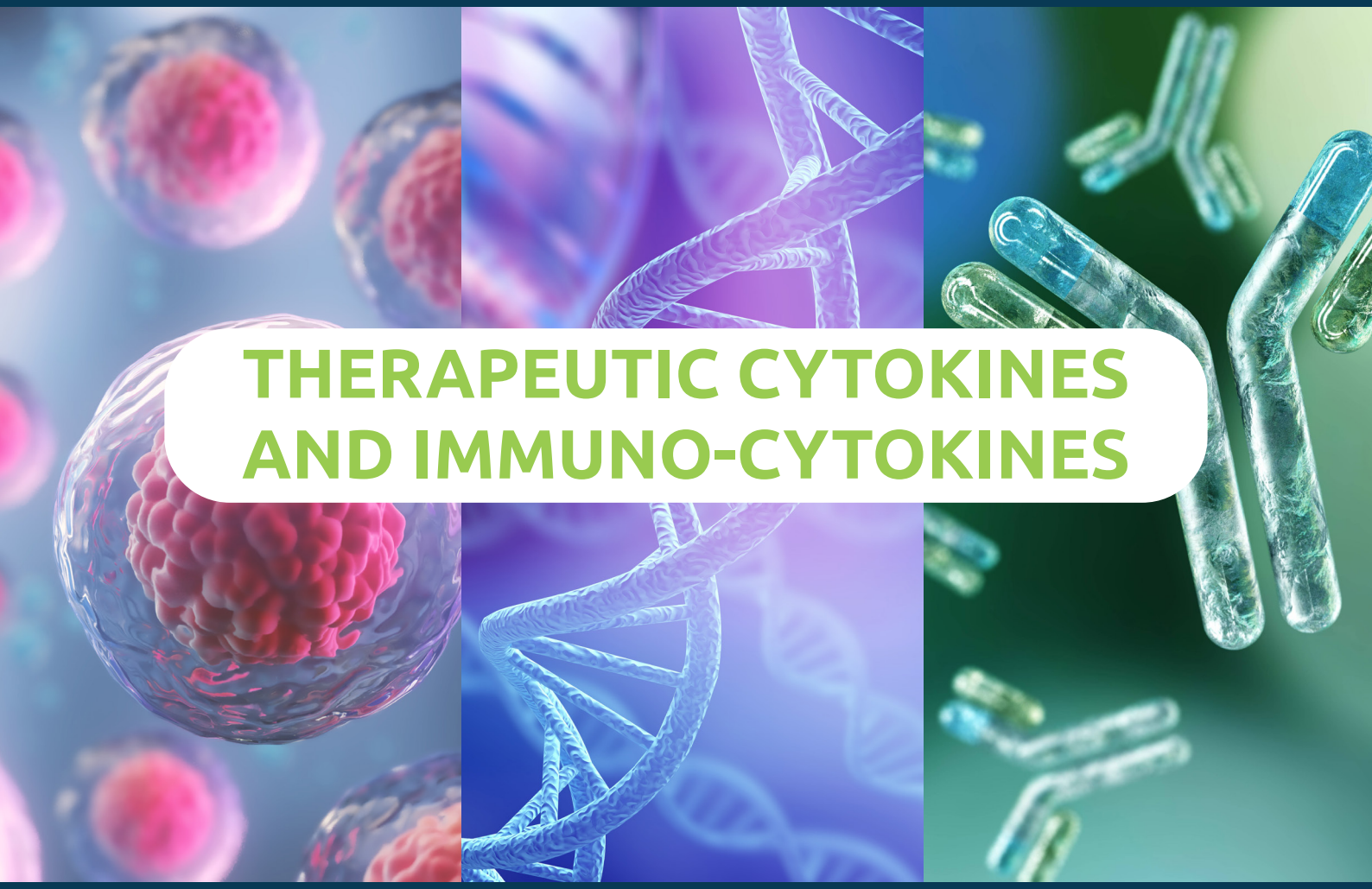
IMMUNOWATCH

EDITION N°8 - 2023



THERAPEUTIC CYTOKINES AND IMMUNO-CYTOKINES

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INTRODUCTION

MabDesign's Immunowatch is a one-of-a-kind information monitoring newsletter in the field of biologics. Its aim is to provide members of our association with the most recent and pertinent data gathered or generated through the key expertise of MabDesign and its collaborators in scientific research, business intelligence, market analysis and intellectual property.

Each edition will focus on trending type of biologics. Its general format includes market study research, financial and economic data, invited contributions from scientific teams working in the industry or in academia and a section dedicated to intellectual property. The content of each edition is decided by an editorial composed of two field experts. Decision concerning the theme and conception of each newsletter is done in-house by the permanent members of our editorial team.

Finally, we would like to acknowledge the support of the Ambition Recherche & Développement (ARD) Biomédicaments 2020 Phase II programme, funded by the Centre Val de Loire region during the initial phases of launching this newsletter.

BIOPHARMACEUTICALS
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Editorial

Nicolas Poirier
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Université de Nantes

Boosting the immune system against tumor cells remains a crucial focus of pharmaceutical research. The multifaced activity of cytokines and their capacity to promote effector function, proliferation and or survival of T cells raise considerable interest in explorating of cytokine-based therapies. Over the past years, a myriad of engineered cytokines have been generated and evaluated in multiple clinical trials attesting to the major enthusiasm and hopes for this new class of therapy for the treatment of cancer or inflammatory diseases.

Several cytokines have been developed with different biological properties, such as IL-2, IL-15, IL-7, and others cytokines used to boost T-lymphocyte activities. For instance, IL-15 plays a crucial role in stimulating the proliferation of effector T cells and NK cells, supporting the differentiation of cytolytic effector cells. While IL-2 exhibits similar anti-tumor activity by activating T cell and NK cell populations, it also induces the proliferation of regulatory immunosuppressive T cells, unlike IL-7, which preferentially expands effectors.

Novel augmented cytokine approaches have been developed to enhance specific activity and increase therapeutic index and efficacy, for example by redirecting cytokine activity on PD-1 + activated T cell population in the tumor microenvironment. Fusion of an anti PD-1 to an attenuated cytokine allows specific cis-delivery of the cytokine and cis-potentiation of the appropriate tumor reactive PD-1+ CD8 T cell population, thus improving anti-tumor efficacy compared to a non-targeted cytokine agent or targeting the tumor antigens to activate immune cells locally in-trans. Cytokine on-demand with, for example, tumor-activable immunocytokine using a protease specific linker between the targeted antibody and the cytokine has been generated and explored now in clinical trials for specific proteolytic release and biological activity of the cytokine into the tumor microenvironment enriched in the selected protease while sparing cytokine activity in non-specific peripheral tissues. Altogether, the cytokine-based drug modality represents a powerful and pleiotropic class of drugs which could be engineered to increase their therapeutic index by increasing activity in the right place and/or right cells while decreasing off-tumor activity associated with toxicity.

This issue of immunowatch reviews some challenges and recent advances of engineered cytokine-based strategies for future clinical application in cancer immunotherapy.

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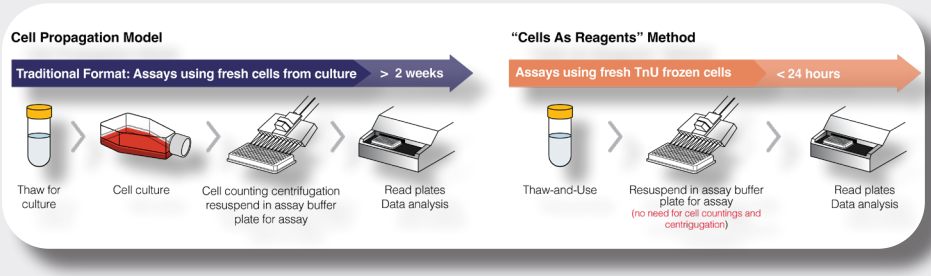

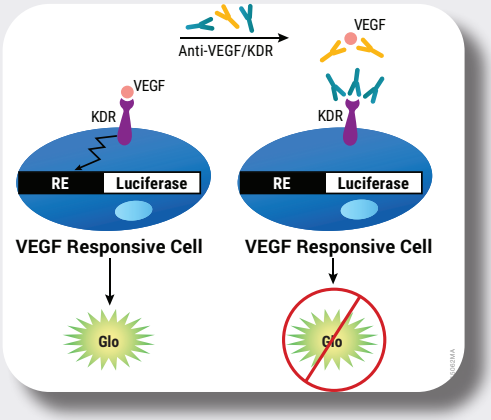

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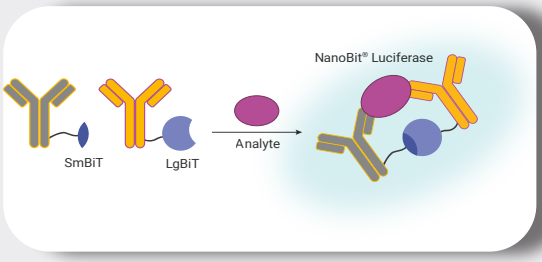

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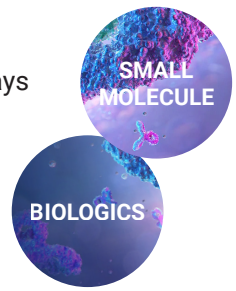

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THERAPEUTIC CYTOKINES OVERVIEW

Discover an introduction by MabDesign about therapeutic cytokines

INTRODUCTION

By MabDesign

Cytokines are soluble, low molecular weight proteins that mediate cell-to-cell communication. Cytokines are produced by a broad range of cells and act as immunomodulators in autocrine, paracrine, or endocrine signaling pathways (Figure 1). Key classes of cytokines involved in cellular communication encompass interleukins (ILs), interferons (IFNs), specific members of the tumor necrosis factor (TNF) superfamily, and various other effector molecules.

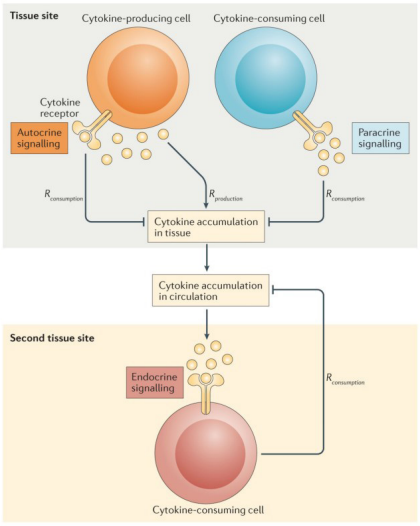


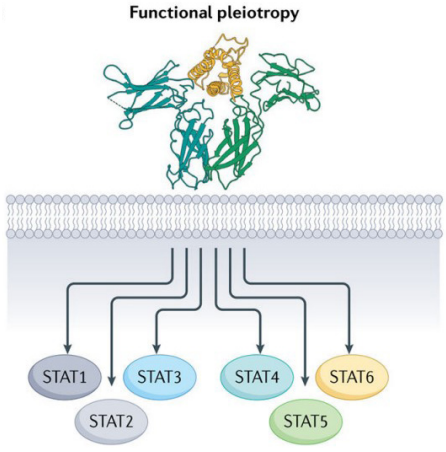
Figure 1: *Three modes of cytokine-mediated cell-to-cell communication*
Cells of the immune system communicate through the exchange of secreted cytokines. Depending on the spatial location and identity of the cytokine-consuming cell, such communication can be autocrine (signaling to self), paracrine (signaling to neighboring cells) or endocrine (signaling globally through the circulation). The key parameters that determine the signaling mode are the rate of cytokine production (Rproduction) and the rate of cytokine consumption (Rconsumption). Recent quantitative studies in systems immunology have begun to clarify how cells switch from one mode of signaling to another.

Adapted from Altan-Bonnet and Mukherjee, Nat Rev Immunol 2019 April

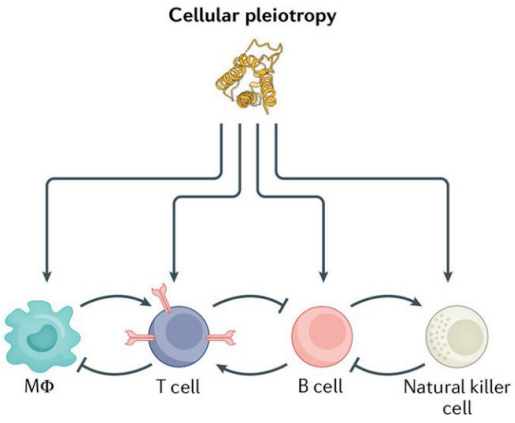
Cytokines regulate immune function by enhancing the cellular immune response or the antibody response. Their ability to have various pro or anti-inflammatory effects on numerous cell types within both the immune system and other tissues allow them to be a key actor in many biological functions, by maintaining physiological homeostasis or modulating pathophysiological processes. These pleiotropic and versatile effects of cytokines (Figure 2) have contributed to the complexity of developing cytokine-based therapeutics, notably in oncology, autoimmune, and infectious diseases.

Figure 2: *Mechanism of cytokine signaling and sources of pleiotropy.*
Many cytokines exhibit functional and/or cellular pleiotropy due to the activation of multiple downstream transcription factors, or due to their ability to signal on diverse cell types, such as macrophages, T cells, B cells or natural killer cells.

Adapted from Saxton et al Nat Rev Drug Discov. June 2023



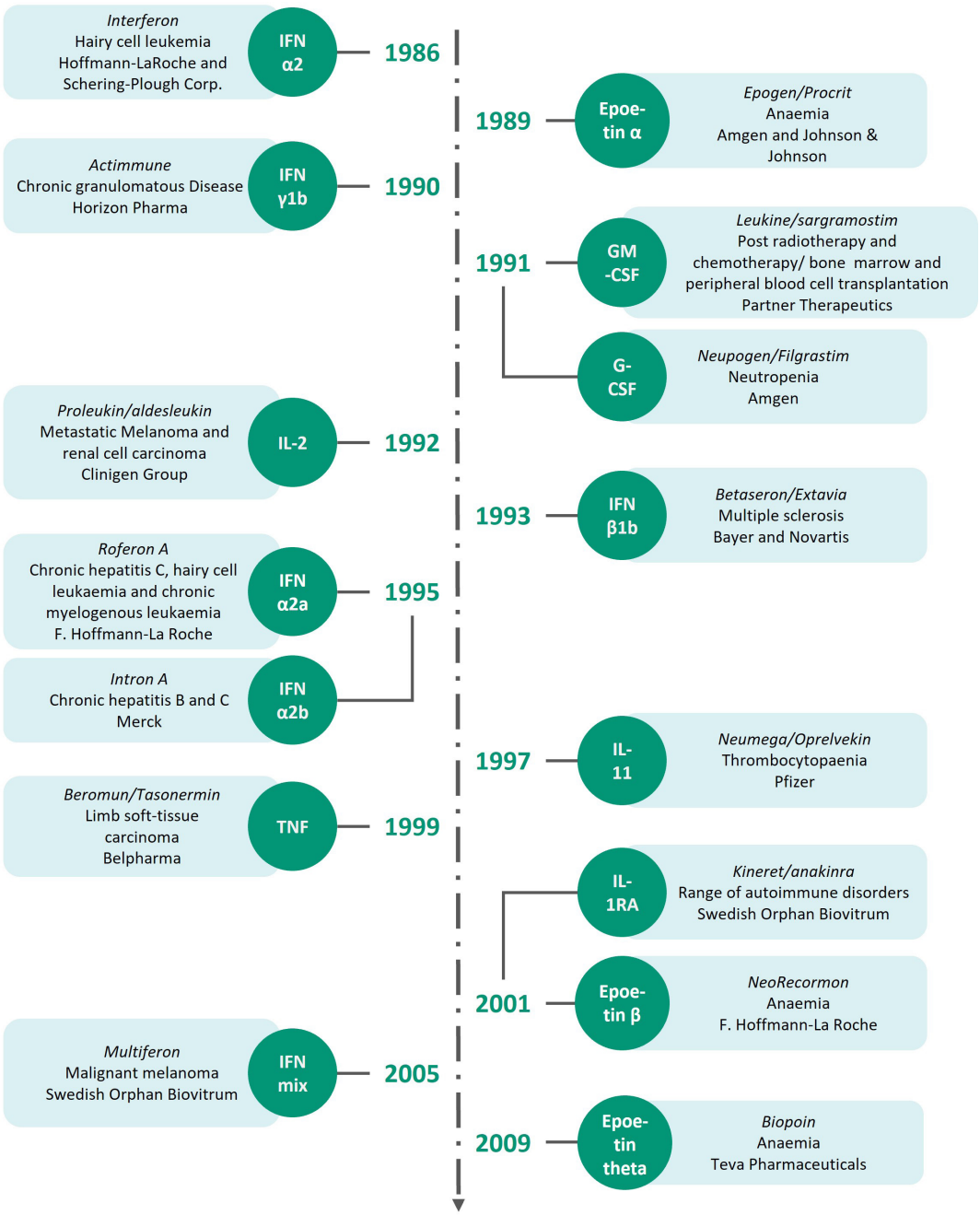
Functional pleiotropy arises from the ability of one molecule to activate multiple signaling pathways, and thereby induce multiple functional responses on a target cell type



Cellular pleiotropy arises from the ability of one molecule to stimulate multiple distinct cell types or tissues

Main successful cytokine-based therapies

The main success in cytokine therapeutics field are represented below on a chronological timeline by first approval date and by cytokine.



First approval date (FDA or EMEA) by cytokine

Adapted from Deckers et al, Nature reviews bioengineering April 2023

Despite these notable successes, many projects have encountered setbacks in clinical development, due to limited clinical benefits, toxicity issues and adverse events. Theses projects concerned specifically the following cytokines: IL-4, IL-6, IL-10, IL-12, IL-15, IL-21 and TRAIL, in various indications within oncology and auto-immune diseases. These difficulties arise from various factors, including the inherent complexities and specificities of cytokines : short half-life in the circulatory system, low biodistribution, pathway redundancy, narrow therapeutic window, prohibitive toxicities, immunosuppressive effects, and context-dependent/pleiotropic effects.

Challenges and new strategies

Over the past five years, a notable resurgence of interest has emerged in both academia and biotech to tackle the cytokine challenges and unlock their full therapeutic potential. Novel approaches are rapidly evolving to modulate cytokine-receptor affinity, extend the cytokine half-life (bioavailability), modulate cytokine biodistribution or initiate cytokine activity on demand. The main strategies developed to reach these objectives are described below, with a few examples of clinical drugs currently in development.

Modulate cytokine-receptor affinity and activity

- **Cytokine engineering**

One of the first engineering strategies used to lessen the pleiotropic effects of cytokines was locus mutation. Different versions of mutated cytokines have been developed, to either increase or decrease cell receptors binding affinity, leading to a more selective effect of the engineered cytokine (Figure 3). Theses protein mutants can be designed by direct evolution, or by rational design. Rational design relies on detailed knowledge of cytokine structure and 3D protein modelling.

Recently, de novo protein design based on computational tools, data driven methods and AI allows to develop customized cytokines. For example, it can be a cytokine agonist with the same function but a completely different sequence, or a synthetic cytokine (synthekine) that engages unnatural receptor domain pairs and therefore activates distinct signaling pathways.

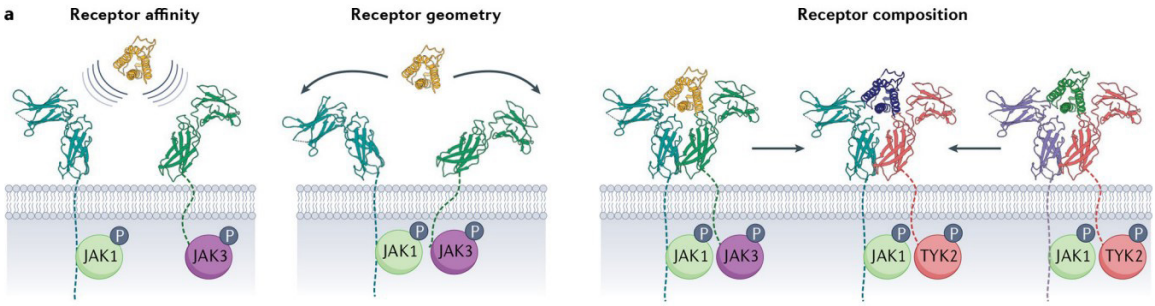


Figure 3: *Model highlighting the three parameters of cytokine receptor signaling that can be controlled through cytokine engineering to influence cytokine function : receptor affinity (a), receptor geometry/orientation (b) and receptor subunit composition (c).*

Adapted from Saxton et al Nat Rev Drug Discov. June 2023

Some example of on-going clinical cytokines engineered therapeutics are available below in table 1.

Name	Engineered approches	Mechanism of action	Tumor type	Clinical Stages	Institutions/ references
KY1043	Immunocytokine; Locus mutation and fused with PD-L1 blocking antibody	Tumour-targeted and selective activation of T lymphocytes	Solid tumours	IND application in 2021	Kymab
Nemvaleukin alfa (ALKS 4230)	Fusion IL-2Ra domain; Circularly permuted IL-2v–IL2Ra fusion protein	Selectivity for NK cells	Solid tumours	Phase III	Alkermes plc
NL-201 (Neo-2/15)	Agonist of the IL2Ra-independent IL-2/IL-15 receptors; computationally designed	Selectively stimulated expansion and tumour infiltration of cytotoxic NK CD8+ T lymphocytes	R/R cancer	Phase I	Neoleukin Therapeutics

Table 1: *Examples of on-going clinical cytokines engineered therapeutics*

- **Altered receptor geometry**

Another way to alter and modify cytokine function is by controlling receptor geometry. Using engineered receptor binding proteins as surrogate ligands, agonists that engage cytokine receptors in distinct geometries have been generated (figure 4). By modulating cytokine receptor topology, one can significantly alter downstream signaling and functional responses, opening an entirely new avenue for the pharmacological control of cytokine function.

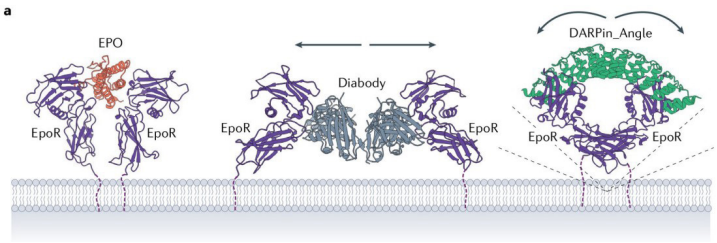


Figure 4: *Crystal structures of the erythropoietin (EPO) receptor (EpoR) in complex with EPO [PDB:1CN4] (left), a diabody [PDB:4Y5Y] (middle) and a homodimeric DARPin [PDB:6MOI] (right)*

Adapted from Saxton et al Nat Rev Drug Discov. June 2023

Different approaches of engineered receptor binding proteins

- Diabody is a novel antibody developed using recombinant DNA technology, consisting of two variable heavy (VH) and two variable light (VL) domains. Each VL domain in a single-chain variable fragment (scFv) is connected with a VH domain via a short linker, resulting in a diabody with two antigen-binding sites pointing in opposite directions.
- DARPins, short for designed ankyrin repeat proteins, represent a novel category of potent, specific, and versatile small-protein therapeutics. These genetically engineered proteins function as mimetics of antibodies, displaying typically high specificity and affinity for target proteins
- Nanobody-based surrogate cytokines : Single-domain antibody fragments (nanobodies) can be raised against various distinct epitopes on cytokine receptors. Nanobodies targeting different cytokine receptors can be paired in a combinatorial manner, providing a panel of potential surrogate cytokines with various receptor binding sites and orientations.

Extend the cytokine half-life / bioavailibility

A common approach for extending a cytokine's half-life is binding it to high molecular weight carrier proteins such as human serum albumin (HSA), fusion with a fragment crystallizable region (Fc) of an immunoglobulin, or conjugation with a polymer (Figure 5). All of these strategies significantly extend the cytokine's half-life.

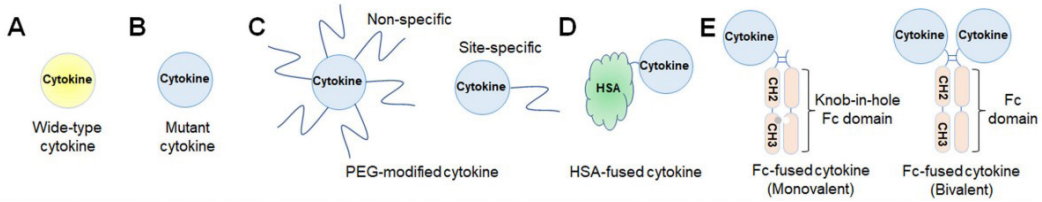


Figure 5: *Different engineering approaches to increase the drug-like properties of cytokine-based therapeutics. (A) Wild-type cytokine. (B) Mutant cytokine. (C) PEG modification methods including nonspecific or site-specific chemical conjugation. (D) Fusion to the N- and/or C-terminus of HSA. (E) Fcfusion strategies. Monovalent ("knob-in-hole" technology) or bivalent cytokine fused to the N-terminus of the IgG Fc-domain.*

Adapted from Zhao et al Front. Immunol. July 2023

Some example of on-going clinical cytokines engineered therapeutics are available in table 2.

Name	Engineered approches	Mechanism of action	Tumor type	Clinical Stages	Institutions /references
SHR-1916	Locus mutation and PEG modification	Promoted the amplification of NK and T lymphocytes	Advanced malignant tumours	Phase I	Hengrui
Nemvaleukin alfa (ALKS 4230)	Fusion IL-2Ra domain; Circularly permuted IL-2v–IL2Ra fusion protein	Selectivity for NK cells	Solid tumours	Phase III	Alkermes plc

Table 2: *Examples of IL based therapeutics*

Targeting and delivery

The following strategies improve cytokines-drugs efficacy and safety by diminishing the off-target effects and improving the therapeutics biodistribution profile.

Cytokine fusion constructs help promote drugs localization and can be used to target specific sites or cell types, particularly in tumour microenvironment.

- Immunocytokines

The category of immunocytokines includes antibody cytokine conjugates, which combine the targeting characteristic of antibodies with the ability of cytokines to trigger a local immune response. Over the past 15 years, they have been extensively investigated in the context of oncology to target tumor microenvironment. A various and numerous formats of immunocytokines are currently investigated (Figure 6). For example, immunocytokines comprising cytokines fused to antibodies targeting tumor or cell type-specific proteins are being explored, using several cytokines including IFNα, IFNγ, IL-2, IL-12, IL-15 and IL-21.

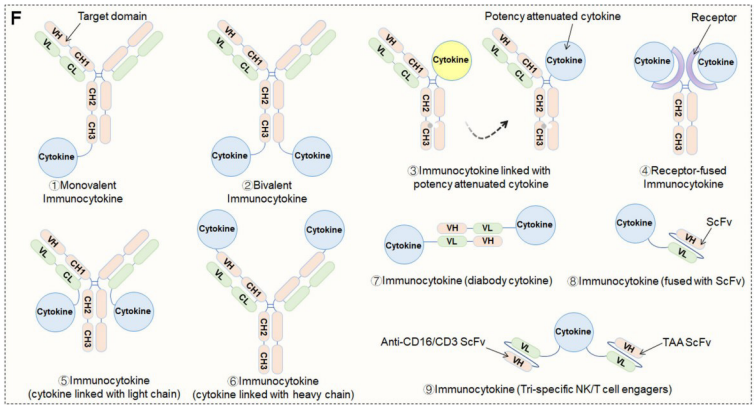


Figure 6: *Various formats of immunocytokines*
1. Monovalent immunocytokine;
2. Bivalent immunocytokine;
3. immunocytokine with potency attenuated cytokine;
4. Receptor-fused immunocytokine;
5. cytokine linked with light chain;
6. cytokine linked with heavy chain;
7. diabody cytokine-based immunocytokine;
8. immunocytokine (fused with ScFv);
9. Tri-specific NK/T-cell engagers).

Adapted from Zhao et al Front. Immunol. July 2023

Two example of immunocytokine candidate on on-going clinical are available below in table 3.

Name	Engineered approches	Mechanism of action	Tumor type	Clinical Stages	Institutions /references
Dodekin (IL12-L19L19)	Immunocytokine ; a vascular targeting antibody fused to IL-12	Tumour-targeted and immune activation	Advanced solid carcinomas; DLBCL	Phase II	Philogen S.p.A. Pharmaceuticals
NHS-IL12	Immunocytokine; two IL-12p70	Necrosis-targeted and immune activation	Solid tumours	Phase I/II	National Cancer Institute (NCI)/ EMD Serono Inc

Table 3: *Examples of immunocytokines*

• Prodrug strategies

Another approach involves the conditional activation of cytokines at target tissues, such as using cytokines fused to protease cleavable auto-inhibitory domains (prodrugs), which are only activated in the presence of proteases that are upregulated in tumor microenvironments. Protease-activatable "masked" cytokines have been prepared with various masking domains, including anti-cytokine antibodies, antibody fragments, peptides, and native cytokine receptors (Figure 7). Preclinical studies have shown promising results for protease-activatable versions of IFNα, IL-12 and IL-15, but these have not yet reached clinical trials. A example of a prodrug candidate currently in phase I are available in Table 4.

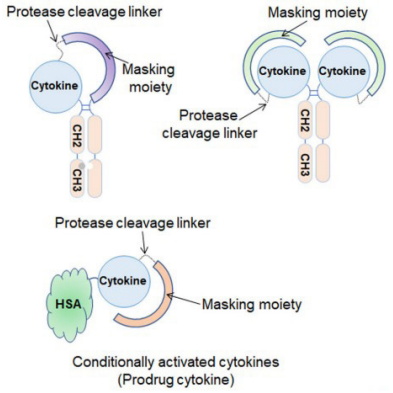


Figure 7: *Prodrug cytokines using conditional activation approaches.*

Adapted from Zhao et al Front. Immunol. July 2023

Name	Engineered approches	Mechanism of action	Tumor type	Clinical Stages	Institutions /references
WTX-124	Conditionally activated wild-type IL-2 prodrug	Selective tumour release and stimulate the immune system	Advanced solid tumours	Phase I	Werewolf Therapeutics

Table 4: *Examples of prodrug therapeutics*

• Routing cytokines with nanomedicine

Nanomedicine approaches have the potential to modulate the bioavailability and biodistribution profile of cytokines by routing them to specific organs or (immune) cells. Cytokines can be presented on the nanoparticle surface to enable direct interactions with their respective receptors, or nanomedicines can be directed to non-cellular targets, such as collagen in the extracellular matrix, where cytokines are released for local interaction with their receptors.

• Cell therapeutics

The re-engineering of cytokines can also be seamlessly integrated into cellular engineering, offering improvements in immune cell therapy and cytokine delivery. For instance, the efficacy of CAR-T cell therapy can be heightened by engineering CAR-T cells to express IL-12. This enables the depletion of tumor-associated macrophages and surmounts the immunosuppressive tumor microenvironment. CAR-T can be programmed to deliver cytokines of interest only upon antigen engagement (so-called 'cytokine armoured CARs'). A representation of a CAR-T membrane payload cytokine is available in Figure 8 with other potential approaches for cytokine target and delivery.

Cytokine engineering proves valuable in overcoming challenges that arise prior to the administration of engineered T cells. For example, the use of a novel IL-2 variant can stimulate ex vivo T cell expansion and maintain them in a more stem-cell-like state. This, in turn, prevents T cells from terminally differentiating before in vivo transfer, ultimately enhancing therapeutic efficiency.

Utilizing cellular engineering, cytokine delivery can be facilitated. For instance, T cells expressing GM-CSF can be directed to target cytokines to central nervous system malignancies, offering potential avenues for delivering cytokines across the blood–brain barrier.

Significant cellular engineering extends beyond T cells, with several ongoing projects focusing on NK cells, dendritic cells, or macrophages.

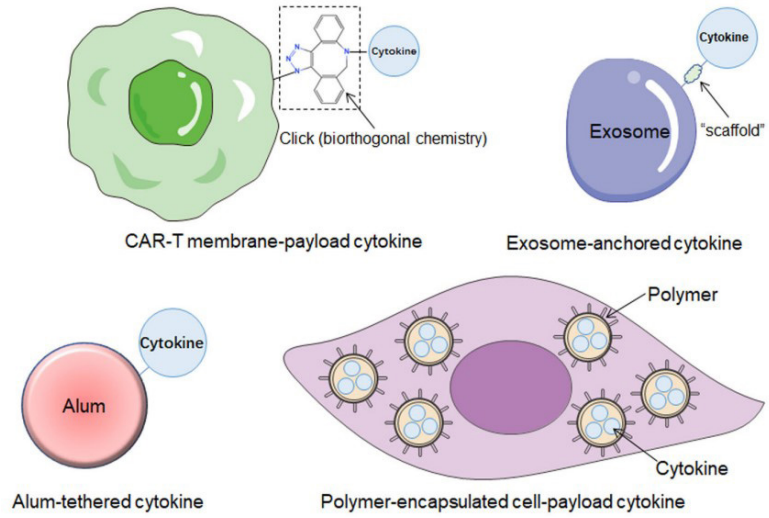


Figure 8: *Other approaches, including CAR-T-cell membrane-payload cytokines, exosome-anchored cytokines, alum-tethered cytokines, polymer-encapsulated cell-payload cytokines, etc.*

Two example of cytokine candidate combined with cell base therapeutics or other payloads are available in table 5.

Name	Engineered approches	Mechanism of action	Tumor type	Clinical Stages	Institutions /references
Alum-tethered IL-12	Recombinant IL-12 bound tightly to the vaccine adjuvant alum	Vaccine adjuvant; long retention in the tumours; substantial IFN-γ mediated T-cell and NK-cell activities	Melanoma tumours	Preclinical	K. Dane Wittrup et al.
exoIL-12	IL-12 fused with PTGFRN (as a scaffold) preformed in exosome	Long-action; improved therapeutic window	Early stage cutaneous T-cell lymphoma	Phase I	Codiak BioSciences
IL-12 INS-CAR T	IL-12-loaded HSA nanoparticles are efficiently conjugated onto CAR T cells	Immune-boosting effects of IL-12 enhance CAR T-cell antitumour abilities	Solid tumours	Preclinical	Luo et al

Table 5: *Examples of cytokine Based therapeutics*

- mRNA-based cytokine therapeutics

The COVID-19 pandemic has accelerated the advancement, approval, and extensive use of mRNA-based vaccines and RNA nanotherapeutics. This has opened up possibilities for therapeutic approaches centered around modulating cellular cytokine expression through intracellular mRNA delivery using nanoparticles. Current strategies for mRNA nanotherapeutics encompass both local and systemic administration routes. The application of these strategies to cytokine therapeutics holds promise for overcoming their existing limitations.

Specifically, local cytokine delivery to tumors involves using lipid nanoparticles encapsulating mRNA-encoding cytokines to induce localized inflammation against the tumor. Additionally, intratumorally injectable mRNA therapies encoding a cytokine cocktail have the potential to reshape the tumor microenvironment. Several companies are currently conducting phase I clinical trials to assess the safety of these innovative mRNA-encoding cytokine-based immuno-oncology treatments.

While local delivery strategies show promise, they face challenges when treating deep-seated tumors,

requiring the exploration of systemic mRNA therapy strategies.

Using systemically administered mRNA therapeutics to modulate the immune landscape can help avoid the toxicities of recombinant cytokine therapies. One strategy for systemic mRNA therapy exploits high LNP accumulation in the liver to express cytokines and turn the liver into a cytokine production 'factory'. This approach improves cytokines' blood half-life and decreases their toxicity with lower and less frequent dosing. Other complex new strategies are in development to expand the systemic RNA administration to other tissue, these could lead to a broader range of therapeutic applications.

Despite encouraging preclinical outcomes, clinical translation of mRNA therapeutics encounters challenges, including immunogenicity triggered by cationic ionizable lipids. However, altering the LNP composition can modify the biodistribution profiles and help to target specific organ.

Outlook

In conclusion, immunotherapy has emerged as a cornerstone in the treatment landscape, offering transformative outcomes for a spectrum of immune-mediated diseases spanning from autoimmune and inflammatory conditions to cancer.

Pioneering approaches like monoclonal antibodies, checkpoint inhibitors, and CAR-T cells have revolutionized patient care, extending and improving lives on a large scale. The application of innovative protein engineering technologies in molecular designs promises to fine-tune cytokine biology. This approach aims to unravel the pleiotropism of cytokines, optimize cytokine drug-like properties, and induce selective proinflammatory effects. These advancements are anticipated to facilitate cytokine-based therapeutics clinical translation, notably enhancing their role in the realm of cancer immunotherapy.

Enthusiasm for innovations around cytokine-based therapies is translating on the market, with notable deals over the past years :

- In 2023, Ankyra Therapeutics entered into a partnership with Merck. The Ankyra platform uses an inert scaffolding composed of aluminum hydroxide, a well-known vaccine adjuvant, and links bioactive immune agents to the anchor ;
- Synerkine Pharma raised additional 12.83 Millions USD in Series A financing in 2023, to accelerate the development of their lead asset SK-01, a therapeutically active cytokine fusion protein;
- In 2020, Boehringer Ingelheim entered into a licensing agreement with Enleofen Bio for 1 billion USD for worldwide exclusive rights to Enleofen's preclinical interleukin-11 (IL-11) platform.

Cytokines now stand out as a focal point for biotech and academic of extensive research, and the advent of engineered cytokine-based therapies represents an exciting new hopes for the future.

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INTERLEUKIN-15, FROM ITS DISCOVERY TO THE CLINICAL TRIALS: KEY DATES

Erwan Mortier[1]

[1]*Nantes Université*

Immunotherapy is recognized for its efficacy in the treatment of inflammatory pathologies (e.g. anti-TNFα...), and has recently seen a boom in the treatment of cancer. It works by modulating the immune system to recognize and attack cancer cells, offering a new approach to cancer treatment, in addition to traditional chemotherapy and radiotherapy. The development of a variety of cancer immunotherapeutic agents, such as recombinant stimulatory cytokines (e.g. IL-2), immune checkpoint inhibitors (e.g. anti-PD-1, anti-CTLA4) and CAR-T cell therapy has revolutionized and continues to revolutionize cancer treatment. One of the most promising targets for cancer immunotherapy is IL-15, particularly since it was considered the most promising candidate by the National Cancer Institute in 2008 (1). Different forms of IL-15 have been developed and have demonstrated significant tumor growth inhibition and anti-metastatic properties in preclinical studies and are currently the subject of clinical research. In this article, we'll briefly tell the story of IL-15, from its discovery to its application in the treatment of cancer.

1994: discovery

IL-15 is a 14–15 kDa glycoprotein belonging to the common gamma chain (γc) cytokine family along with IL-2, IL-4, IL-7, IL-9 and IL-21 (2). These cytokines play a crucial role in regulating the immune system. IL-15 was isolated from CV-1/EBNA cell culture supernatant by Grabstein and coll. (2), and was first called "T cell growth factor". Then called IL-15, it is mainly secreted by dendritic cells (DC), macrophages, and monocytes (3). IL-15 has a twin cytokine, IL-2 which became approved by the United States Food and Drug Administration (FDA) in 1992. Both bind to a dimeric receptor composed of γc and IL-2Rβ receptor chains. The specificity of action is provided by a third alpha chain, IL-2Rα and IL-15Rα. They activate similar signaling pathways and share common biological activities, including stimulation of T and NK cell proliferation and activation, and support of cytolytic effector cell differentiation. However, unlike IL-15, IL-2 promotes the proliferation of CD4 regulatory T cells and can also eliminate self-reactive T cells via activation-induced cell death (AICD) to prevent autoimmunity. On its side, IL-15 promotes not only the maintenance of CD8+ T cells and long-lived memory phenotype NK cells, but also IL-2-induced cell death (4). Due to severe side effects and toxicity, IL-2 therapy has encountered limitations in its clinical application. As a result, IL-15 has gradually emerged as an alternative to IL-2 in cancer therapy.

2002: An original mode of action: the IL-15 trans-presentation

To deliver its signal, IL-15 has three distinct receptor chains, IL-15Rα, IL-2Rβ and γc chains (5). The majority of lymphocytes express IL-2Rβ and γc chains (6). These two chains can combine to form a dimeric IL-2Rβ/γc receptor, which is expressed on the surface of effector cells such as T cells and NK cells and is responsible for signaling when binding to IL-15. IL-15Rα is an IL-15-specific cytokine receptor chain. The distal domain of IL-15Rα, called the sushi domain, mediates the binding to IL-15 with high affinity (Kd ~10-11 M) (7-9), leading to a stable complex. IL-15Rα can form high-affinity trimeric receptors with IL-2Rβ and γc chains, enabling cells to respond to low concentrations of IL-15 (10) (Fig. 1A). IL-15Rα can also form a complex with IL-15 inside producer cells (e.g. DCs, macrophages, epithelial cells), and this complex then emerges at the cell surface (11), where IL-15Rα presents IL-15 in trans to cells expressing the dimeric IL-2Rβ/γc receptor (e.g. T and NK cells), eliciting a response in the latter. This mode of action was called IL-15 trans-presentation (12) (Fig. 1B). Thus, IL-15Rα acts more like a stabilizing molecule for IL-15, assisting IL-15 in its trans-presentation.

2008: IL-15 ranks first most promising drug for cancer treatment

Among the many immune cells that express IL-2Rβ/γc, NK cells and CD8+ T cells are particularly sensitive to IL-15 stimulation (13, 14). One of the main mechanisms by which IL-15 exerts its immunostimulatory

effects is the activation and expansion of NK cells. IL-15 promotes the survival and proliferation of NK cells and CD8+ T cells and favor the secretion of perforin, granzyme B and IFNγ, which is manifested by increased cell killing activity (15). Thus, IL-15 offers an interesting opportunity to improve cancer immunotherapy and was ranked first among the most promising candidates for fighting cancer by the National Cancer Institute in 2008 (1). This is why IL-15 derivatives have attracted attention as potential immunotherapeutic agents against cancer. Several promising IL-15 agonists have been discovered in preclinical studies. Some of these are currently in clinical trials for the treatment of cancer. However, no IL-15 agonist has yet been approved for marketing.

From 2009: First-in-human clinical trial

Firstly, wild-type IL-15 was tested in its recombinant form as a 13 kDa non-glycosylated IL-15 monomer produced in E coli (16). The first phase I human clinical trial of rhIL-15 began in 2009 and was conducted on patients with metastatic malignant melanoma or metastatic renal cell carcinoma. Results showed that rhIL-15 administration was relatively safe and increased the number of peripheral NK cells and CD8+ T cells. However, no significant remission was observed in any of the patients tested (16). Subsequently, various trials were conducted to conclude that the results of these trials showed its shortcomings, notably its short half-life (requiring continuous daily administration) (17). Consequently, the optimal dose and the route and frequency of administration of rhIL-15 for different diseases remain to be determined. In conclusion, rhIL-15 shows promise as a potential cancer treatment, but the nature of the molecule, route and frequency of administration still need to be optimized.

Since 2006: Towards optimizing IL-15 for its use in the clinic

To overcome the shortcomings of rhIL-15, different strategies have been developed.

IL-15 activity enhanced by association with IL-15Rα. Since IL-15 delivers its signal by trans-presentation, researchers have designed IL-15/IL-15Rα complexes to mimic this mode of action. In 2006, three laboratories published results demonstrating that soluble IL-15Rα enhanced IL-15 activity on NK and T cells, notably by stabilizing and increasing IL-15 affinity for the IL-2Rβ/γc receptor (18-20). Since then, numerous molecules have been developed for evaluation in clinical trials, such as Nanrilkefusp alfa (RLI, SO-C101, SOT-101) (18, 21), inbakicept (ALT-803, N-803, anktiva, ImmunityBio) (22), hetIL-15 (NIZ985) (23), P22339 (SHR-1501) (24) (Fig. 2). In addition to IL-15/IL-15Rα complexes, mutations in the IL-15 sequence can modulate its activity. To this end, Altor Bioscience introduced an N72D mutation into IL-15 (25). The resulting non-covalent complex of IL-15 with a homodimeric IL-15Rα-Fc fusion, inbakicept, is slightly more potent than wild-type IL-15/IL-15Rα. This molecule has been evaluated in numerous preclinical models, reinforcing the interest in using IL-15 in cancer. These molecules are currently in clinical trials, either alone or in combination with other drugs.

Prolonging IL-15 action. The short half-life of IL-15 has led researchers to try to achieve slower clearance. The clearance of the IL-15/IL-15Rα complex is slightly slower than that of IL-15 alone (26). Thus, IL-15 or its variants have been fused to an Fc fragment to allow recycling of the protein by interaction with FcRn (inbakicept; RO7310729/XmAb24306/XmAb306) (27). Another approach developed was the bioconjugation of high-molecular-weight PEG, which slows clearance by increasing hydrodynamic size (NKTR-255) (28). However, target lymphocyte cell populations increase following IL-15 administration, generating more target receptors, resulting in massive uptake of injected IL-15. Thus, a recent approach combines reduced affinity for IL-2Rβ/γc via protein engineering offset by a prolonged half-life, which maintains therapeutically relevant drug concentrations in serum, facilitating better lymphocyte expansion in vivo. Finally, instead of using a simple Fc fragment, IL-15 can be fused to a therapeutic antibody (anti-PD1 or PDL1, anti-CTLA-4, anti-LAG3), enabling the creation of multifunctional drugs that currently undergo clinical trials.

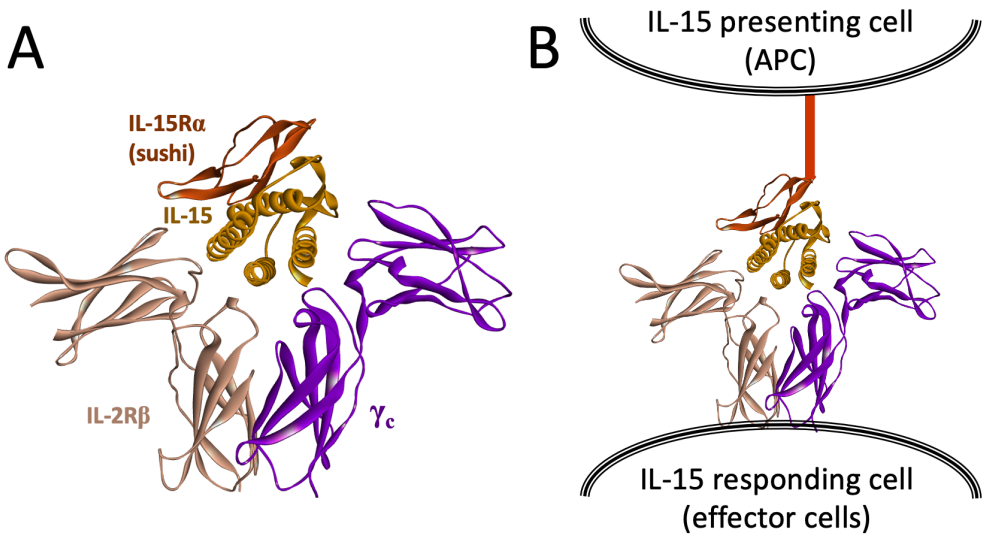

Figure 1: *IL-15 and its receptor. (A) IL-15 bound to its receptor chains, IL-15Ra, IL-2Rβ and γc. (B) IL-15 trans-presentation, a mechanism by which IL-15 is presented in trans via IL-15Ra to neighboring cells expressing the dimeric IL-2Rβ/γc receptor.*

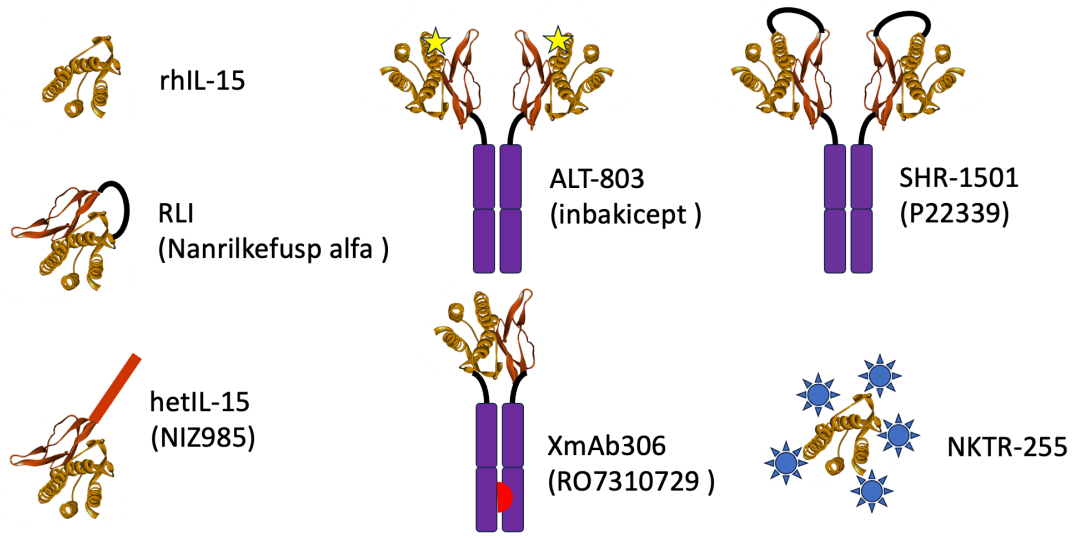

Figure 2: *IL-15 and its derivatives. rhIL-15: recombinant IL-15 from E coli. RLI: Receptor-Linker-IL-15 fusion protein consisting of IL-15 linked to the sushi domain of IL-15Ra. HetIL-15: IL-15 heterodimer with soluble IL-15Ra (entier ectodomain). ALT-803: N72D mutant (yellow star) and human IL-15Ra sushi domain-Fc fusion protein (in purple). XmAb306: monomeric IL-15 / IL-15Ra dimer linked to Fc region of human IgG using Knob into hole technology (in red). SHR-1501: Receptor-Linker-IL-15 fusion proteins are linked to the Fc region of human IgG. NKTR-255: polyethylene glycol-conjugate (in blue) of rhIL-15.*

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ANTI PD-1 CIS-TARGETING CYTOKINES ALLOWS CIS-POTENTIATION OF TUMOR-SPECIFIC ENRICHED PD-1+ T CELLS AND AUGMENTED CYTOKINE ACTIVITY IN THE TUMOR MICROENVIRONEMENT AND LYMPH NODES.

Ose Immunotherapeutics

Anti PD-1 and Anti PD-L1 Immunotherapies have revolutionized cancer patient treatment. But despite remissions, the majority of patients do not respond or acquire resistance to checkpoint blockade treatment leading to tumor relapse. Several biological primaries and acquired resistance mechanisms have been described to limit therapeutic efficacy of immune checkpoint blockade including lack of T cell infiltration of TME, poor T cell survival and inefficient activation or the high immunosuppressive microenvironment. To circumvent these mechanisms, there is need for novel combinatorial immunotherapy strategies, as cytokines-based therapy. Cytokine therapy is one promising approach to provide survival and activating signals to T cells for not only enhancing anti-tumor response but also to improve the quality and durability of T cells responses. High dose of recombinant IL-2 (Aldesleukin) was the first clinically FDA-approved immunotherapy to treat stage IV Melanoma patients and metastatic Renal cell carcinoma patients in 1992 and 1998. However, naked wild-type IL-2 has limited efficacy and high toxicity in patients due to activation of T regulatory cells, very short half-life (elimination range less than 1 hour) and important side effect of the drug (e.g. vascular leak syndrome). To reduce peripheral toxicity, enhance drug disponibility and restrict cytokine activity to a specific T cell subpopulation, novel immunocytokine strategies was designed by fusing an anti PD-1 antibody to a cytokine. The anti PD-1 antibody allows the selective delivery of the cytokine to PD-1 expressing T cells, highly enriched in tumor-specific T cells in cancer patients, in a cis-manner while sparing other immune and non-immune cells (PD-1 negative) (Figure 1). At OSE Immmunotherapeutics, we have developed an anti PD-1/IL-7v bifunctional antibody to durably activate T cells for a sustained long-term antitumor immunity. Several other anti PD-1 Immunocytokine have been designed by different biotech company with other cytokines fused (e.g. IL-2, IL-15 or IL-21) each having distinct biological properties and reviewed briefly below.

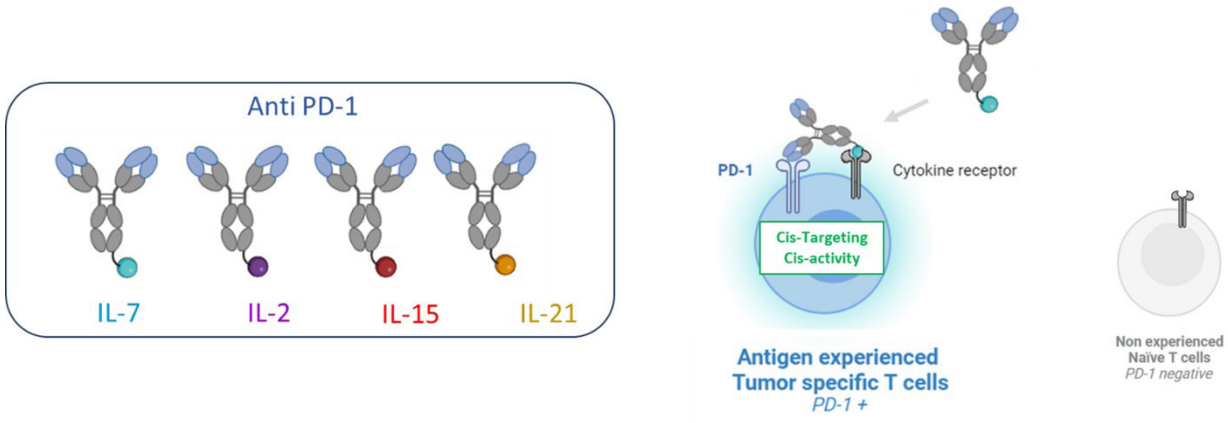

Figure 1: *Selective cytokine cis-delivery to PD-1+ tumor specific T cell*

Anti PD-1/IL-2v

Due to the attractive potent effect of IL-2 cytokine to promote T cell proliferation and activation, IL-2 cytokine was the first cytokine developed in combination with anti-PD-(L)1 blockade in clinic. A mutated or modified form of IL-2 cytokine that does not bind to CD25 receptor was designed to spare Treg while maintaining T effector stimulation. However, it has been recently demonstrated that CD25 engagement plays an essential role for the synergistic activity between IL-2 cytokine and PD-1 blockade since the use of a blocking CD25 with an antibody completely abrogated therapy efficacy in mouse model of chronic

infection1. These preclinical findings may explain the Phase III clinical trial failures of the combination therapy anti PD-1 + bempegaldesleukin (pegylated IL-2 cytokine Nektar therapeutics/BMS not targeting CD25 receptor) with absence of clinical benefit vs standard of therapy 2.

Codarri et al., 2022 (Nature publication)3 demonstrates superior efficacy of the anti PD-1/IL-2v fused immunocytokine in both chronic infection and cancer mouse models compared to the use of 2 separate drugs anti PD-1 + Isotype IL2v due to the ability of anti PD-1/IL2v to activate T cell and deliver IL-2 in cis-manner (on the same cells). By docking/forcing IL-2v onto PD-1+ T cells, anti PD-1/IL2v overcomes the need of CD25 binding on T cells and recovers the ability of IL-2 cytokine to reprogram CD8+ T cells into "better effectors" population with strong cytotoxic functions. In the same experiment, the authors compared the activity of IL-2v immunocytokine presented to T cells in trans-manner using an anti FAP targeting moiety (Anti FAP-IL-2) targeting tumor associated fibroblast into the TME. Contrary to anti PD-1/IL2v, Anti FAP-IL-2v + anti PD-1 combination treatment was not able to expand "better effector T cells" but rather induces accumulation of fully exhausted non-specific tumor T cells with poor anti-tumor activity. This study highlights the superior advantage of selective cis-delivery the cytokine to PD-1+ T cells using PD-1 targeting immunocytokine for activation of the appropriate anti-tumor reactive T cells for an enhanced tumor control as opposed to non-specific trans-delivery of the cytokine having low efficacy. A PD-1 Targeted IL-2 Variant (IL-2v) Immunocytokine (eciskafusp alfa) developed by Roche company is being tested in phase IA/B alone or in Combination with Atezolizumab (Anti PD-L1) to treat Advanced and/or Metastatic solid tumors patients (NCT04303858).

Anti PD-1/IL15v

Other bifunctional immunocytokine has been developed as anti PD-1/IL-15 fusing an antagonist anti PD-1 to an attenuated IL-15 variant (e.g., KD050 also called mut-1N-IL-15/38B or SAR445877). Mutation of IL-15 and fusion to IL5Rα sushi domain confers an optimal activation of PD-1 targeted T cells while lowering non-specific NK and T lymphocyte stimulation and peripheral systemic toxicity induced by a wild-type IL-15 cytokine (AACR Abstract 20224). Anti-PD-1/IL-15 (KD050) demonstrated significant preclinical efficacy with tumor regression in multiple solid tumor mouse models following a single injection without peripheral toxicity observed at this dose regimen 5. A phase 1/2, open label, first-in-human, dose escalation study clinical trial is currently ongoing (ClinicalTrials.gov #NCT05584670) in patient with advanced solid tumors6. Other group also developed an anti PD-1/IL15 immunocytokine strategy and further confirm that cis-delivery IL-15 on PD-1+ TILs achieve the best therapeutic effect while the same IL-15 Immunocytokine targeting tumor antigen (anti-EGFR) was ineffective to control the tumor in preclinical mouse models 7.

Anti PD-1/IL21v

An antagonist anti PD-1 targeting antibody fused with a mutated IL-21 variant (Anti PD-1/IL-21) was generated by Amgen Company (also called AMG256 or Latikafusp)8. Construction of the bifunctional antibody was optimized using a highly attenuated IL-21 mutein variant enhanced specific cis-targeting and cis-potentiation of PD-1+ T cells while reducing off-target signaling into PD-1 negative T cells. Anti PD-1/IL21 variant demonstrated superior efficacy to activate T cell effector functions and anti-tumor immunity in preclinical tumor mouse model refractory to the Anti PD-1 monotherapy treatment8. Additionally, fusion of engineered IL-21 to anti PD-1 antibody improves drug-like properties of IL-21 cytokine (improved serum half-life) allowing for less frequent dosing. To evaluate safety and tolerability, of Anti PD-1/IL21 a phase I study is currently conducted in patients with advanced solid tumors (NCT04362748).

Anti PD-1/IL-7v

OSE immunotherapeutics has created an anti PD-1/IL7v bifunctional antibody by fusing a high-affinity

antagonist anti-PD-1 fused to a single point IL-7 mutein (IL7 variant or IL-7v). Design format of the immunocytokine (PD-1 monovalent format) and affinity for IL7R was improved for better PK profile in vivo and an optimal cis-targeting and cis-activation of PD-1+CD8 T-cells while limiting activation of IL-7R+ PD-1 negative T cells. Long-term anti-tumor efficacy in monotherapy in anti PD-1 partially sensitive or refractory tumor mouse models with complete anti-tumor response > 60% in orthotopic HCC model was observed while anti-PD-1 or IL-7 alone or in combination have no or low anti-tumor efficacy. Our data highlight again the advantage of PD-1 targeted fused immunocytokine for cis and synergistic activation vs 2 separate drugs (Oral presentation AACR 2021, Poirier et al.). Interestingly, Anti PD-1/IL7v can overcome resistance of ICI treatment since anti PD-1/IL7v treatment was able to control tumor growth in a relapsed tumor model post Anti PD-1 or anti PD-L1 treatments9. To further understand the mechanism of activation, we next evaluated the activity of anti PD-1/IL7v using an in vitro chronic antigen stimulation assay recapitulating T cell exhaustion occurring into the TME. Anti-PD-1/IL7v induced a long-term reinvigoration and survival of T cells despite repeated antigen stimulation (> 5 weeks) whereas IL-2 and IL-15 cytokines promoted at short-term proliferative effect with the differentiation of T cells into effector exhausted phenotype and rapid activation induced cell death. In contrast, anti PD-1/IL7v significantly induces proliferation and maintenance of stem-like-memory-TCF1+CD8+T-cells10. TCF1+ stem-like CD8 T cell population has been thoroughly characterized as key T cell subset for an effective ICI response into patient and improved survival11–15. The use of Anti PD-1/IL7v in patient may improve anti PD-1 therapy response by amplifying the right T cell subset and favor their long-term survival. In addition to modify quality of T cells, we also observed that anti PD-1/IL7v can improved biodistribution of T-cells by promoting T-cell migration into the tumor nest whereas anti-PD-1 induced mostly T-cell exclusion in vivo mouse preclinical model10.

To conclude, Anti PD-1 immunocytokines allow the selective cis-delivery of the cytokine to PD-1+ T cells into the TME or lymph nodes and demonstrated an enhanced specific T cell immune response and anti-tumor efficacy compared to non-targeted cytokine or TME-targeted cytokine approaches. These new strategies have great clinical potential in cancer patient that are partially sensitive or refractory to current immune checkpoint therapies to maintain a durable specific T cell immune response.

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TUMOUR ACTIVATED IMMUNOCYTOKINES AND METHODS FOR THEIR IDENTIFICATION.

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Abstract

Cytokines represent potentially the most potent modulators of the immune system, however, their therapeutic application is limited by short half-lives and toxicity at active doses. Considerable effort has been applied to improve the pharmaceutical properties of cytokines including half-life extension and detoxification through modified receptor interactions. We have developed through systematic screening, a method to identify antibody cytokine fusions, wherein the cytokine activity is significantly attenuated (>200x) when fused to the antibody. The inclusion of a protease sensitive linker allows proteolysis between the antibody and the cytokine. Following proteolysis, full cytokine activity is recovered. We have demonstrated in a model system that an antibody fusion protein is preferentially proteolyzed in the tumour compared to the circulation. A range of cytokines, interferons and chemokines have been evaluated in the system to determine those that demonstrate cytokine attenuation when fused to an antibody. Controlled proteolysis and in vitro assays have demonstrated that cytokine activity can be restored in several of the tested fusion proteins. The authors believe that activatable immunocytokines identified from this system have the potential to address the principal problems for the therapeutic application of certain cytokines, targeting the biological activity specifically to a tumour and limiting the activity elsewhere.

Introduction

Recent advances in treatment have improved the outcomes for many cancer patients. Unfortunately, not all patients respond to these treatments and others develop resistance. It is increasingly evident that methods to induce, attract and stimulate the immune system are required to increase the number of patients who can benefit and achieve durable responses. Cytokines, chemokines and interferons, referred to collectively here as cytokines, have long been appreciated for their potential to modulate the immune system. The therapeutic use of cytokines in cancer care and other therapeutic disciplines has been limited due to the poor pharmacokinetics and the frequently observed toxicity associated with biologically active concentrations when administered systemically. Interferon alpha (IFNα) and interleukin-2 have both been approved as recombinant protein therapeutics, but their use has declined due to the limited patient benefit compared to the undesired side effects1,2. A wide range of techniques have been explored to improve the pharmaceutical properties of cytokines for cancer immunotherapy3. To improve poor pharmacokinetics, cytokines have been fused to a range of proteins to prolong half-life including; antibody Fc domains 4–6, albumin 4,7 or collagen binding lumican 8. Alternatively pegylation is currently under clinical evaluation for a range of cytokines including IL-2 9, IL-10 10 and IL-15 11,12 and has been successfully employed for the clinically approved pegylated IFNα2a in the treatment of viral hepatatitis 13. The targeted delivery of cytokines has also been widely explored and is detailed in a series of extensive reviews 14–17. Antibody cytokine fusions, commonly known as immunocytokines, have undergone considerable clinical evaluation 18. Preclinical and clinical activity has been observed for a range of immunocytokines, although to date none have been approved for use in the treatment of cancer 19.

The adverse effects associated with the administration of free cytokines are like those observed in the immunocytokine format, wherein the antibody cytokine fusion is selected specifically to retain cytokine function. Alternative strategies have been evaluated to control cytokine function so it becomes active only where its therapeutic effect is required and not systemically. The overexpression of matrix metallo, serine and cysteine proteases in the tumour microenvironment has been described and their proteolytic capacity has been explored to activate a range of pro-drugs 20 including silenced cytokines 21–23. However, no tumour targeting was present in these fusion proteins.

Alternative strategies have sought to address the short circulating half-life or lack of targeted activity, and others have tried to reduce the adverse effects observed with systemic administration. We have developed an activatable immunocytokine (AIC) platform that can deliver a biologically inactive cytokine to a tumour and exploit the protease rich milieu to release a fully active cytokine. To determine optimal protein fusion sites for both expression and subsequent proteolysis we evaluated heavy and light chain N- and C-terminal fusions to a selection of model antibodies. In the first instance we evaluated a series of 6-8 amino acidpeptide linkers described as sensitive to matrix metaloproteinases (MMP) -2 and or -9 20. We determined that heavy chain fusions, either N or C-terminal, have superior sensitivity to protease mediated cleavage. Fusions at to light chains were generally resistant to proteolysis by MMP-9 or MMP-2.

An initial proof of principle was performed with a model construct consisting in a C-terminal fusion of NanoLuc® luciferase (Nluc) to an antibody with a polypeptide linker sensitive to MMP-9. The stability of this molecule, evaluated in serum and plasma, demonstrated enhanced proteolysis within tumours. Hence, we chose to further explore the concept by testing a panel of cytokines to determine their functional attenuation and protease mediated reactivation. To evaluate the polyvalence of the system, a number of different antibodies were tested with model protein or cytokine payloads. We observed that six out of ten cytokines were at least partially silenced and could regain activity following MMP-9 proteolysis. An additional cytokine could be fully attenuated and regained activity using a urokinase plasminogen activator (uPa) sensitive peptide linker.

Results

The evaluation of MMP9 expression levels in a wide range of solid tumours compared to normal adjacent tissue using the TCGA datasets identified that this gene is overexpressed in a broad range of indications (Figure 1). We generated a series of fusion proteins based on the anti-PD-L1 antibody atezolizumab. Fusions were generated to N or C-terminal extremities of the heavy or light chains of the antibody using a series of five peptide linkers identified from the literature as sensitive to MMP-2/9 and an additional peptide identified as resistant to MMP-2/9 proteolysis (Table 1). Payloads in these initial investigations were the cytokine IL-2 or the bioluminescent reporter NanLuc® Luciferase (Nluc) as it was similar in size to cytokines, and we postulated it would not have biological effects in more advanced physiological experiments. A total of forty-eight fusion proteins were generated and expressed in Expi HEK293 cells . Following protein-A purification, sufficient protein amount was obtained to perform in vitro proteolysis assays with human and murine MMP-2 and MMP-9 (Figure 3).

The C-terminal fusion to the heavy chain constant domain was shown to favour proteolysis by MMP-2 and MMP-9 of human or murine origin. N-terminal heavy chain fusions did demonstrate proteolysis but this was more variable than C-terminal fusion (Table 2). Light chain fusions at either N- or C-terminal sites demonstrated very low levels of proteolysis (Table 2). The linker GIVGPL a scrambled variant of the PVGLIG was resistant to proteolysis and we chose chose to further evaluate these linkers in C-terminal heavy chain constructions.

As the C-terminal heavy chain fusion proteins were sensitive to proteolysis we next evaluated their stability in biological fluids. Initially we determined mass spectrometry conditions that enabled us to confirm the intact nature of the anti-PD-L1-PVGLIG-Nluc protein. In vitro proteolysis demonstrated a unique cleavage site as predicted between the glycine and leucine residues of the PVGLIG linker (Figure 4). Incubation for twenty-four hours in murine serum (Figure 5) or plasma demonstrated no proteolysis (Figure S1). The addition of 10 mM human MMP-9 did not induce proteolysis in any of the tested biological fluids (Figure 5 and Figure S1) indicating rapid inactivation of the enzyme rather than resistance to proteolysis.

Having demonstrated that the anti-PD-L1-PVGLIG-Nluc fusion protein was stable in vitro in biological fluids, we sought to explore if preferential proteolysis was observed in the tumour compartment of syngeneic tumour grafted rodents. The anti-PD-L1-PVGLIG-Nluc construction was injected intravenously to six-week-old BALB/c mice with established MC38 or RENCA tumours. Proteolysis in the circulation compared to the tumour demonstrated a statistically significant difference in the proportion of AIC that had undergone proteolysis in the tumour compartment at all time points. The increased proportion of proteolyzed AIC was observed in both the MC38 and the RENCA models (Figure 6).

Having determined that a model AIC construct was preferentially proteolyzed in the tumour compartment, we evaluated the capacity to produce cytokine fusions and determined the attenuation and recovery of function following controlled proteolysis. All AIC fusions were seen to be sensitive to protease treatment (Figure S2) From a total of nine C-terminal heavy chain fusions, AICs containing IFNα2a, IL-15, IL-18, IL-21 and IL-36γ demonstrated at least partial cytokine attenuation and recovered activity following proteolysis with MMP-9 (Figure 7). The IFNα2a AIC demonstrated partial attenuation when fused to the antibody but recovered full activity after MMP-9 proteolysis. Previous IFNα2a based immunocytokines lacking a protease cleavable linker have described three hundred fold attenuation of the cytokine activity 24, comparable to the levels observed here, however, proteolysis allowed the full recovery of IFNα2a activity (Figure 7G). Initial testing of the IL-18 based AIC demonstrated full attenuation but no recovery of cytokine activity after proteolysis (Figure 7F). Detailed characterization suggested that the two IL-18 molecules present in each AIC existed as a dimer (data not shown). The presence of non-paired surface cysteines were believed to be the source of the dimerization. Attempts to replace

the cysteines with serine residues as previously described 25 did not reduce the dimerization (data not shown). Replacement of the PVGLIG linker with PVGYIG to yield a sequence more coherent with mature IL-18 amino-terminus resulted in cleavage and release but activity was only detected following treatment with TCEP (tris(2-carboxyethyl)phosphine). The IL-21 based AIC demonstrated partial attenuation and regained activity following proteolysis (Figure 7I). Detailed analysis of the expressed protein showed partial proteolysis during the production and purification phase with fraction of the AICs possessing only a single IL-21 (Figure S2), further investigation would be required to determine the extent of attenuation in a fully intact AIC.

The AICs containing CCL4 (Figure 7A), CXCL9 (Figure 7B) and CXCL10 demonstrated no cytokine activity when fused to the antibody, however, following incubation with MMP-9 and despite demonstrated proteolysis, no activity was observed for C-terminal heavy chain fusions. Additional experimentation with a uPA sensitive linker SGRS 26 showed a C-terminal heavy chain fusion of CXCL10 was fully attenuated and regained full activity following proteolysis (Figure 7C).The IL-2 AIC demonstrated no attenuation as a fusion protein and proteolysis with MMP-9 had no impact on the cytokine activity (Figure 7D). Previously described IL-2 immunocytokines all maintain constitutive cytokine activity therefore this was not unexpected 27 . The IL-10 C-terminal heavy chain AIC demonstrated full activity as seen with IL-2 (Figure S3). Detailed characterization demonstrated that the two IL-10 molecules were present as a dimer, the active state for IL-10 (data not shown). To force monomeric presentation of the cytokine an N-terminal heavy chain fusion of IL-10 was evaluated. The N-terminal fusion demonstrated full cytokine attenuation and complete recovery of activity following MMP-9 proteolysis (Figure 7). Partial aggregation of the N-terminal heavy chain IL-10 AIC was observed in the preparation of this protein and may have contributed to the observed result.

The IL-15 AIC demonstrated potent attenuation in the IL-15 bioassay with no detectable activity of untreated AIC at nM concentrations but full activity at pM concnetrations of MMP activated AIC (Figure 7). To further evaluate the activity of the IL-15 based AIC we tested the biological function of these fusion proteins on human immune cells. Three AIC constructs were evaluated based on the antibody to which IL-15 was fused: NHS76, AMG479 and c9G4. IL-15 was included as a C-terminal heavy chain fusion following the PVGLIG linker in each protein. Prior to activation with MMP-9 no activity was detected on human CD3+ Tcells. When activated with MMP-9, all AICs induced expression of CD25 and CD69 equivalent to that observed with recombinant IL-15 (Figure 8). Additionally, we measured the release of IFNγ and observed that prior to activation none of the AICs induced measurable IFNγ release. Following MMP-9 activation IFNγ release was measured from all the tested AICs however the observed levels were variable depending on the antibody to which the IL-15 had been attached. The c9G4 derived IL-15 was less active in this assay. Additionally, recombinant IL-15 had reduced activity following MMP-9 treatment, suggesting a potential degradation of the cytokine, although we had not observed this in previous experiments.

Discussion

We observed that certain immunocytokine fusions including IFNα2a and IL-12 were described with a partial loss of cytokine activity 13. In addition, the IL-1 family of cytokines are predominantly produced with inactivating pro-domains 28. We therefore wanted to determine if the fusion of cytokines to antibodies via a protease sensitive linker would result in an attenuated cytokine. Having established that MMP9 gene expression was elevated in a broad range of tumour samples compared to adjacent normal tissue we tested peptide linkers senstive to MMP-9 that would release linked cytokines. We systematically evaluated all possible fusion sites with a range of protease sensitive linkers and determined that heavy chain fusion at either extremity were highly sensitive to MMP-2 and 9 proteolysis. Paratope masking peptide technologies have previously been described using amino terminal light chain fusions

for tumour protease activation 29,30 although the protease sensitive linker was potentially longer and more accessible than those evaluated here. Model AIC constructs demonstrated that protease sensitive linkers were resistant to proteolysis in biological fluid, the presence of naturally abundant MMP inhibitors such as alpha 2 macroglobulin or TIMP-1 potentially contributing to this in ex vivo experiments as the system was static with no protease production or activation 31. When evaluated in vivo we observed that proteolysis occured rapidly within the tumour, this appeared tumour dependant but could exceed 40% of the tumour resident AIC after three hours, indicating the over expression of proteases overcame the naturally abundant inhibitors. Proteolysis at a much lesser extent was also detected in the circulation with approximately 5% of AIC proteolysed every three hours. As the PVGLIG linker was sensitive to MMP-2 and MMP-9, protease activation was perhaps less restricted than if a single protease sensitivity had been selected. Further investigation would be required to determine which protease should be preferentially exploited for activation based on the expression of these enzymes in patients but may also offer a means of adapting treatments for specific indications.

Having found C-terminal heavy chain fusions were protease sensitive and the majority of previously described immunocytokines are based on this fusion site we chose to further evaluate a range of cytokines for their activity at this position. Initial testing identified that CCL4, CXCL9, CXCL10, INFα2a, IL-15, IL-18, IL-21 and IL-36 were at least partially attenuated when produced as C-terminal fusions. The C-terminal fusions of IL-2 and IL-10 retained full activity. Several IL-2 C-terminal fusions have been described and the IL-2 is always constitutively active 32–35, we also determined that no attenuation of IL-2 activity resulted from the fusion. The structure and function of IL-2 and IL-15 are similar and yet the IL-15 fusion demonstrated complete attenuation. As IL-15 is typically co-expressed with the IL-15 receptor α(CD215) 36, we hypothesised that the absence of this protein may cause the loss of activity, however, following proteolysis full activity was recovered. Thus, the presentation of IL-2 and IL-15 and the potential for interaction with the respective receptors is clearly different when associated with an antibody. We observed variable levels of IFNγ released from human CD3+ cells depending on the AIC used to release IL-15. As the parental antibodies did not effect IFNγ secretion directly, we speculated that proteolysis levels were different between the three fusion proteins and that minor differences in quantities of released IL-15 translated by more marked differences in IFNγ release.

The IL-1 family cytokines; IL-18 and IL-36 were predicted to be attenuated in the AIC format as they contain amino terminal prodomains that naturaly silence these cytokines prior to their proteolytic activation. We observed full attenuation of both cytokines as predicted when produced as AICs, but reactivation was only possible for IL-36. The IL-18 based AIC also demonstrated recovered activity but only following treatment with the reducing agent TCEP thus limiting it's further evaluation. Based on these observations, additional members of the IL-1 family including IL-1β, IL-36Ra, IL-36α, IL-36β, IL-37 and IL-38 will also potentially function as AICs due to their expression as silent pro forms.

Of the three chemokines evaluated CCL4, CXCL9 and CXCL10, all demonstrated full attenuation when produced as AICs. Proteolysis was successful for all the constructions but no activity was regained. As chemokines frequently interact with their receptors through their amino terminal sequence 37–39 we explored if the three additional amino acids resulting from cleavage of the PVGLIG linker was responsible for the loss of activity. Truncation of the chemokines or the linker did not result in cleavable AICs, in addition CXCL10 has been described as sensitive to MMP-9 40 potentially resulting in an antagonistic form of the protein being released, we therefore chose to evaluate peptide linkers sensitive to uPa that theroreticaly result in none or one additional amino acid remaining at the amino termini of the cytokines following proteolysis. A uPa sensitive linker SGRS was found to result in the production of a silenced CXCL10 fusion protein that could be successfully reactivated following proteolysis. This effect was not reproducible for CXCL9 (Figure S4). The evaluation of additional protease sensitive linkers would be required to identify methods of releasing these chemokines in a functional form.

Initial C terminal fusions of IL-10 were found to be constitutively active due to the dimerization of IL-10 into its active form. We had previously determined that amino terminal heavy chain fusions were amenable to MMP-9 proteolysis and we therefore generated an amino terminal IL-10 AIC with the PVGLIG linker. The amino terminal construction was fully attenuated, and proteolysis resulted in full activity. This construction did demonstrate aggregation during the production and purification phase and further optimization and testing would be required to ensure the observed result was not due to these. Similarly, IL-21 demonstrated partial attenuation and fully regained activity but partial proteolysis was detected in the initial AIC. The presentation of a single IL-21 on a fraction of the purified AIC may have resulted in the detected activity that suggested a partial attenuation. However this may also have resulted from the presentation of an active monomer. Further optimization of either the linker or the production and purification conditions would be required to fully evaluate an IL-21 AIC.

The IFNα2a AIC demonstrated approximately three hundred fold attenuation compared to recombinant cytokine as has been previously described 24, the full activity was recovered following proteolysis and thus could represent a potential targeting and detoxification route for this clinically approved cytokine.

We sought to address the major problems encountered with the use of cytokines as therapeutics, namely, systemic toxicity and short half-life. We have identified that the fusion of cytokines to the amino or carboxy terminus of an antibody heavy chain can result in complete or partial attenuation. The positioning of the cytokine was determined empirically in this work but knowledge of cytokine function such as monomer or dimer may educate this choice as was observed for IL-10. Surprisingly IL-2 was the only cytokine tested that did not demonstrate attenuated activity as a fusion protein. The inclusion of protease sensitive linkers adapted to the biology of the cytokine permitted the proteolytic activation of some but not all the tested cytokines. We discovered that the choice of protease sensitive linker could influence cytokine activity, and thus the evaluation of alternative peptide linkers may be required to extend the range of cytokines that can be attenuated and successfully reactivated. The in vivo evaluation of a model AIC demonstrated that proteolysis occurred over a prolonged period with a significant level of proteolyzed material detected in the tumour. We also demonstrated that IL-15 was silenced in the AIC format and regained full activity when treated in vitro with MMP-9. We intend to further evaluate the potential for cytokines that we have identified as attenuated and that recover activity following proteolysis for their therapeutic potential.

Materials and Methods

Matrix metaloproteinase gene expression in tumour tissue

Gene expression levels of matrix metaloproteinase 9 in tumour tissue were determined in comparison to normal adjacent tissue in a range of cancers. TCGA RNASeq data were downloaded from firebrowse (https://www.firebrowse.com). Expression of MMP9 was extracted from all indications and compared between tumour tissus and normal adjancent tissue (Supplementary Table 1). Data were integrated in Spotfire and expression of MMP9 was expressed as log2 transformed data relative to indications.

Construction of vectors for AIC expression

Individual AICs were generated through the synthesis of antibody heavy and light chain genes (GeneArt Ltd) either upstream or downstream of sequences encoding a protease cleavable linker and a protein payload consisting of a cytokine or NanoLuc® Luciferase (Promega) (Figure 2). Complementary heavy or light chain genes lacking payload were also synthetized for production of variants. All antibody constant domains were heavy chain IGHG1*03 and light chain IGKC*01 based on IMGT definitions 41. C-terminal lysine residues were deleted from all heavy chain constructs. Synthetic genes were proceeded by a synthetic Kozak sequence (aagcttgccgccacc), a signal peptide (METDTLLLWVLLLWVPGSTG), and

terminated with a stop codon. All sequences were flanked by HindIII and BamHI restriction sites to facilitate cloning into the pcDNA3.4 mammalian expression vector. Cloned DNA was confirmed by Sanger sequencing prior to transfection of mammalian cells. The resulting genetic constructs tested are shown in Figure 2.

Peptide linkers previously described for protease sensitivity (Table 1) were introduced between antibody heavy or light chain amino or carboxy termini (Figure 2) and the payload fusion protein. The 'GIVGPL' linker described as non-cleavable 42 was used as negative control. Initial testing was performed with the anti PD-L1 antibody atezolizumab. Additional AICs were constructed with antibodies targeting insulin like growth factor receptor-1 (IGF-R1) AMG479 43, histone (NHS76)44 and bacterial flagellin as a control (c9G4) as described in the text, the methods of generation were identical. The following human cytokines were produced as AICs CCL4, CXCL10, CXCL9, hINFα2a, IL-2, IL-10, IL-15, IL-21, IL-36, IL-18. The sequences of each cytokine as used in the AIC are detailed in Table 3. Cytokine signal sequences were not included in the AIC constructs and known prodomains or propeptides were also excluded.

Protein expression and purification

Fusion proteins were obtained by transient protein expression in Expi HEK293 cells (Thermo Fisher Scientific, Cat # A14527) grown to a density of 2.5 106/ml in Expi293 Expression Medium (Thermo Fisher Scientific, Cat #A1435101) and co-transfected with 1.25 µg/ml DNA (HC/LC: 1/1 w/w) using polyethyleneimine (PEI, Polyscience, DNA/PEI ratio: 1/4). 2 mM valproic acid (Sigma-Aldrich, Cat #P4543) was added 3 hours post transfection. Transfected cells were incubated at 37°C with 5% CO2 under agitation at 120 rpm. Supernatants containing the produced fusion proteins were harvested 6 days post-transfection. Proteins were purified by affinity chromatography on HiTrap MabSelect Sure, (GE Healthcare, Cat #11-0034-95) and formulated by overnight dialysis against 25 mM His/His-HCl, 150 mM NaCl, pH 6.5.

Protein integrity

Purified AICs were analyzed by SDS-PAGE in non-reducing and reducing conditions with heating (95 °C x 5 minutes). The monomeric content of each AIC was determined by Size Exclusion Chromatography with Superdex™ 200 Increase 10/300GL (GE Healthcare, Cat #28-9909-44). AIC integrity was also determined by LC-MS on treated or untreated AICs (glycosylated AICs and on deglycosylated AICs after IdeS digestion). Reverse phase separation was performed on an ultra-high-performance liquid chromatography (UHPLC) system (Acquity UPLC H-Class Bio system, Waters) coupled to a Synapt G2si mass spectrometer, instrument control was performed using MassLynx ®software (Waters).

In vitro proteolysis

Human MMP-9 (10 µg, R&D systems, Cat #911-MP-010), human MMP-2 (10 µg, R&D systems, Cat #902-MP-010), murine MMP-9 (10 µg, R&D systems, Cat #909-MM-010) and mouse/rat MMP-2 (10 µg, R&D systems, Cat# 924-MP-010) were activated by incubation for 24 h at 37 °C with p-aminophenylmercuric acetate (APMA, 1 mM, SIGMA Cat #A-9563) in assay buffer: Brij (50 mM TRIS (Gibco Cat # 15567027), 10 mM CaCl2 (Sigma), 150 mM NaCl (Sigma), 0,05% Brij35 (Sigma, Cat #430HG-6), pH 7.5). APMA was removed from samples with Zeba Spin Desalting Columns (Thermo Fisher, Cat #89882). AMPA free MMP was stored at -80 °C.

MMP-9 activity was measured with the Mca-PLGL-Dpa-AR-NH2 Fluorogenic MMP Substrate (R&D systems, Cat #ES001) at 20 µM. Reagents were combined in a 1:1 ratio and measurements were taken immediately using an Infinite M1000 pro (Tecan).

AIC constructs containing the anti PD-L1 antibody with N- or C- terminal fusion to the heavy or light chain

of interleukin-2 or NanoLuc® Luciferase were evaluated for protease sensitivity. 2 µg of purified AIC was incubated in presence of 40 ng recombinant MMP-9 or MMP-2 (molar ratio 25:1) in assay buffer containing 20 mM Tris pH 7.5, 10 mM CaCl2 and 100 mM NaCl in 20 µl total volume. Samples were incubated at 30°C for 2.5 hrs under gentle agitation (300 rpm). The reaction was stopped by addition of loading buffer and reducing agent (4x XT sample Buffer and 20x Reducing Agent, BioRad) and incubation at 90°C for 5min. 15 µl was then run onto Criterion TGX 4-20% Stain Free gels (BioRad, USA) in Tris-Glycine buffer at 300 V for 20 min with protein standards. Protein bands were then visualised using a ChemiDoc Touch Imager.

AIC stability and cleavage site determination

AICs were spiked in buffer (50 mM Tris pH7.5, 150 mM NaCl, 20 mM CaCl2), sera, plasma, or fresh blood at a concentration of 100 µg/ml with or without 12 nM activated MMP-9. Aliquots of 100 µl were incubated for 24h at 37 °C in Protein LowBind Tubes (Eppendorf, Cat #0030108442). Post treatment AICs were immunoprecipitated from biological fluid using M280 streptavidin magnetic beads (Thermo Fisher, Cat #11205D) coated with 4 µg of CaptureSelectTM human IgG-Fc Biotin (Thermo Fisher, Cat #7103322100). After washing steps, AICs were eluted with 0.4% TFA in water and freeze dried. Samples were reconstituted in denaturing buffer (6 M Guanidine, 0.1 M Tris, 2 mM EDTA pH 8.0) and reduced in the presence of DTT for 45 minutes at 56 °C. Acetic acid was added to quench the reaction and samples were analyzed by LC-MS.

Reverse phase separation was performed on an Acquity UPLC H-Class Bio system, (Waters) coupled to a Synapt G2si mass spectrometer, instrument control was performed using MassLynx® software (Waters).

The reduced samples were diluted volume to volume with eluent A and injected on a PLRP-S column heated to 80 °C with a flow rate of 0.5 mL/min. Elution was performed with water as eluent A and acetonitrile as eluent B, both containing 0.05 % TFA. The gradient condition was maintained at 5% B for 5 minutes, ramped to 70 % in 45 minutes and increased to 95 % in 2 minutes. For each peak of MS chromatogram, m/z spectrum was extracted, and mass determined after m/z spectrum deconvolution.

In vivo stability

The stability of the anti PDL1-PVGLIG-Nluc AIC was evaluated in vivo in syngeneic tumour bearing mice. Animals were housed in sterilized filter-topped cages, maintained in sterile conditions and manipulated according to French and European guidelines. Six-week-old immunocompetent BALB/c mice were injected subcutaneously in the hind flank with 0.5x106 RENCA cells (ATCC, Cat #CRL-2947) or 0.5x106 MC38 cells (kindly gift by Pr Pellegrin). When tumours reached 100 mm3, mice (n=6) were administered intraperitoneally with a single dose of 200 µg per mouse of the anti PDL1-PVGLIG-Nluc.AIC. Animals were sacrificed at 0, 3, 6, 24 and 48 hours and blood samples were collected in Na-heparinised tubes by cardiac puncture. Blood samples were centrifuged for 15 min at 1500 g at 4°C and plasma was collected in 96 well U bottomed plates. Tumour samples (200 mg) were recovered in 1 ml of lysis buffer (50 mM Tris-HCl buffer, 150 mM NaCl, 0.5% DOC, 1% Igepal, 1% Triton X100 containing cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche Cat #1873580)). The tumours were disrupted with a Minilys device (Bertin technologies, Cat # P000673-MLYS0-A) and Hard tissue grinding MK28 (Bertin technologies, Cat #P000910-LYSK0-A). three cycles of agitation at 5000 rpm in the presence of steel balls for 15 seconds between cycles tubes were kept on ice for two minutes. Lysates were centrifuged at 11500 g at 4 °C for 10 min. The supernatant was recovered, and protein content measured with Pierce™ BCA Protein Assay Kit (Thermo Fisher, Cat #23227) assay according to the manufacturer's instructions. Plasma and tumour samples were analyzed by Western Blot performed in triplicate. SDS-PAGE was performed with 4–15% polyacrylamide gels loading 0.1 µl of plasma or 2 µl of tumour lysate. Samples were migrated under heated, non-reduced conditions. Proteins were transferred to nitrocellulose membranes using Trans-Blot® TurboTM Midi Nitrocellulose Transfer Packs (Bio-Rad, Cat #1704159) with the Mixed MW

protocol. After a one hour, room-temperature saturation step in Tris buffered saline, 0.05% Tween 20, 1% Blotting-Grade Blocker (Biorad Cat #1706404), membranes were incubated for one hour at room temperature with a 1/50000 dilution of Peroxidase AffiniPure Goat Anti-Human IgG, Fcγ fragment specific (Jackson Immunoresearch, Cat #109-035-098). After three washes, ECL Clarity (Biorad Cat # P10026374/375) was added and membrane labelling was analysed with a ChemiDoc™ Touch Gel Imaging System. Densitometric analysis of western blots was performed with Image Lab (Bio-Rad). Statistical analyzes are carried out with GraphPad Prism6.

Cytokine function evaluation in vitro

The cytokine function when coupled to the antibody or following incubation with activated MMP was evaluated with a range of assays adapted to each cytokine. Tests were initially validated with recombinant cytokine prior to their use to evaluate AICs and MMP activated AICs. In all experiments test samples, cytokine controls and parental antibodies were incubated for one hour in the presence (+ MMP-9) or absence (-MMP-9) of recombinant MMP-9 in an assay buffer containing 50 mM Tris, 150 mM NaCl, 10 mM CaCl2 pH 7.5. Cleavage efficiency was controlled by SDS-PAGE analysis and samples were immediately stored at -20 °C until tested. Assays were performed according to the manufacturer's instructions unless otherwise stated. Data was collected with an Infinite M1000Pro or a Mithras LB940 (Berthold) microplate reader. Data analysis was performed with the Prism 7.01 software (GraphPad).

CCL4

The activity of CCL4 based AICs was evaluated with PathHunter eXpress CCR5 CHO-K1 β-arrestin GPCR assay (DiscoverX, Cat #93-1224E2). Recombinant CCL4 (R&D Systems, Cat #271-BME) was used as a control.

CXCL9 and CXCL10

Sharing a common receptor, CXCL9 and CXCL10 AICs were evaluated with PathHunter eXpress CXCR3 CHO-K1 β-arrestin GPCR assay (DiscoverX Cat #93-0271E2). Recombinant CXCL9 (Peprotech, Cat #300-26) and CXCL10 (R&D Systems, Cat #266-IP) were used as controls.

INFα2a

The activity of three IFNα2a-based AICs was assessed with GloResponse™ ISRE-luc2P/HEK293 (Promega, Cat #E8520). Recombinant IFNα2a (Fisher Scientific, Cat #111012) was used as a control.

IL-2

IL-2 based AIC activity was evaluated with the PathHunter® U2OS IL2RB/IL2RG/IL2RA Dimerization Bioassay Kit (DiscoverX, 93-1003C3). Recombinant IL-2 (R&D systems, Cat # 202-IL) was used as a control.

IL-10

IL-10 activity was evaluated with IL-10 Bioassay cells (Promega, early access program), HEK293 overexpressing IL-10R containing a Firefly luciferase gene driven by a sis-inducible element. Cells were propagated in: DMEM (Gibco, Cat #10566016) with 10% FBS, 100 µg/mL of Hygromycin (Invivogen, Cat #31282-04-9) and 2 ug/mL Blasticidin (Gibco, Cat #A1113903) this was replaced with assay medium: 90% DMEM (Gibco, Cat #10566016) with 10% FBS (Promega, Cat #364574). Cells were seeded at a 4x105 c/mL in a white flat bottom plate and cultured overnight. Samples were added in a 3-fold serial dilution in non-selective assay medium and cultured for 3 hours. The plate was then cooled to room temperature and the Bio-GloTM reagent (Promega, Cat #G7941) was added according to the manufacturer's protocol.

IL-15

The activity of IL-15 based AICs was measured the IL-15 Bioassay (Promega, Cat # CS2018B09). Recombinant IL-15 (Peprotech, Cat #200-15) was used as a control.

IL-18

IL-18 activity was evaluated with HEK-Blue™ IL-18 Cells (Invivogen, Cat #hkb-hmil18). Recombinant IL-18, (R&D systems, Cat #9139-IL-010) was used as a control. The IL-18 released from AIC by MMP-9 was observed to exist as a dimer. Incubation with TCEP (1 mM) following proteolysis was performed prior to functional testing.

IL-21

The IL-21 based AIC (AMG479-PVGLIG-IL-21) was evaluated with PathHunter® eXpress IL21R/IL2RG Dimerization Assay (DiscoverX, Cat #93-1035E3CP0M). Recombinant IL-21 (Peprotech, Cat #200-21) was used as a control.

IL-36

To assess the activity of an IL36γ-based AIC (AMG479-PVGLIG-IL-36) or control molecules were applied as serial dilutions onto squamous carcinoma A431 (ATCC, Cat #CRL-1555) cells and incubated for 24 hours. Then cell culture media were collected, and hIL-8 dosed by Human IL-8/CXCL8 DuoSet ELISA (R&D Systems, Cat #DY208).

Cytokine function evaluation ex vivo

Human peripheral blood mononuclear cells (PBMC) were isolated from fresh healthy donor cytapheresis rings by density gradient centrifugation. CD3+ T cells were purified from PBMCs by negative selection using the human pan T cell isolation kit II (Miltenyi, Cat #130-096-535). CD3+ T cells were seeded at 2x105 cells/well in RPMI1640 (Thermo Fisher, Cat #11875093) culture medium supplemented with, 10% FCS (Sigma, Cat#F7524), 1% L-glutamine (Gibco, Cat #25030-24), 1% Sodium Pyruvate (Gibco, Cat #11360-039) and 1% Penicillin/streptomycin (Gibco, 15140-122), Cat #15140122). Cells were treated with 200 ng/ml human IL-15 (Peprotech, Cat #200-15) or 6 µg/ml of AIC constructions (equivalent concentration of IL-15) that had undergone MMP-9 pretreatment as required. Cells were incubated at 37°C 5% CO2 and culture medium was refreshed after 3 days. The activation markers CD25 and CD69 were measured by flow cytometry (Novocyte, ACEA) after 6 days of culture. Detection antibodies were PE Mouse Anti-Human CD25 (BD Biosciences, Cat #555432) and APC anti-human CD69 Antibody (Biolegend, Cat #310910), isotype controls were PE Mouse IgG1, κ Isotype control antibody (Biolegend, Cat #400112), and APC Mouse IgG1, κ Isotype control (FC) antibody (Biolegend, Cat #400122). Interferon-γ secretion was measured in culture supernatants after two days of incubation by flow cytometry with Human IFN-γ Flex Set cytometric bead array (BD Biosciences, Cat #560111). All experiments were performed in triplicate; three human healthy donors were tested. Data analysis was performed with Prism 7.01 software (GraphPad).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Gene expression analysis was performed by OD. Construction of vectors for AIC expression, protein expression and purification was performed by; JT, AC, MM, CB, SB, KBDF, AV and BB. Protein integrity and in vitro proteolysis was performed by; JT, AC, MM, CB, SB and BB. AIC stability and cleavage site determination was performed by; MCJ. In vivo stability was performed by BA, KL, AV, MM. Cytokine function evaluation in vitro was performed by; JT, AC, CB, SB, LT, BB, NB, CBL. Cytokine function evaluation ex vivo was performed by FBT. Eperiments were conceived by JT, AC, MM, BA, FBT, MCJ, NB, CBL, JFH, NC and PRL. The document was writen by JT, MM, BA and PRL.

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Peptide sequence	Protease sensitivity	Ref
GPLGMLSQ	MMP-2/9	45
GPLGIAGQ	MMP-2	46
GPLGLWAQ	MMP-2/9	45
PVGLIG	MMP-2/9	47
PLGLAG	MMP-2/8/9/14	48
GIVGPL	Scrambled	42
SGRS	uPa	26

Table 1: *Peptide linker sequences evaluated in AIC constructs and described protease sensitivity. MMP: matrix metalloprotease, uPa: urokinase plasminogen activator.*

Fusion site	C-term								N-term							
Ig chain	VH				VL				VH				VL			
Fusion	Nluc		IL2		Nluc		IL2		Nluc		IL2		Nluc		IL2	
Linker \ MMP	9	2	9	2	9	2	9	2	9	2	9	2	9	2	9	2
GPLGMLSQ																
GPLGIAGQ																
GPLGLWAQ																
PVGLIG																
PLGLAG																
GIVGPL																

Table 2: *Proteolysis measured in heavy (VH) or light (VL) chain, carboxy (C-term) or amino (N-term) terminal constructs with NanoLuc Luciferase (Nluc) or interleukin-2 (IL2) following incubation with recombinant human MMP-9 (9) or MMP-2 (2). Green >80%, yellow 50-80% and red <10%.*

Cytokine	Sequence	Amino acids	Length (AA)	Mw (kDa)
CCL4	APMGSDPPTACCFSYTARKLPRNFVVDYYETSSLCSQPAVVFQTKRSKQV-CADPSESWVQEYVYDLELN	24-92	69	7.8
CXCL9	TPVVRKGRCSCISTNQGTIHLQSLKDLKQFAPSPSCEKIEIIATLKNGVQTCLNPDSADVKELIK-KWEKQVSQKKKQKNGKKHQKKKVLKVRKSQRSRQKKTT	23-125	103	11.7
CXCL10	PLSRTVRCTCISISNQPVNPRSLEKLEIIPASQFCPRVEIIATMKKKGEKRCLNPESKAIKNLLK-AVSKERSKRSP	23-98	76	8.5
IFNα2a	CDLPQTHSLGSRRTLMLLAQMRKISLFSCLKDRHDFGFPQEEFGNQFQKAETIPVLHE-MIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLMKEDSI-LAVRKYFQRITLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE	24-188	165	19.2
IL-2	APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA-TELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETA-TIVEFLNRWITFCQSIISTLT	21-153	133	15.4
IL-10	SPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYLG-CQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPCENKSKAVE-QVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN	19-178	160	18.6
IL-15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIHDT-VENLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS	49-162	114	12.8
IL-18	KLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISMYKDSQPRGMAV-TISVKCEKISTLSCENKIISFKEMNPPDNIKDTKSDIIFFQRSVPGHDNKMQFESSSYEGYFLACE-KERDLFKLILKKEDELGDRSIMFTVQNED	40-193	154	17.8
IL-21	KSSSQGQDRHMIRMRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWSAFSCFQKAQLK-SANTGNNERIINVSIKKLKRKPPSTNAGRRQKHRLTCPSCDSYEKKPPKEFLERFKSLLQKMI-HQHLSSRTHGSEDS	26-162	137	15.9
IL-36γ	KPITGTINDLNQQVWTLQGQNLVAVPRSDSVTPVTVAVITCKYPEALEQGRGDPIYLGIQN-PEMCLYCEKVGEQPTLQLKEQKIMDLYGQPEPVKPFLFYRAKTGRTSTLESVAFPDW-FIASSKRDQPIILTSELGKSYNTAFELNIND	21-169	149	16.7

Table 3: *Cytokine sequences as employed in AIC fusion proteins. Amino acid positions relative to parent molecule, length in amino acids (AA) and molecular weight (Mw)of protein as used.*

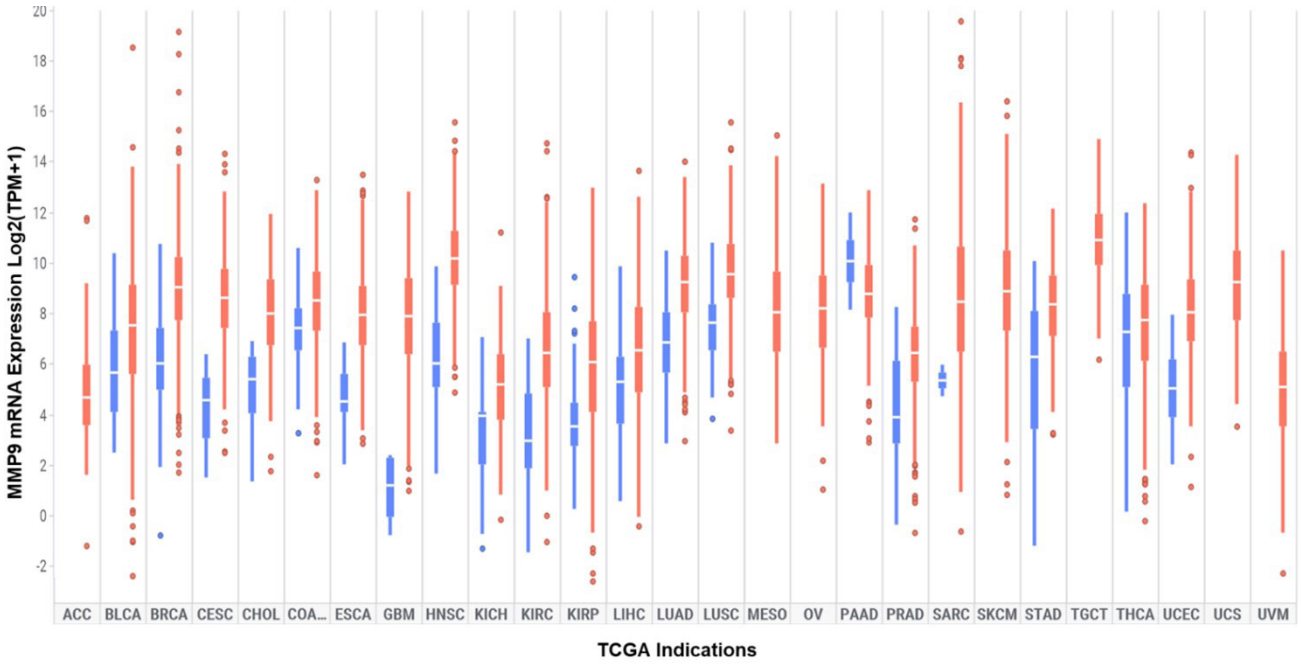

Figure 1: *Caption MMP9 mRNA expression levels in tumour (red) and normal adjacent (blue) tissue: ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma ; BRCA, Breast invasive carcinoma ; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma ; CHOL, Cholangiocarcinoma ; COA Colon adenocarcinoma ; ESCA, Esophageal carcinoma ; GBM, Glioblastoma multiforme ; HNSC, Head and Neck squamous cell carcinoma, KICH, Kidney Chromophobe ; KIRC, Kidney renal clear cell carcinoma, KIRP, Kidney renal papillary cell carcinoma ; LIHC, Liver hepatocellular carcinoma ; LUAD, Lung adenocarcinoma ; LUSC, Lung squamous cell carcinoma ; MESO, Mesothelioma ; OV, Ovarian serous cystadenocarcinoma ; PAAD, Pancreatic adenocarcinoma ; PRAD, Prostate adenocarcinoma ; SARC, Sarcoma ; SKCM, Skin Cutaneous Melanoma ; STAD, Stomach adenocarcinoma ; TGCT, Testicular Germ Cell Tumors ; THCA, Thymoma ; UCEC, Uterine Corpus Endometrial Carcinoma ; UCS, Uterine Carcinosarcoma ; UVM, Uveal Melanoma. Data lacking normal adjacent tissue are underlined.*

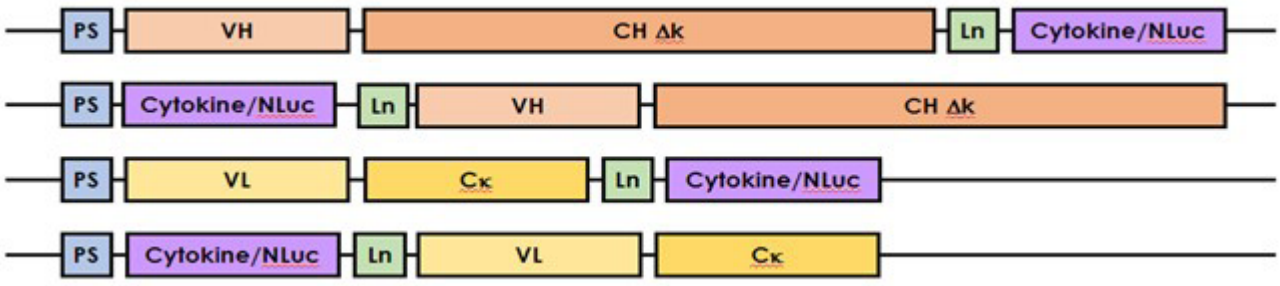

Figure 2: *The 4 different AIC constructions with either the linker (LN) and the cytokine located at the C-Terminus of the heavy (CH) or the light chains (C□) constant domain, or at the N-Terminus between the peptide signal (PS) and the variable heavy (VH) or light (VL) chain.*

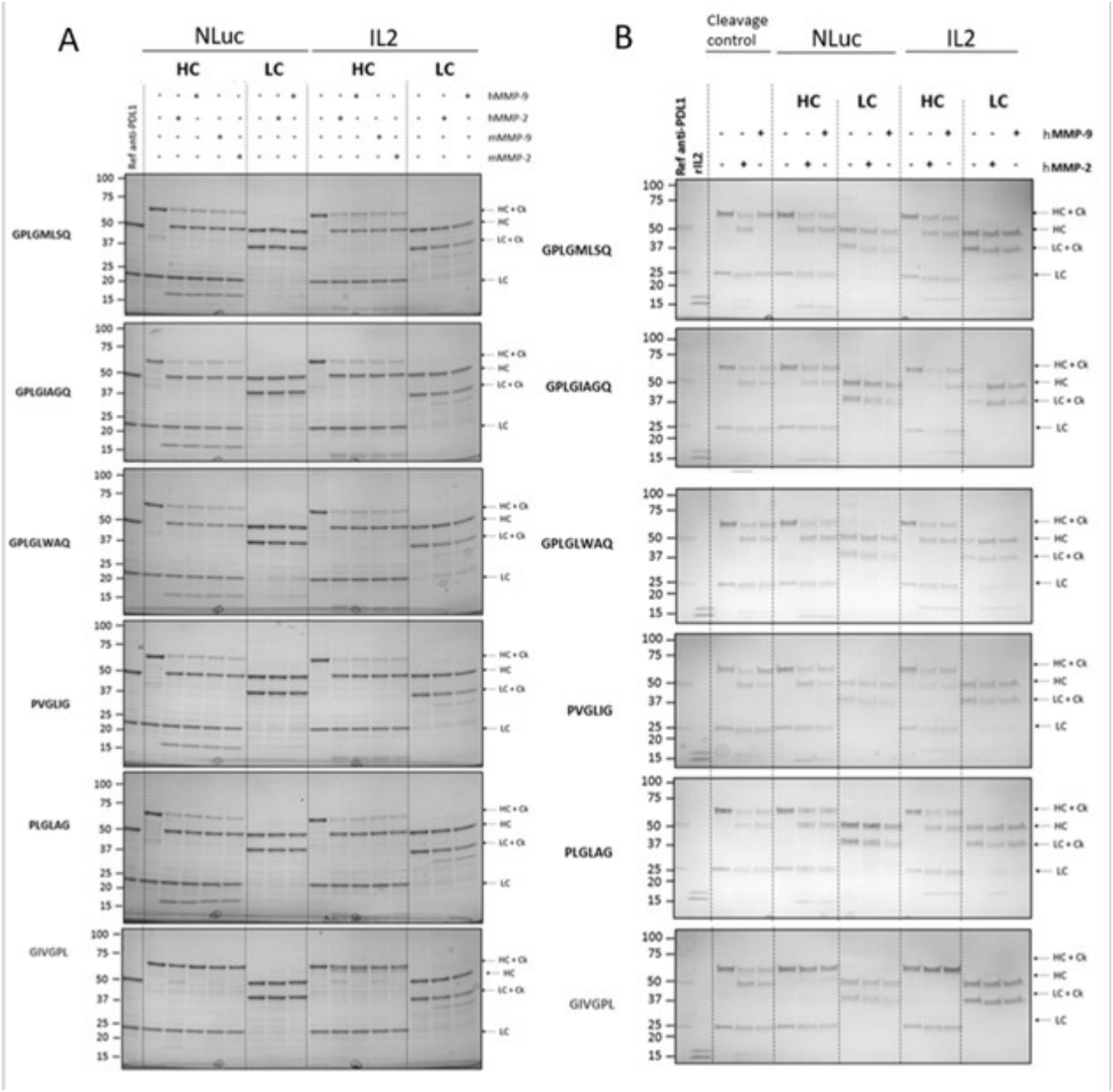

Figure 3: *All of the C-terminal heavy chain fusions contructs were cleaved by human or murine MMP9 or MMP2 except for the negative control peptide GIVGPL. None of the C-terminal light chain fusion contructs were cleaved in these conditions. B: All of the N-terminal heavy chain fusion contructs were partially cleaved by human or murine MMP9 or MMP2 except for the negative control peptide GIVGPL. None of the C-terminal light chain fusion AIC contructs were cleaved.*

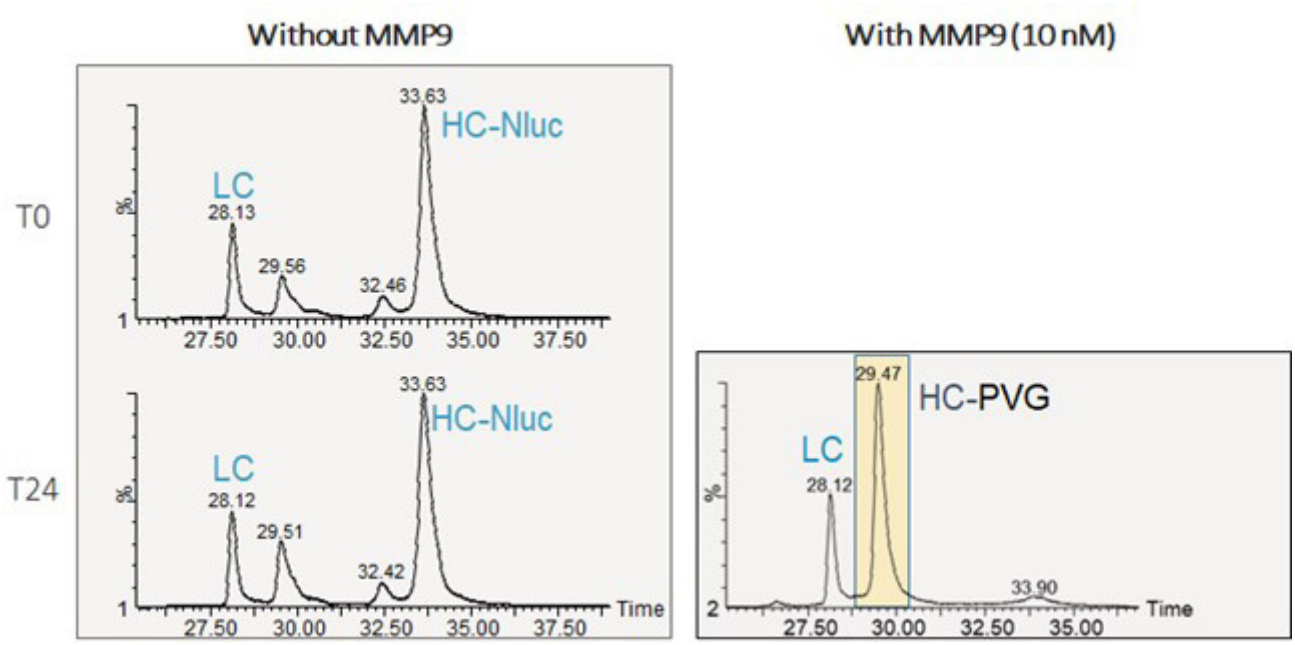

Figure 4: *Mass spectrometry chromatograms showing that heavy chain fusion remaining unchanged (HC-Nluc) after 24h in the absence of MMP9. Following 24h incubation with MMP9 full proteolysis at the predicted cleavage site is observed yielding the heavy chain (HC) and the remaining linker amino acids PVG.*

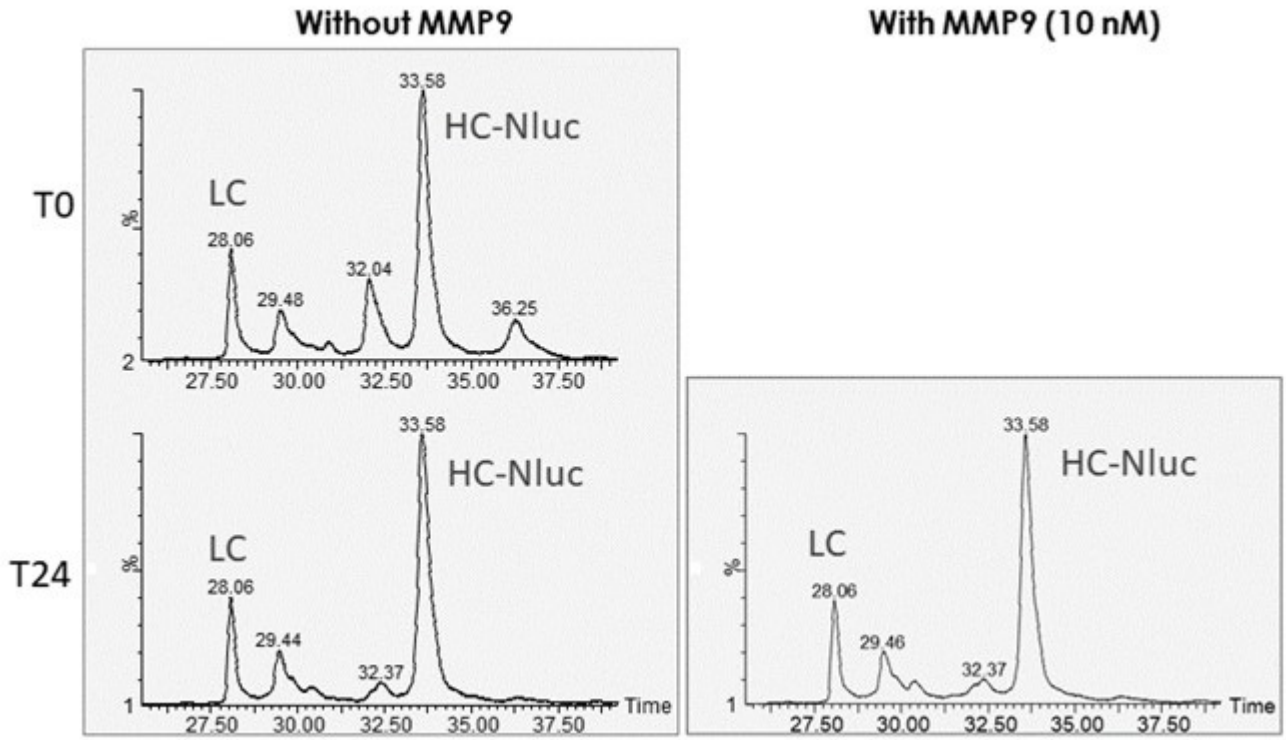

Figure 5: *Mass spectrometry chromatograms showing that heavy chain fusions stay stable without and following addition of MMP9 in mouse serum for 24H.*

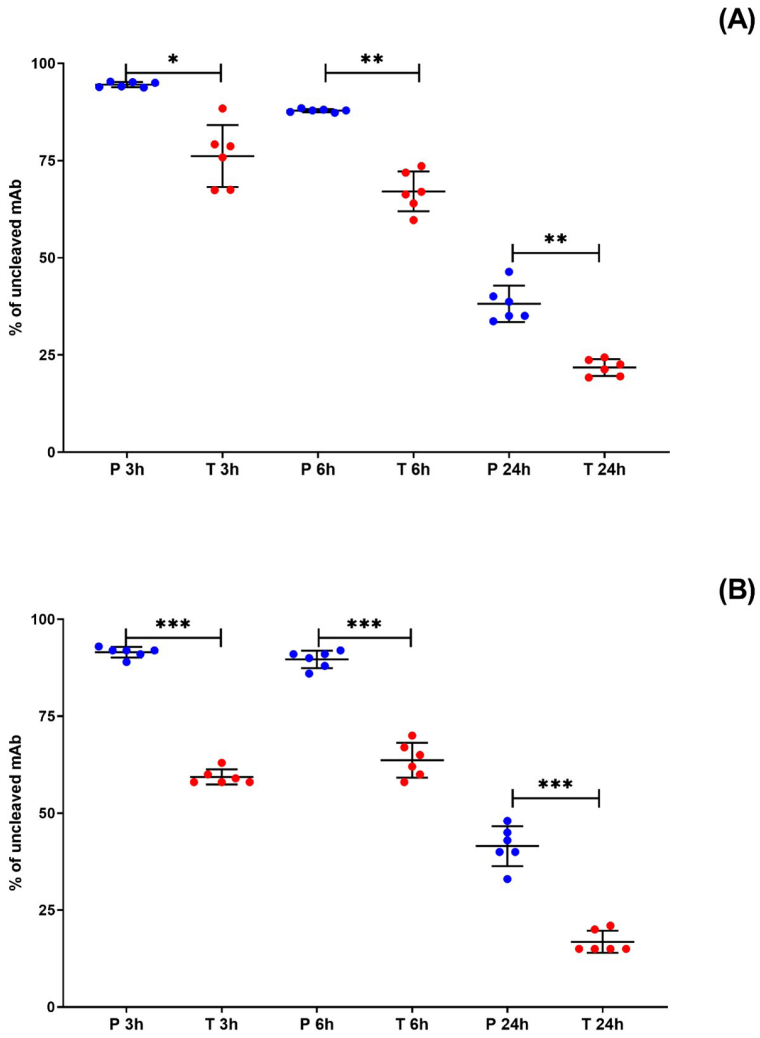

Figure 6: *Percentage of uncleaved AIC is significatively higher in the blood (blue) than in the tumor (red) of mice engrafted with MC38 (A) of RENCA (B) cells from 3 to 24h after injection.*

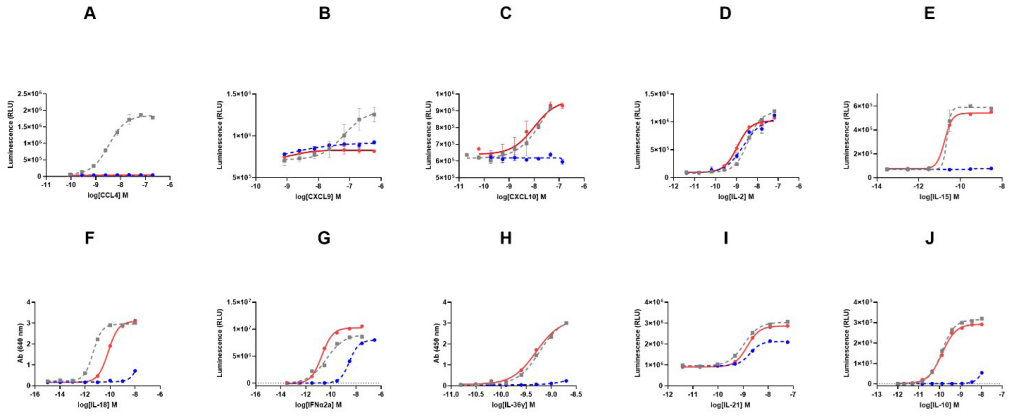

Figure 7: *AICs containing CCL4 and CXCL9 are inactive before and after incubation with hMMP9. D: AIC containing IL2 is active before and after incubation with MMP9. E, H, I: AICs containing CXCL10, IL15, IL36γ and IL10 are inactive and become active after incubation with MMP9. F, G, I: AICs containing IL18, IFNa2a and IL21 have reduced activity prior to MMP9 treatment and this increases after incubation with MMP9.*

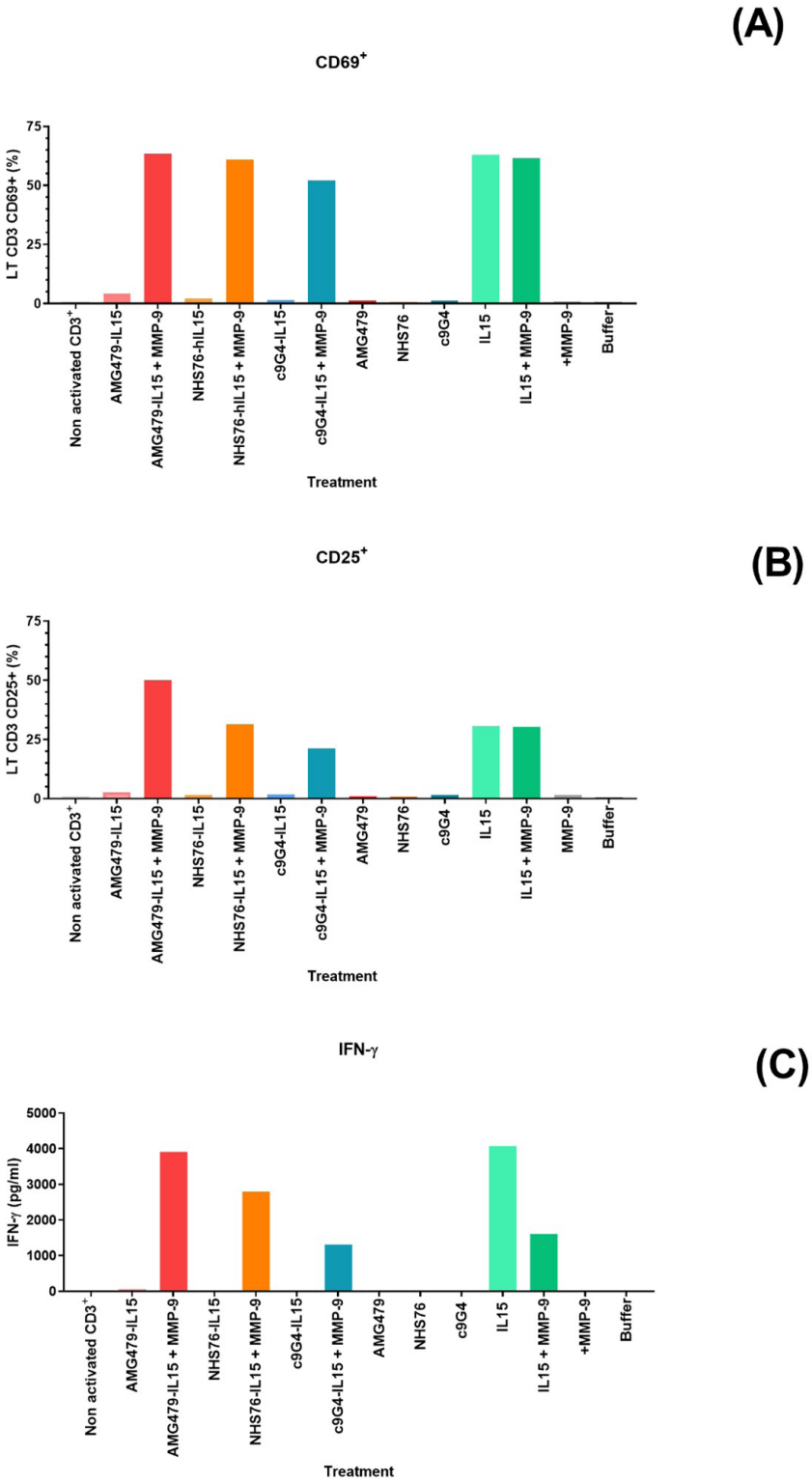

Figure 8: *Treatment with IL15-AIC pre-incubated with MMP9 increases the percentage of human CD3+ T cells expressing CD69 to levels comparable to IL15. Untreated AIC resulted in changes equivalent to control antibodies. B: Treatment with IL15-AIC pre-incubated with MMP9 increases the percentage of human CD3+ T cells expressing CD25 compared to treatment with untreated AIC. C: Treatment with IL15-AICpre-incubated with MMP9 increases the percentage of human CD3+ T cells secreting IFNγ compared to treatment with untreated AIC.*

DETERMINATION OF CYTOKINES PRESENT IN A CAR-T CO-CULTURE ENVIRONMENT BY ALPHALISA AND HTRF TECHNOLOGIES.

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Introduction

Cancer immunotherapy is a treatment option that exploits the body's own immune system to fight against cancer. T-cells protect the human body from infection by pathogens and clear mutant cells through specific recognition by T-cell receptors. Those same T-cells can be genetically modified to contain chimeric antigen receptors (CARs) targeting surface antigens on tumors to help identify and eradicate tumors.[1] CAR design consists of an extracellular domain, typically an scFv from a monoclonal antibody, to recognize the tumor antigen, then a linker or spacer followed by a transmembrane domain (ex CD3ζ) and an intracellular signaling domain that acts in a stimulatory fashion to the T-cell (ex CD28, or 4-1BB). Original CAR-T designs contained only one intracellular signaling domain, later generations contain multiple domains to augment T-cell proliferation and survival and increase cytokine production.[2]

CAR T-cell therapy involves collection and isolation of a patient's T-cells followed by genetic modification to include a CAR targeting a tumor antigen. The CAR T-cells are then expanded ex vivo and re-infused to the patient for treatment. CAR T-cells have been tested in a wide variety of cancer types but have been most effective against hematological malignancies. The FDA has approved anti-CD19 CAR T-cell therapy for refractory acute lymphocytic leukemia (ALL) and diffuse large B-cell lymphoma (DLBCL).[2,3]

T-cells are able to directly trigger apoptosis of cancer cells through granule exocytosis (perforin, granzyme) or death ligand-death receptor (Fas-FasL, TRAIL) systems.[4] Additionally, after binding to the tumor antigen, T-cells become active and release a mixture of Th1 cytokines (e.g. TNFα, IFNγ), pro-inflammatory cytokines (e.g. IL-6, IL-8, IL-12, IL-18, IL-1β), survival cytokines (e.g. IL-2), and granulocyte macrophage colony stimulating factor (GM-CSF).5 Cytokine secretion results in stromal cell sensitization in the tumor microenvironment.6 These cytokines and lytic molecules can be detected in vitro in co-culture cell-based models by several detection methods including ELISA,7 MesoScale,8 AlphaLISA,9 or HTRF10 technologies. This application note will focus on detection by two orthogonal no-wash immunoassays: AlphaLISA and HTRF in an in vitro co-culture model with CAR T-cell and tumor targeted CD19 positive Raji cells.

Materials and methods

Reagents and consumables

- Raji cells (ATCC, #CCL-86)
- RPMI media (ATCC, #30-2001)
- Fetal bovine serum (FBS), heat inactivated (Thermo Fisher, #10082-147)
- CD19 CAR T-cells, CD19scFv-CD28-4-1BB-CD3z (Promab, #PM-CAR1003)
- CAR-T Complete Growth Media (Promab, #PM-CAR2001)
- CellCarrier Spheroid ULA 96-well Microplates (Revvity, #6055330)
- 0.5 mL Eppendorf Tubes (VWR, #89166-278)
- AlphaPlate-384, light gray (Revvity, #6005350)
- Proxiplate-384 plus, shallow well (Revvity, #6008280)
- AlphaLISA kit – IL-8 (Revvity, #AL224C)
- AlphaLISA kit – IFNγ (Revvity, #AL217C)

- AlphaLISA kit – TNFα (Revvity, #AL208C)
- AlphaLISA kit – IL-1β (Revvity, #AL220C)
- AlphaLISA kit – IL-18 (Revvity, #AL241C)
- AlphaLISA kit – IL-2 (Revvity, #AL221C)
- AlphaLISA kit – IL-6 (Revvity, #AL223C)
- AlphaLISA kit – IL-12 (Revvity, #AL3116C)
- AlphaLISA kit – GM-CSF (Revvity, #AL216C)
- HTRF Kit – TNFα (Revvity, #62HTNFAPEG)
- HTRF Kit – IFNγ (Revvity, #62HIFNGPEG)
- HTRF Kit - IL-8 (Revvity, #62HIL08PEG)
- HTRF Kit – GM-CSF (Revvity, #62HGMCSFPEG)

Assay technologies

AlphaLISA® is a bead-based immunoassay technology used to study biomolecular interactions in a microplate format. The acronym "Alpha" stands for amplified luminescent proximity homogeneous assay. Some of the key features are that it is a non-radioactive, no-wash, homogeneous proximity assay. Binding of molecules captured on the beads and excitation of the donor leads to an energy transfer from the donor bead to the acceptor bead, ultimately producing a luminescent/fluorescent signal (Figure 1).

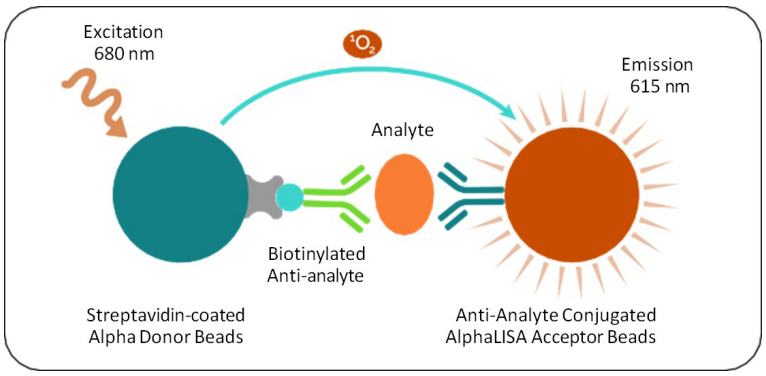

Figure 1: *AlphaLISA Assay Principle. A biotinylated anti-analyte antibody is bound by the Streptavidin-coated Alpha Donor beads, while another anti-analyte antibody is conjugated directly to AlphaLISA Acceptor beads. In the presence of analyte, the donor and acceptor beads come into proximity. Excitation of the donor beads at 680 nm provokes the release of singlet oxygen molecules that trigger a cascade of energy transfer in the nearby acceptor beads, resulting in maximum emission at 615 nm.*
AlphaLISA signal is proportional to the amount of analyte present in the sample.

HTRF® is a TR-FRET based technology and stands for homogeneous time resolved fluorescence. It is based on the fluorescence resonance energy transfer (FRET) between two fluorophores, a donor and an acceptor. These fluorophores can be coupled to antibodies targeting an analyte such that once bound they come in proximity to one another. Excitation of the donor by an energy source (e.g. a flash lamp or a laser) triggers an energy transfer towards the nearby acceptor, which in turn emits specific fluorescence at a given wavelength (Figure 2).

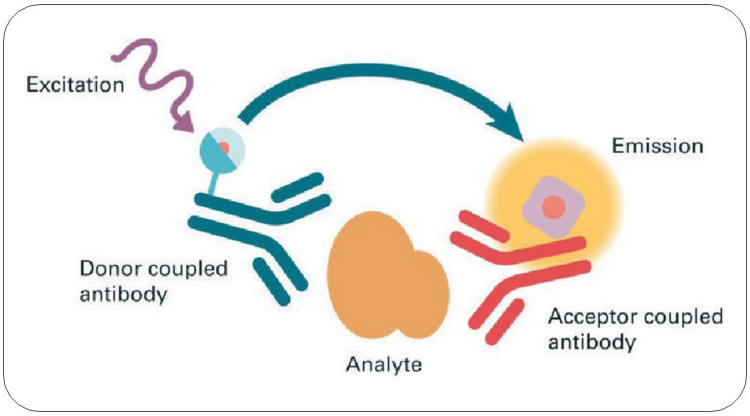

Figure 2: *HTRF Assay Principle. When the labelled antibodies bind to the same antigen, the excitation of the donor with a light source (laser or flash lamp) triggers a fluorescence resonance energy transfer (FRET) to the nearby acceptor, which in turn fluoresces at a specific wavelength. The two antibodies bind to the analyte present in the sample, thereby generating FRET. Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the analyte concentration.*

CAR-T co-culture protocol

CAR T-cell were rapidly thawed and allowed to recover overnight in 5 mL of CAR-T media in a T-25 flask before setting up the co-culture assay. Raji cells grown in RPMI media were harvested and counted on the day of the assay. Effector to target cell ratio was set to 10:1 with 100,000 CD-19 CAR-T to 10,000 Raji cells in each well following Promab's experiment design for CAR-T co-culture with their product. Controls included target cells alone and CAR T-cell alone to determine any baseline level of cytokine release from each independent cell population. 100 µL of target or effector cells was added to each appropriate well. Final assay volume was 200 µL and consisted of a 50:50 mixture of RPMI media and Promab's CAR-T media. Separate wells were setup for supernatant collection at 6 hours and 24 hours. Once plated, cell populations were gently mixed then briefly centrifuged to encourage settling as a cell pellet in the U-bottom assay plate to increase cell to cell contact between the two cell types.[3]

Data collection and analysis

Cell supernatant was carefully sampled from the assay plate so as not to disturb the cell pellet at 6 and 24 hours post cell plating. Collected supernatant was centrifuged to remove any unwanted cell carryover. Supernatant was split into aliquots in 0.5 mL tubes and kept frozen at -20°C prior to testing to avoid unwanted freeze thaw cycles.
All AlphaLISA assays were performed following the recommended protocol for each kit. Each assay required 5 µL of sample in a 50 µL reaction in a 384-well AlphaLISA plate. A fresh aliquot for each test condition (target cell alone, CAR T-cell alone, and co-culture well) was thawed on the day of testing. AlphaLISA signal was measured on a Revvity EnVision® 2105 Multimode plate reader using default values for Alpha detection of the fluorescence label. Data was analyzed in GraphPad Prism using non-linear 4 parameter logistic regression for standard curve fitting with 1/Y2 weighting.

All HTRF assays were performed following each kit's recommended protocol. Each assay required 16 µL of sample in a 20 µl reaction in a 384-well shallow well Proxiplate. A fresh aliquot for each test condition (target cell alone, CAR T-cell alone, and co-culture well) was thawed on the day of testing. HTRF signal was measured on a Revvity EnVision® 2105 Multimode Plate Reader using HTRF settings. Excitation with laser followed emission at 620 nm (europium donor reference signal) and 665 nm (d2 or XL acceptor signal) was recorded. Data is reported as the HTRF ratio of acceptorto donor signal = (665/620)*10,000. Data was analyzed in GraphPad Prism using non-linear four parameter logistic regression for standard curve fitting with 1/Y2 weighting.

Results

Nine cytokine targets, mentioned above, were selected for testing using AlphaLISA technology on the collected supernatants: TNFα, IFNγ, IL-1β, IL-2, IL-6, IL-8, IL-12, IL-18 and GM-CSF. From this set of targets IL-6, IL-12, IL-18 and IL-1β were not detected at levels higher than the calculated lower limit of quantification (LLOQ) under any treatment condition. IL-2 however was determined to be present in the Promab CAR-T media to a high level and therefore additional IL-2 expression in the supernatant could not be accurately determined. Three targets, TNFα, IFNγ and IL-8, were detected in the co-culture supernatant at both 6 h and 24 h (Figure 3) but were not detected in the single cell type control wells (Raji or CD19 CAR-T alone, data not shown) suggesting they are only released from the activated CD19 CAR T-cell in the co-culture condition. GM-CSF had a mild level of release in the non-stimulated CAR T-cells (data not shown) which increased significantly with the co-culture condition (Figure 3).

As an orthogonal assay approach, the four positive targets were tested by HTRF as confirmation of the activity seen in the AlphaLISA assays. Absolute values detected (Figure 4) are not the same as the AlphaLISA values possibly due to different antibodies used for detection in the kits, however the trends over time match (mild accumulation of TNFα at 24 h and much more pronounced increase of IFNγ, IL-8 and GM-CSF) which is shown in Figure 5 as the fold increase in target (pg/mL) over time.

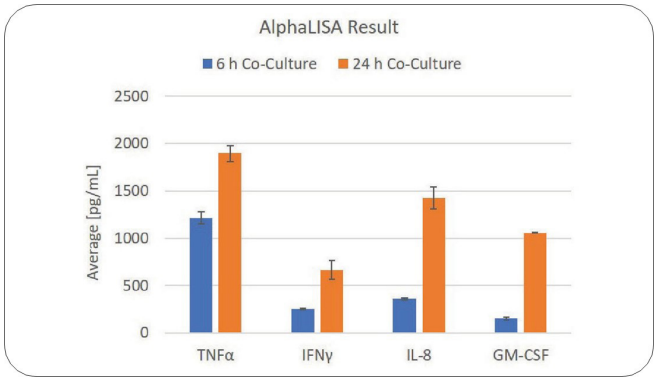

Figure 3: *Detection of cytokines by AlphaLISA. Expression level was detected at 6 hs and continued to accumulate in the 24 h time point. Results are shown as average pg/mL in a 5 µL sample as interpolated from the standard curve.*

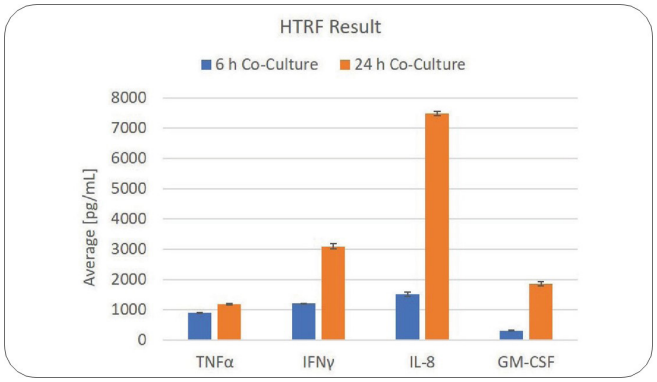

Figure 4: *Detection of cytokines by HTRF. Expression level was detected at 6 hs and continued to accumulate in the 24 h time point. Results are shown as average pg/mL in a 16 µL sample as interpolated from the standard curve.*

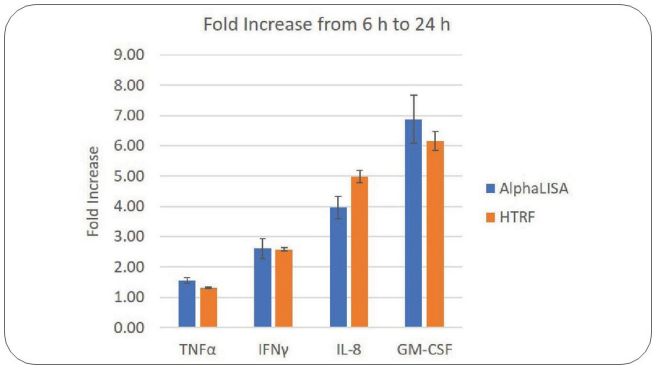

Figure 5: *Fold increase in target (pg/mL) over time. 6 h data set to 1.*

Summary

In the current application note, we demonstrate an orthogonal approach by using AlphaLISA detection and HTRF to probe the landscape of cytokines and chemokines present in the cell supernatant from a CAR-T/Raji cells co-culture model. Cytokine panning from AlphaLISA yielded four targets (TNFα, IFNγ, IL-8, and GM-CSF) which were elevated in the supernatants of the co-culture wells and confirmed in the HTRF assay with quantitated levels correlating well to each other between the two technologies. Fold increase in the targets over time matched between AlphaLISA and HTRF assays. Both technologies offer rapid, no wash homogeneous detection of cytokines in a 384-well format that is amenable to screening efforts as well as automation, in contrast with a traditional ELISA format also commonly used to detect cytokines.

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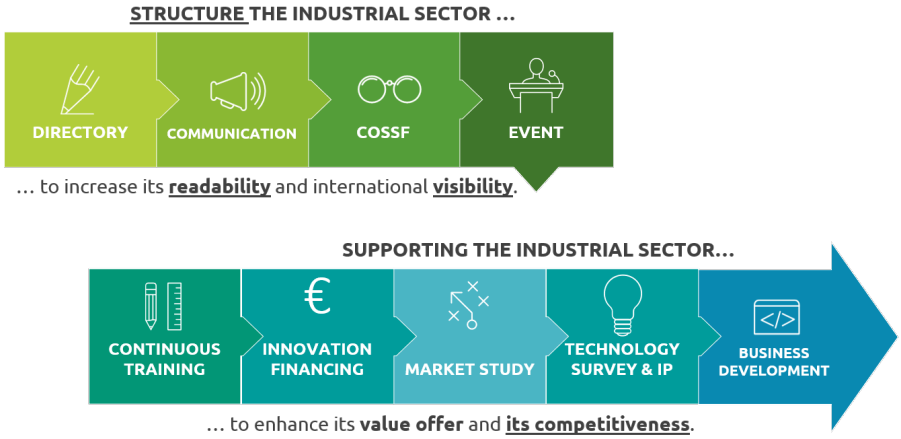

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