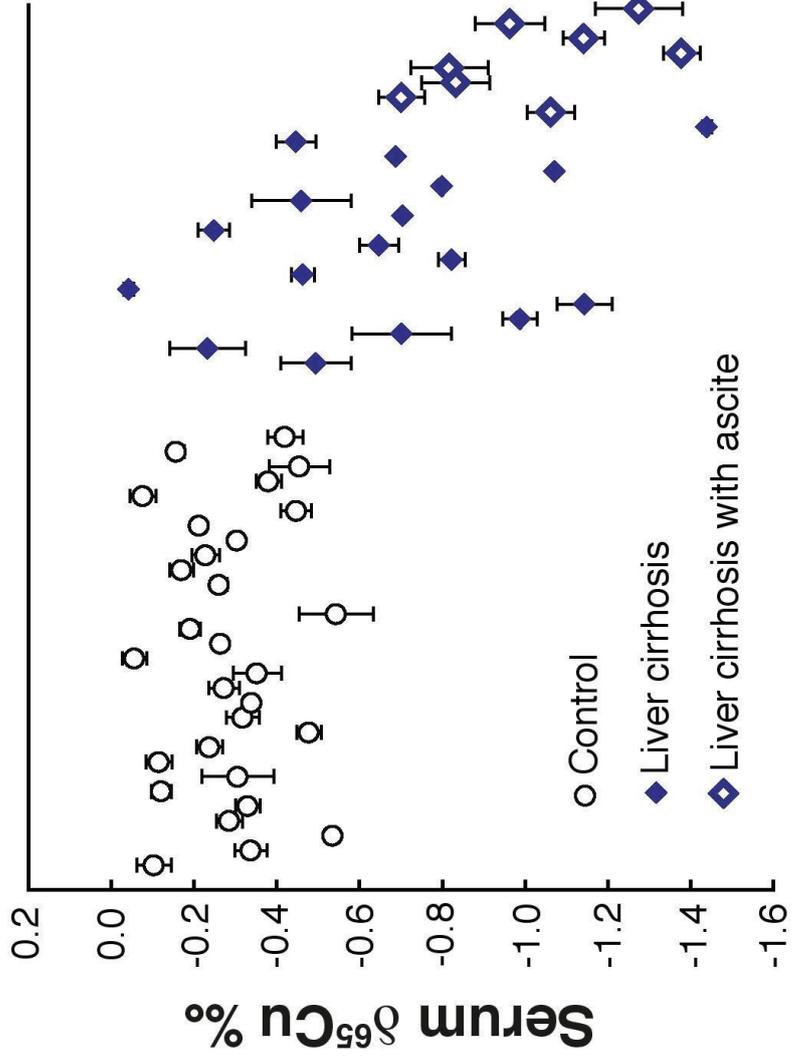
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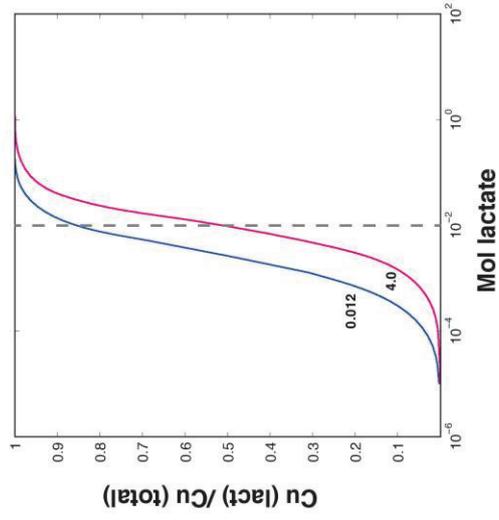


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### 2.3 Zinc (Zn)

Zinc is the chemical element with the atomic number 30. It is the first element of group 12 and it shares with other elements of this group a similar ion size and a common oxidation state (+2). Differently from the other elements of the group 12, Zn is relatively stable in its divalent state and does not undergo redox changes.

Zinc is the 24th most abundant element in Earth's crust and has five stable isotopes that occur in nature.  $^{64}\text{Zn}$  is the most abundant isotope (48.63% natural abundance) with a long half-life and with an ignorable radioactivity, followed by  $^{66}\text{Zn}$  (28%),  $^{68}\text{Zn}$  (19%),  $^{67}\text{Zn}$  (4%) and at last  $^{70}\text{Zn}$  (0.6%). Even if less abundant,  $^{70}\text{Zn}$  conserves characteristics and half-life similar to the most abundant isotopes.

Biologic and public health importance of Zn have been highlighted in different papers and have been reviewed for this work (66). Disturbances in Zn function and metabolism may have serious consequences for health. Actually, this element plays an important role in growth, development and functioning of all living cells. Our aim was to summarize how Zn homeostasis is regulated in the different human cells and tissues and by which mechanisms; moreover, Zn effects on the immune response were described in detail, focusing on those that are implicated in the physiopathology of rheumatoid arthritis (chapter II. Review paper: "Zinc and its role in immunity and inflammation).

### 2.4 Cadmium (Cd)

Another metal of the group 12, Cd, was studied in this work, with particular accent on its effect on the immune system modulation.

It is often stated that Cd is "natural" and this means that it is naturally present, in traces, everywhere in air, water, soils and food. The consumption of Cd has been

increasing for years during industrialization and it cannot be degraded, causing a steady increase of its concentration in the environment(67). Improved technology for the production, use, and disposal of Cd and Cd-containing products is nowadays trying to reduce the emissions.

Even if contaminated water is the first source of Cd, another important source for humans is smoking, as the tobacco plant naturally accumulates relatively high concentration of Cd in its leaves. Cadmium appears as a novel risk factor, independent from smoking, contributing to cardio-vascular diseases (68). It has been recognized in the list of carcinogenic molecules and even if the exposure to high Cd-doses was previously stated as an important risk factor, there are epidemiologic evidences that even long-term exposure to low doses of Cd can have an effect. For these reasons the safe day-exposure dose to Cd according to WHO guidelines is limited to 25 µg/Kg of body weight (69).

Liver is the first target organ following acute Cd intoxication (70–72). High doses of Cd administered to experimental animals lead to morphological and functional changes in these organs by cellular mechanisms that will be explained in the next paragraphs (and increasing liver-damage marker as transaminases or C-reactive protein production). Moreover, Cd target kidney and bones, especially in the case of chronic exposure: kidney is targeted directly, bones can be targeted indirectly via kidney, inducing  $\text{Ca}^{2+}$  excretion in urine, or directly with osteoporosis induction (73).

#### *2.4.1 Transport and effects of cadmium in cells*

Most chemical forms of cadmium are ionic, which prevents passive diffusion through biological membranes. A variety of trans-membrane molecules have been proposed to participate to Cd transport, but in mammalian cells, it mainly uses Zn transporters (74, 75). Cadmium, differently from other metals as Cu and Zn is not

needed by mammalian cells for living; nevertheless, and probably through a process of ionic mimicry, it can substitute other metal ions (mainly  $Zn^{2+}$ ,  $Cu^{2+}$  and  $Ca^{2+}$ ) on the access-mechanisms into the cells and on enzymes inside the cells: this process is at the basis of Cd toxicity.

Zn transport is described in detail in the review paper in the chapter II (66) and the used transporters are the same for Cd. The expression of the Zrt/IRT-like proteins (ZIP) importers is tissue specific. For that reason, Cd absorbed through the respiratory tract, through smoking, or through the gastro-intestinal tract, through water and food, will use different and cell-specific transporters of the tissue on which it deposit. In the case of the synoviocytes the specific importer is ZIP-8 and the cell surface concentration of ZIP-8 seems to follow iron availability by a post-transcriptional mechanism (76).

Once it entered the cell, Cd induces the metal-regulatory transcription factor 1 (MTF-1) which triggers metallothioneins (MTs) transcription. MTs are small, cysteine rich metal binding proteins that function as the regulator of metal homeostasis in cells. The MT molecule is composed of two domains: the  $\alpha$ -domain binds four atoms of metals, while the  $\beta$ -domain binds three atoms. In various organs and intracellular organelles, the different localization and physiological functions of MTs, including those of the metal-free (apo-MT) and metal-bound (holo-MT) forms, impact the redox and energy status of the cells.

In the cell, MTs act as a Zn-chaperone protein by delivering metal to metalloenzymes, which require a constant shuffling of metal ions, and control the response to the stress conditions, in particular detoxification mechanisms in response to heavy metals (77), with a potential protective role for the cell. A recent

work stated that clustered Cd-MTs are more resistant than apo-MTs to proteolysis (78).

All these reactions elicited by Cd inside the cell may lead to death or stress adaptation, survival or cancer. These processes are mediated by signaling pathways which induce up-regulation of various stress-inducing or protective molecules (e.g. glutathione). Moreover, Cd inhibit or damage DNA repair enzymes, cell cycle control proteins, pro-apoptotic and tumor-suppressing proteins (e.g., PARP-1, p53), Zn-containing transcription factors, enzymes mediating DNA methylation, and also E-cadherin (79). Nevertheless, its role on cell proliferation and apoptosis is still not clear and depends on the cell type.

Zinc transporter proteins (ZnTs) facilitate the removal of cytosolic free metal ions either by exporting them through the plasma membrane or by sequestering them in vesicles; ZnT1 is the only transporter expressed ubiquitously and the only one among ZnTs expressed on the cellular membrane and mediating metal export to the extracellular fluid. Zn is known to regulate the expression of this transporter through MTF-1 in several cell types. Nevertheless the expression of this exporter on synoviocytes and the ability of Zn and other metals in modulating its expression need further investigations.

#### *2.4.2 Competitive role of Zn and Cd*

Even if they share many physico-chemical properties and they use the same transporters, Cd<sup>2+</sup> and Zn<sup>2+</sup> ion competition at different levels is still not clear and depends on the model.

ZIP importers, for the uptake into cells, are not selective, except for Cu, which is not transported by ZIPs, but has other specific transporters. This means that the

ratio of Zn/Cd ions transported only depends on the availability of the metal. In this way, one of the metals can influence the uptake and action of the other, depending on their levels.

For intracellular binding sites, Cd may displace Zn in a number of biological processes, due to its higher affinity to several sites. Depending on the biological systems, Cd and Zn are linked to macromolecules, primarily through sulphur (S), oxygen (O) and nitrogen (N) and interact readily with S-, O- and N-donors (80). They bind preferentially to the same proteins — albumin in the bloodstream and MTs and other proteins in tissues (81). Although both metals have a high affinity to biological structures (proteins, enzymes) containing –SH (sulphydryl) groups, the affinity of Cd to S-ligands as well as to N-donors is greater than the one of Zn (67). Thus, many toxic effects of Cd occur through a disruption of Zn-mediated or Zn-dependent metabolic processes, including cellular production of DNA, RNA and protein.

The binding of Zn and Cd to MTs have long been considered isomorph, but the Cd-detoxifying role of MTs occurs through the replacement of Zn-MT by Cd-MT demonstrating a competitive role of the two metals, in favor of Cd binding.

Even if the affinity of Cd for MTs is higher, there are evidences of a protection role afforded by Zn-supplementation against the deleterious effect of Cd. Numerous data, for example, indicate that some of the adverse acute and chronic effects of Cd in laboratory animals can be prevented or markedly reduced by prior, post or concomitant Zn supply (82, 83). This can be due to the reduced import of Cd when a higher level of Zn is given. For the same reason, low dietary Zn is an important factor increasing Cd absorption, retention and accumulation in the organism, or

vice-versa elevated level of Cd in blood are often accompanied by changes in levels of some essential elements, including Zn (84–86).

#### 2.4.3 Cadmium and Zinc levels in biological samples of RA patients

Unpaired levels of metals were measured in biological samples from RA patients in comparison to healthy controls.

The mean values of Arsenic, Lead and Cd were found higher in blood, scalp hair, and urine samples of arthritis patients as compared to those values obtained in age-matched referent subjects (87). The concentration of Zn was lower in the biological samples of rheumatoid arthritis patients of both genders with respect to non-arthritic subjects and negatively correlated with the concentration of Cd (87). Moreover a case-report study described a patient affected by heavy metal intoxication, with evidence of Cd presence, who developed rheumatoid arthritis (88). Zn supplementation potential role in mitigate the effects of Cd intoxication was already proposed (89).

## **II. AIMS OF THE WORK**

The general aim of the work was to investigate how chronic inflammation can modify metal homeostasis using the RA model. Moreover we analyzed the effect of different cytokines in inducing changes in metal homeostasis. Interesting results obtained *in vitro* and on biopsies lead to the development of a new strategy for local treatment of diseases characterized by joint hyperplasia and inflammation.

To do this we specifically aimed to analyze:

- How Zn and Cd can be transported in the cell (in comparison to other metals) and if the transport could be modified by metal level and inflammation
- The differential effect of Cd and Zn for transporter regulation
- The differential effect of mediators of inflammation (IL-17, TNF, IL-1 $\beta$ ) in regulating metal transport in cells
- The effects of metal assimilation by synoviocytes on cell viability and inflammation in the *in vitro* and the *ex vivo* model of rheumatoid arthritis
- Possible therapeutic application of the anti-inflammatory and anti-proliferative effect of Cd on the *in vivo* model of rheumatoid arthritis.

### **III. ZINC AND ITS ROLE IN AUTOIMMUNITY AND INFLAMMATION**

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## Review

Zinc and its role in immunity and inflammation<sup>☆</sup>Paola Bonaventura<sup>a</sup>, Giulia Benedetti<sup>a</sup>, Francis Albarède<sup>b</sup>, Pierre Miossec<sup>a,\*</sup><sup>a</sup> Immunogenomics and Inflammation Unit and the Department of Clinical Immunology and Rheumatology, Hospices Civils de Lyon, EA 4130 University of Lyon 1, Hôpital Edouard Herriot, Lyon, France<sup>b</sup> CNRS UMR 5276 "Laboratoire de Géologie de Lyon", Ecole Normale Supérieure, 46 Allée d'Italie, 69634 Lyon, France

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## ABSTRACT

Zinc (Zn) nutritional importance has been known for a long time, but in the last decades its importance in immune modulation has arisen. This review aims at describing the mechanisms involved in the regulation of Zn homeostasis and their effects on the immune response focusing on those which are implicated in the pathophysiology of rheumatoid arthritis. Zn functions as a modulator of the immune response through its availability, which is tightly regulated by several transporters and regulators. When this mechanism is disturbed, Zn availability is reduced, altering survival, proliferation and differentiation of the cells of different organs and systems and, in particular, cells of the immune system. Zn deficiency affects cells involved in both innate and adaptive immunity at the survival, proliferation and maturation levels. These cells include monocytes, polymorphonuclear-, natural killer-, T-, and B-cells. T cell functions and the balance between the different T helper cell subsets are particularly susceptible to changes in Zn status. While acute Zn deficiency causes a decrease in innate and adaptive immunity, chronic deficiency increases inflammation. During chronic deficiency, the production of pro-inflammatory cytokines increases, influencing the outcome of a large number of inflammatory diseases, including rheumatoid arthritis.

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## Contents

1. Introduction . . . . .	278
2. Transport of zinc and its family members . . . . .	278
2.1. Entry (import) . . . . .	278
2.2. Exit (export) . . . . .	278
2.3. Homeostasis . . . . .	278
3. Zinc functions inside cells . . . . .	278
3.1. Structural, catalytic and co-catalytic roles of Zn . . . . .	278
3.2. Zinc as a neurotransmitter and secondary messenger . . . . .	280
3.3. Zinc regulation of proliferation and apoptosis . . . . .	280
4. Role of zinc in the immune system . . . . .	281
4.1. Zinc and the innate immune system . . . . .	281
4.2. Zinc and the adaptive immune system . . . . .	281
4.3. Zinc and inflammation . . . . .	282
5. Role of zinc in diseases . . . . .	282
6. Conclusions and perspectives . . . . .	283
Conflict of interest . . . . .	284
Take-home messages . . . . .	284
References . . . . .	284

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## 1. Introduction

The 24th most abundant element in the earth's crust, zinc (Zn), is a metallic chemical element, which has "exceptional biologic and public health importance" [1,2]. Zn is referred to as a trace element with a minor plasma pool (13.8–22.9  $\mu\text{mol/L}$ ) and a rapid turnover. Because of the absence of specialized Zn storage in the body [3], a Zn daily intake (20–40% of daily intake depending on diet) is required to achieve the steady-state, maintain it and support all its functions.

Zn is involved in numerous aspects of cellular metabolism and is an integral component of proteins involved in cell structures and stabilization of cell membranes [4]. It plays a role in cellular respiration (carbonic anhydrase), immune functions [5], protein synthesis [6], wound healing [7], DNA synthesis [6,8], and cell division [6]. For these reasons, Zn nutritional defect or over-absorption is linked to a large number of diseases and particularly to immune diseases.

This review describes the importance of Zn at the cell, tissue and system levels and analyzes the effects of its deficiency on the immune system during chronic inflammation. Its role in several pathologies will be reviewed, with focus on the chronic inflammatory disease rheumatoid arthritis (RA).

## 2. Transport of zinc and its family members

Zn is a ubiquitous element in cells, present in the cytoplasm and in most organelles. Zn belongs to the family of transition metals, which have low ionization energies and a wide range of oxidation states, or positively charged forms. Zn, cadmium (Cd) and mercury (Hg) have been classified as "group 12" metals. They share their way of transport in mammalian cells. They are taken up across cell membranes by active [9] or facilitated diffusion and this mode of transport in cells has been described as "ion mimicry" [10].

Mammalian Zn transporters come from two major families, the SLC39 (Zrt-Irt-Protein, ZIPs) family and the SLC30 (Zn-transporters, ZnTs) family. ZIP importers, ZnT exporters, notably ZnT1, and intracellular binding proteins, notably metallothioneins (MTs) (Table 1 and Fig. 1) are the main controllers of intracellular Zn concentration.

### 2.1. Entry (import)

Zn is taken up as a divalent cation. Zn has a single oxidation state and cannot be oxidized or reduced. The  $\text{Zn}^{2+}$  ions are hydrophilic and do not get through cell membranes by non-facilitated-passive diffusion, so they need specialized transporters to get into the cytoplasm. The ZIP family, in mammals, consists of 14 isoforms (ZIP1 to ZIP14) of transporters, which promote metal ion passage from the extracellular fluid or from intracellular vesicles into the cytoplasm [11] (Table 1 and Fig. 1). Most ZIP members have a similar protein structure with eight transmembrane domains and a cavity through which metals are transported [12]. Despite such similarities, different ZIP transporters are expressed specifically on different cell types (Table 1) [13]. The role of Zn in cells is based on its behavior as a divalent cation and therefore, the absorption of Zn is reduced by increased levels of other divalent cations, such as cadmium, copper, magnesium, calcium, nickel and iron [14].

### 2.2. Exit (export)

Flux of Zn away from the cytosol is needed to balance the input. Zn export is controlled by ZnT proteins. ZnT family consists of 10 isoforms (ZnT1 to ZnT10) which contribute to the cytoplasmic Zn balance by exporting Zn out to the extracellular space or by sequestering cytoplasmic Zn into intracellular compartments when cellular Zn levels are too elevated [15] (Table 1 and Fig. 1). ZnT-1 is the only member of the SLC30 family which is ubiquitously expressed and the only Zn transporter involved in Zn efflux across the plasma membrane in many different cells, thus conferring resistance to Zn [16]. Moreover, among all

transporters, ZnT-1 is the most highly regulated, with expression levels increased up to 20-fold during Zn supplementation [17]. Low-dose of Cd induces ZnT1, while ZnT1 silencing enhances Cd toxicity [18]. The other members of the ZnT family are localized on the membrane of intracellular organelles and secretory vesicles/granules. They sequester cytoplasmic Zn into various compartments for secretion, storage or for supplying proteins that require Zn for their structure and function [19, 20]. For example, different ZnTs working together are necessary to supply Zn to alkaline phosphatases and other enzymes important in the early secretory pathway of proteins [21].

### 2.3. Homeostasis

Zn homeostasis is maintained by the regulation of uptake/elimination but also by intra-cellular sequestration in the so-called "zincosome". Metallothioneins, which behave as Zn chaperones, are a major tool of this process: Zn is extensively chelated by MTs in the cytosol thanks to their large Zn-binding capacity [22] (Table 1 and Fig. 1). MTs are small, cysteine-rich metal binding proteins and are involved in the regulation of Zn homeostasis at the cytoplasmic level and possibly act as a Zn pool [23]. Their half-life is short, depending on the tertiary structure of metal-metallothionein complexes and until a maximum of 24 h [24].

MTs have the capacity to bind heavy metals. Because Cd-MT complexes are 100 to 1000-fold more stable than Zn-MT complexes, MTs were initially considered as Cd detoxification molecules [25,26]. It now appears that MTs are involved in, at least, two major types of reactions with Zn: Zn buffering and Zn muffling. Zn buffering is, under steady state conditions, a primary function of cytosolic Zn-binding proteins to buffer the Zn content in the picomolar range. Under non-steady state conditions, muffling reactions can restore the initial value by removing Zn from the cell, or sequester Zn in subcellular compartments. The process of muffling differs from buffering process because it is slow compared with thermodynamic buffering process [27].

MTs, as well as some exporters, are transcribed via the metal-responsive transcription factor-1 (MTF-1) [28,29]. MTF-1 is a pluripotent transcriptional regulator localized in both cytoplasm and nucleus. It is involved in cellular adaptation to various stress conditions, primarily exposure to heavy metals, but also to hypoxia and oxidative stress. When a cell is exposed to stress, MTF-1 accumulates in the nucleus, binds DNA, recruits different co-activators and often relies on other transcription factors for a coordinated target gene expression. Its DNA-binding domain is composed of six Zn fingers of the Cys2His2-type. Since MTF-1 shows an increased DNA-binding upon Zn supplementation, this domain was suggested to mediate the intrinsic Zn sensing of MTF-1 [30]. MTF-1 up-regulates the expression of exporters and scavengers and represses the expression of importers in response to heavy metal exposure, such as Cd [31,32].

## 3. Zinc functions inside cells

### 3.1. Structural, catalytic and co-catalytic roles of Zn

Zn functions can be divided into three large groups, namely structural, catalytic, and co-catalytic. The structural Zn atoms maintain the tertiary structure of an enzyme in a manner analogous to that of disulfide bridges, but their removal may not abolish the enzymatic activity. The role of a structural Zn site is probably twofold: it maintains the structure of the protein in the immediate vicinity of the metal site and in this manner; it may influence enzymatic activity [33]. The catalytic Zn atoms participate directly in the bond-making or breaking-step [34] and the co-catalytic Zn atoms operate in concert to affect catalysis and perhaps stabilization of active site conformation. Various arrangements of Zn-binding domains exist in proteins depending on their function. Among structural domains of proteins, Zn-finger domains have a major physiological relevance, as demonstrated by their presence in

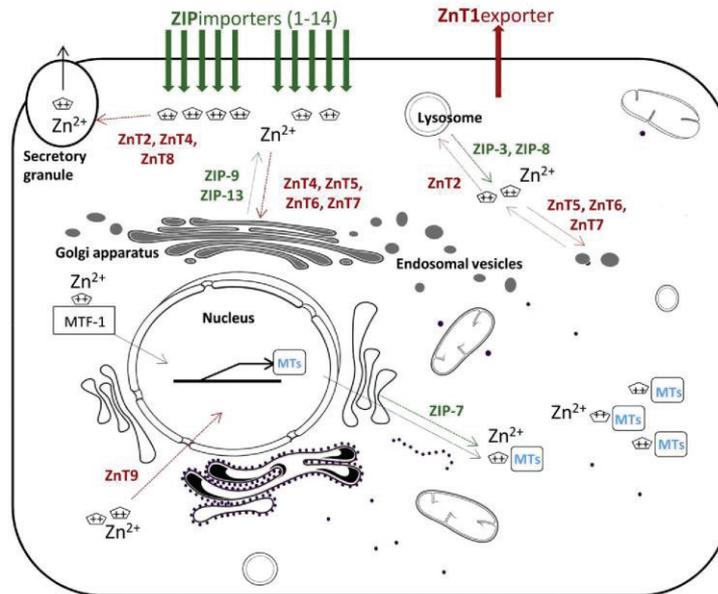
**Table 1**  
Zinc transporters, their distribution, role and involvement in diseases.

Importers	Tissue specificity	Role	Disease implication	Ref.
Zip1	Ubiquitous. Expressed in most adult and fetal tissues	Mediates Zn uptake. Functions as a major endogenous Zn uptake transporter. Rapid uptake and accumulation of Zn in prostate cells	Prostate cancer	[85]
Zip2	Expressed only in prostate and uterine epithelial cells	Uptake of Zn and other divalent cations. May be important in contact inhibition of normal epithelial cells	Possible loss of its effect in tumorigenesis	[86]
Zip3	Bone marrow, spleen, small intestine and liver	Zn influx transporter	–	[11]
Zip4	Kidney, small intestine, stomach, colon, jejunum and duodenum	Zn homeostasis regulated by Zn availability	Acrodermatitis enteropathica	[75]
Zip5	Intestine, pancreas, liver, and kidney cells	Uptake of dietary Zn. Role in polarized cells by carrying out serosal-to-mucosal Zn transport. Tissue Zn homeostasis.	–	[87]
Zip6	Prostate, placenta, mammary gland and HeLa cells	Zn import. Could act as a metalloproteinase. Controls the nuclear localization of the Zn finger transcription factor Snail	Placenta cancer development and metastasis	[88]
Zip7	Mammary gland cells	Zn import. Causes tamoxifen resistance when its expression is increased	Breast cancer	[89]
Zip8	Fibroblasts and chondrocytes	Responsible for Cd transport and toxicity in fibroblasts and chondrocytes	Osteoarthritis	[90–92]
Zip9	Human lymphocytes	Activation of AKT in response to BCR activation		[93]
Zip10	Mammary gland cells	Zn influx	Breast cancer	[94]
Zip11	Testes, digestive tract: stomach, ileum and cecum	Zn transport. Regulated by dietary Zn	Zn deficiencies (low expression)	[95,96]
Zip12	Brain and eye	Zn transport	May be involved in the outbreak of schizophrenia	[97]
Zip13	Bone, teeth and connective tissue	Zn transport. Maturation of osteoblasts, chondrocytes, odontoblasts and fibroblasts	Ehlers–Danlos syndrome-like spondylocheirodysplasia (SCD-EDS)	[98]
Zip14	Mammalian cells	Responsible for Cd transport and toxicity in mammalian cells	Hypozincemia and inflammation	[13]
Exporters	Tissue specificity	Cell localisation and role	Disease implication	Ref.
ZnT1	Ubiquitously expressed	Plasma membrane. Export of metals from cytoplasm to extracellular medium	–	[16]
ZnT2	Mammary gland, prostate, retina, pancreas, small intestine and kidney	Plasma membrane, endosomal/lysosomal/secretory vesicles, and mitochondria	Reduced zinc content in nursing women	[15]
ZnT3	Brain, testes and pancreas	Accumulation of metals in organelles. Synaptic vesicles	Seizures, learning defects and memory loss	[15]
ZnT4	Ubiquitously expressed	Absorption of metal ions	–	[15]
ZnT5, ZnT6, ZnT7	Ubiquitously expressed	Plasma membrane, membrane of the Golgi apparatus, and cytoplasmic vesicles	Bone abnormalities, heart failure, prostate cancer and diet-induced diabetes	[99]
ZnT9	Ubiquitously expressed	Accumulation of metals in vesicles, for transport. Cytoplasm, nucleus	–	[100]
Regulators	Tissue specificity	Cell localisation and role	Disease implication	Ref.
MT-1 (MT-1A, -B, -E, -F, -G, -H and -X)	Ubiquitously expressed, preferentially in fibroblasts, epithelial and endothelial cells	Cytoplasm detoxification from heavy metals ions	Prostate cancer, hepatoma, breast carcinoma and metal-induced toxicity	[101]
MT-2	Ubiquitously expressed	Nucleus and cytoplasm. Role in cell proliferation and apoptosis	Breast cancer, prostate cancer, gastric cancer and cystitis	[101]
MT-3	Primarily expressed in the central nervous system and a small amount in intestine, lung and pancreas epithelial tissues	Nucleus and cytoplasm	Multiple system atrophy and non-small cells lung cancer	[102]
MT-4	Primarily in skin and upper gastrointestinal tract	Nucleus and cytoplasm	Stomach and intestinal inflammation, diarrhea, loss of taste and texture discrimination of the tongue and skin traumas	[103]

Abbreviations: AKT (protein kinase B); BCR (B-cells receptor).

3% of the genes in the human genome [1]. Structurally, Zn is a critical part of Zn-finger domains coordinated by one or more Zn ions, in order to stabilize the protein folding and regulate scores of protein–protein or protein–nucleic acid interactions.

The Zn proteome, referring to the number of potential Zn proteins in a genome, has been estimated at around 3000 proteins [35]. Several of these proteins are known to regulate the immune system (Table 2). However, the functions of numerous other Zn proteins are still unknown.



**Fig. 1.** Subcellular localization of human ZIP/ZnT Zn transporters and of Zn homeostasis regulators. Directions of Zn transport are indicated with arrows. Green arrows indicate Zn entry in the cytoplasmic space (from extracellular space or from organelles) mediated by ZIP importers (depicted in green) and red arrows indicate Zn exit from the cytoplasmic space (to extracellular space or to organelles) mediated by ZnT exporters (depicted in red). MTF-1 accumulates in the nucleus and binds to DNA for the transcription of MTs (depicted in blue), necessary for Zn homeostasis in the cell. Note that this figure only provides a very simplified view of a general cell type, as expression of some ZIP/ZnT proteins are tissue- or cell type-specific, and regulated by Zn and other metal status.

### 3.2. Zinc as a neurotransmitter and secondary messenger

Zn ions act also as intracellular signaling molecules, capable of communicating between cells, converting extracellular to intracellular signals and controlling intracellular events [36]. Zn executes its functions in two different ways: as a neurotransmitter (or the first messenger in a cell–cell communication), or as an intracellular signaling molecule (as the secondary messenger). In the first case, Zn is stored in membrane-enclosed synaptic vesicles and released into the surrounding milieu by exocytosis after pre-synaptic stimulation. It is then taken up into the cytoplasm of neighboring cells through gated Zn channels [37]. In the second case, Zn signaling is activated when extracellular stimuli such as cytokines or growth factors increase nitric oxide (NO) and subsequently the stimulation of  $Zn^{2+}$  release from MTs. This change in intracellular Zn can be either in a transcription independent (early Zn signaling or EZS) [36] or dependent (Late Zn Signaling or LZS) manner [38]. When EZS occurs, Zn levels change rapidly. In LZS, the intracellular Zn levels are altered several hours after extracellular stimulation, through changes in Zn transporter expression. Since many cytosolic proteins may have Zn-binding potential, both EZS and LZS are involved in a wide range of physiological responses, including immune functions, cell development and apoptosis.

### 3.3. Zinc regulation of proliferation and apoptosis

Zn has a key role in the regulation of proliferation and differentiation. Indeed, cell proliferation does not occur in the absence of Zn [9]. Zn is a structural component of different proteins involved in the

transcriptional machinery, such as transcription factors and ribosomes and the presence of Zn is also necessary for DNA synthesis. Furthermore, Zn is a second messenger of mitogenic signaling [39] and a master growth and proliferation factor, involved in the regulation of cell division. Moreover, Zn binds to MTs, which are largely expressed in highly proliferating tissues and translocate into the nucleus during the S-phase of the cell cycle. After nuclear translocation,  $Zn^{2+}$  is released and regulates the completion of the cell cycle [40], probably via affecting Zn-activated gene transcription of critical mitogenic signals [9].

Zn has a key role in apoptosis regulation. Zn chelation in cell culture medium causes apoptosis and subsequent addition of Zn protects cells against the undergoing apoptosis even if it was added to the cell culture only a short time after an apoptotic agent [41]. The cellular Zn buffering capacity determines the threshold between physiological and pathophysiological actions of Zn ions: Zn can become cytotoxic if Zn buffering is compromised, with a large fluctuation of Zn ions, which can injure cells through effects on redox biology and interactions with several proteins. In this case, a breakdown of Zn transporting system occurs leading to the activation of apoptosis [42,43].

Regarding cell differentiation, Zn deficiency inhibits myotube differentiation in myoblasts [42]. In pre-adipocytes, the phase of rapid proliferation after stimulation of differentiation is concomitant with a rise in MTs-binding-Zn import from cytosol to nucleus, explaining the importance of Zn for their differentiation [44]. This Zn–MT system could be a general system used for every cell differentiation.

Thus, Zn is present in all enzyme classes, as well as in transcription and replication factors and is implicated at all levels of cellular signal transduction. As Zn is essential for proliferative cells, its role in the regulation of immune cells is crucial.

**Table 2**  
Major Zn-proteins and their function on the immune system.

Protein	Function	Effector cells	Ref.
<i>Structural domains</i>			
PLZF (promyelocytic leukemia zinc finger)	NKT cell development	NKT cells	[104]
Bcl-6	Proliferative expansion of germinal centers of B cells	B lymphocytes	[105]
Gfi1	T-cells lymphogenesis and pre T-cell development	T-cell precursors	[106]
	Differentiation of myeloid precursors into granulocytes or monocytes	Mature T-cells Granulocytes Monocytes	
<i>B lymphocyte maturation-induced</i>			
protein-1 (Blimp1)	T-cell homeostasis and effector differentiation	T-cells	[107]
Tnfr1	Terminal differentiation of different immune cells	Short-lived CD8(+) cytotoxic T cells	
Tnfr2	Necessary and partly redundant for T-cell differentiation	T-cells	[108]
<i>Catalytic and co-catalytic activity</i>			
The TNF-alpha converting enzyme (TACE)	Proteolytic release from cellular membranes of some cytokines, chemokines, growth factors and their receptors, including TNF- $\alpha$	Macrophages, monocytes, and T-cells for the production of pro-inflammatory molecules	[109]
<i>Calprotectin (S100A8/A9)</i>			
	Heterodimer forms	Myeloid cells in particular neutrophils	[110]
	Zn binding protein expressed on immune cells		
<i>MMPs (1 to 23)</i>			
	Promote chemotaxis by controlling chemokines activity	Innate and adaptive immune system cells	[111]
	Remodeling and repairing tissues		
	Angio- and embryogenesis		
MMP-2	Cleavage of CCL-7 creating chemokine antagonist, Reduction of immune response	Innate and adaptive immune system cells	[112]
MMP 1-3-9-13-14	Cleavage of CCL1-2-3-9-13-14, and CXCL-8 (by MMP-9)	Innate and adaptive immune system cells	[113]
<i>SOD-1</i>			
	Reduction of immune cell recruitment		
SOD-1	Anti-inflammatory activity	Macrophages	[114]
<i>SOD-2</i>			
	Decrease of ROS		
SOD-2	Anti-inflammatory activity	Innate immunity	[115]
	Decrease of ROS		

#### 4. Role of zinc in the immune system

The immune system is highly proliferative, and thus particularly susceptible to Zn deficiency [45]. The immune response can be divided into two major systems: the innate and the adaptive immunity [46]. In the case of pathogens entering the body, the first cells acting in pathogen recognition and elimination are the cells of the innate immune system, notably polymorphonuclear cells (PMNs), macrophages and natural killer (NK) cells. PMNs are the first cells to actively enter the site of infection. They take up pathogens by phagocytosis and kill them through the production of reactive oxygen species (ROS) [47]. The adaptive immune response is orchestrated by highly specialized cells, the T and B lymphocytes. B lymphocytes play a role in the humoral immune response by the production of antibodies specifically directed against an antigen, whereas T-lymphocytes are involved in cell-mediated immune responses by activation of other immune cells (T helper lymphocytes) and by the production of toxic granules in cytotoxic T lymphocytes. Dendritic cells (DCs) serve as a link between the innate and adaptive immune systems. They circulate as immature cells and after coming into contact with the antigen, DCs start expressing major histocompatibility complex (MHC) molecules and co-receptors on their cell surface for the activation and stimulation of T cells.

##### 4.1. Zinc and the innate immune system

Zn regulates several crucial processes implicated in the innate immune response via mechanisms not yet completely understood (Fig. 2). First, Zn ions function as chemo-attractants for some immune cells. Zn deficiency leads to reduced PMN chemotaxis [48] and, inversely, a super-physiological Zn concentration (500  $\mu$ M) induces PMN chemotaxis *in vitro* [49]. Zn deficiency decreases phagocytosis while Zn supplementation has the opposite effect [50]. The effect of Zn on phagocytosis is probably mediated by Zn proteins involved in this process, such as the early endosome antigen 1 (EEA1). EEA1 binds directly to the phospholipid phosphatidylinositol 3-phosphate (PI3K) at its C-terminal and binds to Rab5 via its N-terminal zinc finger domain enabling membrane tethering and fusion critical for phagosome and

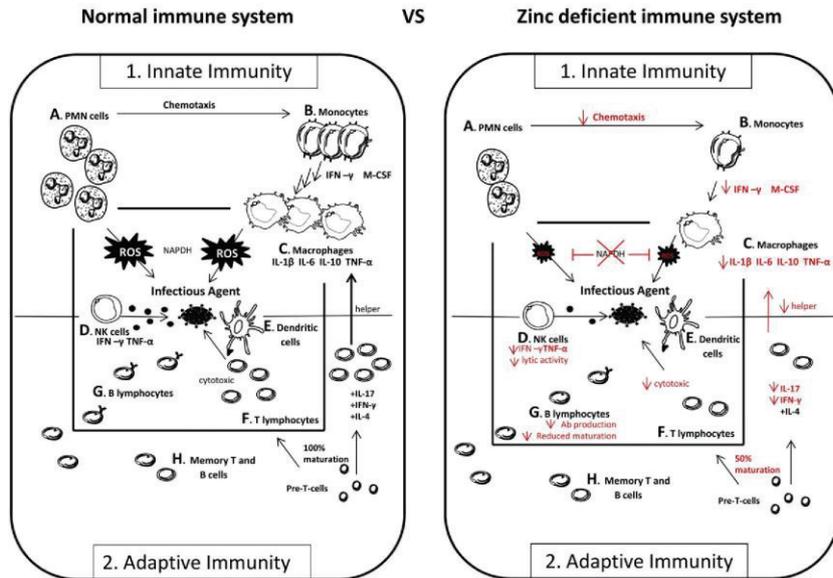
endosome maturation [51,52]. For the neutralization of pathogens, Zn is equally important: both Zn deficiency and Zn excess inhibit nicotinamide adenine dinucleotide phosphate (NADPH), which regulates superoxide anion production responsible for the destruction of pathogens after phagocytosis [53] (Fig. 2A).

Moreover, Zn promotes monocyte adhesion to endothelial cells *in vitro* [54] and is important for the production of pro-inflammatory cytokines such as interleukins IL-1 $\beta$ , IL-6 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (Fig. 2B and C). In an *in vitro* culture of peripheral blood mononuclear cells, Zn deficiency does not affect phagocytosis and oxidative burst of monocytes but only affects the production of IL-6 and TNF- $\alpha$ . This suggests a shift from intracellular communication to basic innate immune functions during Zn deficiency [55]. Zn also modulates the response of NK cells with a decreased recognition and stimulation of their MHC-class I expression [30]. Furthermore, Zn supplementation increases the differentiation of CD34+ cells toward NK cells as well as their cytotoxicity [56] (Fig. 2D).

Zn also affects the maturation of DCs. During LPS-induced DC maturation, dynamic changes in the expression of Zn transporters on the cell surface are observed: ZIP6 and ZIP10 are suppressed while several ZnTs are up-regulated [15]. Moreover, LPS stimulation decreases free Zn in DCs, and increases the expression of MHC-II and co-stimulatory molecules on DCs. Interestingly, treatment with a Zn chelator mimics the LPS effect, while Zn supplementation or overexpression of ZIP6 inhibits the up-regulation of MHC-II and of co-stimulatory molecules [15] (Fig. 2E). These results suggest that a reduction of Zn in DCs, through ZIP-6 down-regulation, is important for DC maturation and subsequent activation of the adaptive immune response.

##### 4.2. Zinc and the adaptive immune system

T cells are especially vulnerable to Zn deficiency, at both developmental and functional levels (Fig. 2F). Zn deficiency causes thymic atrophy and subsequent T-cell lymphopenia. A lack of Zn during T cells maturation leads to a 50% reduction in between "potential" pre-T-cells and "effective" T cells in mice, associated with an increased apoptosis of pre-T-cells [57].



**Fig. 2.** Schematic representation of the innate and adaptive immune system acute response in a normal versus Zn-lacking system. Innate immunity: Zn-lacking innate immune system is characterized by reduced PMN chemotaxis and phagocytosis with reduced NAPDH production and consequent reduced production of ROS for pathogen neutralization (A). Reduced monocyte adhesion and maturation of macrophages (B), as well as reduced NK (C) and DC maturation (E) are also observed during Zn deprivation. The production of cytokines is also modified by Zn deficiency. Adaptive immunity: Zn deficiency causes thymic atrophy with subsequent T-cell lymphopenia, reduced differentiation of T-cells, increased pre-T-cell death, unpaired balance between Th1 (decreased) and Th2, as well as reduced Th17 subset and subsequent reduced IL-17 production (F). B-cell maturation and antibody production are less affected (G). The ratio of memory versus naive T and B cells is also affected by Zn deprivation (H).

Zn is also crucial for the balance between the different T-cell subsets. Its deficiency decreases the production of Th1 cytokines (IFN- $\gamma$ , IL-2 and TNF- $\alpha$ ) whereas the Th2 response (IL-4, IL-6 and IL-10) is less affected, resulting in an imbalance between Th1 and Th2 subsets, which is restored when Zn reaches physiologic levels [30].

The proinflammatory Th17 cells are also negatively affected by Zn deficiency. Th17 cell development is critically controlled by IL-6-induced STAT3 activation during chronic inflammation and Zn suppresses Th17 development via attenuating this activation [58]. For example, in the collagen-induced arthritis (CIA), the mouse model of RA, Zn treatment inhibits Th17 development [58]. Mild nutritional Zn deficiency increases the ratio of cytotoxic T cells versus total T cells and of memory versus naive T cells [30], leading to possible dysfunctions of the immune system and autoimmune reactions (Fig. 2H). Furthermore, Zn regulates T cell activation via stimulating the autophosphorylation of the protein kinase Lck by interaction with the cytoplasmic tails of CD4 and CD8 [59].

Many important cytokine-driven functions in T cells are regulated by Zn–MT binding. MTs regulate the phosphorylation levels of the transcription factors STAT1 and STAT3, necessary for T cell survival and expansion. Zn binding to MTs could disrupt the phosphorylation process resulting in increased levels of IL-10, produced by a class of non-FoxP3 Treg cells called Tr1. IL-10 modulates the process of autoimmunity and is known to suppress some autoimmune processes [60].

B cell development and functions are less affected by Zn changes than T cells. Still, Zn deficiency causes a reduction of B cells, affecting the development of immature and pre-mature B cells [61] and affecting antibody production [62] (Fig. 2G). Zn addition has been evaluated as supplement for vaccination in deficient individuals leading to controversial results [63,64] (Table 3).

#### 4.3. Zinc and inflammation

Altered production of cytokines during Zn deficiency can lead to inflammation. It induces IL-1- $\beta$  release and subsequently inhibits the inflammation depending on the transcription factor NF- $\kappa$ B [65]. These data suggest that Zn deficiency contributes to diseases in which there is an IL-1 $\beta$ -dependent inflammatory response and that Zn supplementation could potentially have a beneficial effect [66]. Moreover, simultaneous evaluation of circulating cytokines and Zn status showed that the reduced circulating Zn correlates with increased IL-6, IL-8 and TNF- $\alpha$  levels [67]. In addition, cytokine signaling pathways are influenced by the Zn status. For example, the proliferative response of T and B lymphocytes following IL-6 and IL-2 stimulation increases in Zn deficiency, whereas it adversely influences the IL-4 signaling, causing an impairment of the immune system [68].

In summary, Zn deficiency affects all aspects of the immune system indicating that the availability of Zn is essential for the proper development and function of the immune system, even if some mechanisms of action are not yet decrypted. Consequently several pathologies are characterized by imbalanced Zn homeostasis, as described in the following section.

#### 5. Role of zinc in diseases

Malnutrition is one of the major public health challenges and the most common immunodeficiency disease. Micronutrient deficiencies (as Zn and vitamin A) are the major risk factors for malnutrition, which are responsible of more than one third of death in children under five in developing countries [69]. Moreover, malnutrition

**Table 3**  
Chronic inflammatory diseases characterized by Zn-deficiency and models of Zn supplementation.

Disease	Dysregulation	Pathway	Zn supplementation	Ref.
Dermatitis, acrodermatitis enteropathica	Zn decrease leads to an increased inflammatory status	TEWL increase, IgE increase, impaired Th1/Th2 balance, reduction in NF- $\kappa$ B, and increased TNF- $\alpha$ , IL-1 $\beta$ and IL-8 levels	Human study, not yet Zn supplementation; possibility to use Zn supplementation as long-term treatment	[116]
Arthritis RA Spondylarthritis	Reduced Zn assimilation leads to increased inflammatory status, alteration of the immune system, degradation of extracellular matrix components, Zn-mediated disruption of the Th1/Th2 balance with increase of Th17 and reduction of phagocytic function of PMNs	Increased secretion of IL-1 $\beta$ , IL-8 and TNF- $\alpha$ ; increased production of MMPs; increased production of ROS; reduced production of T <sub>H</sub> 1 cytokines (IFN- $\gamma$ , IL-2)	Zn supplementation reduced LPS-mediated production of pro-inflammatory cytokines in RA synoviocytes	[117]
Chronic asthma	Zn deprivation makes airway epithelial cells highly susceptible to apoptosis	Cellular apoptosis induced by oxidants or by the FAS death receptor pathway with caspase-3 activation, proteolysis of E-cadherin and $\beta$ -catenin	<i>In vitro</i> . Zn supplementation inhibited both apoptosis and paracellular leak	[118]
Atherosclerosis	Marginally Zn-deficient diet increases apoptosis of vascular smooth muscle cells (VSMC) important to maintain atherosclerotic plaque integrity	Decreased activation of extracellular signal-regulated kinase (ERK)1/2, which maintains BAD phosphorylation as a pro-survival mechanism	Rat model of VSMC apoptosis. Rabbit model for Zn supplementation fed with high cholesterol diet: delay in the progression of atherosclerosis	[119,120]
Ulcerative colitis, Crohn's disease, inflammatory bowel disease	Zn deficiency leads to increased apoptosis of intestinal cells	TNF- $\alpha$ Zn-dependent survival pathway with nuclear translocation of NF- $\kappa$ B and transcription of cIAP2 mRNA (apoptosis inhibitor)	Normal levels of Zn inhibit apoptotic pathways	[121]
Systemic lupus erythematosus	Zn decrease leads to increased inflammatory status; alteration of PMN phagocytic function	Dysregulation of MMP-9	Human. Increased phagocytic function of PMNs	[122]
Vasculitis	Reduced Zn intake, increased plasma lipoprotein levels, increased vascular inflammation and vascular lesions	Increased secretion of IL-1 $\beta$ and IL-6	AEKO mouse model; Reduced arterial lesions	[123]
Alzheimer disease	Abnormal compartmentalization of Zn	Failure of Zn homeostasis proteins in neurons (MTIII-ZnT1-3)	Mouse model. Efficacy dependent on additional nutrients	[124]

increases the risk of infections and affects every part of the immune system. Although dietary Zn deficiency was originally thought to be of rare occurrence, it is now estimated to affect more than 25% of the world's population [70]. According to a World Health Organization report (WHO 2002), Zn deficiency ranks fifth amongst the most important health risk factors in developing countries and eleventh worldwide [71]. As Zn is widely required in cellular functions, abnormal Zn homeostasis causes a variety of health problems reaching different levels of severity. If chronic, severe and untreated, Zn deficiency can be fatal through immune defects leading to infections. Less drastic symptoms include infections, hypogonadism, weight loss, growth defects, dermatitis, alopecia, and delayed wound healing [72].

Unfortunately, the importance of Zn in humans was only appreciated in the 60s; before, only animal studies were conducted on this subject. An increased susceptibility to infections in patients was associated with Zn deficiency, indicating its importance for host immunity [41]. Zn deprivation is associated with a decline of the immune system with increased inflammation leading to chronicity [73]. Several Zn supplementation studies were then conducted in Zn deficient patients affected by different disease types (viral, bacterial and parasitic infections or autoimmune diseases) [74], or as a supplement in vaccinations [62] (Table 3).

Acrodermatitis enteropathica is one of the first diseases to provide the hallmarks of Zn importance [72]. This rare genetic disorder is due to a recessive mutation in the gene encoding ZIP4, responsible for Zn uptake [75]. This mutation leads to low plasma Zn concentration and to hyper-pigmented skin lesions, defective growth and, of major importance, to thymic atrophy and lymphopenia. Several other diseases including infectious diseases, cancer, chronic diseases such as bronchial asthma and Alzheimer disease and autoimmune diseases are related to alterations in Zn status [72] (Table 3).

Between others, RA, characterized by a chronic inflammatory reaction leading to progressive destruction of the joints, has been associated with deficiencies of Zn homeostasis. The first study linking Zn to RA reported improvements in joint swelling, morning stiffness and walking in RA patients taking Zn supplements [76]. However, these results on

the anti-rheumatic effect of Zn were not confirmed by two other studies [77,78]. But Zn supplementation was shown to modulate *ex-vivo* phagocytosis and oxidative burst in phagocytes from RA patients [78]. Nevertheless, patients affected by RA show reduced Zn levels in sera, which negatively correlate with the levels of the monocyte secreted pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  [79]. These studies were supported by *in vitro* studies. Zn deficiency increases the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 in a monocyte-macrophage cell line [80] and Zn supplementation inhibits the LPS-mediated release of TNF- $\alpha$  and IL-1 $\beta$  in monocytes [81]. Furthermore, as mentioned before, Zn treatment in CIA mice was shown to inhibit T<sub>H</sub>17 development in part via inhibition of STAT3 activation [58].

Besides its effects on the immune system, Zn regulates several other pathways involved in the development and perpetuation of RA. Zn is a component of matrix metalloproteases (MMPs), which are responsible for matrix remodeling and have a central role in bone destruction in RA. Lack of Zn and the related increase in IL-1 $\beta$  production, stimulate the production of MMPs with consequent degradation of extracellular matrix components [82]. Lack of Zn has also been associated with an increased oxidative stress and an increased ROS production [83]. Furthermore, Zn deficiency leads to an absence of super oxide dismutase production [84] and subsequently, to a reduction in MT induction. Interestingly, local control of RA has been obtained following intra-articular administration of SOD [29]. Zn also inhibits the activation of the transcription factor NF- $\kappa$ B resulting in a decrease in the transcription of pro-inflammatory cytokines and adhesion molecules [74]. However, the beneficial effects of Zn have not yet been directly demonstrated in RA patients.

## 6. Conclusions and perspectives

The nutritional importance of Zn is well known. Zn requires daily intake, and to achieve a steady-state level, the homeostasis of Zn inside the cells is tightly regulated by different transporters and regulators. Zn functions as a key structural or catalytic component of more than 300 enzymes and it is implicated at all levels of cellular signal transduction.

In this way, Zn regulates also communication between cells, cell proliferation, differentiation and survival. Furthermore, Zn involvement in the regulation of immune cells and inflammation contributes to pathologies in which both Zn deficiency and inflammation are observed.

However, several mechanisms about Zn action on the immune system are still unclear. Further studies on the expression and regulation of transporters or on the interactions of Zn with other ions will also help to better understand the pathogenesis of Zn-related diseases, such as RA, leading to potential new and innovative therapeutic avenues in the immunological field.

#### Conflict of interest

The author declares no financial or commercial conflict of interest.

#### Take-home messages

- Zinc is a key component of critical proteins such as enzymes.
- Zinc has major effects on both the innate and acquired immune system.
- Zinc deficiency contributes to and results from chronic inflammation.

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#### **IV. A FEEDBACK LOOP BETWEEN INFLAMMATION AND ZINC UPTAKE**

Bonaventura P, Lamboux A, Albarède F. and Miossec P.

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Objective: investigate how IL-17/TNF induced inflammation could modify Zn metabolism at the synoviocyte level eventually contributing to chronicity.

The essential trace element Zn is required for human health (90) and it has a major role in the regulation of the immune system and inflammation as described in our previous review paper (66). Zinc is involved in essential cell mechanisms and it is part of the structure of numerous proteins (91). Although Zn importance in the pathogenesis of chronic inflammatory diseases is fully appreciated systemically (45, 46), its implication at the cellular level is not well known and needs further investigations.

As Zn cannot be imported as a divalent ion, it is imported in synoviocytes mainly through the importer ZIP-8. The zinc pool in cells is regulated by MTs and the export in the extracellular fluid is mediated by the ZnT1 exporter on the cellular membrane. The correct function of these mechanisms is necessary to maintain the correct homeostasis of Zn for the survival of the cell.

Based on this knowledge this work aimed to describe how Zn is regulated by IL-17/TNF induced inflammation at the cellular level, eventually contributing to chronicity. Moreover a model of diffusion-controlled Zn transport was developed to

account for the observed variations of the Zn flux in the inflammatory condition against normal conditions.

Results:

### **Zinc uptake is increased by the combination of IL-17 and TNF- $\alpha$**

The uptake of Zn was measured in the medium and in the cells (both OA and RA synoviocytes) exposed to 0.9 ppm of Zn alone or in combination with the pro-inflammatory cytokines IL-17 and TNF- $\alpha$ . The effect of cytokine addition was not measurable in the medium, while there was evidence of a higher Zn accumulation in cells exposed to the cytokine combination, after 120 hours. The equilibrium of the  $^{70}\text{Zn}$  tracer between cells and medium indicated an isotopic equilibrium between intake and efflux

### **TNF- $\alpha$ and IL-17 combination enhances zinc importer gene expression and modifies other trafficking molecule gene expression**

The gene expression of the importer ZIP-8 was quantified after overnight exposure to IL-17A and TNF- $\alpha$  alone or in combination demonstrating that there is a synergy between those cytokines in inducing ZIP-8 expression.

The gene expression of other molecules involved in Zn trafficking were analyzed after exposure to cytokines, Zn, or both. ZIP-8 expression was enhanced by inflammation only, while the exporter ZnT1 by Zn. MT expressions were increased by Zn exposure but inflammation enhanced this effect.

### **Exposure to zinc and pro-inflammatory cytokines synergistically enhances IL-6 production by synoviocytes**

In agreement with earlier experiments addition of the combination of IL-17 and TNF- $\alpha$  stimulated the production of IL-6 relative to control. The comparison of the results without IL-17 and TNF- $\alpha$  addition showed that Zn addition enhanced IL-6 expression. The presence of both Zn and cytokines amplified the inflammatory effect on RA synoviocytes.

### **A model of cytokine-induced zinc homeostasis regulation**

Using the data obtained, a mathematical model of the cell was developed with the aim to extend the findings to other cell types. In simple terms, the mathematical model expresses that inflammation opens the gates for Zn uptake and prepares the cell for Zn storage, regardless of the amount of Zn actually present in the extracellular medium. On the contrary, inflammation does not prepare the cell to excrete Zn rapidly, leading to the accumulation and potential intoxication of the concerned tissue

## RESEARCH ARTICLE

# A Feedback Loop between Inflammation and Zn Uptake

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## Abstract

### Objective

Zinc (Zn) has major effects on the immune system and inflammation is associated with systemic Zn deficiency. The aim of this work was to investigate how inflammation modifies Zn metabolism at the cellular level. Rheumatoid arthritis (RA) synoviocytes exposed to cytokines were used as a model of chronic inflammation. Osteoarthritis (OA) synoviocytes were used as control.

### Methods

Zn levels were measured in medium and inside cells by Induced Coupled Plasma-Mass Spectrometry (ICP-MS), in the presence of minute quantities of stable spike <sup>70</sup>Zn isotope and the addition or not of the pro-inflammatory cytokines interleukin-17 (IL-17) and tumor necrosis factor alpha (TNF-α). Gene expression of ZIP-8 importer, ZnT1 exporter and the homeostasis regulators metallothioneins (MTs) was evaluated after pre-exposure to cytokines, with or without exogenous Zn addition at increasing concentrations. IL-6 production was used as a marker of inflammation and measured by ELISA.

### Results

Exposure to IL-17 and TNF-α enhanced expression of the Zn-importer ZIP-8, regardless of the concentration of Zn in the culture medium. In contrast, the expression of the Zn-exporter ZnT1 and of the MTs was primarily dependent on Zn levels. Addition of Zn also increased the production of IL-6, thus further stimulating the inflammatory response.

### Conclusion

IL-17/TNF-mediated inflammation enhanced the intracellular Zn uptake by synoviocytes, further increasing inflammation. These observations document the existence of a feedback loop between inflammation and Zn uptake. Based on these results, a mathematical model was developed to represent the cytokine-mediated Zn homeostasis alterations.

**Competing Interests:** The authors have declared that no competing interests exist.

## Introduction

Zinc (Zn) is an essential trace element, which is ubiquitous at low levels in the environment and required for human health [1]. Low levels of Zn contribute to the immune defects associated with malnutrition. Conversely, exposure to high doses of Zn e.g., in the context of mining or manufacturing activities, has toxic effects with adverse consequences on the immune system [2].

At the cellular level, Zn is involved in cell metabolism, survival, and immune response mechanisms [3,4]. Zn is a component of numerous proteins including the metallo-proteases (MMPs), involved in matrix remodeling [5], the carbonic anhydrases in cell respiration [6] and the TNF- $\alpha$  converting enzyme (TACE) in the proteolytic release of cytokines, such as TNF- $\alpha$  [7]. As part of these important mechanisms for cell survival and inflammatory response, Zn has a major role in inflammation.

Zinc transport through cells is controlled by the SLC39 importers (Zrt-Irt-Proteins, ZIPs 1 to 14), by the SLC30 exporters (Zn-Transporters, ZnTs 1–10, with ZnT1 as only membrane exporter) and the homeostasis regulators metallothioneins (MT-1 and -2). MTs are low molecular weight, cysteine-rich, heavy metal-binding proteins. Binding Zn, MTs act as a Zn pool in cells and modulate the immune system [8], as well as the response to stress conditions, including the exposure to heavy metals.

Although Zn importance in the pathogenesis of chronic inflammatory diseases is fully appreciated systemically, its implication at the cellular level is not well known [4,9–12]. Synovial fibroblasts, isolated from rheumatoid arthritis (RA) patient biopsies, referred to as synoviocytes, were selected as a model of chronic inflammation, illustrating how stromal cells could contribute to the persistence of inflammation through the acquisition of defective apoptosis. Synoviocytes from osteoarthritis patients (OA) were used as control, as representing a less inflammatory state of the synovium.

Fibroblasts express some of the Zn transporters (ZIP-8 [13], MTs-1 [14]) and respond rapidly to inflammation [15]. They are active participants in the immune response, interacting with, promoting the activation of T cells [16,17], and thereby influencing the switch from acute to chronic inflammation [18]. In chronic inflammation, an infiltration of immune cells at the disease site leads to self-sustained production of pro-inflammatory cytokines, notably interleukin-17 (IL-17) and tumor necrosis factor alpha (TNF- $\alpha$ ). These two cytokines often act synergistically on synoviocytes, inducing high IL-6 production, which in turn triggers the activation of inflammatory cells (B cells, T cells, synoviocytes, and osteoclasts) and their highly aggressive phenotype [19]. IL-6 has been shown to be a central pro-inflammatory cytokine involved in RA development and IL-6 blockade with a humanized anti-IL-6 receptor antibody has proved its efficacy either as monotherapy or in combination with disease-modifying anti-rheumatic drugs [20].

The aim of our work was to investigate how the combination of IL-17 and TNF- $\alpha$  can modify cellular Zn homeostasis in synoviocytes, eventually contributing to chronicity. The use of minute quantities of stable  $^{70}\text{Zn}$  provided a novel perspective to study the metal kinetics in cells. A model of diffusion-controlled Zn transport was developed to account for the observed variations of Zn flux through cells under inflammatory vs. normal conditions.

## Materials and Methods

### Primary cell isolation, cell culture, and experimental design

Synoviocytes were grown from fresh synovial tissue samples aseptically isolated from RA and osteoarthritis (OA) patients' joints. The RA patients fulfilled the American College of

Rheumatology criteria for RA [21]. All patients signed an informed consent form and the study was approved by the ethics committee of the hospitals of Lyon. The synovial tissue, minced in small pieces, was allowed to adhere to plastic plates and maintained in DMEM medium (Eurobio, Courtaboeuf, FR) supplemented with 10% FBS (Life Technologies by Thermo Fischer scientific, Grand Island, NY, USA), 2% Penicillin-Streptomycin, 1% L-glutamine and 1% Amphotericin B (all Eurobio) until cells reach 90% confluence. Synoviocytes were used between the fourth and ninth passages to ensure the cell specificity. All data are the results of at least three separate experiments ( $n = 3$ ) and patients.

### Cytokines and Zn exposures

Synoviocytes were pre-exposed overnight to a combination of IL-17A at 50 ng/ml (R&D systems, Minneapolis, MN, USA) and TNF- $\alpha$  at 0.5 ng/ml (R&D systems), prior to Zn exposure. Normal medium contains between 0.2 and 0.3 ppm of Zn. Zn was added in nitrate form to increase bulk concentration to 0.9 ppm or 3.6 ppm of Zn. In order to assess the extent of exchange between the cells and the ambient medium, negligible amounts of isotope  $^{70}\text{Zn}$  were added (10 ppb).

### Zinc kinetics and $K_D$ by ICP-MS

Synoviocytes ( $5 \times 10^5$ ) were cultured for 5 days with and without cytokines. Supernatants were collected at 6, 48, 96, and 120 hours, while cells were collected, and counted at day 5. Samples were mineralized with  $\text{HNO}_3$  0.5N plus  $\text{H}_2\text{O}_2$  (15–20%) at 100°C and re-dissolved in a 5%  $\text{HNO}_3$  solution in deionized water. Metal ions in the samples were measured on a single collector ICP-MS platform ELEMENT 2 (Thermo Finnigan, Ringoes, NJ, USA). Zn found in cells was adjusted to the number of cells. Zinc fractionation constant ( $K_D$ ) between cells and medium was calculated as  $K_D = (^{64}\text{Zn}/^{70}\text{Zn})_{\text{cells}} / (^{64}\text{Zn}/^{70}\text{Zn})_{\text{medium}}$ .

### Zinc-transporter quantitative RT-PCR analysis

Synoviocytes were exposed or not to cytokines overnight followed or not by a six-hour Zn exposure. Total RNA was extracted using the RNeasy Mini Kit (Qiagen<sup>®</sup>, Hilden, GE), quantified with the Quant-it kit assay (Invitrogen<sup>™</sup> by Thermo Fisher Scientific, Grand Island, NY, USA) and RNA was reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen<sup>®</sup>, GE). PCR amplification was performed on a CFX96 Real-Time PCR Detection System (BioRad, Hercules, USA) using the QuantiFast SYBR Green PCR Kit (Qiagen<sup>®</sup>, GE). The expression of the genes was normalized to the expression of GAPDH.

### Measurement of the production of the pro-inflammatory cytokine IL-6

IL-6 production was quantified with standard ELISA kit (R&D system, San Diego, CA, USA) in the supernatants of synoviocytes exposed to cytokines and Zn at day 1, 5 and 8.

**Statistical analysis and modeling.** Statistical significance of changes in data (mean  $\pm$  standard error of the mean, SEM) was determined by GraphPad Prism<sup>™</sup> using non-parametric statistical methods. A two-way ANOVA test was used in the case of exposure to both Zn and cytokines. A Mann-Whitney test or Wilcoxon matched-pairs signed rank test were used in the case of single exposure. p-values inferior or equal to 0.05 were considered statistically significant. A model of diffusion-controlled Zn transport was constructed to describe the variation of Zn flux through the cells with or without IL-17 and TNF- $\alpha$  addition.

## Results

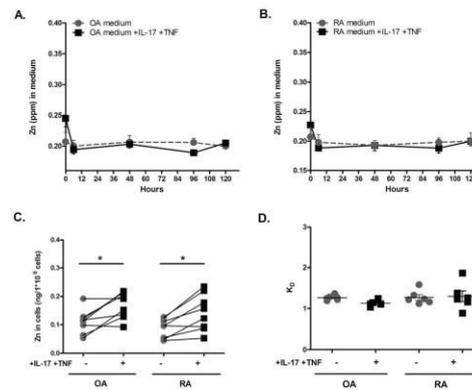
### Zinc uptake is increased by the combination of IL-17 and TNF- $\alpha$

To evaluate the effect of inflammation on the homeostasis of Zn, Zn content was measured in culture medium and intracellularly in OA and RA synoviocytes exposed or not to the combination of IL-17 and TNF- $\alpha$  for up to 120 hours. A transient Zn decrease in medium was seen during the first 6 hours of culture in both cell types (Fig 1A and 1B; S1 Table). The effect of cytokine addition was not measurable in the medium, while there was evidence of Zn accumulation in cells after 120 hours (Fig 1C; S1 Table). In both types of synoviocytes, the accumulation of Zn was increased by the combination of IL-17 and TNF- $\alpha$  in comparison to the control situation (Fig 1C, OA synoviocytes:  $0.11 \pm 0.01$  ng/ $10^6$  cells without cytokines vs.  $0.17 \pm 0.02$  ng/ $10^6$  cells with cytokines,  $p = 0.04$ ; RA synoviocytes:  $0.09 \pm 0.01$  ng/ $10^6$  cells without cytokines vs.  $0.14 \pm 0.02$  ng/ $10^6$  cells with cytokines,  $p = 0.02$ ; S1 Table). RA synoviocytes had a tendency for a more pronounced heterogeneity between cell lines from different patients (Fig 1C).

Zn  $K_D$  represents the isotopic fractionation constant between cells and medium (see materials and methods). Values of  $K_D$  of  $\sim 1$  indicate an isotopic exchange equilibrium, whereas  $K_D > 1$  indicates that the exchange did not still reach the equilibrium.  $K_D$ s of Zn in both types of synoviocytes were  $\sim 1$ , indicating a nearly perfect Zn isotopic equilibrium between cellular intake and efflux (Fig 1D, see the analytical model; S1 Table).  $K_D$ s in RA synoviocytes exposed to pro-inflammatory cytokines reproduced the heterogeneity already observed with the cell content (Fig 1C).

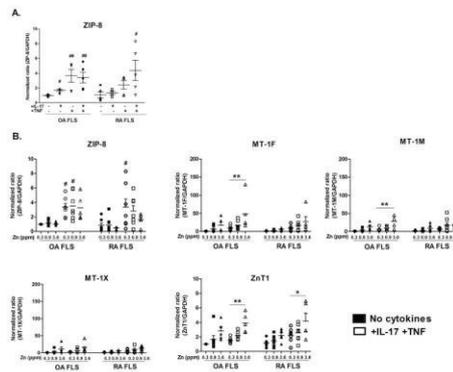
### TNF- $\alpha$ and IL-17 combination enhances zinc importer gene expression and modifies other trafficking molecule gene expression

To identify which mechanism increased Zn cellular content during inflammation, the expression of ZIP-8 importer was examined in synoviocytes exposed to IL-17 and TNF- $\alpha$ . The effect



**Fig 1. Zinc is more absorbed by synoviocytes under inflammatory conditions.** (A) Zn concentration in the OA synoviocyte culture experiment as a function of time. Dashed line: control experiment with no cytokine addition. Solid line: cytokines added to the culture medium. (B) Zn concentration in the RA synoviocyte culture experiment as a function of time. Dashed and solid line as in A. (C) Zn uptake calculated from Zn concentration measured in the cells expressed in ng per  $10^6$  cells at time  $t = 120$  h. (D)  $K_D = (^{66}\text{Zn}/^{70}\text{Zn})_{\text{cells}} / (^{66}\text{Zn}/^{70}\text{Zn})_{\text{medium}}$  at 120h after addition of ultra-trace amounts of  $^{70}\text{Zn}$ . At equilibrium,  $K_D = 1$ . Data are the mean of at least three independent experiments. Data are presented as mean  $\pm$  SEM; \*  $p < 0.05$ .

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**Fig 2. TNF- $\alpha$  and IL-17 enhance zinc importer gene expression and modify other trafficking molecule gene expression.** (A) The gene expression of the importer ZIP-8 was quantified after overnight exposure to IL-17A and TNF- $\alpha$  alone or in combination. (B) The gene expression of several Zn transporters was quantified by q-RT-PCR in synoviocytes under control conditions and in synoviocytes exposed overnight to a combination of recombinant IL-17A (50ng/mL) and TNF- $\alpha$  (0.5ng/mL) in the presence of increasing Zn concentrations.

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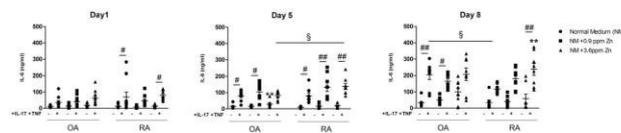
of IL-17 alone on ZIP-8 gene expression was limited in OA (Fig 2A,  $1.00 \pm 0.05$  vs.  $1.69 \pm 0.14$ ,  $p < 0.05$ ) and absent in RA synoviocytes. In OA synoviocytes, TNF- $\alpha$  alone significantly enhanced the expression of ZIP-8 ( $1.00 \pm 0.05$  vs.  $3.67 \pm 0.85$ ;  $p < 0.01$ ), but addition of both IL-17 and TNF- $\alpha$  had no further effect (Fig 2A). In RA synoviocytes, the presence of TNF- $\alpha$  alone was not sufficient to enhance ZIP-8 expression (Fig 2A, control  $1.08 \pm 0.40$  vs. TNF- $\alpha$  alone  $2.43 \pm 0.57$ ) but the combination of IL-17 and TNF- $\alpha$  enhanced ZIP-8 expression synergistically (Fig 2A, control:  $1.08 \pm 0.40$  vs. combination of IL-17 and TNF- $\alpha$ :  $4.38 \pm 1.35$ ;  $p < 0.05$ ) (S2 Table).

Once demonstrated the strong impact of the combination of IL-17 and TNF- $\alpha$  on RA synoviocytes, the expression of other Zn trafficking molecules was studied after exposure to both cytokines at different Zn concentrations.

**Importer.** The addition of Zn did not result in any change in ZIP-8 gene expression in OA synoviocytes, in the absence or presence of IL-17 and TNF- $\alpha$ . In contrast, in RA synoviocytes, Zn addition inhibited the effect of cytokines on ZIP-8 expression in a dose dependent fashion (Fig 2B; S2 Table).

**Regulators.** Addition of Zn to synoviocytes increased gene expression of MTs, the metal-binding proteins that regulates metal homeostasis. MTs expression was further enhanced with the combination of IL-17 and TNF- $\alpha$  (Fig 2B; S2 Table), while the presence of the highest concentration of Zn affected less the expression of MTs in RA than in OA synoviocytes (i.e. in the presence of 3.6 ppm of Zn, MT-1F was  $5.56 \pm 1.49$  in RA cells vs.  $17.03 \pm 8.50$  in OA cells).

**Exporter.** The combination of cytokines had no significant effect on the expression of the exporter ZnT1 in both OA and RA synoviocytes. The expression of ZnT1 was slightly enhanced in proportion to Zn in both cell types (Fig 2B). Its expression was significantly increased only in the presence of the highest concentration of Zn (3.6 ppm) and the IL-17 and TNF- $\alpha$  combination in both OA and RA synoviocytes (Fig 2B, OA cells: +IL-17 and TNF- $\alpha$ :  $1.5 \pm 0.2$  without Zn addition vs.  $3.9 \pm 0.6$  with 3.6 ppm Zn addition,  $p < 0.01$ ; same conditions for RA cells +IL-17 and TNF- $\alpha$ :  $2.3 \pm 0.4$  vs.  $4.2 \pm 1.1$ ,  $p < 0.05$ ; S2 Table).



**Fig 3. Exposure to Zn and pro-inflammatory cytokines synergistically affect IL-6 production in RA synoviocytes.** OA and RA synoviocytes were treated overnight with IL-17A (50 ng/mL) and TNF- $\alpha$  (0.5 ng/mL) followed by the addition of different Zn concentrations (0.9 and 3.6 ppm). Supernatants were collected at day 1, 5 and 8. IL-6 production was quantified by ELISA. Statistical significance was assessed by a two-way Anova. Data are presented as mean  $\pm$  SEM; # refers to the analysis of cytokine exposure, \* to the analysis of Zn exposure, § to the analysis of differences between OA and RA synoviocytes. \*/#/§ p<0.05, \*\*/##/§§ p<0.01.

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Results are normalized with GAPDH expression and are presented as fold changes compared to control. Data are the mean of at least three independent experiments. For data in which the effects of both cytokines and Zn are assessed, a two-way Anova test was used (A). For data for which only the effect of cytokines is assessed, a Mann-Whitney test was used (B). Data are presented as mean  $\pm$  SEM; \*/# p<0.05, \*\*/## p<0.01, # refers to the analysis of cytokine exposure and \* refers to the analysis of Zinc exposure.

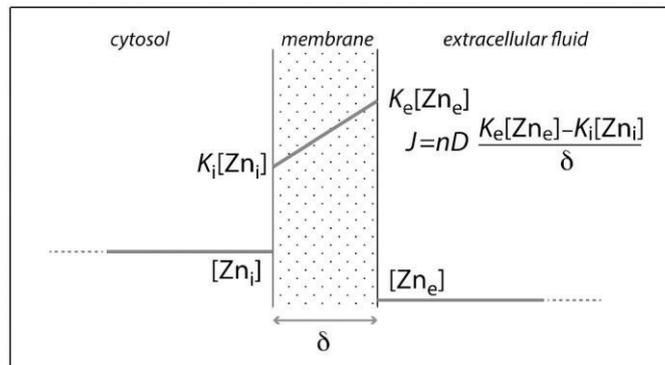
### Exposure to zinc and pro-inflammatory cytokines synergistically enhances IL-6 production by synoviocytes

Zinc is known to regulate inflammation through the alteration of the immune system and systemic Zn deficiencies have been associated with several immune deficiencies and autoimmune diseases [4]. Therefore, the effect of Zn supplementation on the production of IL-6, the main pro-inflammatory cytokine secreted by fibroblasts, was evaluated at the cellular level in both inflammatory and non-inflammatory conditions. In agreement with earlier experiments [22], addition of the combination of IL-17 and TNF- $\alpha$  stimulated the production of IL-6 relative to control (Fig 3; S3 Table). The comparison of the results without IL-17 and TNF- $\alpha$  addition showed that Zn addition enhanced IL-6 expression. The results were particularly demonstrative for OA synoviocytes, especially after 8 days (99.7  $\pm$  25.3 ng/ml for OA cells treated with 3.6 ppm of Zn, vs. 33.0  $\pm$  9.2 ng/ml without). The presence of IL-17 and TNF- $\alpha$  made the effect of Zn addition more difficult to interpret for OA cells, while the effect was clear for RA cells. In RA synoviocytes, both the combination of IL-17 and TNF- $\alpha$  and 3.6 ppm of Zn induced the highest level of IL-6, with Zn amplifying the effect of the added cytokines (106.4  $\pm$  15.65 ng/ml, with cytokines only vs. 237.4  $\pm$  35.58 ng/ml with both cytokines and Zn, p<0.01).

### A model of cytokine-induced zinc homeostasis regulation

In the presence of pro-inflammatory cytokines, Zn uptake was enhanced in synoviocytes as consequence of the increased expression of the ZIP-8 importer relative to the lack of change in ZnT1 exporter gene expression. We therefore analyzed the results of Zn changes in synoviocytes and the modulation by inflammation signals, through a simple transport model. Quantitative models of cellular Zn homeostasis have been previously proposed by Colvin et al. [23,24], but the number of analyzed parameters remains very large. Using the variables studied here in vitro, we have set up a simple model of Zn transport, in which the respective roles of cytokines and Zn concentration were taken into account.

The diffusion-controlled model represents a cell as an inner cytosolic fluid separated from the extra-cellular medium by a membrane (Fig 4). Here, the number of Zn importer and



**Fig 4. Model of diffusion-controlled Zn transport across the cellular membrane.** A schematic model of Zn transport in cells takes into account the role of cytokines and Zn concentration. Fick equation describes the flux.  $J$ : total Zinc flux in cells through transporters;  $n$ : number of transporters;  $D$ : diffusion coefficient;  $k$ : partition coefficient, proportional to Zn concentration, where  $e$  and  $i$  stand for external and internal compartment respectively;  $\delta$ : membrane thickness.

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exporter channels per cell is characterized by a single number,  $n$  for ZIP-8, and  $n'$  for ZnT1, respectively. Based on the  $K_D$  determination, we assume a steady-state transfer by diffusion across the membrane, which has a thickness of  $\delta$ . The respective diffusion coefficients for Zn transfer are  $D$  and  $D'$ . We further assume that the proportion of occupied transporters, inside and outside the membrane, is proportional to the intra- and extra-cellular Zn concentrations. The proportionality constant is equivalent to a partition coefficient  $K$ . The total Zn fluxes  $J$  and  $J'$  across the transporters, can now be represented by Fick equations:

$$J = nD \frac{K_e[Zn_e^{2+}] - K_i[Zn_i^{2+}]}{\delta}$$

$$J' = n'D' \frac{K'_i[Zn_i^{2+}] - K'_e[Zn_e^{2+}]}{\delta}$$

in which subscript 'e' stands for the extra-cellular, and 'i' for the intra-cellular compartments. Non-primed and primed symbols refer to ZIP-8 and ZnT1, respectively.

Results on concentrations and isotopic abundance can be used to support the assumption that steady state was achieved very rapidly and that Zn is already linked to methionine, also at steady state. Therefore  $J$  and  $J'$  are equal. The two previous equations can be recast into:

$$[Zn_i^{2+}] = \frac{nDK_e + n'D'K'_e}{nDK_i + n'D'K'_i} [Zn_e^{2+}]$$

In synoviocytes, the effect of IL-17 and TNF- $\alpha$  was largely independent of the Zn concentration. Since the  $K$  values reflect a difference in thermodynamic potentials and  $D$  values potential gradients across the membrane, the present results indicate that cytokines affect the number of channels  $n$  and  $n'$ . They indicate that  $n$  is enhanced independently of Zn concentration [5]. Since  $n$  is affected by cytokines to a greater extent than  $n'$ , the cell is primed by cytokines to control the Zn concentration level in the extracellular fluid.

The small overexpression of ZnT1 by cytokines and its dependence on Zn concentration indicates that the excess of Zn taken up by the cell to be stored by metallothioneins will eventually be excreted and the excretion rate of Zn will be slower than the uptake, leading to final intra-cellular accumulation.

Zinc residence time  $\tau_{Zn}$  within a cell of volume  $V$  is:

$$\tau_{Zn} = \frac{V[Zn_i^{2+}]}{J'} = \frac{\delta V}{n'([Zn_e^{2+}]D/(K_t - (K_t[Zn_e^{2+}]))) / ([Zn_i^{2+}])}$$

where  $n'$  (ZnT1) is dependent on Zn concentration. The cell controls the concentration gradient between the intra- and the extra-cellular compartment by enhancing Zn export in response to a large excess of cytosolic Zn, which could induce toxicity.

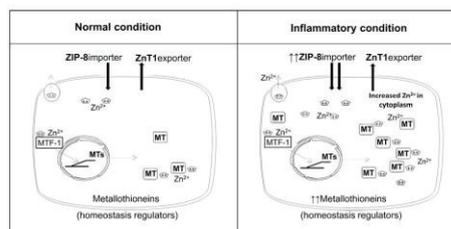
Zn excess inside cells is stored in cytosolic metallothionein. Mammal metallothionein consists of two domains: a  $\alpha$ -domain sequestering three  $Zn^{2+}$  ions, and a  $\beta$ -domain sequestering four  $Zn^{2+}$  ions [25]. The strong dependence of metallothionein expression reflects Zn storage requirements. The maximum Zn storage capacity by metallothionein is expressed as a sum of free  $Zn^{2+}$  and of the different complexes made with apo-metallothionein, i.e.,

$$[Zn_{total}] = [Zn_i^{2+}] + \beta_1[MT][Zn_i^{2+}] + \beta_2[MT][Zn_i^{2+}]^2 + \dots$$

where [MT] stands for the apo-metallothionein concentration in the cytosol and the  $\beta$ -values represent the stability constants of the  $Zn_i$  MT compounds ( $i = 1$  to 7). Free Zn ions in the cytosol should be negligible [25]. Zn-excess in the extracellular medium therefore requires massive production of metallothionein, which is reflected by the Zn-dependent expression of the MT1 genes.

## Discussion

The combination of the in vitro results and their mathematical analysis shows that the pro-inflammatory cytokines IL-17 and TNF- $\alpha$  over-express Zn transporters, leading to a net export/import ratio in favor of Zn accumulation inside the cell (Fig 5). This concept seems sufficiently general to be applicable to other cell types. Our results suggest that both the up-regulated expression of the importer ZIP-8 and of the homeostasis regulators MTs, in the presence of inflammation, contribute to Zn accumulation in cells (Fig 5). Other studies support our



**Fig 5. Zn transport in mesenchymal cells with and without inflammation.** A simplified schematic view of Zn transport in mesenchymal cells. Arrows from cytoplasmic space to intracellular space indicate Zn entry by the ZIP-8 importer. Arrows from intracellular space to cytoplasmic space indicate Zn exit mediated by the ZnT1 exporter. MTs are transcribed via MTF-1 and MTs are necessary for Zn homeostasis in the cell. Non-inflammatory condition on the left panel is compared to inflammatory condition on the right panel. The picture shows an increase of ZIP-8 importer and of MTs during inflammation, correlating with an increased quantity of Zn inside the cell.

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results of inflammation triggering Zn-regulator gene expression and Zn accumulation in human cells [26,27]. This may lead to the discovery of new therapeutic options, targeting Zn-binding groups [28] to control inflammation [29].

An important consequence is the induction of inflammation associated with the accumulation of Zn inside cells. We focused on the production of IL-6, the main pro-inflammatory cytokine produced by synoviocytes. The effect of Zn on IL-6 production indicated a feedback loop, where the production of IL-17 and TNF- $\alpha$  upon original inflammation increases Zn uptake, leading to excess accumulation of Zn that promotes IL-6 production. Such self-sustained inflammation therefore has the potential of triggering chronicity. Interestingly, IL-17 and TNF- $\alpha$  stimulation of IL-6 production was faster for RA synoviocytes, but higher for OA than RA synoviocytes at the late stage. These results confirm that RA synoviocytes are early-responders and can easily adapt to the inflammatory stimuli. The use of OA cells as control for RA mainly rests on the notion that OA represents a non-/lower-inflammatory state [30]. No significant difference for Zn absorption was found between OA and RA synoviocytes, rather representing a continuum [30]. Further investigations should address the differences between OA and RA synoviocytes regarding the kinetics of Zn uptake and the factors defining the maximum Zn uptake by each type of cell.

Even if no change in Zn absorption was observed between the two cell types, Zn-induced mechanisms were different. ZIP-8 gene expression was increased by the IL-17 and TNF- $\alpha$  combination in both cell types. The combination of IL-17 and TNF- $\alpha$  was necessary for the increased expression of ZIP-8 in RA but not in OA synoviocytes. This results from a sequential effect where IL-17 primes RA synoviocytes to respond to TNF- $\alpha$  by enhancing the expression of the TNF- $\alpha$  receptor TNFR1 [31]. MTs isoforms were less expressed in RA than in OA synoviocytes and their expression was sensitive to inflammation. MTs expression was enhanced by Zn alone in OA more than in RA synoviocytes.

In conclusion, independently of the cell type, inflammation from IL-17 and TNF- $\alpha$  combination plays a major role in altering Zn homeostasis in cells, even in the absence of added Zn. The changes in Zn homeostasis induced by inflammation then contribute to its self-activation and may play a strong role in the transition to the chronic stage of the disease.

In simple terms, the mathematical model expresses that inflammation opens the gates for Zn uptake and prepares the cell for Zn storage, regardless of the amount of Zn actually present in the extracellular medium. In contrast, inflammation does not efficiently prepare the cell for rapid excretion of metal excess. The price of increased cytokine production may signal the potential intoxication of inflamed tissues if metal levels in the extra-cellular medium become too high.

The same conclusion may apply to other metals of the Zn family (including some heavy metal as cadmium and mercury) and mimic Zn kinetics at the cellular level, accumulating in the cells linked to MTs. These results with Zn highlight the potential influence of the environmental factors associated with metals on the induction and progression of inflammation.

## Supporting Information

**S1 Table. Data table of zinc quantification in media and cells after exposure or not to IL-17 and TNF- $\alpha$  combination.**

(XLSX)

**S2 Table. Data table of transporter gene expressions after exposure or not to Zn and TNF- $\alpha$  and IL-17.**

(XLSX)

**S3 Table. Data table of IL-6 production in RA synoviocytes after exposure to Zn and pro-inflammatory cytokines.**  
(XLSX)

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### Author Contributions

Conceived and designed the experiments: PB FA PM. Performed the experiments: PB AL. Analyzed the data: PB AL FA PM. Contributed reagents/materials/analysis tools: FA PM. Wrote the paper: PB FA PM.

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## **V. INFLAMMATION-INDUCED CADMIUM UPTAKE BY SYNOVIOCYTES PROTECTS JOINTS FROM ARTHRITIS DESTRUCTION**

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PNAS 2016 (submitted)

Objective: The aim of this work was to study the inflammation-induced alterations of Cd balance in RA *in vitro* and *ex vivo* models and using the Cd effects as a local treatment against hyperplasia and inflammation *in vivo*.

Zinc (Zn) and cadmium (Cd) belong to the group 12 of divalent metals and they are closely associated for their chemical properties (92) as well as for their specific transport mechanisms in cells. However, while Zn is known to be necessary for the normal function of the immune response and for cell proliferation (66), the role of Cd on those pathways is still not clear regarding the induction of cell proliferation *versus* apoptosis (93, 94).

In the previous chapter we showed how IL-17/TNF dependant inflammation can enhance the entry and the accumulation of Zn inside the cells (95). In this paper the analysis will be enlarged to Cd, always using RA as the chronic inflammation model.

RA is characterized by hyperproliferation of synoviocytes at the site of inflammation, which are refractory to apoptosis. The inflammation in joints is sustained by a continuous production of IL-17, TNF (by the immune cells) and IL-6 (by synoviocytes). Synoviocyte selectivity for Cd will be used to reduce

inflammation and hyperplasia and was tested as potential local treatment on the model of rat adjuvant-induced arthritis.

Results:

### **Cd is preferentially absorbed from synoviocytes in comparison to zinc and other metals**

Synoviocytes were exposed to a cocktail of metals in order to measure their differential intake. Zinc was added to cultures in a 'tracerless' form using minute quantities of a stable  $^{70}\text{Zn}$  spike. The affinity ratio ( $K_D$ ) of metals, between cells and medium are referred to  $^{70}\text{Zn}$  isotope as tracer. The obtained values of  $K_D$  indicated that Cd is highly absorbed by synoviocytes in comparison to Zn and other metals.

### **ZIP-8 and MT1 drive Cd accumulation in synoviocytes exposed to IL-17 and TNF- $\alpha$ combination**

An increased absorbance of Cd in synoviocytes exposed to IL-17 and TNF- $\alpha$  in comparison to Cd alone was measured. In both conditions, even after cell wash with normal medium, Cd was not excreted. The increased Cd content in cytokine-stimulated cells was due to the increased expression of the ZIP-8 importer and MTs after IL-17/TNF- $\alpha$  exposure. Cadmium binds to MTs with a high affinity (78). Even if the exporter ZnT1 was overexpressed in the presence of Cd, it was not excreted but stayed linked to MTs, making ZnT1 export non-functional.

### **Low-dose Cd exposure reduces cell viability of inflammatory synoviocytes and decreases the production of IL-6 in cultures and biopsies**

RA synoviocytes are refractory to apoptosis and their proliferation and invasiveness is increased in the presence of pro-inflammatory cytokines (41), as well as their production of IL-6.

After 5 days of low-dose Cd exposure a reduction of cell viability was measured, which was further decreased in the presence of the pro-inflammatory cytokines IL-17 and TNF- $\alpha$ . After IL-17/TNF- $\alpha$  activation, Cd addition also reduced cell viability in co-cultures of synoviocytes and PBMCs and cell proliferation from *ex vivo* cultured biopsies of synovial tissue.

Cadmium addition was found to reduce IL-17/TNF- $\alpha$  induced IL-6 production of synoviocytes cultured alone or in co-culture with PBMCs and of synovial biopsies.

### **Zinc reduces Cd-induced effects on synoviocytes**

The competition between Zn and Cd is well-known, especially the protective role of Zn on Cd toxicity (96–98). The addition of Zn to Cd treated cultures partially reversed the effects of Cd on cell viability and reduced Cd-induced detachment of cells from the plate layer.

The simultaneous exposure of synoviocytes to Zn and Cd resulted in a reduced expression of MTs relative to Cd-only exposed cells, probably due to the higher resistance of the clustered Cd-MT. Moreover, the exposure to Zn in the presence of Cd reduced the effects of Cd on IL-6 production in both cultures of synoviocytes and co-cultures of synoviocytes with PBMCs.

### **Cadmium intra-articular injection improves adjuvant induced arthritis in the Lewis rat model**

A model of rat adjuvant induced arthritis was used to evaluate the potential of Cd as local treatment. Cadmium dissolved in a physiological solution of 0.9% NaCl was injected into the rat ankles at the stage of active arthritis resulting in a reduction of clinical scores and ankle perimeters in comparison to controls.

The impact of Cd on bone loss was evaluated, as the reduction of bone loss and joint destruction remains the key target when applied RA patients (99). Cd injection stopped bone loss in ankle bones and the increased density was proportional to the concentration of Cd injected

## **Inflammation-induced cadmium uptake by synoviocytes protects joints from arthritis destruction**

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Key words: Cadmium, Zinc, Inflammation, IL-17, TNF- $\alpha$ , synoviocytes

## Abstract

Cadmium (Cd) shares with Zinc (Zn) many physico-chemical properties and cell transporters. However, they have contrasting effects on the immune system. Rheumatoid arthritis (RA) is characterized by synoviocytes refractoriness to apoptosis and their uncontrolled proliferation. The continuous secretion of pro-inflammatory cytokines (IL-17/TNF- $\alpha$ ) contributes to disease chronicity.

The work aims to study inflammation-induced alterations of Cd balance and to use Cd effects as a local treatment against hyperplasia and inflammation *in vivo*.

Results show that Cd entered synoviocytes more efficiently than Zn. After IL-17/TNF- $\alpha$  exposure, Cd content in cells was increased through the enhanced gene expression of importers and metallothioneins. Synoviocytes were sensitized toward apoptosis by Cd/cytokine exposure and IL-6 production was reduced in cultures, co-cultures with immune cells and synovium biopsies. Intra-articular Cd injection improved arthritis, reducing inflammation, clinical scores and bone destruction, indicating a Cd-selective effect on synoviocytes. This may represent a new local therapeutic approach for arthritis treatment

## Introduction

Zinc (Zn) and cadmium (Cd) belong to the group 12 of divalent metals and are closely associated for their chemical properties, homeostasis and oxidative stress induction mechanisms<sup>1,2</sup>. Their transporters through the cell are Zrt-Irt-Proteins (ZIPs 1-14) importers, Zn-Transporters (ZnTs 1-10, with ZnT1 as the only membrane exporter) and the metallothioneins (MT-1 and -2), cysteine-rich, heavy metal-binding proteins controlling metal homeostasis in cells<sup>3</sup>. Metal regulatory transcription factor (MTF-1) induces MTs transcription, and in particular of MT-1s in response to metal exposure, oxidative stress and hypoxia<sup>3-5</sup>.

Bonds of Cd and Zn with MT-1 has long been considered isomorphous and Cd-detoxification action takes place through the replacement of Zn by Cd<sup>3</sup>. Although Cd-MTs affinity is the strongest, the far more abundant supply of Zn has a protective role against Cd-exposure<sup>6</sup>.

In contrast with Zn, necessary for the normal immune response and for cell proliferation<sup>7,8</sup>, Cd inhibits the activity of antioxidative enzymes and the mitochondrial electron transport-chain<sup>9</sup>, interferes with metalloproteins<sup>10</sup> and eventually enhances apoptosis<sup>11,12</sup> with a role on immune system enhancement or suppression<sup>13,14</sup>.

Rheumatoid arthritis (RA) is a chronic inflammatory disease. Synovia are maintained in a chronically-inflamed status, by the production of IL-17, TNF- $\alpha$  and IL-6<sup>15,16</sup> from infiltrated immune cells. Synoviocytes develop an aggressive behaviour characterized by hyper-proliferation and resistance to apoptosis<sup>17</sup>. IL-17 synergistically with TNF- $\alpha$  induces synoviocytes to produce IL-6, contributing to disease chronicity<sup>18</sup>. Systemic therapy, targeting inflammation mediators, is still the gold standard for RA<sup>19,20</sup>. Nevertheless, there

is increasing evidence of patients' remission attainment using systemic and intra-articular therapy combination.

Having first studied Zn alteration effects on the regulation of inflammation in a RA model<sup>21</sup>, the effect of its binding-competitor Cd will be compared. The aim of our work was to understand Cd-turnover through its transporters and investigate Cd immune-modulatory and anti-proliferative effects in the inflammatory context of arthritis. Results provide new insights for local treatment in RA.

## Results

### **Cd is preferentially absorbed by synoviocytes in comparison to Zn and other metals**

Synoviocytes were exposed to a cocktail of indium (In), tin (Sn), lead (Pb), bismuth (Bi), copper (Cu), cadmium (Cd) and gold (Au), in order to measure the relative affinity of these metals for the intracellular medium. Zinc was added to cultures in a 'tracerless' form using minute quantities of a stable <sup>70</sup>Zn spike, a very minor natural Zn isotope (0.6% of the total of the other isotopes), which does not noticeably modify the original amount of Zn present in the culture. The affinity ratios ( $K_D$ ) of metals between cells and medium are referred to <sup>70</sup>Zn isotope as tracer (see materials and methods). A Zn  $K_D$  value near 1 unity (isotopic equilibrium) indicates that the proportion of all isotopes is the same in the cells and in the medium, and therefore that cells efficiently exchange Zn with the medium (Fig.1a). Other elements can be divided into three groups according to their relative affinity with respect to Zn. The high-affinity group is made of Cu and Cd. The  $K_D$  for Cu is 6-8 in the absence of cytokines in both OA and RA synoviocytes.  $K_D$  for Cd reaches 70-100, which is at the low

end of literature values<sup>22,23</sup>, but still high for a toxic metal. The low-affinity group, made of In and Pb, has a  $K_D < 1$ , which indicates that the cells efficiently discriminate against these metals. The indifferent group (Au, Sn, Bi), with a  $K_D \sim 1$ , suggests that metal importers do not recognize them as being different from Zn (Fig. 1a). This relative affinity scale is consistent with the scale recognized for metallothioneins<sup>24</sup>.

### **ZIP-8 and MT1 drive Cd accumulation in synoviocytes exposed to IL-17 and TNF- $\alpha$ combination**

Cadmium kinetic experiments showed that IL-17 and TNF- $\alpha$  combination highly increased RA synoviocyte absorption of this metal relative to those exposed to Cd only (Fig. 1b). In both inflammatory and non-inflammatory conditions, absorption from the medium began after 12-hour exposure to Cd, resulting in a significant difference between the two conditions after 48 hours ( $0.07 \pm 0.01$  ppm vs.  $0.09 \pm 0.01$  ppm;  $p < 0.05$ ). Even after cell wash, synoviocytes kept absorbing residual Cd from the medium, showing a net positive bias of synoviocytes in favour of Cd, even at small doses. The intracellular amount of Cd was evaluated at the end-point in synoviocytes, after their 48-hour exposure to Cd, followed by a 96-hour wash in normal medium (see materials and methods). As expected, Cd accumulated in cells and its amount was increased in the Cd- plus IL-17/TNF- $\alpha$ -exposed synoviocytes in comparison to Cd-exposed synoviocytes in the absence of cytokines ( $0.34 \pm 0.09$  ppm in inflammatory condition versus  $0.18 \pm 0.06$  ppm in non-inflammatory condition;  $p < 0.05$ ). Moreover, Cd accumulation was not reversible since Cd was not excreted in the medium during the 96-hour wash.

The expression of Zn/Cd trafficking molecules showed that IL-17/TNF-induced-inflammation had a pivotal role in the expression of the importer ZIP-8 in both OA and RA synoviocytes (Fig. 1c). The presence of Cd reduced the effect of cytokines on ZIP-8 expression relative to the Cd-free experiment. Observations confirm the detoxifying role of cytokines: the expression of MT-1M and MT-1X was enhanced by a factor of 40 and 60 by exposure to Cd in comparison to control ( $p < 0.01$ ) in both cell types. MT-1F expression was only marginally up-regulated by Cd, but the presence of cytokines enhanced this effect (Fig. 1c). The expression of the exporter ZnT1 was significantly enhanced in OA, and to a slightly lesser extent in RA synoviocytes exposed to Cd, by a factor of ~5 to 7 ( $p < 0.05$ ) and further increased by IL-17 and TNF- $\alpha$  combination by a factor of ~7 to 10 ( $p < 0.01$ ). Those results taken together show a general trend for an increased intracellular uptake and storage of Cd by synoviocytes under inflammatory conditions.

#### **Low-dose Cd exposure reduces cell viability of inflammatory synoviocytes and decreases the production of IL-6 in cultures and biopsies**

To understand the effect of low-dose Cd on cell viability and proliferation, synoviocytes were exposed to 0.1 ppm of Cd for 8 days, in the presence or in the absence of IL-17 and TNF- $\alpha$ . Cell viability was then quantified at different points in time (day 1, 5 and 8). For cells not exposed to Cd, the data showed a peak of cell viability at day 5, followed by a viability decrease from day 5 up to day 8. In contrast, synoviocytes exposed to Cd showed an early-stage reduced viability at day 5 relative to control experiments ( $p < 0.05$ ). At day 8, the number of Cd-treated viable cells was lower than 10% of the control viable cells.

Interestingly, cell death was delayed in RA synoviocytes exposed to Cd-only in comparison to those exposed to both Cd and IL-17/TNF- $\alpha$  combination (Fig.2a,  $p < 0.01$ ).

Co-cultures of synoviocytes and PBMCs were used to reproduce the cell interactions in the synovium. In the absence of Cd and in the presence of non-activated PBMCs, cell proliferation clearly increased for RA relative to OA synoviocytes (Optical density:  $0.52 \pm 0.06$  for RA versus  $0.28 \pm 0.06$  for OA synoviocytes,  $p < 0.05$ ). On the contrary, cell viability tended to decrease between 25-30% when RA synoviocytes were cultured with PHA-activated PBMCs for 5 days. Exposure of the co-cultures to low-dose Cd induced cell death, whether PBMCs in co-cultures were PHA-activated or not (Fig.2b). This result suggested that cell interaction alone provides the pro-inflammatory microenvironment sufficient to sensitize synoviocytes to Cd-induced cell death.

Once demonstrated the functional effect of Cd on synoviocyte proliferation in cultures and co-cultures in the inflammatory context, an *ex-vivo* model was developed to better reproduce the *in vivo* condition and to confirm these results. Small pieces of synovial biopsies adherent to plastic dishes were exposed to different Cd doses, with or without addition of a combination of IL-17 and TNF- $\alpha$ . The addition of 0.1, 1.0 or 10.0 ppm of Cd reduced cell proliferation from the biopsy to the plate-layer in a dose-dependent manner (Fig.2c).

Cadmium addition was found to reduce the production of IL-6 induced by IL-17 and TNF- $\alpha$  in both in OA and RA synoviocytes cultured alone and in co-culture with PBMCs. The same effect was tested on synovial biopsies after *ex vivo* IL-17/TNF- $\alpha$  activation (Fig.2d). In control supernatants, the level of IL-6 was continuously increasing from day 1 to 8 up to a concentration of 750 ng/ml. Using Cd at 0.1 ppm, there was no significant reduction of IL-6 relative to controls. Higher concentrations of Cd (1 and 10 ppm) strongly reduced IL-6

production at day 8 in comparison to the control condition, as low as ~150 ng/ml with Cd used at 1 ppm ( $p < 0.05$ ) and ~10 ng/ml with Cd used at 10 ppm ( $p < 0.01$ ). This result shows that the reduction of IL-6 production is proportional to the amount of Cd absorbed per cell, resulting in cell death.

### **Zinc reduces Cd-induced effects on synoviocytes**

Competition between Zn and Cd is known to account for the protective role of Zn against Cd toxicity<sup>6,24,25</sup>. Zn was therefore added to Cd-treated cultures to clarify its possible enhancing or competitive role on cell viability and inflammation. Zn addition partially reversed the effect of Cd on cell viability in both OA and RA synoviocytes. At day 5, the viability score of Cd-Zn treated cells was twice that of Cd-only treated cells, whether cytokines were present or not (Fig. 3a).

Cadmium-induced synoviocyte detachment from the plate and loss of adherence of synoviocytes have previously been described<sup>26</sup>. Combined addition of IL-17 and TNF- $\alpha$  increases this effect (Fig. 3b). Reduction of Cd-induced synoviocyte death upon Zn addition (0.9 ppm, meaning 3-fold classic DMEM amounts) was clearly visible in phase contrast photographs taken at day 10 (Fig. 3b). Likewise, the simultaneous exposure of synoviocytes to Zn and Cd resulted in a reduced expression of MTs relative to Cd-only exposed cells (Fig. 3c). The effect was important for MT-1X expression in both OA and RA synoviocytes: control-relative Cd-induced MT-1X overexpression in both RA (fold change  $45.95 \pm 9.90$ ) and OA (fold change  $49.29 \pm 6.41$ ) cells, was reduced after Zn addition, in both RA ( $14.46 \pm 8.00$ ) and OA (fold change  $26.42 \pm 3.29$ ) ( $p < 0.05$ ) cells, indicating that the phenotypical changes in cells were associated with deep changes in transporter molecular mechanisms.

Moreover, exposure to Zn reduced the effects of Cd on IL-6 production in both cultures of synoviocytes and co-cultures of synoviocytes with PBMCs (Fig. 3d). In the presence of cytokines, RA synoviocytes produced  $120 \pm 14$  ng/ml of IL-6 without Cd and  $44 \pm 25$  ng/ml ( $p < 0.01$ ) with Cd. RA synoviocytes exposed to a combination of Cd and Zn in the presence of cytokines produced  $260 \pm 30$  ng/ml of IL-6 ( $p < 0.01$  in comparison to Cd-only exposed synoviocytes). The presence of Zn could not only restore the production of IL-6 in comparison to Cd-only treated cells, but increased IL-6 over the control situation. The IL-6 production by OA synoviocytes could also be restored to the control situation by Zn addition. Likewise, Cd reduced the production of IL-6 ( $p < 0.01$  relative to controls) in co-cultures of synoviocytes and PBMCs, while Zn addition restored it ( $p < 0.05$ ).

In conclusion, increasing Zn by a factor of 3 relative to the standard culture medium limited the entry of Cd in the cell through ZIP-8. The reduced Cd uptake down-regulated the expression of MT-1s, MT-1X in particular. Thus, Cd, but not Zn, was the limiting factor for MT-1 expression, in line with the role of metallothionein in cell detoxification.

### **Cadmium intra-articular injection improves adjuvant induced arthritis in the Lewis rat model**

Adjuvant induced arthritis (AIA) was induced in 24 six-week-old female Lewis rats at day 0 according to the protocol described by Marotte et al.<sup>27</sup>. Cadmium dissolved in a physiological solution of 0.9% NaCl was injected into the rat ankles using different concentrations (0.1 ppm, 1 ppm and 10 ppm) at the stage of active arthritis at day 14, while a control group received the physiological solution only.

Ankle perimeters and clinical scores were used as a measure of disease activity. No difference in paw perimeters was observed between groups on the day of AIA induction, day 0. At day 20, 6 days after Cd-intra-articular injection, a negative correlation was established between rat ankle perimeters and the concentration of injected Cd. At day 23 the mean perimeters were  $26.57 \text{ mm} \pm 1.27$  in the 10 ppm Cd-treated group,  $28.57 \text{ mm} \pm 0.77$  in the 1 ppm Cd-treated group and  $30.68 \text{ mm} \pm 0.57$  in the 0.1 ppm Cd-treated group, indicating a significant reduction of ankle perimeters after Cd-treatment compared to the untreated AIA group perimeter mean ( $34.94 \text{ mm} \pm 0.80$ ; Fig. 4a). These results showed a reduction of swelling and inflammation in the treated groups directly proportional to the amount of injected Cd.

To confirm the results, other clinical parameters were measured (Fig. 4b). Both the arthritic and the mobility-loss score had a reduction of 2 out of 4 points with Cd at 10 ppm and 1.5 out of four with Cd at 1 ppm ( $p < 0.01$  in comparison to the AIA group). The decrease in the arthritic score indicated that Cd addition induced a reduction of the swelling and erythema and the decreased mobility-loss index an improved use of joints. Rat weight remained unchanged among the experimental groups before or after Cd injection, indicating a general healthy status of all animals (Fig. 4c).

Although the reduction of inflammation is already of interest, reduction of joint destruction and bone loss remains the key target when applied to RA patients<sup>28</sup>. To functionally verify if the Cd-induced reduction of inflammation could protect from bone loss and that Cd-treatment did not alter bone homeostasis, rat ankles were scanned by micro-computed tomography ( $\mu$ -CT) at necropsy (Fig. 4). The evaluation of bone architecture by image-construction indicated that Cd injection stopped bone loss in ankle bones such as navicular,

cuboid, talus, and calcaneus. The increased bone density was proportional to the concentration of injected Cd.

## Discussion

In the present study, the effect of exposure to Cd was studied in the cellular model of the chronic inflammatory disease rheumatoid arthritis, using cells and synovial tissue from RA patients and in the AIA rat model. On the *in vitro* cellular model with synoviocytes, exposure to Cd had an anti-proliferative and immune-modulatory effect, resulting in reduced cell viability and inflammation. The results were confirmed using synovium tissue where the reduced proliferation of cells from biopsies exposed to Cd was associated with a lower IL-6 production. Finally the intra-articular injection of Cd in the arthritic rat model resulted in a less inflammatory disease and moreover in reduced bone loss and joint destruction.

The first important result demonstrated that inflammation, and not metal levels, drives the import of Cd in synoviocytes through ZIP-8. At the cell level, the exposure to IL-17 and TNF- $\alpha$  combination increased the expression of the ZIP-8 importer and of the homeostasis regulator MT-1s. The increased entry of Cd inside the cell induced even a stronger production of MT-1s compared to the non-inflammatory condition, resulting in an irreversible accumulation of Cd inside the cells. Absorption of residual Cd in the ppb range continued after cell wash, indicating an absence of release of Cd in the extracellular medium. The overexpression of the exporter ZnT1 is triggered by the Cd-dependent MTF-1 activation<sup>29,30</sup> but it is not effective for Cd export as long as Cd is linked to MTs.

Taken together these results change the traditional assumption of the environmental factor playing an incepting role in RA<sup>31</sup>, but imply a stronger susceptibility of subjects undergoing inflammatory events to the environmental factor (i.e. heavy metal toxicity)..

The consequence of Cd accumulation inside synoviocytes in cultures and in biopsies was associated with reduced cell viability and proliferation, and with reduced IL-6 levels in supernatants. The reduced inflammation, associated with annexin V staining (supplementary information Fig. 1) demonstrates that cells undergo apoptosis, without the release of additional pro-inflammatory cytokines typical of other processes of cell death, such as pyroptosis.

Addition of Zn to the Cd-treated cultures could partially reverse the effects of Cd on synoviocytes as cell viability was increased and the production of IL-6 restored. Our results are in agreement with several studies, demonstrating the protective effect of Zn in preventing Cd-induced local or systemic alterations<sup>32-34</sup>. Moreover, MT-1 levels were reduced in the presence of 0.9 ppm of Zn and 0.1 ppm of Cd. This result indicated that the importer ZIP-8 is not selective between the two metals, importing each of them in a concentration dependent manner. Conversely, MT-1 expression was reduced because of its Cd specificity. In summary, there is a continuous flux of Zn through cells. Inflammation causes an increase of Zn import and storage. When Cd is added to the medium under inflammatory condition, it is also imported but will remain inside the cell.

To verify our findings on the immune-modulatory effects of low-dose Cd, an *in vivo* model of arthritis was used. In our model the local intra-articular injection of Cd was performed after AIA development to evaluate the therapeutic anti-proliferative and immuno-modulatory effects, as would be the case in a patient with arthritis. The results showed a clear effect on inflammation with increasing Cd concentration, always keeping exposure at sub-toxic doses (according to the WHO recommendation). One of the major sites of Cd accumulation is liver<sup>35</sup>. Cd liver concentration was detectable only in the experimental group treated with 10 ppm

(data not shown). Levels of transaminases remained unchanged suggesting that Cd therapy would remain harmless to the liver (data not shown).

In conclusion, as already demonstrated for Zn<sup>36</sup>, Cd accumulation in cells is dependent on inflammation. The use of low dose Cd had an anti-inflammatory and anti-proliferative effect on synoviocytes, contributing to the resolution of the disease *in vivo*. As the use of the intrinsic toxicity of nanomaterials for anticancer therapy is an emerging concept<sup>37</sup>, this approach could be extended to RA and other diseases such as pigmented villonodular synovitis (PVNS), mono-articular arthritis. The local administration of Cd at the sites of inflammation and/or of cell hyper-proliferation could represent an interesting therapeutic approach.

## **Materials and methods**

### **Biopsies, isolation and culture of synoviocytes and co-cultures**

Biopsies of synovial tissue samples have been aseptically isolated from RA and OA patients' joints. The RA patients fulfilled the American College of Rheumatology criteria for RA. The synovial tissue was minced in small pieces, which were allowed to adhere to plastic plates. Those samples were maintained in Dulbecco Modified Eagle Medium (DMEM, Eurobio, Courtaboeuf, FR) supplemented with 10% FBS (Life Technologies by Thermo Fischer scientific, Grand Island, NY, USA), 2% Penicillin-Streptomycin, 1% L-glutamine and 1% Amphotericin B (all Eurobio) until cells colonized the plastic dishes. At 90% confluence, tissue pieces were removed and cells were trypsinized. Synoviocytes were used between the fourth and ninth passages to ensure the cell specificity.

For co-culture assays, peripheral blood mononuclear cells (PBMCs) were added to standard cultures in a 5:1 ratio, after synoviocytes adhesion to plate. PBMCs and then co-cultures are cultured in complete RPMI (Eurobio, Courtaboeuf, FR) supplemented with 10% human serum (Etablissement français du sang (EFS), Lyon, FR), 2% Penicillin-Streptomycin, 1% L-glutamine.

All data are the results of at least three separate experiments (n=3) using cells taken from three different patients for each type (OA and RA).

### **Cytokines, metals and PHA exposure**

Synoviocytes and biopsies were pre-exposed overnight to a combination of IL-17A (50 ng/ml; R&D systems, Minneapolis, MN, USA) and TNF- $\alpha$  (0.5 ng/ml; R&D systems), prior to metal exposure. The day after, multiple metals were dissolved in nitric acid (0.1 part per million (ppm) of Cu, Pb, In, Sn, Bi; 0.01 ppm of Cd; and 0.001 ppm of Au), or Cd alone (0.1 ppm), or Cd (0.1 ppm) and Zn (0.9 ppm) combination were added to the culture medium, in the presence or not of cytokines.

PBMCs were activated or not with Phytohaemagglutinin PHA (5 $\mu$ g/ml) and exposed to metal conditions, before cell-cell interaction.

In order to assess the extent of exchange between the cells and the ambient medium, negligible amounts of isotope  $^{70}\text{Zn}$  were added (0.01 ppm).

### **Metal fractionation constant between medium and cells, Cd cell content and kinetics by ICP-MS**

Synoviocytes ( $5 \times 10^5$ ) were cultured with cytokines and metals being optionally added. Supernatants (2ml) and cells were uptake at the end-point (day 14 for metal cocktail and day 5 for Cd only) to analyse metal fractionation constants  $K_D$ .  $K_D$  between cells and medium was calculated as  $K_D = (\text{Me}/^{70}\text{Zn})_{\text{cells}} / (\text{Me}/^{70}\text{Zn})_{\text{medium}}$ . Values of  $K_D$  of  $\sim 1$  indicate an isotopic equilibrium, whereas  $K_D > 1$  indicates that exchange reactions are still on their way.

To analyse Cd kinetics and cell content, 2ml of supernatant were collected at 6, 12, 24, 48 hours. Cells were than washed with PBS and fresh complete DMEM was substituted to the Cd-enriched medium, to study the possible exit of Cd from cells. Two ml of medium were collected immediately after the wash, at 60, 72, 96 and 120 hours, while cells were collected and counted at the endpoint (120 hours). All the samples were then mineralized with  $\text{HNO}_3$  0.5N plus  $\text{H}_2\text{O}_2$  (15-20%) at  $100^\circ\text{C}$ . Prior the analysis, the mineralized samples were re-dissolved in a 5%  $\text{HNO}_3$  solution in deionized water in an ultrasonic bath. Metal ions in the samples were then measured on a single collector ICP-MS platform ELEMENT 2 (Thermo Finnigan, Ringoes, NJ, USA) which allows metals to be measured at concentrations levels as low as  $10^{-12}$  units, i.e. in the part per trillion (ppt) range. The detector receives an ion signal proportional to the metal concentration.

### **Quantification of the gene expression of Zn transporters by quantitative real-time PCR**

OA and RA synoviocytes were plated at a density of  $2,5 \times 10^5$  cells/cm<sup>2</sup> in 12-well plates and were then exposed or not to cytokines overnight followed or not by Cd exposure for 6

hours. After 6 hours of treatment, total RNA was extracted using the RNeasy Mini Kit (Qiagen®, Hilden, GE) and quantified with the Quant-it kit assay (Invitrogen™ by Thermo Fisher Scientific, Grand Island, NY, USA) following manufacturer's instructions. cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen®) according to the manufacturer's instructions. SYBR green-based real time qRT-PCRs were performed on the CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA, USA) using the QuantiFast SYBR green kit and QuantiTect primers (Qiagen®). Cycle threshold values were normalized with respect to the endogenous control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative expression of the genes in treated cells versus control cells was determined using the comparative threshold cycle method as described by the manufacturer.

#### **Cell viability quantification by neutral red assay**

The neutral red assay determines the accumulation of the neutral red dye in the lysosomes of viable cells as described by Borenfreund and Puerner (1). Synoviocytes were plated in duplicates at a density of  $10^4$  cells/cm<sup>2</sup> in 96-well plates. After optional exposure to cytokines, metals (Cd alone or Cd plus Zn) and PBMCs (activated or not) for 1, 5 and 8 days, cells were incubated for 150 min with neutral red dye (80 µg/ml 0.33%; Sigma-Aldrich, St. Louis, MO, USA) at pH 6.5 in serum free DMEM. Cells were then washed with PBS followed by 10 min incubation in 200 µl of elution medium (ethanol/acetone, 50%/1% in deionized water). Absorbance at 540 nm was measured using VICTOR™ X4 plate reader (Perkin Elmer, Waltham, MA, USA) and results were obtained by subtracting the background read at 690 nm and the 540 nm-absorbance in medium without cells.

### **Measurement of the production of the pro-inflammatory cytokine IL-6**

IL-6 production was quantified in synoviocytes, co-cultures and biopsies supernatants of day 1, 5 and 8 by standard ELISA techniques according to the manufacturer's instructions (R&D system, San Diego, CA, USA). Absorbance at 450 nm was measured using the VICTOR™ X4 plate reader and results were obtained by subtracting the background read at 540 nm.

### **Cell/biopsy imaging**

Phase-contrast photographs were taken at day 5 with a Nikon ECLIPSE S100 microscope in order to observe morphologic changes in cells after exposure to metal.

### **Induction of adjuvant induced arthritis (AIA)**

Twenty-four 5-weeks old female Lewis rats (from Charles River Laboratories, L'Arbresle, France) were injected subcutaneously at the base of the tail with 300 µl (5 mg/ml) of lyophilized *Mycobacterium butyricum* (Difco labs, Detroit, MI, US) in sterile mineral oil, defined as day 0. Clinical arthritis occurred synchronously 12 days after injection in 23 out of the 24 rats, and the remaining rat was discarded from the experiment. At day 14, during arthritis acute phase of arthritis, 50 µl of Cd at increasing concentrations (0, 0.1, 1 and 10 ppm, N=6 each, except for 1 ppm, N=5) were locally injected in rats' hind ankles, arthritic prominent sites. Starting from a solution of Cd(NO<sub>3</sub>)<sub>2</sub> 5% 1000ppm, dilutions were made in physiological serum.

Clinical parameters (articular index, loss of mobility function index, ankle circumference, and body weight) were measured at different time points (day 0, 12, 14, 16, 20, 23) as already

described<sup>27</sup>. Briefly, all index scores were recorded for each hind joint by a consistent observer blinded to the treatment regimen and then averaged for each animal and for each experimental group. Arthritis and mobility index scoring were performed on a 0–4 scale where for arthritic index 0= no swelling or erythema, 1= slight swelling and/or erythema, 2= low to moderate oedema, 3= pronounced oedema with limited joint usage and 4= excess oedema with joint rigidity and for mobility index 0= normal use of paws 1= reduced support on the paw, 2= support only on the frontal part of the paw 3= reduced support of the frontal part of the paw, 4= complete loss of paw support.

Animal studies were conducted in accordance with the legislation of the European Community and approved by the Ethical Committee for Animal Experiments of Saint Etienne University (authorization #CU14N14).

### **Tridimensional tomography imaging**

Animals were sacrificed at the end of standard acute phase in this model (day 23). Right ankles were collected at necropsy and scanned by micro-computed tomography ( $\mu$ -CT; viva-CT40, Scanco, Brütisellen, Switzerland). X-rays beam features included: energy: 55 kVp, intensity: 145  $\mu$ A, diameter: 25.6 mm, voxel size: 12.500  $\mu$ m<sup>3</sup>, resolution: high. 3D images were constructed with the following segmentation parameters: Gauss sigma: 3.4, Gauss support: 9, Lower threshold: 316, Higher threshold: 1000).

### **Statistical analysis**

Data are expressed as the mean  $\pm$  standard error of the mean (SEM). Statistical significance of changes was determined by GraphPad Prism™. A two-way ANOVA test,

followed by multi-parametric analysis, was used in the case of exposure to both Cd and cytokines. A non-parametric Mann-Whitney paired test was used in the case of single exposure, with pairing between same patients cells. Differences resulting in p-values inferior or equal to 0.05 were considered statistically significant.

### **Annexin V staining**

Annexin V is used as a probe to detect cells that have expressed phosphatidylserine (PS) on the cell surface, based on the observation that soon after initiating apoptosis, cells translocate the membrane PS from the inner face of the plasma membrane to the cell surface. Synoviocytes were plated in quadruplicates at a density of  $10^4$  cells/cm<sup>2</sup> in 96-well plates. After optional exposure to cytokines and Cd cells were incubated with Annexin V-FITC (2µg/ml) and fluorescence was measured after 5 days exposure.

Images (20x) were taken using a Nikon™ camera installed on an Axiovert 200 microscope.

Figures were analysed on Cell-profiler™ and the stained area was calculated with a ration on the total cell area.

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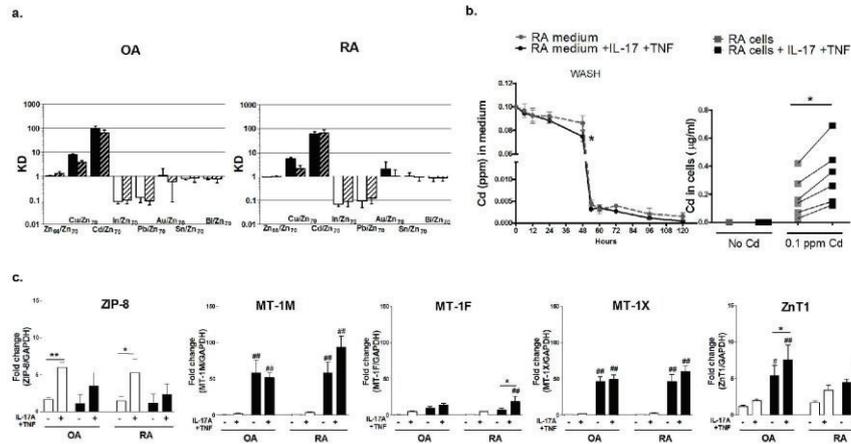
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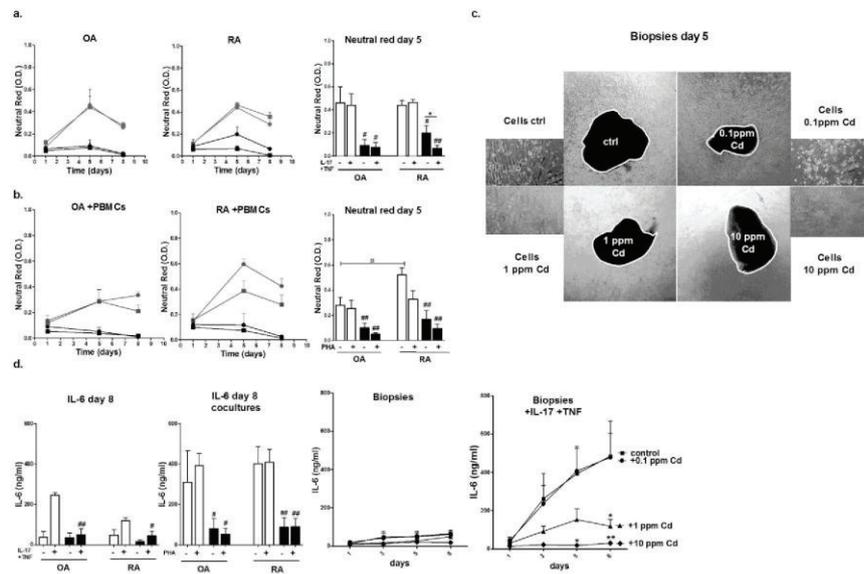
## FIGURES AND FIGURE LEGENDS



**Figure 1: Increased absorption of Cadmium by synoviocytes under IL-17 and TNF driven inflammatory conditions**

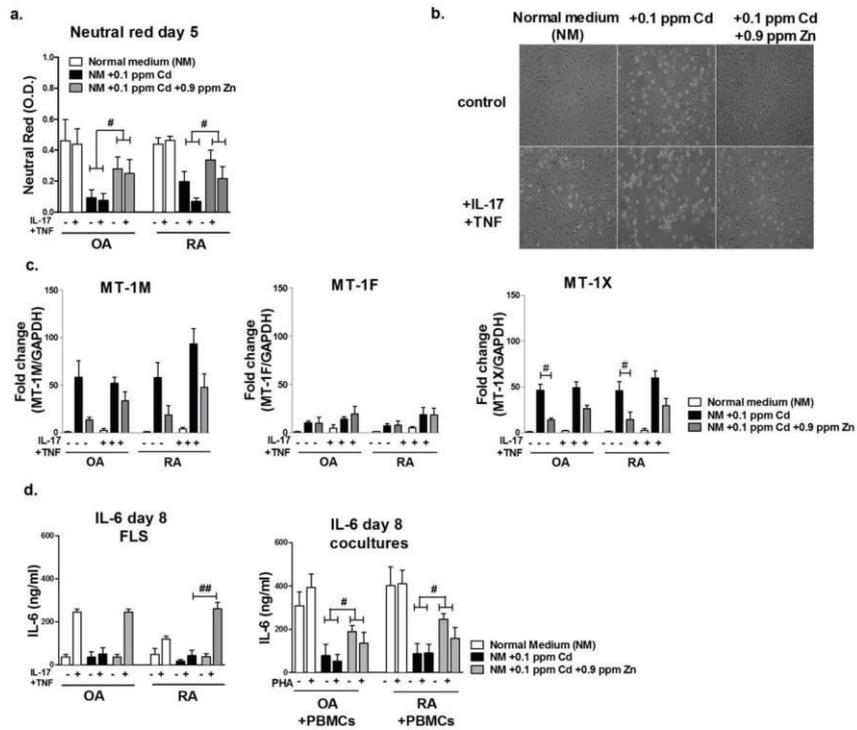
(a)  $K_D = (Me/^{70}Zn)_{cells} / (Me/^{70}Zn)_{medium}$  at 120h after addition of ultra-trace amounts of  $Zn_{70}$  and metal cocktail (In, Sn, Pb, Bi, Cu, Zn, Cd, Au). At equilibrium,  $K_D = 1$ . Striped pattern: IL-17A (50ng/mL) and TNF- $\alpha$  (0.5ng/mL) added to the culture medium. (b) Cd concentration in medium as function of time and in synoviocyte culture at 120 hours. Gray dashed line: control experiment with no cytokine addition. Vertical dashed line at 48 hours: wash. (c) Gene expression of Cd transporters in comparison to GAPDH. Black histograms: exposure to Cd.

Data are the mean of at least three independent experiments. Data are represented as mean  $\pm$  SEM; \* shows differences between non-inflammatory and inflammatory conditions, # shows differences due to Cd addition; \*/#  $p < 0.05$ , \*\*/##  $p < 0.01$



**Figure 2: Low-dose Cd addition to RA synoviocyte cultures and biopsies, in inflammatory conditions, reduces cell viability and IL-6 production**

(a) Cell viability of synoviocyte cultures assessed by neutral red assay at day 1, 5 and 8 after exposure to Cd. (b) Cell viability of co-cultures of synoviocytes and PBMCs assessed by neutral red assay at day 1, 5 and 8 after exposure to Cd. (c) Phase contrast photographs of biopsies (4x) and cells (10x) after exposure to IL-17 and TNF combination in the presence of increasing concentrations of Cd. (d) IL-6 production measured in supernatants of cultures, co-cultures and biopsies after exposure to IL-17 and TNF combination with or without Cd addition. Data are the mean of at least three independent experiments. Data are represented as mean  $\pm$  SEM; Black lines: Cd exposure. \* indicates differences between non-inflammatory and inflammatory conditions, # differences due to Cd addition only in comparison to control; \*/#  $p < 0.05$ , \*\*/##  $p < 0.01$ .

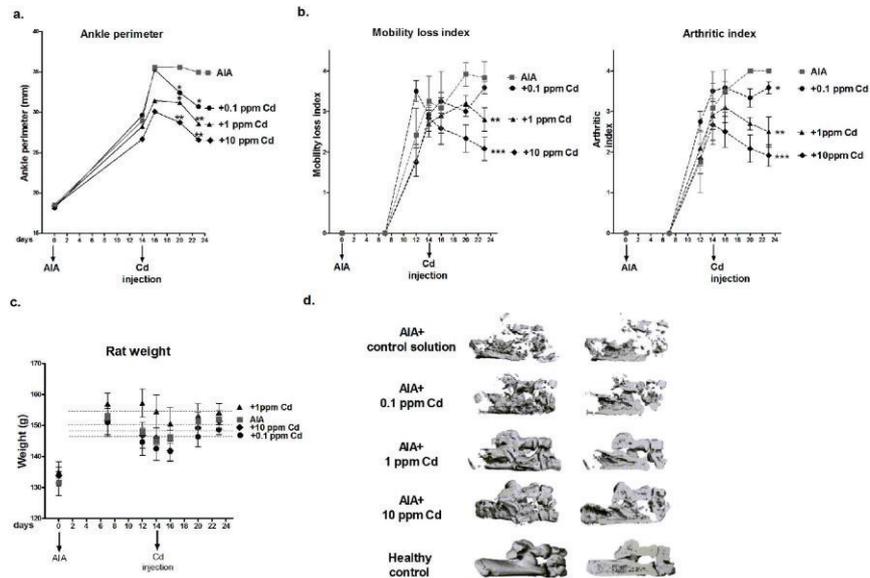


**Figure 3: Zinc partially reduces Cd-induced effects on synoviocytes**

(a) Cell viability of synoviocyte cultures assessed by neutral red assay at day 5 after exposure to Cd (black histograms) or Cd and Zn (gray histograms) in the presence or not of IL-17 TNF induced inflammation. (b) Phase contrast photographs (4x) of synoviocytes after a 10-day exposure to Cd or Cd/Zn in the presence or not of IL-17 TNF combination. (c) MT-1s gene expression after 6-hour exposure of synoviocytes to Cd or Cd/Zn enriched media in the presence or not of IL-17 and TNF induced inflammation. (d) IL-6 quantification in

supernatants of synoviocyte cultures or co-cultures of synoviocytes and PBMCs after 8days exposure to Cd or Cd/Zn enriched media.

Data are the mean of at least three independent experiments. Data are represented as mean  $\pm$  SEM; Black histograms: Cd exposure; Gray histograms: Cd/Zn exposure. # differences due to Zinc addition; # p<0.05, ## p<0.01;



**Figure 4: Cd intra-articular injection improves clinical scores and reduces bone loss in AIA rats**

(a) Ankle perimeters of rat hind paws in function of time. Day 0 AIA induction, day 14 Cd intra-articular injection. (b) Clinical scores of AIA; Left panel: mobility loss index from 0= normal use of paws to 4= complete loss of paw support; right panel: arthritic index from 0= no swelling or erythema to 4= excess oedema with joint rigidity. (c) Rat weight (g) in function of time. (d) 3D-images reconstructed after micro-computed tomography analysis of hind ankle bones. Data are the mean of two independent measures on at least 5 animals per group. Data are represented as mean  $\pm$  SEM; \* $p$ <0.05, \*\* $p$ <0.01.

## **DISCUSSION**

The general scope of this study was to provide new information on the mechanisms that can play a role on changes in metal homeostasis, which are altered during chronic inflammation. At the same time, after studying the differential effect of Zn and Cd on the immune system function, we could provide an innovative tool to control synoviocyte contribution to inflammation.

In the first study the positive role of IL-17/TNF inflammation on the increased uptake and accumulation of Zn was demonstrated. The changes in Zn homeostasis induced by IL-17/TNF inflammation then contribute to inflammation self-activation and may play a strong role in the transition to the chronic stage of the disease. This concept seems sufficiently general to be applicable to other cell types, for that reason the results obtained were summarized in a mathematical model.

In the second study, the effect of exposure to Cd was tested in the cellular model of the chronic inflammatory disease rheumatoid arthritis, on synovial tissue from RA patients and in the AIA rat model. On the *in vitro* cellular model using synoviocytes, exposure to Cd had an anti-proliferative and immune-modulatory effect, resulting in reduced cell viability and inflammation. The results were confirmed using synovial tissue, where the reduced proliferation of cells from biopsies exposed to Cd was associated with a lower IL-6 production. Finally the intra-articular injection of Cd in the arthritic rat model resulted in a less inflammatory disease and moreover in reduced bone loss and joint destruction.

Our results suggest that both the up-regulated expression of the importer ZIP-8 and of the homeostasis regulators MTs, in the presence of inflammation, contribute to Zn accumulation in cells. Our mathematical model expresses that inflammation

prepares the cell for metal storage by opening the gates of metal import, regardless the amount of Zn actually present in the medium. Several previous studies demonstrated the role of inflammation in triggering importers and homeostasis regulators expression in human cells, in agreement with our results (100, 101). This may lead to new therapeutic options by targeting Zn-binding groups to control inflammation.

Moreover, ZIP-8 importer is not selective for the metal it imports. This means that the ratio of Zn ions imported only depends on Zn ions availability, in comparison to the availability of other metal ions present in the extracellular fluid. In this way, the presence of other metals can influence the uptake and action of Zn, and vice-versa depending on their levels.

In contrast inflammation seems not preparing the cell efficiently for metal excretion, but only the metal excess does. This results at early stage in the increased metal content in the inflammatory cells. The increased cytokine production may signal the potential intoxication of the inflamed tissues if metal levels become too high in the extra-cellular fluid. This result indicates that cell mechanisms for metal excretion only respond to metal excess and, importantly, that those are late-stage mechanisms, which reduce the efficacy in protecting the cell in the case of metal intoxication

Nevertheless, ZnT1 dependent Zn export is effective: after inflammation-induced import, cell metal sensing can bring the status back to the equilibrium of import/export through ZnT1, without the formation of a real Zn pool in the cell.

On the basis of the results obtained in the first paper, experiments were performed exposing synoviocytes to Cd in the presence or not of inflammation. As

inflammation increase the ZIP-8-dependent import, Cd accumulates inside the cell. Moreover the expression of MT was highly enhanced by Cd exposure and further increased by inflammation. As a recent work stated, clustered Cd-MTs are more resistant than apo-MTs to proteolysis and tend to persist (78). This, as in our case, may exacerbate Cd persistence in mammalian cells.

The exposure to Cd, as well as Zn exposure, could slightly increase the expression of the exporter ZnT1. Nevertheless its increased expression resulted non-functional in our experiments: after 48-hour exposure to Cd and the following cell wash, no Cd was excreted in the medium but even residual traces of the metal were absorbed. This was not the same for Zn, which can be partially re-excreted in the medium after wash (data not shown). This can be due to the lower affinity of Zn for MTs in comparison to other metals, as Cd.

The reduced accumulation of Cd in cells in the presence of Zn in comparison to Cd-alone was previously demonstrated (102). The simultaneous addition of both Cd and Zn in the extracellular medium did not change the expression of the importer and the exporter but reduced the expression of MTs compared to the expression of Cd-only exposed cells. This meant that ZIP-8 imported the two metals in a 1 to 1 ratio, but the MTs that persisted in the cell were mainly in the Cd-MTs clustered form. Quantification of MTs at earlier time points could be useful to understand better this mechanism.

In conclusion inflammation drives the accumulation of metals inside the cells in a non-selective way and helps in the regulation of MTs. Metals are the main regulator of MTs, but their persistence in the cell depends on metal-MTs affinity. Also the export depends on metal levels but it can result non-functional depending on the preceding step of metal machinery, MT binding. Moreover taken together these

results could imply a stronger susceptibility of subjects undergoing inflammatory events to the environmental factor, depending on its metal enrichment.

Another important point of our work consisted in analyze the effect of metal accumulation in cells on their viability and inflammation. An association was found between the accumulation of Zn in the cell and the induction of inflammation while cell viability was not modified at low doses (data not shown). We focused on the production of IL-6, the main pro-inflammatory cytokine produced by synoviocytes. The effect of Zn on IL-6 production indicated a feedback loop, where the production of IL-17 and TNF- $\alpha$  upon original inflammation increases Zn uptake, leading to excess accumulation of Zn that promotes IL-6 production. Such self-sustained inflammation therefore has the potential of triggering chronicity.

On the contrary, Cd accumulation in the cell led to reduced cell viability, measured as apoptosis. This is stated as a consequent exacerbation of the toxic effect of the formation and persistence of clustered Cd-MTs, recently described in literature in substitution of the classical detoxifying role always attributed to MTs (57). Regarding inflammation, Cd addition reduced IL-6 levels in supernatants of cultures and co-cultures, an effect probably linked to the reduced cell viability and confirming that cells undergo apoptosis without the release of other pro-inflammatory cytokines typical of other process of cell death as pyroptosis.

Interestingly, addition of Zn to the Cd-treated cultures could partially reverse the effects of Cd on synoviocytes as cell viability was increased and the production of IL-6 restored. This protective effect is a consequence of the non-selective ZIP-8 import, which, in the presence of the two metals, led to a reduction of the final concentration of Cd in the cell and a consequent reduction of the anti-proliferative/anti-inflammatory effects.

The same and higher concentrations of Cd were used on synovial biopsies to establish a dose response. As the number of cell in the biopsy is very large in comparison to cultures, higher doses of Cd are needed to achieve the expected results: the reduction of cell proliferation from the biopsy to the plate layer and a strong reduction of IL-6 production could be measured with Cd used at 1 and 10 ppm.

To verify our findings about the immune-modulatory effect of low-dose Cd, an *in vivo* model of arthritis was used. In our model the local intra-articular injection of Cd was performed after AIA development to evaluate the therapeutic anti-proliferative and immuno-modulatory effects, as would be the case in a patient with arthritis. The results showed a clear effect on inflammation reduction with increasing Cd concentration, always keeping exposure at sub-toxic doses. The recommended tolerable weekly intake for Cd according to the WHO guidelines is fixed at 25 µg/kg/body weight/week. This concentration is 4 fold higher of the effective dose we used for the local injection. Further pharmacokinetics studies need to be performed to know the duration of action of Cd-treatment and to better evaluate the potential toxicity.

Nevertheless a previous screening of the possible toxic effect was verified on liver, one of the major sites of accumulation after acute exposure to Cd (103). Cd liver concentration was detectable only in the experimental group treated with 10 ppm (data not shown). Levels of transaminases remained unchanged suggesting that Cd therapy would remain harmless to the liver (data not shown). This represents a favorable starting point for further studies to establish the toxic dose.

Another important result is the reduction of bone loss in the Cd-treated group, as the reduction of joint destruction and bone loss remains the key target when applied

to RA patients. This result may mean that there is no specific toxic effect on osteoblasts and osteocytes and that the principal target of Cd remains synoviocytes.

As the use of the intrinsic toxicity of nanomaterials for anticancer therapy is an emerging concept (104) this approach could be extended to RA and other diseases such as pigmented villo-nodular synovitis (PVNS), or mono-articular arthritis. The use of Cd-enriched quantum-dots (QDs) linked to a specific antibody could help to target specifically synoviocytes. The magnetic properties of QDs could help in the regulation and maintaining at the site of inflammation as well as the development of viscous preparations able to maintain the particles in situ, until the absorbance by synoviocytes is completed.

**ANNEXE 1**



---

**IL-17A and TNF- $\alpha$  increase the expression of the anti-apoptotic adhesion molecule Amigo-2 in arthritis synoviocytes**

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Review



1 **IL-17A and TNF- $\alpha$  increase the expression of the anti-apoptotic**  
2 **adhesion molecule Amigo-2 in arthritis synoviocytes**

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4

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In review

12 **Abstract**

13 Rheumatoid arthritis (RA) is a chronic inflammatory disorder, characterized by a persistent  
14 immune cell infiltrate in the synovium accompanied by high levels of inflammatory mediators  
15 and by synovial hyperplasia. Despite significant therapeutic advances, RA remains an  
16 important unmet medical need. To discover potential new genes controlling inflammation and  
17 apoptosis in synoviocytes, genes induced by the two pro-inflammatory cytokines tumor  
18 necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 17A (IL-17A) were systematically searched. We  
19 identified Amphoterin-induced gene and ORF 2 (Amigo-2), a novel anti-apoptotic adhesion  
20 molecule, as synergistically up-regulated by the IL-17A/TNF combination specifically in RA  
21 synoviocytes. In addition, when RA synoviocytes were co-cultured with immune cells, Amigo2  
22 expression was significantly increased in both fibroblasts and immune cells. This induction  
23 persisted in RA synoviocytes even after the removal of the immune cells. Amigo2 induction  
24 was ERK-dependent and on the contrary, inhibited by JNK. Furthermore, Amigo2 expression  
25 levels correlated with apoptosis of the cells when exposed to the pro-apoptotic agent cadmium  
26 (Cd). Interestingly, exposure of the cells to HMGB1 in inflammatory conditions increased  
27 synergistically Amigo2 expression and significantly reduced Cd-mediated cellular toxicity.  
28 Our findings support a model whereby cell-cell contact with immune cells and exposure to the  
29 combination of both inflammatory cytokines and HMGB1 in the joints of RA patients increases  
30 Amigo2 expression in synoviocytes in an ERK-dependent manner which, in turn, enhances  
31 cellular adhesion and promotes cell survival and cellular proliferation.

32

33 **Keywords:** Rheumatoid arthritis, Amigo-2, IL-17A, TNF- $\alpha$ , HMGB1, ERK, synoviocyte  
34 survival and proliferation

35

36 The authors have no conflict of interest to declare.

37

## 38 Introduction

39 Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovium  
40 hyperplasia leading to progressive joint destruction and bone resorption (Feldmann et al.,  
41 1996;McInnes and Schett, 2007). The synoviocytes present in the synovial intimal lining are  
42 key contributors to RA pathogenesis. They produce cytokines that perpetuate inflammation  
43 and secrete proteases contributing to cartilage destruction. Their excessive proliferation and  
44 apoptosis resistance are the cause of the synovial hypertrophy and their migratory and invasive  
45 properties exacerbate joint damage (Bottini and Firestein, 2013). So far, treatment of RA is  
46 based on targeting the immune system with no direct effect on synoviocytes. Removing the  
47 hypertrophic pathologic synovial tissue by surgical, chemical or radiation relieves arthritis for  
48 a more prolonged time but is complex to use in a polyarticular situation (Nakamura et al.,  
49 2004;Das, 2007). Therefore, new molecules controlling synovial hyperplasia need to be  
50 discovered for the development of more synoviocyte-targeted therapeutic solutions.

51 The two pro-inflammatory cytokines tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 17A  
52 (IL-17A) are important contributors to RA chronicity. They both induce the production of  
53 several inflammatory mediators in the diseased synovium. Furthermore, these two cytokines  
54 synergize to induce several anti-apoptotic molecules in RA synoviocytes (Lee et al.;Youn et  
55 al., 2002;Bai et al., 2004;Toh et al., 2010;Benedetti and Miossec, 2014) and a large amount of  
56 neutrophilic mediators, perpetuating the primary inflammatory response (Keffer et al.,  
57 1991;Youn et al., 2002;Lubberts et al., 2005;Izquierdo et al., 2011;Benedetti and Miossec,  
58 2014). To discover new apoptosis and inflammatory regulators in RA synoviocytes, we  
59 searched for genes induced by the pro-inflammatory cytokines TNF- $\alpha$  and IL-17A in a  
60 previously performed 12-hour transcriptomics analysis. We identified Amphoterin-induced  
61 gene and ORF 2 (Amigo-2) which was synergistically up-regulated by the IL-17A/TNF  
62 combination.

63 Amigo2, also known as Alivin-1, is part of a novel family of genes encoding for type I  
64 transmembrane proteins with two other members, namely AMIGO and AMIGO-3. All  
65 AMIGOs share a similar protein structure composed of an extracellular domain containing six  
66 leucine rich repeats (LRRs) mediating cell-cell interaction followed by an immunoglobulin  
67 domain, a transmembrane domain and an intracellular domain with several possible  
68 phosphorylation sites. They form homo- and heterodimers and potentially lead to signal  
69 transduction in the cells (Kuja-Panula et al., 2003;Kajander et al., 2011). Interestingly,  
70 AMIGO-2 was shown to inhibit apoptosis and to promote the survival of electrical active  
71 neuronal cells (Ono et al., 2003). AMIGO-2 also increased the migration and invasion  
72 capacities of gastric cancer cells. Stable AMIGO-2 knock-down in gastric adenocarcinoma  
73 cells affected the morphology, the ploidy, the chromosomal stability and the cellular adhesion  
74 and migration of the cells and almost completely abrogated tumorigenicity in nude mice  
75 (Rabenau et al., 2004). In addition, the closely related family member AMIGO was found to  
76 be induced by the heparin-binding protein amphoterin, also known as HMGB1 (Kuja-Panula  
77 et al., 2003). Excessive extracellular HMGB1 levels have been detected in joints and serum of  
78 RA patients with higher levels found in the regions where proliferating synovial tissue invaded  
79 cartilage and bone. Furthermore, direct injection of HMGB1 into murine knee joints initiated  
80 persistent inflammatory responses and synovitis and antagonistic HMGB1 therapies  
81 ameliorated arthritis (Andersson and Harris, 2010).

82 In this study we showed that the IL-17A/TNF combination synergistically increased  
83 Amigo2 expression specifically in RA synoviocytes. Amigo2 was also up-regulated when RA  
84 synoviocytes were co-cultured with peripheral blood mononuclear cells (PBMC) in both cell

85 types and this induction persisted in RA synoviocytes even after the removal of the immune  
86 cells. Furthermore, we demonstrated that the IL-17A/TNF-mediated Amigo2 induction was  
87 promoted by ERK and by HMGB1 but was inhibited by JNK. In addition, Amigo2 expression  
88 levels correlated with apoptosis of the cells when exposed to the pro-apoptotic agent Cd and  
89 HMGB1-mediated Amigo2 induction protected the cells against Cd toxicity in inflammatory  
90 conditions. In conclusion, our study showed, for the first time, that cell-cell contact with  
91 immune cells and exposure to the combination of both inflammatory cytokines and HMGB1  
92 increases the expression of the gene Amigo2 in RA synoviocytes which promotes cell survival.

93

## 94 **Material and methods**

### 95 *Isolation and culture of synoviocytes*

96 Synoviocytes were grown from synovial tissue samples obtained from healthy donors or  
97 patients suffering from osteoarthritis or rheumatoid arthritis undergoing joint surgery. Each  
98 individual signed an informed consent and the protocol was approved by the committee for  
99 protection of persons participating in biomedical research under the number AC-2010-11-64.  
100 The RA patients fulfilled the American College of Rheumatology criteria for RA (Arnett et al.,  
101 1988). Briefly, synovial tissue was minced in small pieces which were allowed to adhere on  
102 plastic plates. Those samples were maintained in DMEM medium (Eurobio, Courtaboeuf,  
103 France) supplemented with 10% FBS (Life Technologies), 2% Penicillin-Streptomycin  
104 (Eurobio), 1% L-glutamine (Eurobio) and 1% Amphotericin B (Eurobio) until cells grew out  
105 of the tissue and colonized the plastic dishes. After cells reached confluence, tissue pieces were  
106 removed and cells were trypsinized. Synoviocytes were used between passages 4 and 9.

### 107 *Culture conditions*

108 Cells were exposed to IL-17A 50ng/mL (R&D systems, Minneapolis, MN, USA), TNF- $\alpha$   
109 0.5ng/mL (R&D systems) or a combination of both cytokines. Exposure to the apoptotic agent  
110 cadmium 0.1ppm (kindly provided by Pr. Albarède, Geology laboratory, ENS, Lyon, France)  
111 was performed after treating the cells overnight with a combination of IL-17A and TNF- $\alpha$  or  
112 with the vehicle. HMGB1 exposure at 50 ng/mL (R&D systems) was done in the presence or  
113 not of the combined IL-17A/TNF- $\alpha$  treatment. Exposures to the mitogen activated protein  
114 kinases (MAPKs) inhibitors SP6000125 30 $\mu$ M (Calbiochem), SB203580 5 $\mu$ M (Calbiochem)  
115 and U0126 50 $\mu$ M (Calbiochem) were done one hour prior cytokine addition. Cells were treated  
116 for 12 hours or 6 hours for mRNA extraction or several days for the functional assays.

### 117 *Co-cultures of synoviocytes and peripheral blood mononuclear cells*

118 Peripheral blood mononuclear cells (PBMC) from healthy donors were separated by Ficoll-  
119 Hypaque density-gradient centrifugation. PBMC were cultured in complete RPMI medium  
120 (Eurobio) supplemented with 10% AB-human serum (Invitrogen, Saint Aubin, France), 2%  
121 Penicillin-Streptomycin (Eurobio) and 1% L-glutamine (Eurobio). Co-cultures were performed  
122 in flat-bottomed 96-well culture plates in which 10<sup>4</sup> synoviocytes/well were seeded and  
123 allowed to adhere to the plate for several hours. 5\*10<sup>4</sup> PBMC were then added to the  
124 synoviocytes in the presence or not of 5 ug/mL phytohaemagglutinin (PHA, Sigma Aldrich,  
125 Saint-Quentin Fallavier, France). After 24 hours, supernatants were collected and cells were  
126 washed with PBS, followed by almost complete removal of the PBMC with incubation of the  
127 cells with a 1mM EDTA-PBS solution at 37°C for a brief time. Cells were then lysed for the  
128 24 hours timepoint. Complete RPMI medium in the presence or not of PHA was then added to  
129 the cells for 24 or 48 hours more before collection of the supernatants and lysis of the cells.

130 For each condition, twelve wells were combined in order to obtain sufficient material to  
131 perform gene expression quantifications.

#### 132 *Cell death assays*

133 Cell death was determined by flow cytometry and cell cycle analysis. Cell cycle analysis  
134 was performed by fixating the cells in ethanol during at least 24 hours and by subsequent  
135 staining of the cells with 3.3 $\mu$ M of 4,6-diamidino-2-phenylindole (DAPI). Fluorescence was  
136 analyzed on a Navios flow cytometer (Beckman Coulter, Brea, CA, USA) and the amount of  
137 dead cells in sub-G0/G1 was determined using the Kaluza® flow analysis software (Beckman  
138 Coulter, Brea, CA, USA).

#### 139 *Quantitative real-time PCR*

140 Total RNA was isolated from cells using an RNeasy® Mini kit (Qiagen, Hilden, GE)  
141 according to the manufacturer's protocol. cDNA was synthesized using the QuantiTect reverse  
142 transcription kit (Qiagen) following manufacturer's instructions. Reactions were performed on  
143 the CFX96 Real-Time PCR Detection System (Biorad, Marnes-la-Coquette, France) using the  
144 QuantiFast SYBR green kit and QuantiTect primers (Qiagen). Cycle threshold (Ct) values were  
145 normalized to the expression levels of the housekeeping gene GAPDH. The relative expression  
146 of the genes in treated cells versus control cells was determined using the  $\Delta$ Ct method.

#### 147 *ELISA*

148 TNF- $\alpha$  and IL-17A production (R&D system) were quantified in the supernatants of  
149 exposed synoviocytes or of co-cultures after the indicated culture duration and according to the  
150 manufacturer's instructions. Absorbance at 450 nm was measured using the multilabel plate  
151 reader VICTOR™ X4 (by Perkin Elmer, Waltham, MA, USA) and results were obtained by  
152 subtracting the background read at 540 nm.

#### 153 *Statistical analyses*

154 All data are expressed as mean  $\pm$  standard error of the mean (SEM). RT-PCR data were log2  
155 transformed before statistical analyses. Statistical significance was determined by GraphPad  
156 Prism using a two-way Annova with Bonferroni post-tests. The level of confidence is  
157 represented by P-values indicated in the figures.

158

## 159 **Results**

### 160 *Amigo2 is up-regulated more specifically in RA synoviocytes in inflammatory conditions*

161 To identify new inflammatory and apoptosis regulators in RA synoviocytes, genes induced  
162 by the pro-inflammatory cytokines TNF- $\alpha$  and IL-17A were systematically searched. To do so,  
163 a microarray dataset previously validated and derived from RA synoviocytes exposed for 12  
164 hours to TNF- $\alpha$ , IL-17A or their combination was used (Zrioual et al., 2009a; Hot et al., 2012).  
165 The gene Amigo2 was identified as synergistically up-regulated with the IL-17A/TNF  
166 combination (Figure 1A). The regulatory effect of IL-17A and TNF- $\alpha$  on Amigo2 expression  
167 was validated by RT-PCR and showed that the gene is already up-regulated by TNF- $\alpha$  and IL-  
168 17A alone and that their combination synergistically up-regulates its expression to up to 7 fold  
169 in RA synoviocytes (Figure 1B). In order to know whether this Amigo2 up-regulation was  
170 specific to RA synoviocytes, its expression was also examined in synoviocytes originating from  
171 healthy donors and OA patients. In control situation, Amigo2 expression was higher in RA  
172 synoviocytes than in OA and healthy synoviocytes (Figure 1C). Furthermore, when cells were

173 exposed to the IL-17A/TNF combination, a significant higher gene induction was observed in  
 174 RA synoviocytes (9 fold) in comparison to both healthy and OA synoviocytes (4 and 2 fold  
 175 respectively, Figure 1C). These results demonstrate that Amigo2 induction in inflammatory  
 176 conditions is more specific to RA synoviocytes.

177

178 *Co-culture of RA synoviocytes with immune cells increases Amigo2 expression in both cell*  
 179 *types*

180 In order to better mimick the *in vivo* inflammatory situation where synoviocytes interact  
 181 with immune cells, Amigo2 gene expression was also evaluated in RA synoviocytes co-  
 182 cultured with PBMC activated or not with PHA. Before lysis of the cells for RNA extraction,  
 183 PBMC were removed with EDTA in order to quantify Amigo2 expression only in the  
 184 synoviocytes or the PBMC alone and not in the mixture of synoviocytes with PBMC. Co-  
 185 culture of RA synoviocytes with resting PBMC for 24 hours already induced Amigo2 gene  
 186 expression to up to 5 fold (Figure 2A). This coincided with a very weak IL-17A secretion with  
 187 levels below 10 pg/ml (Figure 2C) and with an increased secretion of TNF- $\alpha$  (Figure 2D).  
 188 Amigo2 gene induction was significantly further enhanced (up to 10 fold) in synoviocytes co-  
 189 cultured for 24 hours with activated PBMC (Figure 2A). This was associated with the secretion  
 190 of larger amounts of IL-17A with levels above 80 pg/ml (Figure 2C). The production of TNF-  
 191  $\alpha$ , however, did not change after PHA stimulation in comparison to the control situation (Figure  
 192 2D). Interestingly, Amigo2 expression also increased in PBMC co-cultured with RA  
 193 synoviocytes in control condition at a much higher level (up to 40 fold) than in synoviocytes  
 194 (Figure 2B). However, PHA stimulation did not affect Amigo2 expression (Figure 2B) in  
 195 PBMC co-cultured with RA synoviocytes in comparison to the control situation. These results  
 196 indicate that the cellular interactions between RA synoviocytes and immune cells trigger the  
 197 induction of Amigo2 expression in both synoviocytes and PBMC. The cytokines TNF- $\alpha$  and  
 198 IL-17A secreted by PBMC in the presence of both cell-cell contact and of an activation signal,  
 199 in turn, synergize to induce even further Amigo2 expression in synoviocytes.

200 *Amigo2 induction in RA synoviocytes co-cultured with immune cells remains stable even*  
 201 *after immune cell removal*

202 Since RA synoviocytes have been described to retain their aggressive phenotype months  
 203 after removal from the RA synovial milieu (Kriegsmann et al., 1996; Muller-Ladner et al.,  
 204 1996; Bottini and Firestein, 2013), the induction of Amigo2 expression was evaluated in the co-  
 205 cultures over time after removing the immune cells at 24 hours. When RA synoviocytes were  
 206 co-cultured with resting PBMC, Amigo2 expression was enhanced at 24h to up to 5 fold and  
 207 its expression continued to raise to more than 10 fold even after PBMC removal (Figure 3A).  
 208 This increase in Amigo2 expression occurred despite the barely detectable levels of both IL-  
 209 17A (Figure 3B) and TNF- $\alpha$  (Figure 3C) after PBMC removal. However, at 72h Amigo2  
 210 expression dropped back to the levels observed at 24h (5 fold, Figure 3A). In the co-cultures  
 211 of RA synoviocytes with activated PBMC, Amigo2 expression increased to almost 15 fold after  
 212 24h and slowly decreased over time after PBMC removal (Figure 3A). At 72 hours, Amigo2  
 213 expression levels remained higher than the ones observed with the co-cultures performed with  
 214 resting PBMC (10 fold vs 5 fold respectively, Figure 3A). These results indicate that induction  
 215 of Amigo2 expression in RA synoviocytes that have been in contact with activated immune  
 216 cells remains even in the absence of the cellular interaction and the inflammatory environment.

217 *Amigo2 expression is regulated by MAPKs and HMGB1 in inflammatory conditions*

218 Since nothing is known yet about the regulation of Amigo2 expression in synoviocytes, the  
219 implication of several potential regulators was investigated. It was previously reported that  
220 MAPKs are involved in the regulation of several processes in RA synoviocytes including  
221 apoptosis (Bartok and Firestein, 2010) and their role in the signaling cascade of Amigo2 is yet  
222 unknown. Therefore, the effect of the three MAPKs c-jun N-terminal kinase (JNK),  
223 extracellular signal regulated kinase (ERK) and p38 on Amigo2 expression was examined by  
224 inhibiting them one hour prior the addition of cytokines. JNK inhibition led to a significant  
225 induction in Amigo2 expression already in control conditions (4 fold) which was enhanced to  
226 up to 8 fold in the presence of cytokines (Figure 4A). On the contrary, ERK inhibition led to a  
227 decrease in Amigo2 expression in control condition and completely abrogated the cytokine-  
228 mediated induction in Amigo2 expression (Figure 4A). p38 inhibition did not affect Amigo 2  
229 expression (Figure 4A), indicating that it is not involved in the regulation of its expression.  
230 These results demonstrate that JNK and ERK regulate Amigo2 expression in opposite manners  
231 with JNK acting as an inhibitor of Amigo2 expression and ERK acting as an activator.

232 The related family member AMIGO was first discovered in a systematic screen searching  
233 for genes induced on HMGB1-coated matrix (Kajander et al., 2011). Since HMGB1 has been  
234 implicated in RA pathogenesis, the regulation of Amigo2 by HMGB1 was investigated in RA  
235 synoviocytes. Amigo2 expression was therefore quantified after exposure of the cells for  
236 twelve hours to HMGB1 alone or in combination with IL-17A and TNF- $\alpha$ . HMGB1 alone  
237 increased Amigo2 expression to more than 3 fold (Figure 4B). Furthermore, the combination  
238 of both HMGB1 and cytokines led to a significant synergistic induction (39 fold, Figure 4B).  
239 These results demonstrate that alike the closely related family member Amigo, Amigo2  
240 expression is regulated by HMGB1 and that HMGB1 can synergize with cytokines to further  
241 enhance its expression.

#### 242 *Amigo2 expression levels correlate with cell death*

243 Since Amigo2 is involved in the survival of other cell types (Zrioual et al., 2009a; Hot et al.,  
244 2012), the correlation between its expression and the apoptosis outcome of the cells was  
245 investigated. Synoviocytes from different clinical settings were treated with a combination of  
246 TNF- $\alpha$  and IL-17A followed by their exposure to a low dose of the cytotoxic agent cadmium  
247 (Cd). Preliminary experiments indicated that Cd could induce significant apoptosis at  
248 concentration as low as 0.1ppm in inflammatory conditions. Amigo2 gene expression was then  
249 evaluated after a 6-hour exposure to Cd (Figure 5A), a time point at which cell death did not  
250 yet occur and cell death was evaluated after a week (Figure 5B). As demonstrated before,  
251 Amigo2 induction with cytokines was significantly higher in RA synoviocytes than in healthy  
252 and OA synoviocytes (Figure 5A). Exposure of the synoviocytes to Cd alone did not affect  
253 Amigo2 expression (Figure 5A) and did not induce any significant cell death (Figure 5B).  
254 Interestingly, Cd significantly inhibited the cytokine-mediated Amigo2 induction in both OA  
255 and RA synoviocytes (Figure 5A). This corroborated with an increased cell death in cells  
256 exposed to Cd in inflammatory conditions (Figure 5B). Healthy synoviocytes presented a slight  
257 Amigo2 induction with cytokines which was not affected by Cd (Figure 5A). However,  
258 Amigo2 expression levels were as low as the ones observed in OA and RA synoviocytes which  
259 could explain their sensitivity to Cd-induced toxicity in inflammatory conditions (Figure 5B).  
260 These results indicate that cell death correlates with the expression levels of the anti-apoptotic  
261 gene Amigo2 in synoviocytes. No difference was observed in Bcl2 expression in synoviocytes  
262 from the different clinical settings in those conditions (data not shown) indicating that Bcl2 is  
263 not involved in the resistance to Cd-induced apoptosis in inflammatory conditions.

264 Since Amigo2 expression levels correlated with cell death, the effect of HMGB1 in  
265 inflammatory conditions was also evaluated at the cell death level. RA synoviocytes were pre-  
266 exposed to HMGB1 alone or in combination with cytokines overnight followed by Cd addition  
267 and cell death was evaluated after a week. Addition of HMGB1 reduced by half the Cd-induced  
268 apoptosis in inflammatory conditions (Figure 5C). These data indicate that HMGB1 protects  
269 RA synoviocytes against Cd-induced apoptosis in inflammatory conditions in part by up-  
270 regulating Amigo2 expression.

271

## 272 Discussion

273 Despite the variety of current treatments in use for RA, none of them completely cure it but  
274 rather slow the progression of the symptoms. Indeed, RA pathogenesis is complex and involves  
275 many cell types including several immune cells as well as the resident fibroblasts. These cells  
276 play a key role by proliferating excessively and by producing cytokines that perpetuate  
277 inflammation and proteases that contribute to cartilage destruction. In the present study, we  
278 systematically searched for genes controlling inflammation and apoptosis regulated by the two  
279 pro-inflammatory cytokines IL-17A and TNF- $\alpha$  in synoviocytes. Using this approach, we  
280 identified the novel anti-apoptotic regulator Amigo2.

281 We demonstrated that Amigo2 expression is regulated by several players in RA  
282 synoviocytes. First, the combination of IL-17A and TNF- $\alpha$  synergistically increased its  
283 expression specifically in RA synoviocytes. These two cytokines are already known to  
284 synergize to induce the production of several pro-inflammatory mediators including IL-6, IL-  
285 1 $\beta$  and IL-8 as well as anti-apoptotic molecules such as synoviolin in RA synoviocytes  
286 (Nakamura et al., 2004). The underlying molecular mechanisms of such synergy are still  
287 unclear. One explanation is the ability of IL-17A to stabilize post-transcriptionally the mRNA  
288 of some of the TNF-induced genes (Cai et al., 1998; Witowski et al., 2000; Hata et al.,  
289 2002; Hartupee et al., 2007; Hamilton et al., 2012). Another explanation is the enhancing effect  
290 of the IL-17A/TNF combination on the expression of the TNF type II receptor (Zrioual et al.,  
291 2009b). It is not sure which of these mechanisms controls the synergistic effect observed on  
292 Amigo2 expression.

293 We also showed that Amigo2 expression was regulated in opposite manner by the two  
294 MAPKs JNK and ERK. ERK promoted Amigo2 expression while JNK suppressed it. It has  
295 been previously showed that ERK predominantly regulates RA synoviocytes proliferation  
296 (Lacey et al., 2003) while JNK controls the expression of matrix metalloproteinase 3 (MMP3)  
297 in RA synoviocytes and thereby joint destruction (Han et al., 2001). Therefore, it seems that  
298 Amigo2 expression is tightly regulated by these two MAPKs and we could speculate that a  
299 small shift in this tight regulation could drive the cells toward a specific fate. Increased Amigo2  
300 expression would lead to excessive proliferation whereas decreased Amigo2 expression would  
301 promote the invasive phenotype of the cells.

302 In addition to the MAPKs, we demonstrated that cell-cell contact between immune cells  
303 and the synoviocytes promoted Amigo2 expression in both cell types. This induction was  
304 further enhanced when the immune cells were activated by PHA. We previously revealed that  
305 interaction of PBMC with RA synoviocytes promoted the activation and expansion of Th17  
306 cells through caspase 1 activation (Eljaafari et al., 2012). Here we show for the first time that  
307 cell-cell contact promotes also the up-regulation of anti-apoptotic molecules such as Amigo2.  
308 Since the co-cultures were performed with PBMC, containing various types of immune cells,  
309 it cannot be sure which immune cells express the most Amigo2. It could be that several of them  
310 express the gene following the example of the anti-apoptotic mediator synoviolin expressed in

311 both Th17 cells and B cells (Toh et al., 2010). However, this remains to be determined.  
 312 Interestingly, the induction in Amigo2 expression persisted in RA synoviocytes even after the  
 313 removal of immune cells. It was already shown that RA synoviocytes present imprinted  
 314 anomalies, such as mutations or epigenetic changes (Bai et al., 2004), and that they can destroy  
 315 human cartilage many months after removal from the RA synovial milieu when engrafted into  
 316 mice with severe combined immunodeficiency (SCID) (Arnett et al., 1988; Cai et al., 1998).  
 317 Here we show that the up-regulation in Amigo2 expression after cell-cell contact with activated  
 318 immune cells can be maintained at least until more than 72. We can therefore speculate that  
 319 the constant cellular interactions between the synoviocytes and the immune cells infiltrating  
 320 the synovium maintain high levels of Amigo2 in the synoviocytes of RA patients.

321 Finally, we showed that Amigo2 expression is synergistically up-regulated by the IL-  
 322 17A/TNF combination and the heparin-binding protein HMGB1. HMGB1 is known to be  
 323 implicated in RA pathogenesis. It is present in excessive levels in joints and serum of RA  
 324 patients and antagonistic HMGB1 therapies ameliorate arthritis in murine models (Andersson  
 325 and Harris, 2010). HMGB1 induces synergistic interactions by forming complexes with certain  
 326 other pro-inflammatory molecules such as lipopolysaccharide (LPS) or IL-1 $\beta$  (Sha et al., 2008).  
 327 In this study, we showed for the first time that HMGB1 also enhances the effect of IL-17A and  
 328 TNF- $\alpha$  in synoviocytes. This enhancing effect is most likely indirect via the activation of the  
 329 toll-like receptors (TLRs) which, in turn, activate the transcription factor nuclear factor  $\kappa$  B  
 330 (NF- $\kappa$ B) leading to the transcription of more TNF- $\alpha$  (Andersson and Harris, 2010).

331 Furthermore, we demonstrated in this study that Amigo2 expression levels correlated with  
 332 the cellular outcome of the cells. Indeed, when cells were exposed to the cytotoxic agent Cd  
 333 in inflammatory conditions, Cd inhibited the IL-17A/TNF-mediated induction in Amigo2 which  
 334 sensitized the cells towards cell apoptosis. Moreover, the increase in Amigo2 expression by  
 335 the HMGB1/IL-17-A/TNF combination protected the cells against cd-induced toxicity.  
 336 However, the direct effect of Amigo2 on cell survival remains to be proven. siRNA-mediated  
 337 knock-down of Amigo2 in RA synoviocytes only led to a 25% knock-down efficiency in our  
 338 hands and did not affect the cellular outcome of the cells (data not shown). AMIGO2 is a  
 339 transmembrane protein known to form hemophilic and heterophilic interactions with other  
 340 AMIGO family members (Hot et al., 2012). It is possible that the other members of the family,  
 341 although very weakly expressed in RA synoviocytes, could compensate when Amigo2 is  
 342 partially depleted. Since Amigo2 is an adhesion molecule, we believe that an increase in its  
 343 expression leads to an enhancement in the cell-cell contacts protecting the cells against cellular  
 344 stress and hypoxia and promoting cellular proliferation. We suggest a model whereby cell-cell  
 345 contact with immune cells and the presence of pro-inflammatory cytokines and HMGB1 in the  
 346 joints of RA patients promote Amigo2 expression in an ERK-dependent manner and enhance  
 347 their resistance to apoptosis (Figure 6).

348

#### 349 **Author Contributions**

350 GB: conception of the work, acquisition and interpretation of the data, elaboration of the  
 351 manuscript; PB and FL: acquisition of data, revising and approval of the article; PM:  
 352 conception of the work, interpretation of the data, critical revision and final approval of the  
 353 article.

354

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In review

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In review

483       Legends of figures:

484       Figure 1: Amigo2 is up-regulated more specifically in RA synoviocytes in inflammatory  
485 conditions.

486       Synoviocytes were exposed to IL-17A or TNF- $\alpha$  alone or to a combination of both for 12  
487 hours. Amigo2 expression was assessed by both transcriptomic analysis (A) and quantitative  
488 real-time PCR (B and C). Amigo2 expression was compared between the different treatments  
489 and is represented as fold changes compared to control in RA synoviocytes (A and B). Its  
490 expression was also evaluated in synoviocytes from different clinical settings (healthy, OA and  
491 RA) and is expressed as fold changes compared to healthy synoviocytes exposed to vehicle  
492 (C). Data are the mean of at least three independent experiments  $\pm$  SEM. # Comparison with  
493 control situation, \* comparison between different cytokine combinations (B) or between cell  
494 types (C). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*, ###  $P < 0.001$ .

495       Figure 2: Co-culture of RA synoviocytes with immune cells increases Amigo2 expression  
496 in both cell types.

497       RA synoviocytes were co-cultured with PBMC from healthy donors in the presence or not  
498 of PHA for 24h. In the co-cultures, PBMC were separated from synoviocytes by EDTA  
499 addition prior cell lysis. Amigo2 expression was assessed by quantitative real-time PCR and  
500 was expressed as fold changes compared to synoviocytes cultured alone and exposed to vehicle  
501 (A and B). Amigo2 expression was evaluated in both synoviocytes (A) and PBMC (B) cultured  
502 alone or together. The production of IL-17A (C) and TNF- $\alpha$  (D) by the co-cultures was  
503 quantified by ELISA. The production of these cytokines was not detectable in synoviocytes  
504 cultured alone. Data are the mean of at least three independent experiments  $\pm$  SEM. \*  $P < 0.05$ ,  
505 \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

506       Figure 3: Amigo2 induction in RA synoviocytes co-cultured with immune cells remains  
507 stable even after immune cell removal.

508       RA synoviocytes were co-cultured with PBMC from healthy donors in the presence or not  
509 of PHA for 24h. In the co-cultures, PBMC were separated from synoviocytes by EDTA  
510 addition. Synoviocytes were cultured for 24h or 48h more after PBMC removal. Amigo2  
511 expression was assessed at different time-points by quantitative real-time PCR and is expressed  
512 as fold changes compared to RA synoviocytes cultured alone and exposed to vehicle (A). The  
513 production of IL-17A (C) and TNF- $\alpha$  (D) by the co-cultures was quantified by ELISA. The  
514 production of these cytokines was measured in co-cultures with no immune cell removal (co-  
515 cultures) or with immune cell removal by EDTA (co-cultures EDTA). Their production was  
516 not detectable in synoviocytes cultured alone. Data are the mean of at least three independent  
517 experiments  $\pm$  SEM. # Comparison with control situation, \* comparison between synoviocytes  
518 (A) or PBMC (B) alone and co-cultures, § comparison between co-cultures with or without  
519 EDTA addition. \*, #, §  $P < 0.05$ , \*\*, ###, §§  $P < 0.01$ , \*\*\*, ###  $P < 0.001$ .

520       Figure 4: Amigo2 expression is regulated by MAPKs and HMGB1.

521       RA synoviocytes were pre-exposed to MAPKs inhibitors for one hour followed by the  
522 addition of a combination of IL-17A and TNF- $\alpha$  (A) or co-exposed to HMGB1 and the TNF/IL-  
523 17A combination (B). After 12 hours, Amigo2 expression was assessed by quantitative real-  
524 time PCR and is expressed as fold changes compared to control situation. SP6000125: JNK  
525 inhibitor, SB203580: p38 inhibitor and U0125: ERK inhibitor, Ctl: control, Cyt: cytokines and

526 H: HMGB1. Data are the mean of at least three independent experiments  $\pm$  SEM. # Comparison  
527 with control situation, \* comparison with cytokine treated cells. \*, #  $P \leq 0.05$ , \*\*  $P \leq 0.01$ .

528 Figure 5: Amigo2 expression levels correlate with cell death.

529 Healthy, OA and RA synoviocytes were pre-exposed to a combination of IL-17A and TNF-  
530  $\alpha$  overnight before addition of a low dose of the pro-apoptotic agent Cd (A, B). Amigo2  
531 expression was then evaluated 6 hours after Cd addition by quantitative real-time PCR and is  
532 expressed as fold changes compared to healthy synoviocytes exposed to vehicle (A). Cell death  
533 was determined by cell cycle analysis after a week (B). To evaluate the effect of HMGB1 on  
534 cell death, RA synoviocytes were pre-exposed to a combination of IL-17A and TNF- $\alpha$  in the  
535 presence or not of HMGB1 followed by Cd addition. Cell death was quantified by cell cycle  
536 analysis after a week and is represented as fold changes compared to cells treated with Cd and  
537 cytokines (C). Data are the mean of at least three independent experiments  $\pm$  SEM. Cyt:  
538 cytokines, H: HMGB1. # Comparison with control situation, \* comparison with cytokine  
539 treated cells, § comparison between cell types. \*, #  $P \leq 0.05$ , \*\*, §§  $P \leq 0.01$ , \*\*\*, ###, §§§  
540  $P \leq 0.001$ .

541 Figure 6: Model of the regulation of Amigo2 expression which promotes cell survival and  
542 proliferation.

543 A model was proposed whereby cellular interaction with immune cells and exposure of the  
544 cells to the pro-inflammatory cytokines IL-17A and TNF- $\alpha$  as well as to HMGB1 increases  
545 Amigo2 expression in an ERK-dependent manner leading to enhanced cellular adhesion and  
546 promoting cell survival and proliferation.

Figure 1.TIF

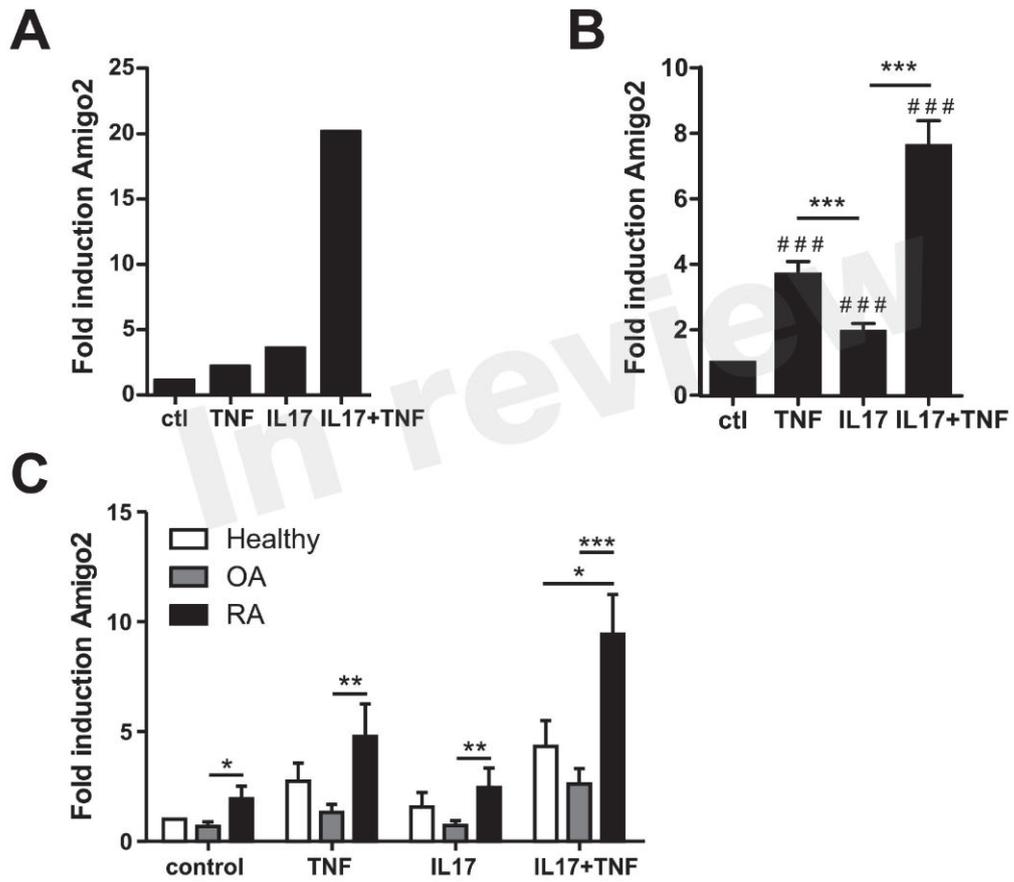


Figure 2.TIF

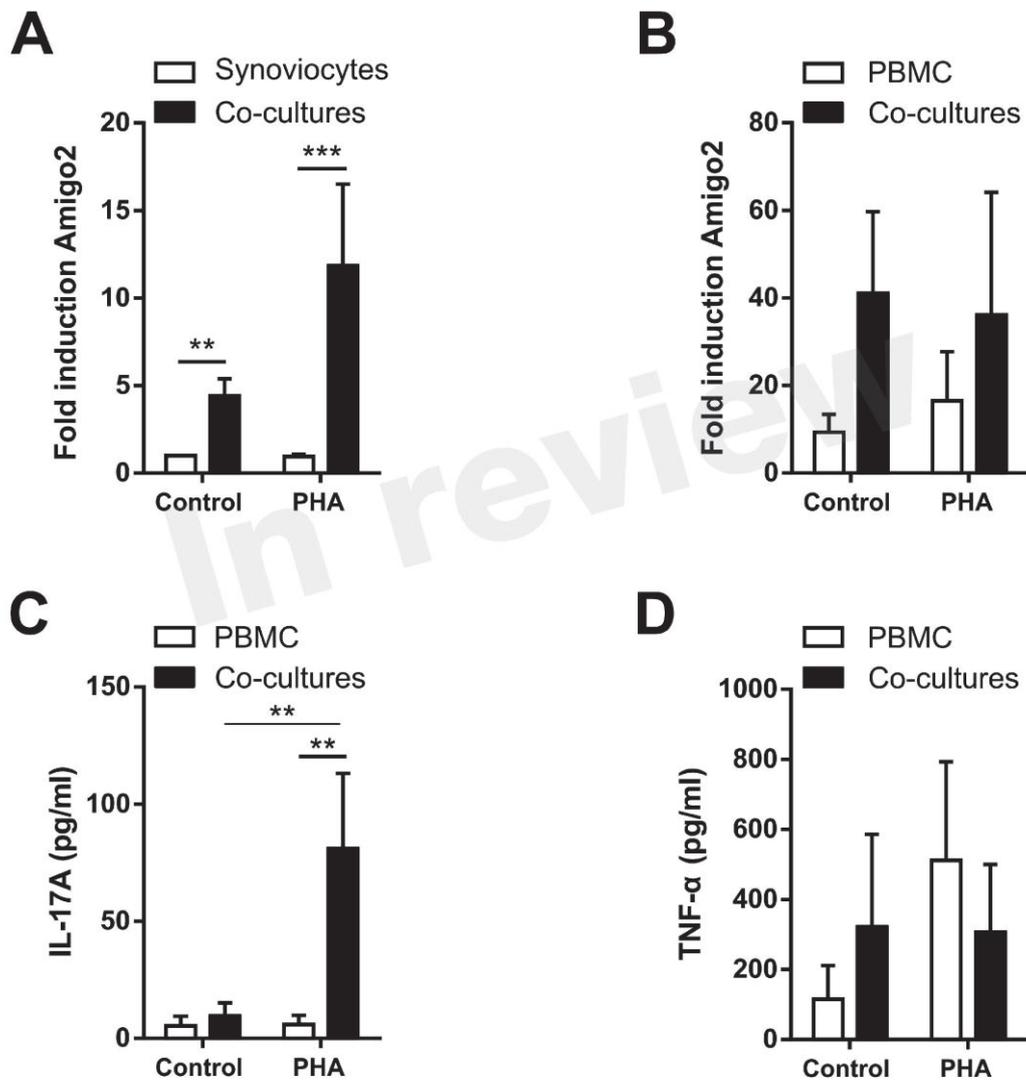


Figure 3.TIF

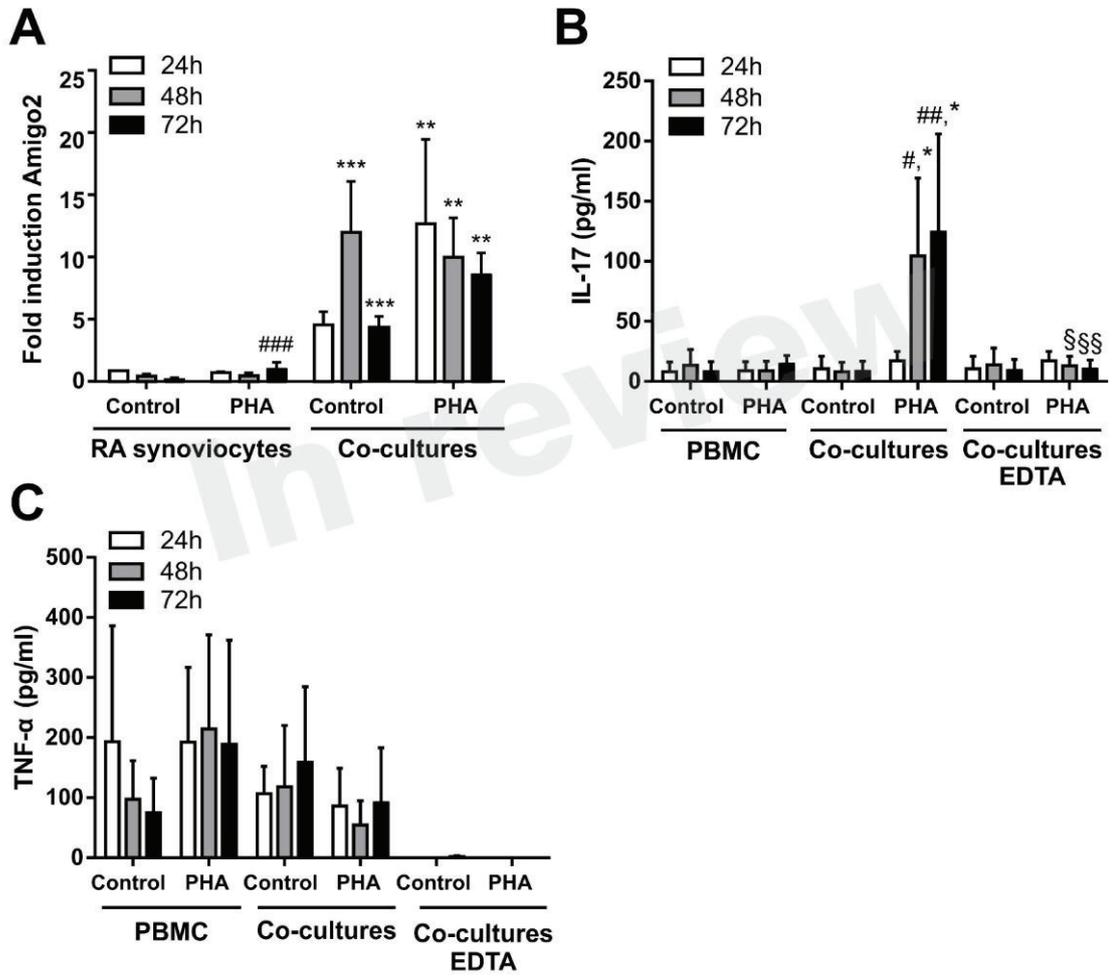


Figure 4.JPEG

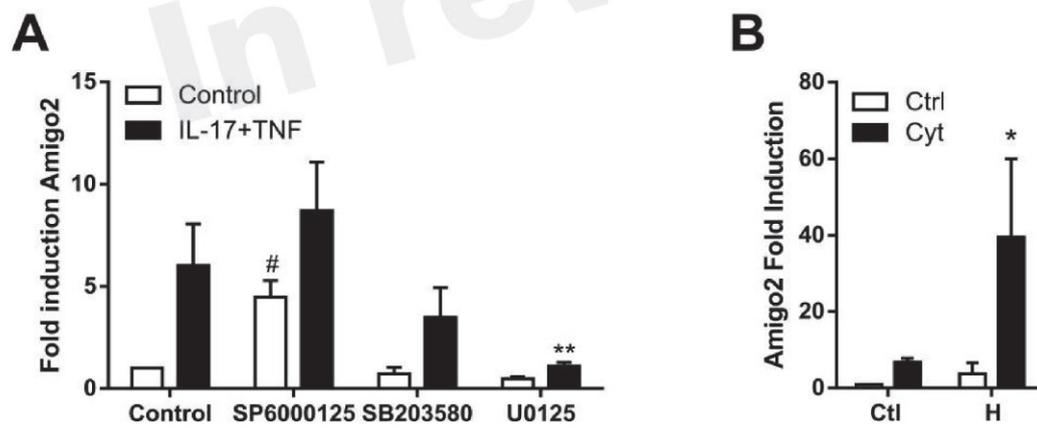
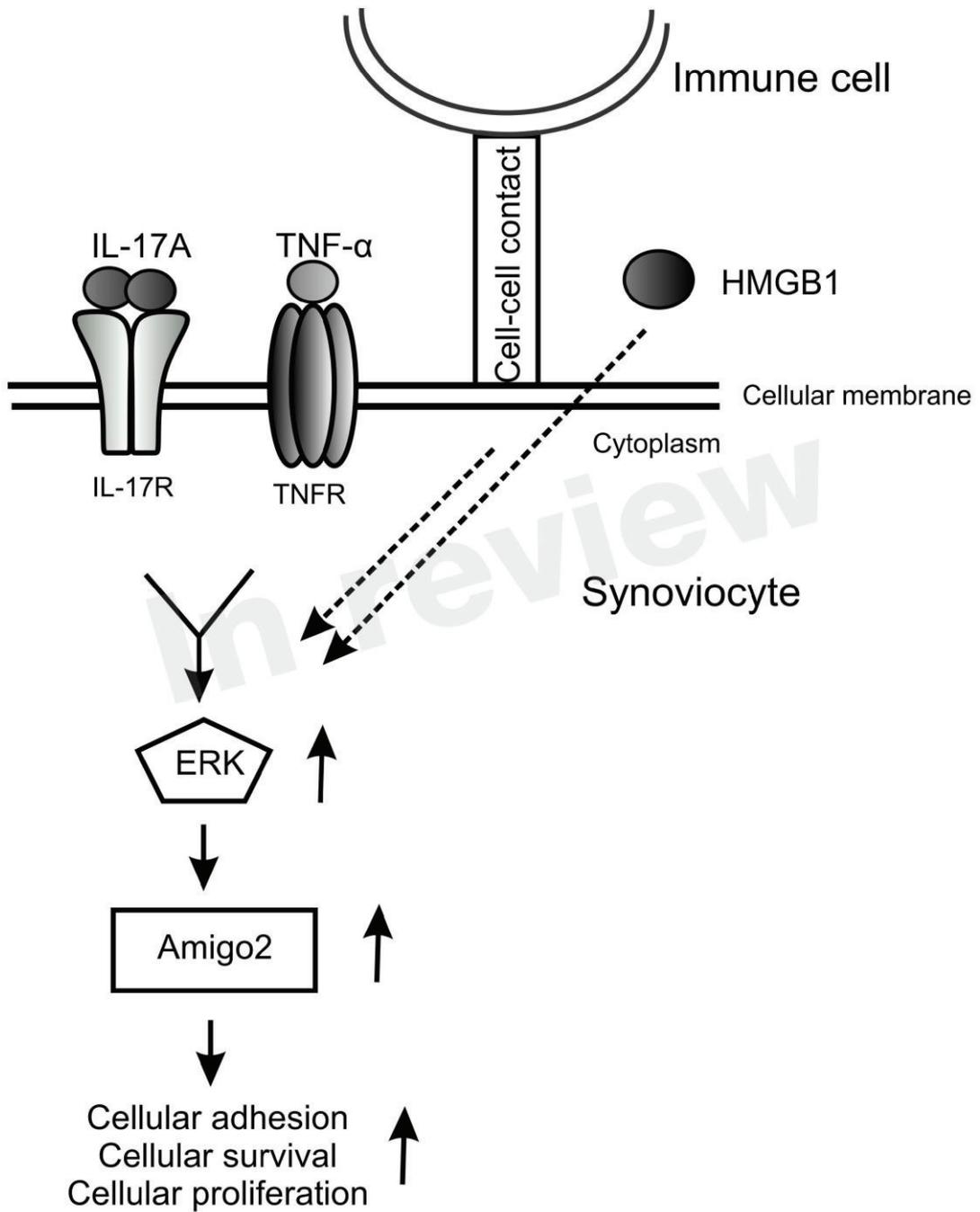




Figure 6.TIF



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