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In vitro Cell-Based Assays for Potency Testing of Anti-TNF- α Biological Drugs

Sara Žigon-Branc, Ariana Barlič and Matjaž Jeras

Abstract

Human cell-based assays for *in vitro* testing of drugs in preclinical and research studies, as well as in clinical practice, are gaining greater importance especially in view of personalized medicine, which is tailored to the individual needs and benefits of a patient. This chapter begins with an overview of contemporary cell-based assays, routinely used for a comparative *in vitro* potency testing of anti-TNF- α innovator biologics and their biosimilars. In sequel, based on the results of our original work, we will further discuss the establishment and use of 2D normal and osteoarthritic primary chondrocyte monolayer cultures and 3D microspheroidal articular cartilage tissues, prepared in hanging drops from osteoarthritic chondrocytes and chondrogenically differentiated mesenchymal stem cells. Both 2D and 3D cultures will be presented as models for assessing the neutralizing potency of the three well-known anti-TNF- α biological drugs: adalimumab, etanercept, and infliximab.

Keywords: *in vitro* cell-based assays, anti-TNF- α biologics, human articular chondrocytes, mesenchymal stem cells, 2D monolayer cultures, 3D cell cultures, gene expression

1. Introduction

Following the discovery and characterization of tumor necrosis factor (TNF) in the mid-1980s, this pleiotropic proinflammatory cytokine continues to be the focus of numerous studies and represents an important therapeutic target [1, 2]. The venue of anti-TNF biological drugs has revolutionized treatment of autoimmune and inflammatory diseases like rheumatoid arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, psoriasis, Crohn's disease, ulcerative colitis, and others [2]. Although expensive, biological drugs (biologics) at the moment represent the best-selling group of pharmaceuticals. Nowadays, following the expiry of originators patents, a plethora of less expensive biosimilar drugs (biosimilars) are available to patients. In order to confirm the biocomparability of original and biosimilar products and to prove their quality, safety, and efficacy, the use of reliable and standardized bioassays relevant in assessing their modes of action is of crucial importance.

In this chapter, after a short introductory review of TNF biology, anti-TNF biological drugs and their mechanisms of action, we will present a selection of *in vitro* cell-based tests used either for general or personalized potency testing of anti-TNF biologics and their biosimilars.

2. A short overview of TNF biology

TNF is produced in various cell types, mainly immune cells such as monocytes and macrophages, microglia, neutrophils, natural killer cells (NK), T lymphocytes, and also in neuronal cells, keratinocytes, and fibroblasts [2, 3]. The cytokine exists in two biologically active forms. The first being a transmembrane protein (tmTNF), which can be cleaved by the metalloproteinase TNF- α -converting enzyme (TACE) (also known as disintegrin and metalloproteinase domain-containing protein 17 (ADAM17)) into its second form, a homotrimeric soluble TNF (sTNF) [2].

There are two TNF-binding homotrimeric transmembrane receptors, namely the TNF receptor 1 (TNFR1 or CD120a) and the TNF receptor 2 (TNFR2 or CD120b) [2]. While the TNFR1 is constitutively expressed on a vast majority of nucleated cells, the TNFR2 expression is inducible and tightly regulated, preferentially on endothelial, hematopoietic, neural, and immune cells [2, 4]. TNFR2 is also expressed on tumor cells where it is supposed to function as a tumor oncogene [5, 6].

Interestingly, tmTNF can induce signals in a bipolar way, as it acts as a ligand of both receptor types and as a receptor itself in cell-to-cell contacts [2, 4]. This means that tmTNF- α -expressing cells transmit signals to cells bearing TNFR1 and/or TNFR2. This phenomenon is called “outside-to-inside” or “reverse signaling,” the function of which has not been completely clarified yet [2, 4]. The receptor function of tmTNF has been demonstrated in human monocytes, macrophages, NK cells, and T lymphocytes [4].

While TNFR1 is activated by both tmTNF and sTNF, TNFR2 can only be triggered by tmTNF. Both types of membrane-bound receptors are prone to TACE cleavage, resulting in fragments termed soluble TNF receptors (sTNFR) [2]. In turn, sTNFR may contribute to the regulation of cellular TNF responses by capturing and neutralizing circulating TNF (intrinsic TNF inhibitors). Additionally, due to increased receptor shedding, the number of functional signaling membrane TNFRs decreases. Consequently, this leads to a state of transient TNF desensitization [2].

3. Anti-TNF biological drugs and their mechanisms of action

Among currently available Food and Drug Administration (FDA)- and European Medicines Agency (EMA)-approved originator and biosimilar anti-TNF drugs, there are three full-length monoclonal antibodies (mAbs); these are infliximab (IFX), a chimeric mouse/human mAb (Remicade[®] and its biosimilars: Remsima[®], Inflectra[®], Flixabi[®], Ixifi[®], Renflexis[®], and Zessly[®]), adalimumab (ADA), a fully humanized mAb (Humira[®] and its biosimilars: Cyltezo[®], Imraldi[®], Amgevita[®], Solymbic[®], Hyrimoz[®], Hulio[®], Halimatoz[®], and Heyifa[®]), and golimumab, another fully humanized mAb (Simponi[®]) (**Figure 1**) [2, 4]. The additional two anti-TNF biological drugs, which are not mAbs, are etanercept (ETA) (Enbrel[®] and its biosimilars: Erelzi[®] and Benepali[®]), a fusion protein consisting of two extracellular parts of the human TNFR2 and the Fc portion of human IgG1, and certolizumab pegol (Cimzia[®]) composed of a human Fab' fragment, covalently attached to two cross-linked 20 kDa polyethylene glycol chains (**Figure 1**) [2, 4].

Although all anti-TNF biologics neutralize the same target (sTNF and tmTNF), they are not equally effective in treatment of certain inflammatory pathologies, for example, Crohn's disease. This is due to differences in their characteristics (structure and binding affinities) and mechanisms of action (**Figure 1**) [2, 4]. Besides all of them being efficacious in neutralizing both forms of TNF, infliximab additionally induces “outside-to-inside” signaling via binding to tmTNF, thereby triggering apoptosis of tmTNF-expressing immune cells [2]. Being full-length mAbs,

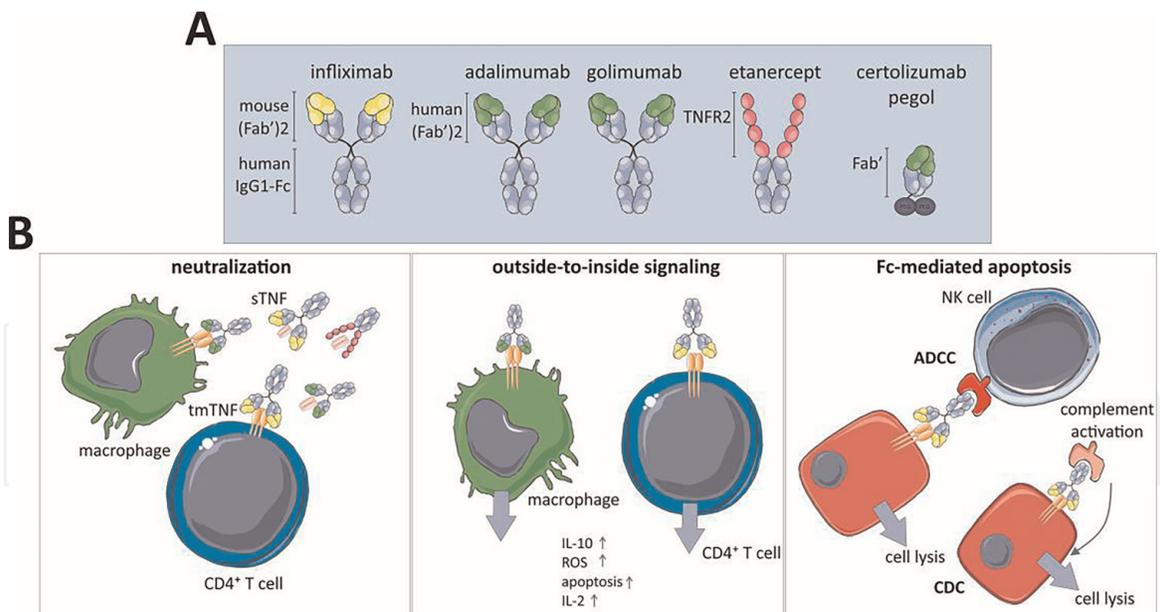


Figure 1. Structures (A) and mechanisms of action (B) of the five FDA- and EMA-approved anti-TNF biologics; original figure used with the authors' permission under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>) [2].

adalimumab, golimumab, and infliximab can, after binding to cells expressing tmTNF via their effector Fc regions (IgG1), induce antibody-dependent cytotoxicity (ADCC) of NK cells and activate the classical complement pathway, resulting in a complement-dependent cytotoxicity (CDC) and apoptosis [2, 4]. While ADCC and CDC are also induced by etanercept, which contains a truncated form of IgG1 Fc domain (lacking a CH1 constant region), certolizumab pegol, due to its Fc domain missing structure, acts differently. In treating inflammatory bowel disease with anti-TNF mAbs, another mechanism of their action is based on the interaction between IgG1 Fc domains of therapeutic mAbs and macrophage Fc γ receptors (Fc γ R), resulting in increased numbers of regulatory M2 macrophages (CD206⁺). These cells in turn inhibit T cell proliferation [2, 7]. Additionally, in rheumatoid arthritis (RA), adalimumab enhances the expression of tmTNF on monocytes, thereby promoting the interaction between tmTNF and TNFR2 present on regulatory T cells (Tregs), which subsequently increase their immunosuppressive activities [2, 8]. Also in RA, infliximab promotes the generation of natural Tregs (CD4⁺CD25^{high}FoxP3⁺), which inhibit a proinflammatory cytokine production and replenish a defective pool of these cells, typically found in this autoimmune disease [2, 9]. In RA patients, the adhesion molecules and chemokines are upregulated on their joint vasculature endothelium. Blockage of TNF- α with adalimumab, golimumab, infliximab, or etanercept deactivates inflamed vascular endothelium, thereby decreasing the numbers of inflammatory immune cells entering synovial joints and additionally improving the generation of new synovial blood vessels by increasing the circulating levels of vascular endothelial growth factor (VEGF) [10, 11].

4. In vitro cell-based bioassays for general potency assessment of anti-TNF biologics

Numerous well-established and standardized cell-based assays are available for assessing and comparing potencies of anti-TNF biologics and their biosimilars. **Table 1** contains some basic information regarding the most frequently used routine TNF- α neutralization (A), ADCC (B), and CDC (C) tests. The majority of data

on bioassays presented in **Table 1** (see next page) were summarized from two publications describing the establishment of the first infliximab and etanercept World Health Organization (WHO) International Standards [12, 13]. These were performed within international collaborative studies, confirming their high degree of relevance and analytical laboratory utility.

Additionally, the capability of anti-TNF biological drugs to downregulate E-selectin adhesion molecules expressed on inflamed vascular endothelium can be determined on *in vitro*-cultured human umbilical vein endothelial cells by using appropriately labeled anti-E-selectin detection antibodies [10].

Other bioassay readout approaches, like flow cytometry and measurement of induced endogenous gene expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR), are also being applied [16–18].

The reason why various human and murine cell lines are used in these assays is that such tests can be standardized and their results can be compared between laboratories. However, the use of different types of primary cells in such general tests is less appropriate due to their high interindividual differences and in certain cases also weak responsiveness to anti-TNF biologics. Therefore, the results obtained in this way can hardly be compared [10].

5. Two-dimensional (2D) and three-dimensional (3D) primary cell cultures for personalized *in vitro* potency testing of anti-TNF biologics

Primary cells are indispensable for determining personal responses of patients to a given anti-TNF biologic, thereby generating important information for planning and performing optimal and cost-effective therapies. For this purpose, different cell types, especially those isolated from a patient's disease-affected tissues or *in vitro* differentiated autologous stem cells, can be used. In general, it is well established that in comparison to cells grown in 2D, those cultured in a 3D environment better mimic the scenarios *in vivo*. A number of cellular processes, that is, proliferation, differentiation, morphology, gene, and protein expressions, as well as responsiveness to external stimuli, are significantly affected by the physical aspects of the 3D environment [19–22].

In 2D cell cultures (monolayers), nutrients are evenly accessible to cells, but the communication between cells via secreted soluble molecules is restricted to their diffusion within the fluid, unless the medium is mixed or stirred regularly [23]. On the other hand, in dense multicellular 3D cell constructs prepared and cultured *in vitro*, nutrients and other soluble molecules, as well as oxygen supply, are limited by the mass transport, which is restricted by the construct's thickness/diameter and cell density per volume [23].

In the following subchapters, we will present our results after establishing 2D and 3D *in vitro* models for potency testing of anti-TNF biologics by using primary normal chondrocytes (NCs) and osteoarthritic chondrocytes (OACs), as well as chondrogenically differentiated bone-marrow-derived MSCs obtained from OA patients, with qRT-PCR gene expression assessment and protein secretion readout measurements.

5.1 Establishment of a 2D primary human chondrocyte-based cell model for *in vitro* testing of anti-TNF- α biologics

Cartilage, which covers joint surfaces, is one of the most affected tissues in RA and other inflammatory arthritic diseases. Its only living constituents are chondrocytes, which produce and maintain a cartilaginous matrix mainly consisting

A

| Cell types | rhTNF- α | Assay | Readout | Ref. |
|-------------|--|---------------|--|----------------------|
| L929 | 10-86 IU/ml 7.2-20 IU/ml | Cytotoxicity | Absorbance: MTS, CCK-8 Fluorescence: Resazurin Luminescence: CellTiter-Glo [®] | [12] [13] |
| WEHI-164 | 100 IU/ml 2-100 IU/ml | Cytotoxicity | Absorbance: MTS, CCK-8/WST-8 | [12] [13] |
| WEHI-13 VAR | 5 IU/ml | Cytotoxicity | Absorbance: MTS, CCK8 | [12] |
| KD4 Cl21 | 4.3 IU/ml | Cytotoxicity | Absorbance: MTS | [12] |
| KYM-1D4 | 0.1 ng/ml | Cytotoxicity | Absorbance: MTT | [10] |
| KYM-1D4 | htmTNF- α expressed on mouse K2 cells | Cytotoxicity | Absorbance: MTT | [10] |
| U937 | 20-151.5 IU/ml 40-60 IU/ml 40 ng/ml | Apoptosis | Luminescence: Caspase-Glo [®] 3/7 | [12] [13] [14] |
| HEK 293 | 17.2 IU/ml 4 ng/ml | Reporter gene | Luminescence: Steady-Glo [®] | [13] [14] |
| KJL | 40 IU/ml | Reporter gene | Luminescence: Steadylite plus [™] | [12] [13] [15] |

B

| Effector cells | Target cells | E:T ratio | Readout | Ref. |
|---|---|-----------|---|--------------|
| NK92 expressing human Fc γ RIIIa | 3T3 expressing htmTNF- α | 1:1 | Luminescence: CytoTox-Glo [®] | [12] [13] |
| Jurkat expressing human Fc γ RIIIa and NFAT-induced luciferase reporter gene | CHO-K1 expressing htmTNF- α | 4:1 | Luminescence: Bio-Glo [™] | [12] [13] |
| Jurkat expressing human Fc γ RIIIa linked to a luciferase reporter gene | HEK-293 expressing htmTNF- α | 6:1 | Luminescence: Dual-Glo [®] | [12] [13] |
| NK3.3 | HEK-293 expressing htmTNF- α | 10:1 | Fluorescence: Calcein | [14] |

C

| Target cells | Complement | Assay | Readout | Ref. |
|---------------------------------------|-------------|-----------|---|--------------|
| Jurkat expressing htmTNF- α | Human serum | Viability | Luminescence: CytoTox-Glo [®] | [12] [13] |
| Jurkat expressing htmTNF- α | Human serum | Viability | Absorbance: CCK-8 | [12] [13] |
| Jurkat expressing htmTNF- α | Human serum | Viability | Luminescence: CellTiter-Glo [®] | [14] |

rhTNF- α —recombinant human TNF- α . Cells \rightarrow CHO-K1: Chinese hamster ovary cells expressing human transmembrane TNF- α (htmTNF- α); HEK 293: human embryonic kidney cell line, transfected with the TNF- α -responsive NF κ B-regulated Firefly luciferase reporter gene construct or expressing htmTNF- α ; Jurkat: human acute T cell leukemia lymphocytes expressing htmTNF- α , resistant to TACE cleavage, human Fc γ RIIIa or TNF- α -responsive nuclear factor of activated T cells (NFAT) transcription factor-regulated Firefly luciferase reporter gene construct; K2: murine cells expressing the uncleavable htmTNF- α ; KD4 Cl21: human rhabdomyosarcoma cell line; KJL: human erythroleukemic K562 cells transfected with the TNF- α -responsive NF κ B-regulated Firefly luciferase reporter gene construct, together with the Renilla luciferase reporter gene under the control of a constitutive minimal thymidine kinase promoter; L929: murine fibroblast cell line; NK3.3: human natural killer (NK) cell line cloned from peripheral blood; NK92: NK lymphoblast cells from a malignant non-Hodgkin's lymphoma patient, expressing Fc γ RIIIa; 3T3: murine embryonic fibroblasts expressing htmTNF- α ; U937: human histiocytic lymphoma cell line; WEHI-13 VAR and WEHI-164: murine rhabdomyosarcoma cell lines. Readout reagents (absorbance) \rightarrow CCK-8/WST-8: 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; luminescence detection kits: Bio-Glo[™], Caspase-Glo[®] 3/7, CellTiter-Glo[®], CytoTox-Glo[®], Dual-Glo[®], Steady-Glo[®] (all from Promega), and Steadylite plus[™] luminescence reporter gene system (Perkin Elmer). Assays \rightarrow TNF- α neutralization (cytotoxicity, apoptosis, reporter gene): measuring the extent of residual TNF- α -induced cytotoxicity and apoptosis in the presence of anti-TNF- α biologics; TNF- α -induced ADCC: measuring the extent of effector cell cytotoxicity on htmTNF- α expressing target cells, in the presence of anti-TNF-biologics; TNF- α -induced CDC: measuring the extent of cytotoxicity in the presence of human serum as a source of complement and anti-TNF biologics.

Table 1. Most frequently used routine cell-based bioassays for assessing the TNF neutralization potency (A), ADCC (B), and CDC (C) of anti-TNF biologics and their biosimilars [14, 15].

of collagen and proteoglycans [24]. *In vitro*-cultured chondrocytes have already provided useful models to study their response to microenvironment alterations [25]. However, we have extended their *in vitro* use to efficacy testing of anti-TNF- α drugs [26–28]. First, we have established a 2D *in vitro* model by culturing human primary chondrocytes in monolayer cultures and later upgraded it to a 3D cell model, which better mimics the organization of these cells in native cartilage. For this purpose, we chose a combination of physiologically relevant cell sources and a gene expression assessment technique (qRT-PCR), which enables analyses of up- or downregulated genes in comparison to measurable changes in secreted proteins or cell numbers [29]. We have selected and screened 42 genes involved in immune responses, extracellular matrix remodeling, stress response, signaling pathways, expression of adhesion and other molecules, responding to a pathogenic inflammatory environment that was artificially created with the addition of rhTNF- α .

For the establishment of our 2D model, two types of cells were used. Normal, healthy chondrocytes (NCs) were obtained from surplus cartilage biopsies of patients scheduled for an autologous chondrocyte implantation procedure or were acquired postmortem from donors with healthy cartilage, in accordance with National Medical Ethics Committee approvals. On the other hand, osteoarthritic chondrocytes (OACs) were obtained from cartilage samples of patients undergoing total knee replacement surgery, in accordance with National Medical Ethics Committee approval. Following chondrocyte isolation and cultivation, confluent cell cultures were incubated in serum-free conditions with 1 ng/mL of rhTNF- α (PeproTech, USA) \pm 1 μ g/mL of each of the two anti-TNF- α biologicals tested, infliximab (IFX; Remicade[®], Centocor, Netherlands) and etanercept (ETA; Enbrel[®], Wyeth Pharmaceuticals, UK). After 24 h of incubation, chondrocytes and cell culture media were sampled for gene and protein expression analyses, respectively. In experiments using OACs, only the most relevant genes were selected and analyzed. Names and symbols of screened genes are presented in **Table 2**. Data were analyzed by applying the $2^{-\Delta\Delta C_q}$ formula (ABI PRISM[®] 7700 Sequence Detection System User Bulletin #2) with the nontreated chondrocyte samples used for normalization. Results are presented as relative quantities (RQ) or Log₂ relative quantity values (Log₂ RQ). For protein expression analysis, a custom antibody array (RayBiotech, USA) was designed to detect interleukin-1 receptor antagonist (IL-1Ra), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8), matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-3 (MMP-3), matrix metalloproteinase-13 (MMP-13), monocyte chemoattractant protein-1 (MCP-1), tissue inhibitor of metalloproteinase-2 (TIMP-2), and vascular cell adhesion protein 1 (VCAM-1). All protein analysis data were normalized to nontreated controls.

The results of the first set of experiments obtained after stimulation of cultured NCs and OACs with rhTNF- α and after their preincubation with a combination of rhTNF- α and IFX or ETA are presented in **Figure 2** (graphs A and B, respectively). Upon TNF- α stimulation of NCs, the highest gene upregulation was observed for *IL8* and *MMP1* with a >1000-fold change. A very high upregulation (\geq 80-fold change) was also observed for *IL6*, *IL32*, *MMP3*, *MMP13*, *TLR2*, and *MCP1* genes (**Figure 2**, graph Aa). We considered the differences between treated and nontreated cells as biologically significant whenever the calculated fold change was \geq 2, which equals a Log₂-fold change of \geq 1 unit on a logarithmic scale. Next, we examined the neutralization efficacy of IFX and ETA by monitoring a decrease in TNF- α -induced gene expressions. Although IFX reduced the expression of TNF- α -upregulated genes, some of them remained more expressed when compared to nontreated cell samples (**Figure 2**, graph Ab). On the other hand, ETA completely abolished the TNF- α -mediated up- and downregulation of the tested genes (**Figure 2**, graph Ac). Altogether, our results revealed differential sTNF- α

| Gene Symbol | Gene Name | Gene Symbol | Gene Name |
|-------------|--|-------------|---|
| ACAN | aggrecan | MMP1 | matrix metalloproteinase 1 (interstitial collagenase) |
| ADAM17 | ADAM metalloproteinase domain 17 | MMP10 | matrix metalloproteinase 10 (stromelysin 2) |
| ADAMTS4 | ADAM metalloproteinase with thrombospondin type 1 motif, 4 | MMP13 | matrix metalloproteinase 13 (collagenase 3) |
| ADAMTS5 | ADAM metalloproteinase with thrombospondin type 1 motif, 5 | MMP3 | matrix metalloproteinase 3 (stromelysin 1, progelatinase) |
| BGN | biglycan | NFKB1 | nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 |
| CCL13 | chemokine (C-C motif) ligand 13 | NOS2 | nitric oxide synthase 2, inducible |
| CD44 | CD44 molecule (Indian blood group) | PTGS2 | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) |
| CHUK | conserved helix-loop-helix ubiquitous kinase | RUNX2 | runt-related transcription factor 2 |
| COL11A2 | collagen, type XI, alpha 2 | SOX9 | SRY (sex determining region Y)-box 9 |
| COL12A1 | collagen, type XII, alpha 1 | TIMP1 | TIMP metalloproteinase inhibitor 1 |
| COL9A3 | collagen, type IX, alpha 3 | TIMP2 | TIMP metalloproteinase inhibitor 2 |
| COMP | cartilage oligomeric matrix protein | TLR1 | toll-like receptor 1 |
| DCN | decorin | TLR2 | toll-like receptor 2 |
| HAPLN1 | hyaluronan and proteoglycan link protein 1 | TLR4 | toll-like receptor 4 |
| IL1B | interleukin 1, beta | TNF | tumor necrosis factor |
| IL1RA | interleukin 1 receptor antagonist | TNFRSF1A | tumor necrosis factor receptor superfamily, member 1A |
| IL32 | interleukin 32 | TNFRSF1B | tumor necrosis factor receptor superfamily, member 1B |
| IL6 | interleukin 6 (interferon, beta 2) | VCAM1 | vascular cell adhesion molecule 1 |
| IL8 | interleukin 8 | VEGFA | vascular endothelial growth factor A |
| MAP2K3 | mitogen-activated protein kinase kinase 3 | | |
| MAPK1 | mitogen-activated protein kinase 1 | | |
| MMP1 | matrix metalloproteinase 1 (interstitial collagenase) | | |
| MCP1 | chemokine (C-C motif) ligand 2 | | |

Table 2.
 List of genes analyzed in qRT-PCR experiments.

neutralizing potency of IFX and ETA at the level of gene expression patterns. The observed changes in gene expression were then also confirmed with a protein expression assay.

Because NCs are difficult to obtain, we performed the same IFX and ETA neutralization experiments with rhTNF- α -treated OACs, however, to a lesser extent. A selected group of the most responsive genes were tested using OAC biological samples from four donors (**Figure 2B**). We observed a similar response to NCs when OACs were treated by rhTNF- α alone (**Figure 2**, graph Ba) and after their preincubation with a combination of rhTNF- α and IFX or ETA (**Figure 2**, graphs Bb and Bd, respectively). In **Figure 2**, graphs Bc and Be show the responses of OACs after their exposure to each individual biological drug. The cartilage of OA patients represents a biological waste material, which can be obtained in joint replacement surgeries. Despite changes in gene expression observed during the *in vitro* cultivation of OACs, the stimulation with rhTNF- α reverted them back to their inflammatory phenotype. Our results, employing anti-TNF- α biologics,

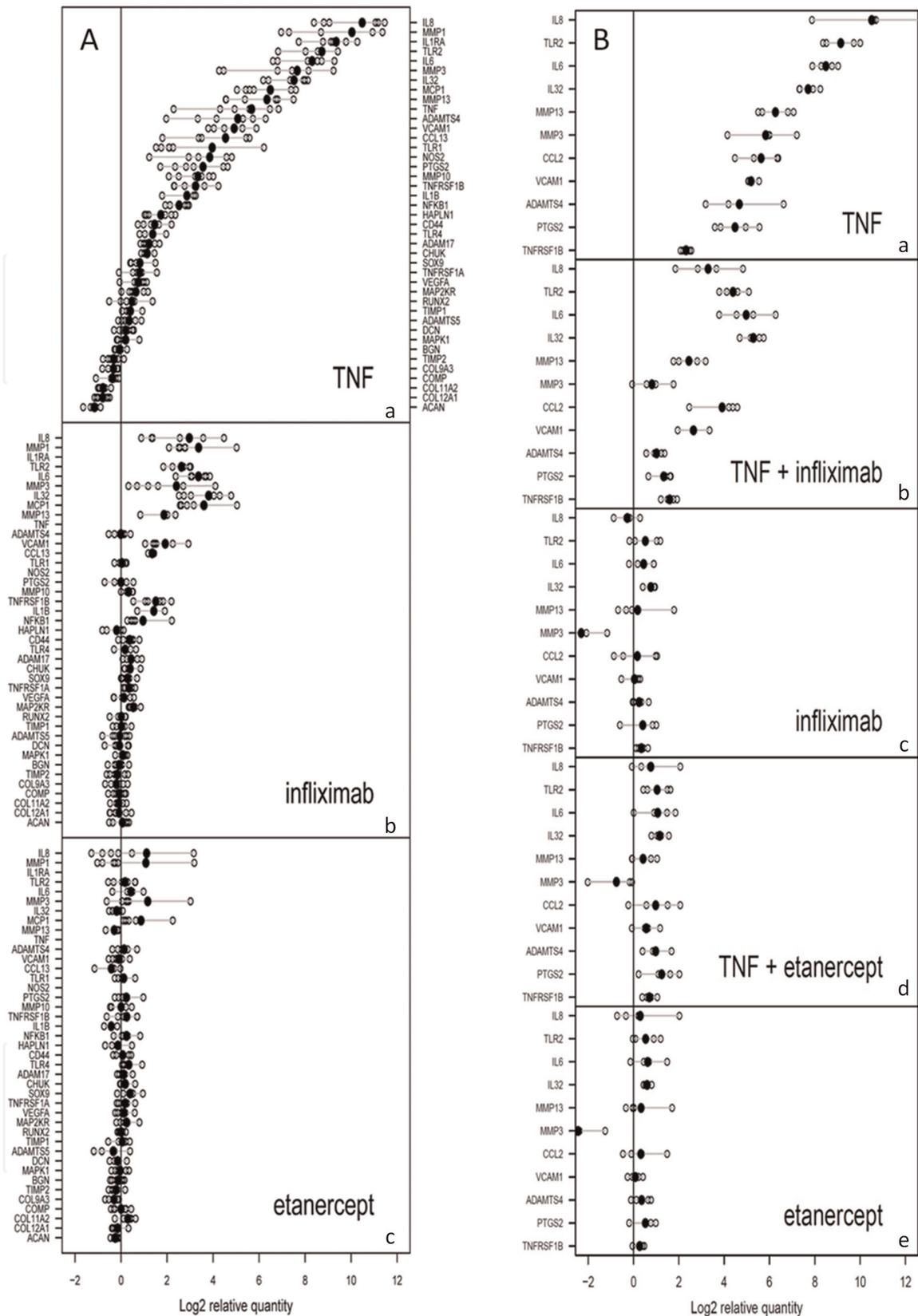


Figure 2. Exposure of in vitro-cultured normal healthy chondrocytes (NCs) (A) and osteoarthritic chondrocytes (OACs) (B) to rhTNF- α markedly increased the expression of various genes coding for interleukins, matrix metalloproteinases, and other factors involved in inflammation and stress response (graphs Aa and Ba). TNF- α neutralizing effect of IFX (graphs Ab and Bb) and ETA (graphs Ac and Bd) is reflected in significantly decreased gene expressions. Graphs Bc and Be show the responses of OACs following their exposure to each individual biological drug tested. All gene expressions were calibrated to nontreated cells. Log2 RQ values of individual biological samples (○) and their corresponding geometrical means (●) are shown. Original figures used with authors' permission under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>) [27, 28].

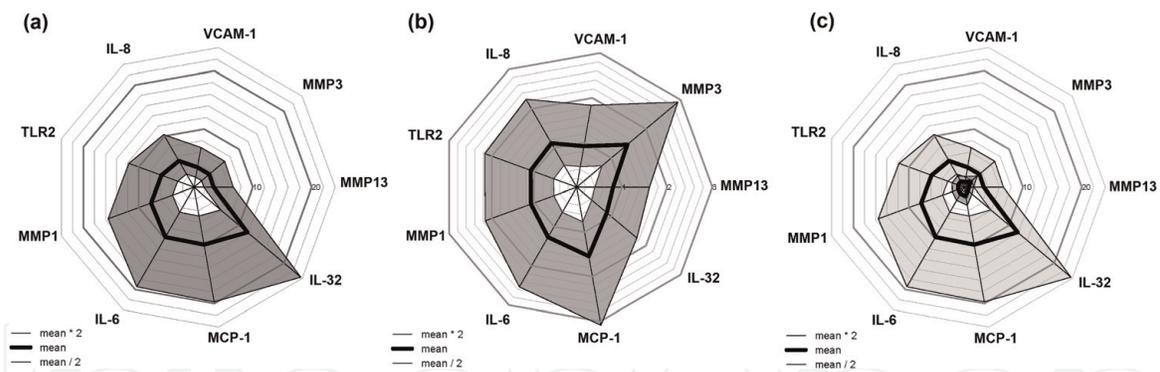


Figure 3. Graphical representation of results obtained with the statistical model for evaluation of sTNF- α neutralization efficacy of biological drugs. Radar graphs for IFX (a), ETA (b), and both of them (IFX and ETA) (c) present geometrical means of RQ values obtained with six NC biological samples. Corresponding twofold area changes are presented. The overlap of twofold rims is only seen in two (MMP3 and VCAM-1) out of nine genes, indicating higher sTNF- α neutralization efficacy of ETA. Value 0 in the center of the radar graphs denotes total inhibition of gene expression. Please note the difference in scales: (b) intervals of 0.2 units, (a) and (c) intervals of 2 units. Original figure used with authors' permission under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>) [28].

confirmed that OACs and NCs can be interchangeably used for obtaining valuable preliminary information regarding the neutralization efficacy of these drugs [27].

With the data obtained, we were able to establish a statistical model for the evaluation of IFX and ETA TNF- α neutralization efficacy. Expressions of the nine most representative genes were chosen for a graphical presentation of results. Geometrical means of RQ values were plotted on radial axes of radar graphs and connected by a polygon, forming a distinctive shape. A comparison of shapes obtained with IFX and ETA revealed differences in their inhibition of gene expressions. Value 0, depicted in the center of graphs, represents total gene inhibition. For easier comparisons of results, shaded areas of twofold changes were plotted as well. Arbitrary fold-change cutoffs >2 (0.5 for down- and 2 for upregulated genes) were considered biologically significant. In our experimental conditions, the twofold change rims only overlapped in case of VCAM1 and MMP3 gene expressions, indicating that both IFX and ETA inhibit these two genes to a similar extent. However, in the case of MMP13, IL32, MCP1, IL6, MMP1, TLR2, and IL8, the inhibition efficacy of ETA was significantly more pronounced. Altogether, in our 2D NC-based model, ETA exhibited higher sTNF- α neutralization efficacy than IFX (Figure 3).

The presented statistical model is also suitable for a comparative neutralization efficacy determination of new bioactive molecules and biosimilars relative to well-established and approved biologics, according to effective criteria for the assessment of biosimilarity, nonsimilarity, and incomparability.

5.2 Establishment of a 3D human osteoarthritic model for *in vitro* efficacy testing of anti-TNF- α biologicals, using primary human osteoarthritic chondrocytes and mesenchymal stem cells

As discussed in the introduction, 2D and 3D cell culture conditions have different impacts on cell phenotype and biological behavior, which were also confirmed for primary chondrocytes and chondrogenically differentiated MSCs [30–35]. In the last decade, cell-based research shifted toward 3D tissue/organ models, providing more physiologically realistic biochemical and biomechanical microenvironments. However, besides their biological relevance, in order to meet the expectations of the pharmaceutical industry, drug screening assays should be high-throughput, widely applicable, and low cost. With this in mind, we established a new *in vitro* 3D

chondrogenic tissue model which, combined with the qRT-PCR readout method, can be used for preclinical or patient-specific potency assessment of anti-TNF- α and anti-interleukin-1 β biological drugs (anti-IL-1 β) [26]. For establishing this model, we used human OACs and chondrogenically differentiated MSCs.

As already stated, OACs represent an attractive source of cells for cell-based models as besides being rather easily accessible and free of ethical concerns, they are also genetically stable during their long-term *in vitro* expansion [36, 37]. Reports show that MSCs isolated from bone marrow of OA patients are capable of producing hyaline cartilage suitable for tissue repair. MSCs obtained from OA and RA patients possess similar chondrogenic potential as those from healthy individuals [38–41]. Therefore, we used paired samples of MSCs and OACs from two donors and a set of genetically mismatched biological samples of patient's OACs and commercially available MSCs. The paired cell sampling approach allowed us to reduce the high patient-to-patient variability, which influences the chondrogenic potential of both OACs and MSCs [42].

Among the numerous commercially available 3D cell culture systems, we have chosen Perfecta 3D[®] scaffolds (3D Biomatrix Inc., USA) to create tissues in hanging drops. Generation of scaffold-free spheroids of micrometric dimensions (microspheroids) by gravity-enforced self-assembly in hanging drops allows cell aggregation and tissue formation in a natural manner, without interference from the scaffold material [19, 32]. This technique has important advantages, especially the drop size control and consequent uniformity of formed microspheroids. Moreover, it is compatible with automated liquid handling systems, a prerequisite for high-throughput screening in drug discovery. The microspheroid formation in hanging drops mimics the condensation process of MSCs, which is one of the earliest phases of *in vivo* cartilage development [32].

Isolated OACs were first expanded in 2D monolayer cultures and then, from passage 2 and on, 10,000 cells were transferred into each hanging drop. In this way, the loss of chondrogenic phenotype of OACs in 2D was restored in 3D conditions, as already reported [30, 43]. Similarly as in our previously described 2D primary chondrocyte model, the TNF- α neutralizing efficiencies of ADA (Humira[®], Abbott Laboratories, USA), ETA (Enbrel[®], Immunex Corp., USA), IFX (Remicade[®], Janssen Biotech, USA), and the anti-IL-1 β drug anakinra (ANA; Kineret[®], Swedish Orphan Biovitrum AB, Sweden) were assessed with both cell types by determining the extent of downregulation of six selected genes (*IL6*, *IL8*, *MCP1*, *MMP1*, *MMP13*, and *VCAM1*) [27, 28]. Gene expression was determined after a 24 h incubation of microspheroids in a medium supplemented with 1 ng/mL of an appropriate inflammatory cytokine (rhTNF- α or rhIL-1 β ; both from PeproTech, USA) or working macrophage conditioned medium (MCM) solution, combined with 1 μ g/mL of each individual biological drug tested (**Figure 4**).

According to our criteria, $\text{Log}_2 \text{RQ} \geq 1$ and ≤ -1 , TNF- α significantly upregulated the expression of *IL6*, *IL8*, *MCP1*, *MMP1*, *MMP13*, and *VCAM1* genes in the 3D microspheroidal model as well (**Figure 4a**). The same was true when IL-1 β or MCM was added to microspheroids. MCM was obtained from cell cultures of the human monocytic cell line THP-1 (ATCC, USA) and represented a rich source of inflammatory cytokines with 0.05 ng/mL TNF- α and 0.45 ng/mL IL-1 β , and numerous other growth factors. In terms of influencing gene expression, IL-1 β was the most potent inflammation inducer, followed by MCM and then TNF- α . The inflammatory process triggered by each of these three inducers could always be reversed by ADA, IFX, or ETA, as well as ANA (**Figure 4a**). When inflammation was triggered by TNF- α , all tested anti-TNF- α biologics extraordinarily suppressed the expression of monitored genes, sometimes even reaching their constitutively expressed levels ($\text{log}_2 \text{RQ} = 0$). Similarly, in the presence of IL-1 β , ANA markedly reversed the

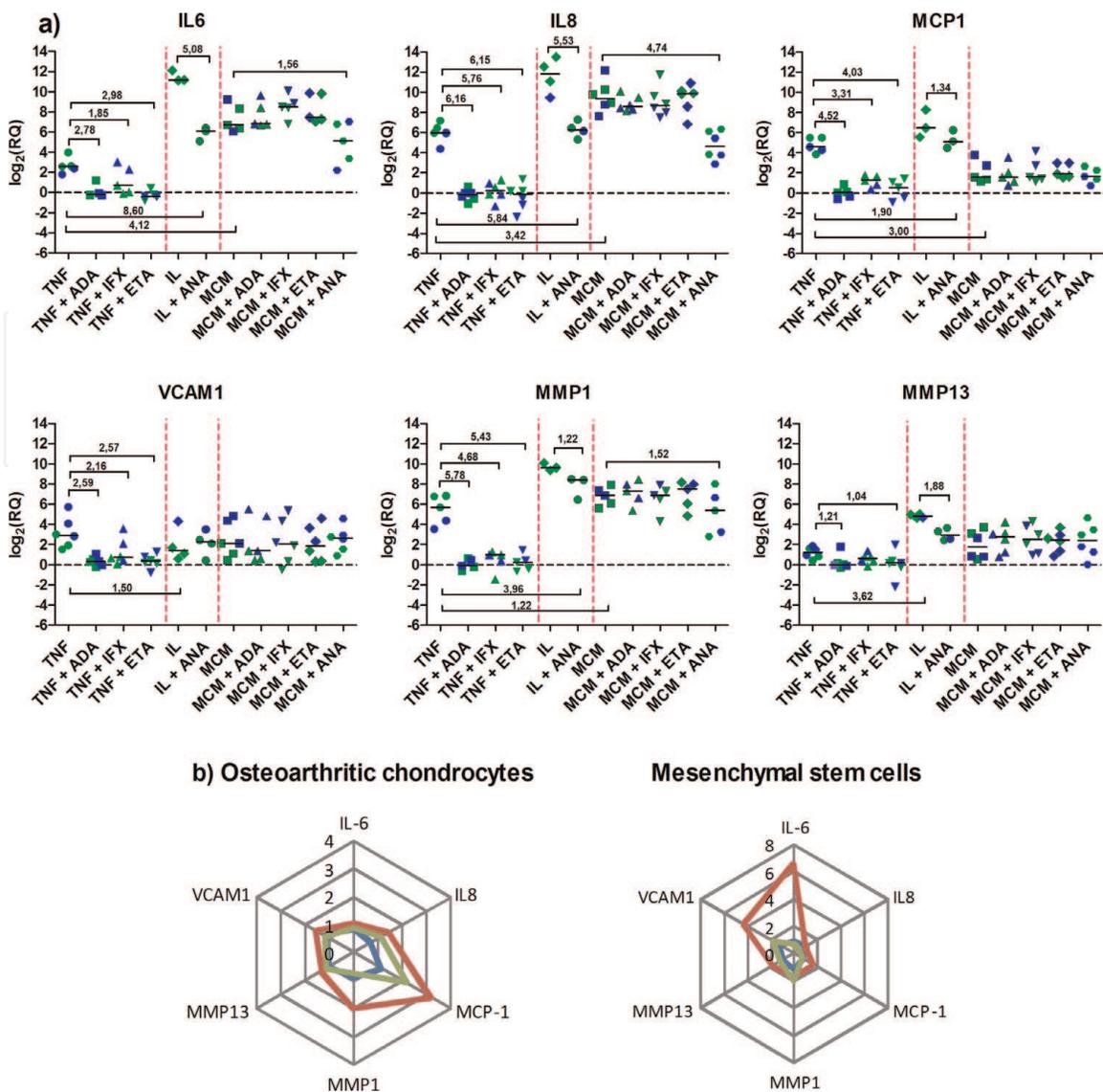


Figure 4.
 (a) Gene expression profiles following the addition of inflammatory mediators TNF- α , IL-1 β , or MCM working solution and anti-inflammatory biological drugs ADA, IFX, ETA, and ANA. Blue and green dots represent values obtained in microspheroidal chondral tissues made of MSCs and OACs (three donors), respectively. Statistically significant changes, that is, $\text{Log}_2 \text{RQ} \geq 1$ and ≤ -1 are outlined with median values for all groups. (b) Radar graphs representing anti-TNF- α neutralization efficacies of ADA (blue), IFX (red), and ETA (green). Mean RQ values of three biological samples are shown for OAC- and MSC-derived microspheroids. Value 0 in the center of each radar graph represents total inhibition of gene expression. Original figure used with authors' permission under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>) [26].

inflammation process in microspheroids. However, when microspheroids were incubated with MCM, none of the three tested anti-TNF- α drugs were successful in diminishing its inflammatory effect. Conversely, ANA could downregulate the expression of *IL6*, *IL8*, and *MMP1* genes. The described changes at the gene level were accompanied by significant differences in the expression of IL6, IL8, and MCP1 proteins, detected in supernatants of microspheroid cultures, 24 h after their incubation with a given inflammatory agent \pm selected anti-inflammatory biological drug [26]. Moreover, these results were additionally supported by the amount of glycosaminoglycans present in chondral spheroids composed of 100,000 OACs treated with various combinations of a particular inflammatory agent \pm a given anti-inflammatory biologic, for a period of 3 weeks.

When microspheroids were incubated with MCM, a superior anti-IL-1 β neutralization capacity of ANA compared to the three tested anti-TNF- α biologics was observed. This difference was probably due to the fact that MCM contained a much

higher concentration of IL-1 β (0.45 ng/mL) than TNF- α (0.05 ng/mL). Nevertheless, these concentrations of both cytokines are much higher than those measured in synovial fluids of OA and RA patients (0.028 ng/mL TNF- α and 0.1 ng/mL IL-1 β) [44]. Although MCM proved to be an excellent *in vitro* inducer of inflammation, its use for potency testing of anti-inflammatory biologicals targeting a specific cytokine is questionable. In fact, from the multiple synergistic proinflammatory effects evoked by different biogenic factors present in MCM, it is very hard to define the potency of a biological targeting a single inflammatory factor.

In our 3D microspheroidal rhTNF- α -induced inflammation model, the neutralization capacity of ADA was superior over that of ETA and the even weaker IFX (**Figure 4b**). Similar results were obtained with both microspheroids, regardless of whether they were made of OACs or chondrogenically differentiated MSCs. The observed differences in neutralizing efficiencies of ADA, ETA, and IFX can be attributed to differences in their molecular structures and sTNF- α -binding affinities [45]. The superior anti-TNF- α efficacy of ADA over ETA and IFX has already been reported together with data, showing that the sTNF- α -binding affinity of ADA is higher for ADA ($K_d = 7.05 \times 10^{-11}$) than ETA ($K_d = 2.35 \times 10^{-11}$) and IFX ($K_d = 1.17 \times 10^{-10}$) [46–48]. However, according to our criterion, a particular biologic would be statistically more efficient than the compared one if it would cause a ≥ 2 -fold decrease in a selected gene expression. This was not the case in any of our 3D microspheroidal model experiments. Consequently, we assumed that the observed differences in TNF- α neutralizing potency of ADA, ETA, and IFX were comparable (**Figure 4b**). Interestingly, although we showed in our 2D OACs model that ETA was significantly more efficient than IFX, the same kind of experiments carried out in a 3D microspheroidal model did not confirm this finding [26, 27]. We assume that compared to the 2D model, the diffusion of tested biologics in our 3D microspheroidal model was much slower and limited. Undoubtedly, the 3D model better resembles *in vivo* conditions and therefore has a higher relevance. Thus, we concluded that 2D cell culture models may be useful for obtaining preliminary data regarding the anti-inflammatory effects of a particular biological drug, while 3D microtissue models enable more relevant insights in drug-tissue interactions and possible outcomes *in vivo*. The results obtained with our 3D microspheroidal model are also supported by the outcomes of clinical studies conducted on patients with RA, where the efficacies of anti-TNF- α biologicals proved to be comparable [49].

We found that OACs and chondrogenically differentiated MSCs are suitable sources for hanging drop chondral 3D microspheroid cultures formation, which are useful for the assessment of neutralization potencies of anti-inflammatory biologics [26]. Although the use of these two types of microspheroids resulted in different gene expression profiles following their incubation with tested combinations of rhTNF- α , and each of the three tested anti-TNF- α biological drugs (**Figure 4b**), these differences were rather small. Therefore, we concluded that MSCs can be used as an alternative and probably even more accessible cell source for *in vitro* testing of neutralization potency of anti-TNF- α biologics. The main advantages of our 3D model are the use of small amounts of human cells and cytokines, personalized testing approach, and the possibility of automation. In addition, the presented approach can also be used as a platform for testing other anti-inflammatory biologics with different mechanisms of action, as shown for ANA, the antagonist of IL-1 β .

6. Conclusion

Cell-based assays are complex analytical tools, susceptible to multiple variables that are virtually impossible to control. Therefore, they have to be precise, reliable,

and well standardized so that the results are reproducible and can be compared among different laboratories. When used for drug potency testing, such assays usually rely on the use of reference standards. Recently, the WHO has prepared two international standards for the two anti-TNF- α biologics, etanercept and infliximab. These have been tested by several laboratories within an international collaborative study using a number of different cell-based assays [12, 13]. In this chapter, we have presented an overview of the most routinely used tests for potency testing of anti-TNF- α biologics, which measure *in vitro* responses of nonmanipulated or genetically engineered human and animal cell lines, with various readout systems.

Nowadays, with an expanding personal medicine approach, laboratory assay-guided pharmacotherapeutical strategies are becoming more and more important. In order to obtain relevant data on drug potencies for a particular patient, these kinds of tests should be based on the patient's own, that is, autologous primary cells, as these can significantly reduce costs and enable safer and more effective therapies. Therefore, we dedicated a part of this chapter to our experience in establishing *in vitro* 2D monolayer cultures consisting of normal and OA chondrocytes and 3D microspheroidal chondral tissues, formed from OACs or chondrogenically differentiated bone-marrow-derived MSCs, and their use for testing anti-TNF- α efficacy of adalimumab, etanercept, and infliximab. The qRT-PCR technique was applied as a readout system for assessing differences in selected gene expressions. The obtained data led us to the establishment of an original statistical method, which was used for the evaluation and comparison of results.

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Conflict of interest

The authors declared that no competing interests exist.

Appendices and nomenclature

| | |
|--------------|---|
| 2D | two-dimensional |
| 3D | three-dimensional |
| ADA | adalimumab |
| ANA | anakinra |
| ETA | etanercept |
| IFX | infliximab |
| IL-1 β | interleukin 1 β |
| MCM | macrophage conditioned medium |
| MSCs | mesenchymal stem cells |
| NCs | normal human articular chondrocytes |
| OACs | osteoarthritic human articular chondrocytes |
| RQ | relative quantity of gene expression |

| | |
|-----------------|--|
| rhTNF- α | recombinant human tumor necrosis factor α |
| sTNF- α | soluble form of tumor necrosis factor α |
| tmTNF- α | transmembrane form of tumor necrosis factor α |

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