

REVIEW

# Antibodies as tools in cytokine discovery and usage for diagnosis and therapy of inflammatory diseases

Jo Van Damme<sup>1,§</sup>, Ghislain Opdenakker<sup>2,§</sup>, Sam Van Damme<sup>3</sup>, Sofie Struyf<sup>1</sup>

<sup>1</sup> Laboratory of Molecular Immunology, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, KU Leuven, Herestraat 49—Box 1042, 3000 Leuven, Belgium; jo.vandamme@kuleuven.be;

<sup>2</sup> Laboratory of Immunobiology, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, KU Leuven, Herestraat 49—Box 1044, 3000 Leuven, Belgium; ghislain.opdenakker@kuleuven.be

<sup>3</sup> Health Care Lab, VIVES University of Applied Sciences, Doorniksesteenweg 145, 8500 Kortrijk, Belgium; sam.vandamme@vives.be;

§ Equally contributed

**Correspondence:** S. Struyf  
<sofie.struyf@kuleuven.be>

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**ABSTRACT.** Polyclonal antisera from patients have been at the basis of the description of autoimmune diseases and today monoclonal antibodies are widely used in the therapy of cancer and many inflammatory diseases. How antisera and antibodies in combination with traditional *in vitro* and *in vivo* biological test systems have been instrumental reagents for the discovery of new cytokines is illustrated here for interleukin-1, -6 and -8. Furthermore, widely used immunological detection/quantification systems, such as ELISAs and multiplex assays, based on the use of either polyclonal or monoclonal antibodies, are often fraught with misinterpretations, because the results are affected by the possible occurrence of posttranslational modifications (PTMs) of the analytes. Cytokines and chemokines are present *in vivo* as mixtures of proteoforms with different amino- or carboxytermini or carrying heterogeneous glycan chains and possibly also being subject to citrullination, pyroglutamination and other PTMs. Increased knowledge about the specificities of antibody (cross)reactivities with cytokine ligands have improved diagnosis and treatment of many diseases, with inflammatory processes, including cancer-associated inflammation, at the frontline.

**Key words:** cytokines, chemokines, interferons, antibody therapy, immunoassay, bioassay, proteoforms

Since SARS-CoV-2 virus infection has reached the pandemic status, vaccination has been the key solution to reduce viral spread, morbidity and mortality rates in susceptible hosts. However, accumulation of mutations in the virus genome generates amino acid substitutions affecting antigenicity and, hence recognition by antibodies elicited by the original viral S1 glycoprotein. Thus, the appearance of additional variants diminishes the success of the present vaccination strategy and this has become a major problem. Indeed, polyclonal antisera raised by vaccination with the original Wuhan corona virus strain insufficiently protect against the more infectious Omikron variant, hence reoccurrence of the disease in hosts who have seroconverted against the original virus strain. This is due to the structural differences in S1 glycoproteins between the virus variants and specific antisera in individual vaccinated hosts, which only partially cross-react [1]. This phenomenon of antibody/antiserum specificity has been used in numerous discovery studies in immunology as a tool to discriminate between various endogenous inflammatory mediators. For example, interferons are the first discovered cytokines which exert “interference” with viral infections and have been classified as different subtypes based on antibody specificity. Indeed, interferon (IFN)

produced by virally infected leukocytes is structurally distinct from that induced in fibroblasts. This was first evidenced by the observation that antisera raised against these two (partially purified) cytokines did not cross-react, in fact did not cross-neutralize, in a biological assay testing antiviral activity [2]. In particular, antisera/antibodies against fibroblast-derived IFN did not neutralize leukocyte IFN in an *in vitro* assay measuring the protective effect of IFN against viral infection of fibroblasts. Hence, despite their structural and functional similarities, these cytokines were classified as IFN- $\alpha$  and IFN- $\beta$ , derived from leukocytes and fibroblasts, respectively. Indeed, the multiple leukocyte IFN- $\alpha$  and the unique fibroblast IFN- $\beta$  genes most likely evolved by gene conversions and duplications (and are syntenic on human chromosome 9) and encode proteins with a certain degree of conservation in structural folding. Both interferon species, collectively named Type I interferons, exert their antiviral activity following binding to the same heterodimeric cellular receptor and the induction of identical intracellular signal transduction pathways [3]. However, and exactly in the same way as variable cross-neutralization of the Wuhan and Omikron strains of SARS-CoV-2 may exist with an antiserum from a vaccinated host, the affinities of single

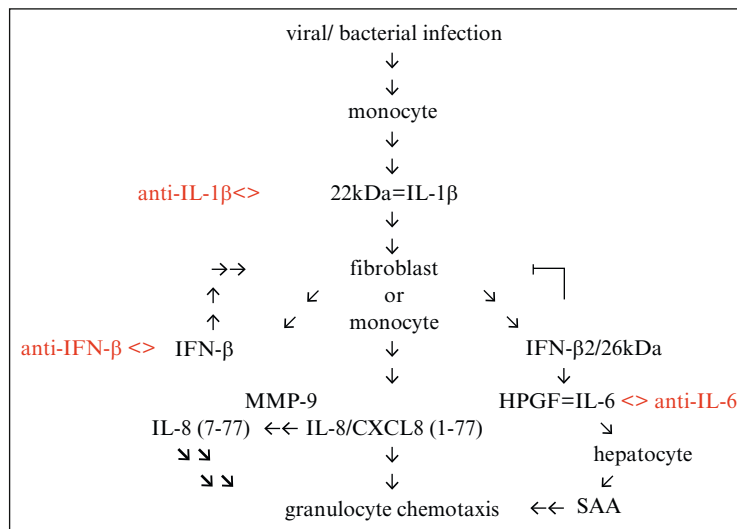
type I interferons (IFN- $\alpha$  or IFN- $\beta$ ) towards their shared receptor differ considerably [3].

Subsequently, a different type of IFN (i.e. Type II or immune IFN or IFN- $\gamma$ ), encoded by a different gene on human chromosome 11, with other receptors and with no serological relationship to the type I IFNs has been discovered [4]. Finally, interferon- $\lambda$  is the latest addition to the interferon family [5].

### Discovery of interleukin-1 $\beta$ as cytokine inducer using a non-specific antiviral assay and a specific interferon- $\beta$ antibody

Preparations of partially purified immune IFN with a molecular size of 45 kDa, produced by mitogen-stimulated leukocytes, were found to contain an additional antiviral component with a MW of 22 kDa, that showed an unexpected serological relationship with fibroblast-derived IFN- $\beta$ . In particular, its antiviral activity detected on fibroblasts infected with various animal viruses, such as Vesicular Stomatitis Virus could be neutralized by antibodies specific for IFN- $\beta$  suggesting that the 22 kDa protein might represent an IFN- $\beta$  variant. However, the latter odd 22 kDa protein showed biochemical characteristics (molecular weight, isoelectric point) that differed from those of authentic IFN- $\beta$ . In addition, antibody preparations raised against this variant

protein purified to homogeneity did not neutralize the antiviral activity of IFN- $\beta$  on virus-infected fibroblasts. The lack of serological relationship between the 22 kDa protein and IFN- $\beta$  was in contrast with the presumed neutralization of the antiviral activity of this protein by an anti-IFN- $\beta$  antibody in the same test system. As a consequence, the hypothesis was formulated that the 22 kDa protein did not represent an IFN- $\beta$  variant. Instead it turned out to be a structurally unrelated factor, which induced IFN- $\beta$  production in fibroblasts and hence, its antiviral effect was serologically undistinguishable from that of IFN- $\beta$  (*figure 1*). It must be stressed that this represented a novel insight, because at that time distinct biological functions were attributed to individual molecules and it was not commonly accepted that a given cytokine could induce the production of another one, a process now known as the cytokine cascade or cytokine network. This enigma was only resolved when the primary protein structure of the 22 kDa protein, purified to homogeneity, was identified by amino acid sequencing and subjected to comparison with human interferon sequences. By this approach, it was proven that the 22 kDa factor was not an IFN- $\beta$  variant but an at that time unidentified cytokine. Further biological characterization of the pure protein with other, yet unidentified, inflammatory mediators demonstrated that it was related to endogenous pyrogen



**Figure 1**

**Antibody-based identification of cytokines.** Last century, a large number of inflammatory mediators has been discovered. These have been classified as cytokines. At the beginning of the cytokine era, the technologies for molecular cloning and the development of monoclonal antibodies were not yet available. The classical approach at identifying new molecules consisted of protein production and purification with the help of biological *in vitro* or *in vivo* test systems. Interferons were the first cytokines to be isolated and purified to homogeneity based upon their antiviral activity. Different types of IFN were distinguished with specific polyclonal antiserum/antibody preparations, which did not cross-react in neutralizing their antiviral activity.

Unexpectedly, a 22 kDa protein with IFN-like antiviral activity could be purified from leukocytes and this biological activity was neutralizable with a specific antibody against pure fibroblast IFN (IFN- $\beta$ ), whereas an antibody against 22 kDa protein did not cross-react with IFN- $\beta$ . This dilemma was solved by the demonstration that the 22 kDa protein is an IFN- $\beta$  inducer, which could be identified as IL-1 $\beta$ . Alternatively, an antiserum against impure fibroblast IFN did cross-react with a 26 kDa protein lacking any IFN activity but was co-induced with IFN in fibroblasts by IL-1 $\beta$ . Furthermore, this 26 kDa protein was identical to a newly discovered B cell growth factor, now designated IL-6. IL-1 $\beta$  was later found to be a potent inducer of many other members of the cytokine network, including colony stimulating factors and chemotactic factors called chemokines. One prototypic chemokine for neutrophils, namely IL-8, alias CXCL8, can be processed by MMP-9 into a more potent proteoform IL-8 (7-77). Finally, SAA, induced by IL-6, synergizes with IL-8/CXCL8 in granulocyte chemotaxis. Arrows indicate induction, processing capacity, chemoattractant or antiviral activity; < > indicates neutralizing capacity of the polyclonal antibody in the biological assay; T indicates lack of activity; bold black arrows indicate increased biological activity. HPGF: hybridoma plasmacytoma growth factor; IFN: interferon; IL: interleukin; MMP: matrix metalloproteinase; SAA: serum amyloid A;

and leukocyte endogenous mediators. Finally, it was demonstrated that the latter biological activities and those of the 22 kDa protein did reside in the same molecule, designated interleukin-1 $\beta$  (IL-1 $\beta$ ), which had been historically named endogenous pyrogen sixty years earlier [6-8].

#### Identification of cytokine-induced interleukin-6 with a non-specific interferon- $\beta$ antibody and a specific B cell growth assay

During the attempts to clone human IFN- $\beta$  cDNA, it was found that virally-induced fibroblasts expressed, aside IFN- $\beta$ , an unidentified 26 kDa protein (*figure 1*). This 26 kDa protein was recognized by polyclonal antiserum against partially purified IFN- $\beta$ . Recombinant expression of 26 kDa cDNA and production of 26 kDa protein in Chinese hamster ovary cells yielded in one laboratory a molecule with antiviral activity. It was therefore designated IFN- $\beta$ 2, as a subtype of authentic IFN- $\beta$  [9]. However, in another laboratory no antiviral activity was detected with this protein [10] and the dispute about the biological function of 26 kDa remained unresolved for a considerable period of time. Only when it was found that the IFN- $\beta$ -inducing IL-1 $\beta$  (*vide supra*) was a potent co-inducer of 26 kDa protein [11], the problem was resolved. The so-called antiviral activity of the 26 kDa protein was caused by co-induction of IFN- $\beta$ , contaminating the misnamed IFN- $\beta$ 2 preparations. The antiviral activities of IL-1 $\beta$  and also of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), being inducers of both IFN- $\beta$  and the 26 kDa protein, are therefore not caused by the 26 kDa molecule but instead fully explained by IFN- $\beta$  production [12].

Independently from this line of investigation on inflammatory cytokines, innate immunity research at that time in the 1980's was focused on the characterization of growth factors for B lymphocytes, such as fibroblast- and endothelium-derived, IL-1 $\beta$ -inducible factors [13, 14]. For example, the originally characterized B cell stimulatory factor-2 (BSF-2) [15] turned out to be the same protein as the B cell hybridoma/plasmacytoma growth factor (HPGF) [16]. Even more surprisingly, the amino acid sequence of these B cell growth and differentiation factors were identical to that of the previously identified IFN- $\beta$ 2/26 kDa protein. In addition, no IFN-like antiviral activity was ascribed to pure natural HPGF. In contrast, a preparation of recombinant IFN- $\beta$ 2 showed only weak antiviral activity, whereas it boosted HPGF activity. It became evident that the IFN- $\beta$ 2 effect [9] was an artefact, possibly due to contaminating hamster IFN from the producer cells [17]. Since this novel B cell growth/differentiation factor was also found to be a potent inducer of acute phase proteins [18], it was designated by the International Cytokine Nomenclature Commission as interleukin-6.

#### Characterization of interleukin-8 as a chemokine induced by interleukin-1 $\beta$ using *in vivo* inflammatory test systems

One of the many biological activities attributed to IL-1 $\beta$  was chemotactic activity [19]. However, 22 kDa factor/IL-1 $\beta$ , purified from stimulated peripheral blood

mononuclear cells, failed to show *in vitro* chemotactic activity for granulocytes, whereas it did cause skin reactivity and neutrophil infiltration *in vivo*. This apparent contradiction was solved by serendipity when testing all chromatography fractions obtained after the final purification step of natural 22 kDa factor/IL-1 $\beta$ . Indeed, unexpected findings were observed: not the IL-1 $\beta$ -containing, but a distinct chromatography column fraction provoked an early skin reaction (within a few hours), whereas IL-1 $\beta$  caused a late reactivity (after one day). The fraction with early reactivity was shown to contain a single 7 kDa protein also chemoattracting neutrophils *in vitro*. This allowed for two important discoveries. First, the 7 kDa protein could be identified as a novel cytokine, i.e. IL-8 [20]. Second, IL-1 $\beta$  is inducing IL-8 *in vivo* (which explains its late skin reactivity compared to the rapid direct effect of IL-8), whereas IL-1 $\beta$  is not able to effectively induce IL-8 in the short (30 min) *in vitro* chemotaxis assay on granulocytes. Similarly, IL-8 was found to cause rapid granulocytosis upon intravenous injection, preceding granulocyte mobilization into the blood circulation induced by IL-1 $\beta$ . The IL-8-inducing capacity of IL-1 $\beta$  was confirmed *in vitro* on endothelial cells and fibroblasts [21, 22]. By using an antibody raised against fibroblast-derived IL-8, it was shown that leukocyte-derived IL-8 was the same gene product as that from fibroblasts, unlike fibroblast IFN and leukocyte IFN [22].

#### Interludium: risks and benefits in getting scientifically caught in the cytokine network

It may be concluded from the previous sections that neutralizing antibodies/antisera have been key reagents in the identification process of many novel endogenous mediators of inflammation. Specific antisera against IFN- $\beta$  allowed for the discovery of IL-1 $\beta$ , whereas polyclonal antisera against IFN- $\beta$  led to the identification of IL-6. However, for the discovery of IL-1 $\beta$ , it took about 5 years of intensive research with many control experiments and progressive and foremost critical thinking to shift from one hypothesis to another to reach the scientific endpoint: from 22 kDa protein, to IFN- $\beta$ , to IFN- $\beta$  variant, to IFN- $\beta$  inducer, to the new protein sequence of endogenous pyrogen, alias IL-1 $\beta$ . At each step, the then young scientists, involved in the practical experimentation, were confronted with critical evaluations of their data by senior supervisors, who claimed that, based on their own experience, the whole project and data sets might be built on an artefact. To complement the situation historically, a decade of critical investigation and a considerable number of publications in international scientific journals were necessary to evidence that IFN- $\beta$ 2 was a real artefact for IL-6. Nevertheless, in both cases the research was executed in established laboratories by respected scientists and supervisors. Although the experimental work was solid, it sometimes lacked full quality control and experimental settings to exclude contaminants. Other aspects, that remain actual today with the publications of claimed novel biological activities, was the absence of pure material and quality control thereof. These "stories" are remindful of a reverberant public speech given in 1985 by a microbiologist, founder of the Rega Institute and

university rector Piet De Somer in front of Pope Johannes Paulus II visiting KU Leuven. De Somer spoke out that scientists need to receive the freedom of making errors in order to do their experimental work with the guarantees for all possibilities of their hypothetical thinking. Even until today, scientific errors based on misinterpretation of obtained results still occur. Often, this is due to the use of poorly quality controlled reagents made commercially available. For example, some inflammatory properties ascribed to the acute phase protein serum amyloid A (SAA), induced by IL-1 $\beta$  or IL-6, were in fact mediated by contamination of commercial SAA with bacterial products including lipoproteins and lipopolysaccharides (LPS) [23, 24]. The exclusion of contamination of protein preparations with the exogenous pyrogen LPS were indeed for about sixty years a hurdle in the identification of the endogenous pyrogen IL-1 $\beta$  [19]. The unique situation that SAA should activate cells via both Toll-like receptors (TLRs) and G protein-coupled receptors (GPCRs) needs therefore to be confirmed with pure reagents. Finally, young investigators should be constantly made aware of the LPS problem and must carefully control the quality of all the reagents used for experimental work. Moreover, all scientists should remain critical about their proper results as well as those from others published in international journals, even those with a decent review system.

#### **Polyclonal versus monoclonal antibodies in detecting cytokine bioactivity versus immunoreactivity**

Above we consciously referred to antisera/antibodies to draw the attention on the fact that, in the past, serological reagents were polyclonal antisera. These antisera were crucial for the purification and identification of new mediators. A complementary situation was evident by the identification of a whole class of diseases on the basis of purified (glyco)proteins, namely autoimmune diseases. The historical identification of Hashimoto disease by Ivan Roitt and colleagues as the first proven autoimmune disease caused by antibodies against thyroglobulin was with polyclonal antisera from patients and purified thyroglobulin [25, 26]. The use of antisera from patients to define new autoimmune diseases continues till today with the finding of type I interferon autoantibodies in a considerable fraction of patients suffering from severe SARS-CoV-2 infection [27, 28]

An antiserum raised against a pure glycoprotein is per definition polyclonal: it reacts with many different epitopes of the used antigen. Like with the spike protein of SARS-CoV-2, cross-reactivity with (similar) molecules sharing epitope structures, may be a problem and cause misinterpretations. It does not take much imagination to critically appraise scientific data when such antisera were raised against contaminated (impure) glycoproteins. For these and other reasons, the development of technologies to generate and mass-manufacture monoclonal antibodies was a blessing for biomedical research [29].

At a further stage, the generation of monoclonal antibodies is essential for the development of specific immunoassays for individual cytokines. These tests are not just complementary but also more specific than

biological assays, which do not always discriminate between distinct cytokines as it was the case for IFN- $\beta$  and IL-1 $\beta$  in the antiviral assay (*figure 1*). This problem became most obvious upon the discovery of IL-8 as a member of a large family of structurally related chemotactic cytokines, designated chemokines [20, 30-32]. Originally, much disbelief circulated among reviewing immunologists about the existence of endogenous chemotactic factors, because the existing, erroneous, dogma was that endogenous mediators of the complement system (C3a and C5a) amply explained endogenous chemotaxis. Anyhow, with pure IL-8 and with recombinant protein by expression of its freshly cloned cDNA at hand it was indisputably proven that, aside C3a and C5a, additional endogenous chemotactic proteins exist. Later it was established that chemokines active on neutrophils have a conserved CXC motif and, hence, were renamed CXC ligands (CXCL). In this new nomenclature, IL-8 has been renamed CXCL8. Some chemokines individually activate cells via several distinct GPCRs, whereas a single GPCR can be recognized by several structurally different chemokines, rendering the chemokine network rather complex. Nevertheless, each chemokine has a unique pattern of biological activities, indicating that each family member exerts a proper role in inflammatory and other diseases [33, 34]. Nowadays, selective and simultaneous detection of multiple chemokines/cytokines in body samples of patients can be done with the use of commercially available multiplex immunoassay kits based upon monoclonal antibodies. However, such advanced cytokine/chemokine detection system still suffers from the fact that only immunoreactivity (of specific epitopes) and no biological activity (of an intact molecule interacting with receptors) is measured. This makes the measured levels of immunoreactivity in patients less relevant in relation to clinical situations. Indeed, chemokines are post-translationally modified, e.g. by proteolytic cleavage or by citrullination. Whereas these modifications may have drastic effects on the biological activity of these molecules [35], their individual recognition by monoclonal antibodies remains unchanged [36].

#### **Immunoassays do not discriminate between biological activity of intact and posttranslationally modified chemokines**

Selective removal of a few amino acids from intact chemokines due to proteolytic cleavage can render a chemokine either more active or lead to a loss of activity and even convert it into a chemokine receptor antagonist. For example, matrix metalloprotease-9 (MMP-9) cleaves off only six NH<sub>2</sub>-terminal amino acids from IL-8/CXCL8 in front of its structurally important CXC motif (*figures 1 and 2*). Nevertheless, this type of posttranslational modification, however small, results in a ten-fold increase in receptor affinity and biological activity and has therefore been named proteolytic potentiation [37]. It needs to be noticed that these drastic alterations of biological activities occur without any change in recognition by monoclonal antibodies against shared epitopes in the various

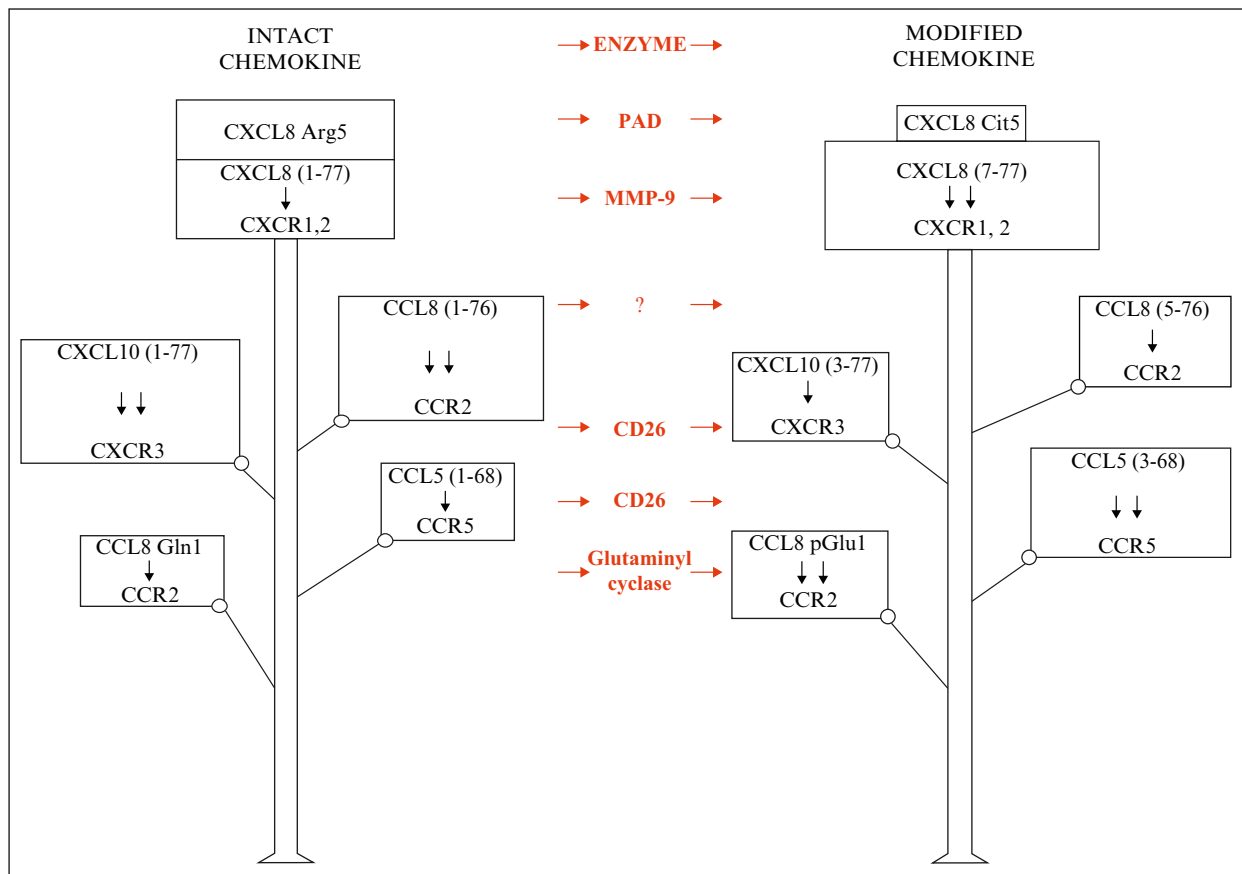


Figure 2

**Posttranslational modifications altering biological effects of chemokines without affecting their immunoreactivity.** Upon secretion by their producer cells (monocytes, fibroblasts, endothelial cells,...) chemokines become sensitive to extracellular membrane associated (CD26) or soluble (MMP-9) proteases which are also upregulated during the inflammatory response. The chemokine family represents a large number of structurally related chemotactic cytokines merely classified as CXC or CC ligands, depending on the positioning of conserved cysteine residues in their NH<sub>2</sub>-terminal region. Dipeptidylpeptidase IV/CD26 cleaves off two aminoterminal residues from chemokines without any effect on their recognition by monoclonal antibodies used in standard immunoassays but with drastic consequences for their biological activities. Depending on the chemokine, such minor modification can decrease or enhance its receptor (CXCR, CCR) affinity and hence its intracellular signaling capacity and chemotactic potency.

The left tree shows branches with distinct authentic chemokines and their corresponding receptors, the tree on the right side illustrates their posttranslationally variant forms modified by enzymes indicated in the middle. For example, the lymphocyte chemoattractant CXCL10(1-77) is converted by CD26 into CXCL10(3-77) which shows impaired binding and signaling through its receptor CXCR3 thereby lacking chemotactic activity (indicated in the figure by reduction in size of the corresponding box and by the lower number of arrows pointing to the receptor). Similarly, the monocyte chemoattractant CCL8(1-76) is NH<sub>2</sub>-terminally cleaved (? means protease unknown) into CCL8(5-76) converting this chemokine into a CCR2 antagonist. In contrast, CCL5(1-68) is processed by CD26 to become a better CCR5 agonist with more antiviral activity against HIV. Cleavage of CXCL8(1-77) by MMP-9 into CXCL8(7-77) results in enhanced receptor affinity and neutrophil chemotactic potency, whereas citrullination of CXCL8 yields reduced tissue inflammation *in vivo*. Finally, pyroglutamination of the NH<sub>2</sub>-terminal residue of CCL2 is necessary in order to exert monocyte chemotactic activity. Since present-day immunoassays for these chemokines do not discriminate between intact and all the NH<sub>2</sub>-terminally processed forms, chemokine quantification in body fluids by ELISA is not representing the real inflammatory status in clinical samples from patients and in preclinical studies with animal models of diseases. CCL: CC chemokine ligand; CCR: CC chemokine receptor; CXCL: CXC chemokine ligand; CXCR: CXC chemokine receptor; DPPIV: dipeptidylpeptidase IV; MMP: matrix metalloproteinase; PAD: protein arginine deiminase;

proteoforms. As another example with a completely different biological effect, soluble or membrane-bound CD26/dipeptidylpeptidase IV removes the two penultimate residues from the CXCR3 agonistic chemokines CXCL9, CXCL10, CXCL11 (figure 2) and this proteolytic event results in a nearly complete loss in receptor recognition and therefore also in lack of lymphocyte chemotactic activity of these chemokines [38]. Again, such minimal posttranslational modification does not influence the immunoreactivity of the truncated chemokines with most monoclonal antibodies. Furthermore, minimal NH<sub>2</sub>-terminal truncation of the monocyte chemotactic protein-2 (MCP-2/CCL8) can convert this chemokine into an inhibitor [39]. Indeed,

by still binding to, but no longer signaling through its GPCR, an altered chemokine may block a receptor and prevent biological activity of the intact molecule, as well as other chemokines signaling through the same blocked receptor [40, 41]. However, truncation by a different molecule such as CD26, a proteinase expressed on the surface of most cell types, can render a chemokine also more active, as is the case for CCL5 (figure 2), which obtains higher affinity for its receptor CCR5, resulting in enhanced lymphocyte chemotactic activity [35, 36]. Finally, even the chemical or enzymatic modification of a single amino acid, e.g. by citrullination [42], pyroglutamination [43] or glycosylation may have significant impact on chemokine activity [36]. As

a consequence, the amount of detected chemokine immunoreactivity with monoclonal antibodies in body fluids does not necessarily reflect the biological status of a patient. Recently, a new method – immunosorbent tandem mass spectrometry proteoform analysis/ISTAMPA – based on the combined use of specific antibodies and mass spectrometry has been developed, allowing to quantitatively discriminate between different post-translationally modified forms of a single chemokine in body fluids [44].

### **The use of cytokines and monoclonal antibodies against cytokines in inflammatory diseases**

The discovery of beneficial endogenous immune-modulating factors raised great hopes that these would become useful for therapy. Interferons were the first cytokines clinically tested to protect against viral infection and to treat cancer, but with less success than expected. Fortunately for patients with multiple sclerosis, the anti-inflammatory properties of type I interferons were successfully exploited and initiated the hope for other forms of immunotherapies. As mentioned above, possible pre-existing or induced neutralizing antibodies need to be carefully monitored as preceding autoimmune reactions or prior immunization with recombinant cytokines might prevent therapeutic responses to cytokine treatments [27, 28, 45].

Certain pro-inflammatory cytokines, such as IL-1 $\beta$  and TNF- $\alpha$  have disease-promoting effects. Therefore, blocking or neutralization of their pathogenic activity by specific monoclonal antibodies was considered an option for the treatment of inflammatory diseases such as rheumatoid arthritis (RA). This reasoning has been met with much success [46]. The use of anti-TNF- $\alpha$  monoclonal antibodies has not only transformed the treatment of patients with various forms of arthritis [47], it also became standard therapy for those with inflammatory bowel disease (IBD) [48]. For patients who are refractory to anti-TNF- $\alpha$  therapy, new drugs are being developed and attempts are made to target other pro-inflammatory cytokines or their subunits, in particular IL-17 and IL-23 [48]. In particular, patients with psoriasis have benefited from the development of monoclonal antibodies against IL-17. For this cytokine too, individual patient refractoriness and side-effects of this novel biological treatment are noticed and need to be addressed [49]. Notably, additional cytokine-inhibiting therapies, including the IL-1 antagonist Anakinra and anti-IL-6 receptor monoclonal antibodies developed to treat patients with RA and IBD, respectively, now also have been proven to efficiently control the cytokine storm syndrome that critically ill SARS-CoV-2 patients experience [50-53]. So far, cytokine and cytokine-directed monoclonal antibody therapies are parenteral and are supposed to act systemically. Above, we mentioned that IFN- $\beta$  may be neutralized by (auto)antibodies in a fraction of COVID-19 patients. To overcome loss of IFN- $\beta$  bioactivity by such neutralizing antibodies, nebulized IFN- $\beta$  has been administered through the oropharynx after SARS-CoV-2 infection and this treatment prevented the development of severe disease [54].

Two critical conclusions may be formulated. First, the discovery of new cytokines and their biology, together with the tools of monoclonal antibody technology, has led to major therapeutic advances for the benefit of patients with various infectious and inflammatory diseases. Second, it must be noted that pro- and anti-inflammatory cytokines protect a host against microorganisms and may have homeostatic and restorative functions. Therefore, the use of neutralizing antibodies against pro-inflammatory cytokines and also parenteral administration of recombinant cytokines come at the expense of diminished/altered antimicrobial defense and may also lead to severe side-effects in susceptible patients [46].

### **Antibodies against cytokines: the good, the bad and the ugly**

It is evident that clinical application of properly humanized monoclonal antibodies against inflammatory cytokines is providing a “good” medical tool to combat autoimmune and inflammatory diseases, including RA, IBD and psoriasis. Alternatively, monoclonal antibodies recognizing a cytokine constitute good reagents for specific immunoassays allowing to detect levels of cytokine proteoform mixtures in various body fluids from patients. However, sometimes the “ugly” limitations need to be considered (*figure 3*). Indeed, immunological tests do not measure biological activity and do not discriminate between minor posttranslational modifications of individual cytokines which may have a drastic impact on their biological activity. The latter can only be determined in often complex and less reproducible *in vitro* or *in vivo* bioassays with the “bad” connotation that these tests are not specific for a single cytokine. However, bioassays become more relevant if used in combination with neutralizing (monoclonal) antibodies against the specific cytokine under investigation. The combination of an ugly non-specific antiviral bioassay and a good specific antibody against IFN- $\beta$ , allowed for the discovery of IL-1 $\beta$ . Alternatively, even the usage of a non-specific and bad antibody, recognizing an IFN- $\beta$ -contaminating 26 kDa protein, led to the identification of IL-6. In addition, the usage of complex *in vivo* bioassays were key tests in discovering IL-8. However, a major drawback in using biological rather than immunological tests is the interference of exogenous inflammatory mediators such as bacterial LPS. As a consequence, the most dangerously error-prone reagents are those that are still commercially sold without good quality control, e.g. for the absence of contaminants, such as LPS.

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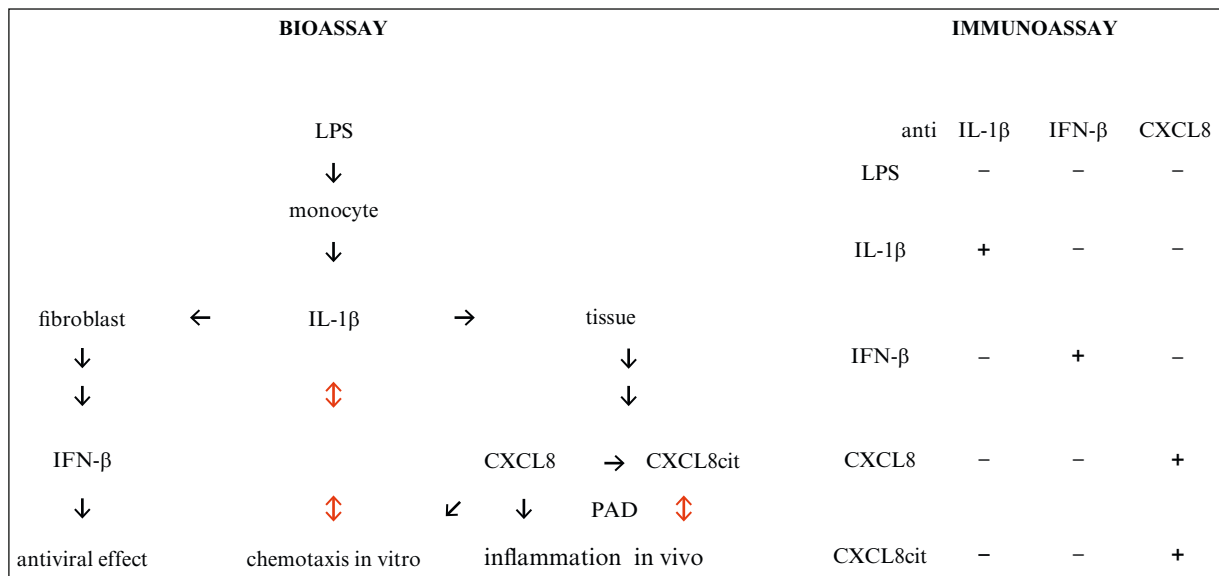


Figure 3

**Endotoxins mimic the cytokine network in bioassays, but not in immunotests.** Bioassays are attractive because they detect the biological outcome of a cytokine *in vitro* or *in vivo*. However, biologically testing of an impure cytokine preparation is a burden due to interference of endogenous (other cytokines) or exogenous (LPS) contaminants. Indeed, LPS can mimic IL-1 $\beta$  or chemokines by inducing these cytokines *in vivo* but also in the test cells during an *in vitro* bioassay. Test samples must therefore not only be endotoxin-free throughout, but should also be purified to contain only one particular cytokine. However, even in this optimal situation, a single cytokine (IL-1 $\beta$ ) can interfere biologically (chemotaxis) with another (CXCL8) *in vivo* activity by inducing the latter during the biotest, whereas this is not the case with a short (45 min) *in vitro* chemotaxis assay using a single cell type (neutrophils). Nevertheless, IL-1 $\beta$  can exert antiviral activity via IFN- $\beta$  induction on fibroblasts during the 24h lasting *in vitro* bioassay. Finally, one should take into account that the cytokine under test can interact with a contaminating or endogenously induced cytokine in a synergistic or antagonistic way. In any case, it is crucial to test the cytokine in the bioassay in the presence of specific neutralizing antibodies against the molecules involved. Such strategy has led to the discovery of IL-1 $\beta$  and CXCL8.

Immunoassays based on monoclonal antibodies have the major benefit that they can measure single cytokines easily in a quantitative way and are therefore used predominantly to detect cytokine levels in body fluids from patients. There is certainly no interference with LPS contamination and even structurally related cytokines can easily be distinguished. However, immunotests do not discriminate between protein variants derived from the same gene which are minimally different. Indeed, minor posttranslational modifications have a major impact on chemokine activity (figure 2). For example, when the neutrophil chemoattractant CXCL8 becomes citrullinated, it fails to recruit cells after intraperitoneal injection unlike intact CXCL8. The fact that immunoassays do not take into account biological activity is a major disadvantage of such assays.

+ indicates immunoreactivity; - indicates no immunoreactivity; black arrows indicate induction or activity;  $\updownarrow$  indicates no activity. IL: interleukin; IFN: interferon; LPS: lipopolysaccharide; PAD: protein arginine deiminase.

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