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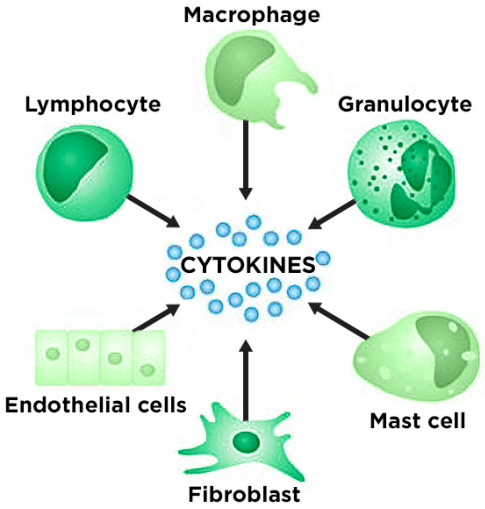
# THE IMPORTANCE OF CYTOKINE RELEASE ASSAYS TO DERISK THE DEVELOPMENT OF IMMUNOMODULATORY DRUGS

New immunomodulatory drugs have the potential to cause serious immune-related adverse events, including cytokine release syndrome (CRS). CRS is a systemic inflammatory response characterized by the release of pro-inflammatory cytokines from immune cells, which results in fever, fatigue, and possibly multiple organ failure.

Drugs that are likely to require cytokine release assays (CRAs) as part of derisking their development include, but are not limited to:

- Immune checkpoint inhibitors
- Gene therapies
- Oncolytic viral therapies
- Cytokine-based therapies
- Nanoparticle-based therapies
- Therapeutics targeting autoimmune diseases
- Vaccines (traditional and mRNA)
- Antibody-Drug Conjugates (ADCs)
- Chimeric Antigen Receptors cells (e.g., CAR T cells)

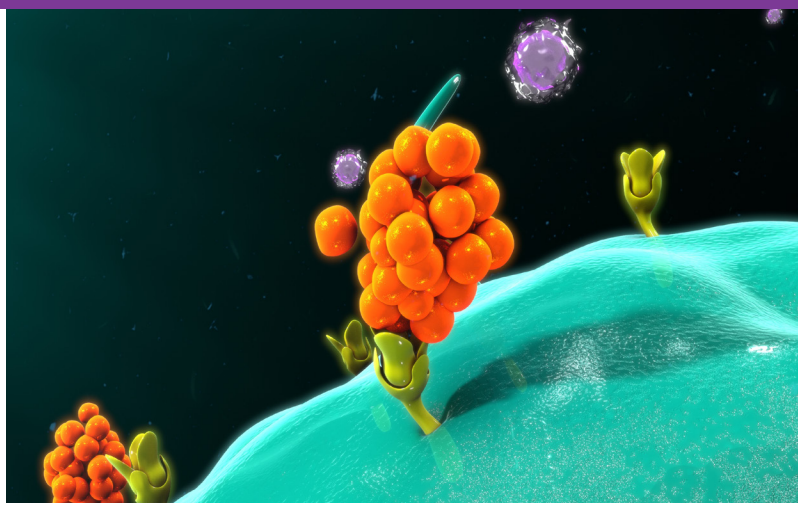



https://www.cusabio.com/cytokines/What-Cells-Release-Cytokines.html

## IN THIS ISSUE

We review strategies, including those implemented at Altasciences, for developing, qualifying, and implementing CRAs to assess the safety of test articles *in vitro* to complement *in vivo* preclinical safety assessment.

# THE IMPORTANCE OF NONCLINICAL CYTOKINE RELEASE EVALUATION



Nonclinical safety assessment serves as a cornerstone in drug development, providing essential data to identify early signs of potential toxicity and inform subsequent clinical trial design. One important aspect of toxicity studies is determining whether the drug produces undesired immune effects. The focus is to ensure that the drug does not have any potential to induce an excessive immune response, particularly cytokine storm, which can lead to severe adverse effects and impede clinical development.

During nonclinical safety assessment, we investigate whether there is an increase or decrease in cytokine production in the serum of dosed animals compared to pre-dose and vehicle controls over time. If there are indications *in vivo* alluding to a specific trend, such as a dose-dependent increase of specific cytokines in several or all treated animals, a CRA may be conducted to confirm these results *in vitro*. Alternatively, if a drug's mechanism of action or the patient population being targeted poses a higher risk for undesired immune effects, the CRA may be included in the development pathway prior to starting first-in-human studies even in the absence of any immunotoxicity trends during nonclinical safety testing.

## Triggers for *In Vitro* Confirmation

Below is a list of triggers that can lead to *in vitro* CRA confirmation.

- **Significant Elevation of Pro-Inflammatory Cytokines:** If levels are unusually high in dosed animals compared to pre-dose or vehicle control animals, showing a clear trend, these could indicate that the elevated pro-inflammatory cytokines in dosed animals are related to the test article. In these cases, additional *in vitro* assays may be needed to characterize this immune response and assess its relevance prior to moving to first-in-human studies.
- **Severe Clinical Reactions in Animals:** If animals exhibit strong signs of cytokine storm (e.g., systemic inflammation, hypotension, or multi-organ failure), additional *in vitro* testing in the same animal species to confirm the findings is warranted.
- **Species-Specific Differences:** If a response is observed in animals but human immune relevance is uncertain, testing in human-derived immune cells may clarify translatability.
- **Previous Data Suggesting High Risk:** If prior studies (e.g., receptor binding or known immune-modulating mechanism) indicate that the drug could induce cytokine release, *in vitro* testing could confirm findings before initiation of clinical trials.

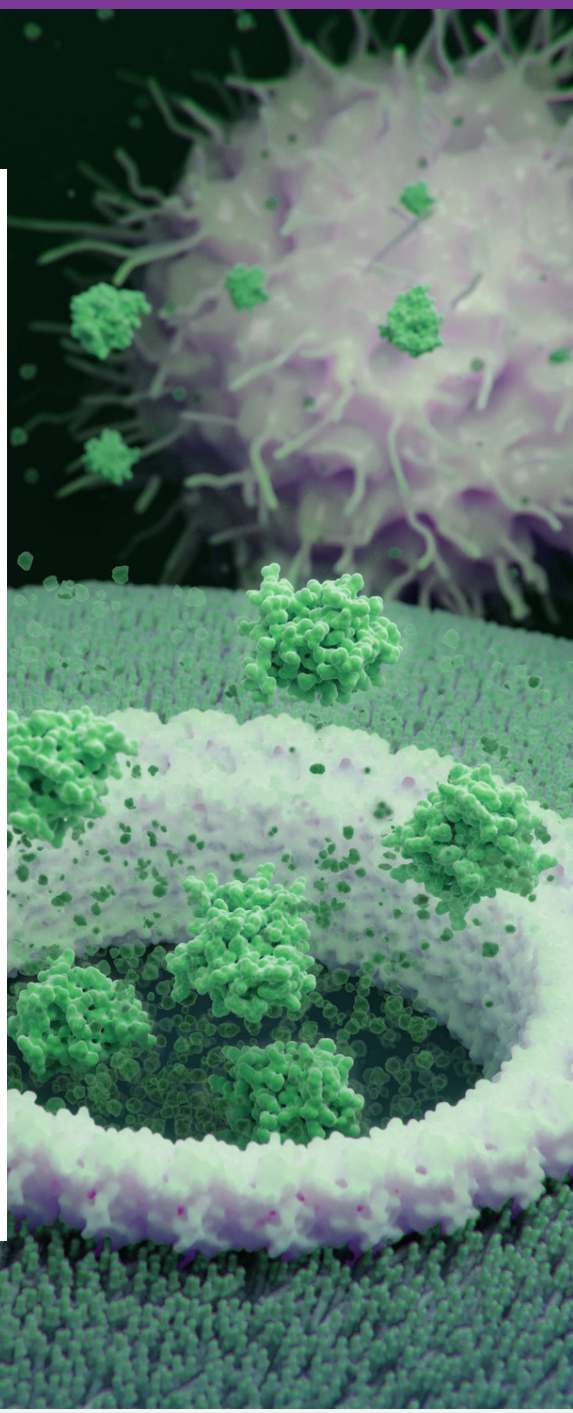
# CUSTOMIZED APPROACHES TO CRA USE IN DRUG DEVELOPMENT

In a CRA, the test article (TA) or antibody therapeutic is co-cultured with human immune cells such as peripheral blood mononuclear cells (PBMCs), whole blood, or other appropriate cells. The amount of inflammatory cytokines produced is measured and provides critical data for hazard identification.

Currently, there is no published regulatory guidance specific to CRA development, validation, or use. The industry has therefore developed several approaches in performing the CRA assay based on the drug type and its mechanism of action, which we explore below.

Using a deep understanding of immunology, experienced laboratories are able to develop approaches specific, to each TA, adapted to the disease indication, mechanism of action and type of molecule.

As the context of use is crucial for CRA implementation, the industry has developed several considerations in performing the CRA assay based on the drug type and its mechanism of action.



## 1 *In Vitro* Approaches to CRA

**PBMC Assays:** PBMC assays involve incubation of the drug with PBMCs to measure cytokine release profiles. This approach is used when the target cell population is abundant and well-represented following the isolation procedure of PBMCs, such as T or B cells. PBMC assays offer precise control over the number of cells used per sample, resulting in less variability compared to whole blood assays as well as higher cytokine levels.

**Whole Blood Assay:** Whole blood assays are a more physiologically relevant system using fresh human or nonhuman primate whole blood. Whole blood will contain factors at physiological concentrations that may influence mechanisms involved in the CRA, such as red blood cells, neutrophils, or components only present in serum that play an important role in target engagement.

## 2 Solid Phase (SP) or Aqueous Phase (AQ)

Depending on the biological properties of a given drug product, its mode of action, and specific target(s), CRAs should be developed on SP or AQ formats, as appropriate. For instance, drug-target cross-linking might be a critical step that triggers cytokine release *in vivo*. A CRA that accurately mimics this drug-target interaction would require the drug to be bound on the surface of the plate. Therefore, CRAs that do not employ the appropriate assay format can miss potential risks of identifying cytokine release *in vivo*. This is exemplified by TGN1412—a monoclonal antibody designed to activate T cells by targeting CD28 receptors—that caused severe cytokine storm in patients participating in the Phase I clinical trial, while results from the initial *in vitro* CRA failed to identify this phenomenon. Indeed, the assay relied on an AQ presentation of the monoclonal antibody to human lymphocytes; the absence of cytokine release is suggested to be associated with the lack of localized cell receptor clustering and target engagement.

## 3 Choosing the Right Cytokines to Test

The selection of cytokines to test in the CRA depends on the expected immune activation profile. For example, pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, which are early indicators of systemic inflammation, are produced by a wide variety of immune cells, while other cytokines are more cell-specific. IFN-γ is associated with T or NK cell activation and IL-8 is secreted predominantly by neutrophils. IL-4, IL-5, and IL-13 are useful markers for monitoring adaptive immunity and Th2 allergic reactions. Additional considerations for cytokine selection are also based on the class of drug and mechanism of action. For example, IFN-γ, IL-2, and TNF-α are important cytokines to monitor for immune checkpoint inhibitors.

## 4 Appropriate Timepoints for Cytokine Measurement

The timing of cytokine measurement is essential to capture both early and late immune responses. Cytokines such as TNF-α, IL-1β, and IL-6 are usually secreted within six hours of stimulation, while IL-4 and IL-17 typically require at least 48 hours. The choice of the timepoints also depends on the drug's mechanism of action. For example, monoclonal antibodies like anti-PD-1 can elicit a large spike in cytokine levels within six to 24 hours post treatment, while CAR T cells may require up to seven days of stimulation. It is also important to note that often several cytokines will be monitored in the CRA and thus a careful consideration of the stimulation period will need to be made to obtain meaningful data from all the cytokines in the panel.

## 5 Choosing the Appropriate Concentration of the Drug Product

Selecting the correct TA concentrations is crucial to mimic physiological exposure while avoiding non-specific toxicity. A high concentration (10-100x $C_{max}$) will inform on the maximum activation potential, while a clinically relevant concentration (1x $C_{max}$) would mimic the typical drug exposure profile. Low concentrations (0.1x $C_{max}$) may confirm whether the effect observed with higher doses is dose-dependent. In general, the information required from the CRA assay will also drive the number of concentrations to test.

## 6 Appropriate Controls for Cytokine Release Assays

A robust experimental setup requires multiple control groups.

Negative controls represent the baseline immune state of a given sample analyzed in the CRA. These controls should be selected by choosing compounds that are related to the TA under investigation (e.g., isotype controls for monoclonal antibodies, and empty capsids for viral-based therapies). Similar drug products that are already on the market and have been shown to be safe in humans may also be suitable negative controls. These controls will set the baseline of acceptable activation levels in each sample that will be compared to TA activation.

It is important to include positive controls that ensure assay performance, irrespective of the TA mechanism of action. It is usually a non-specific activation which will impact a large number of cytokines. As an example, LPS is a strong inducer of monocyte/macrophages, while PMA/ionomycin causes a strong T cell activation.

Additionally, in order to compare the level of activation with the TA to a known reference control drug, a second positive control is usually included. Typically, a reference drug within the same class as the TA is used to measure increases in specific cytokines, using the same timepoints for TA stimulations and concentrations.

The National Institute for Biological Standards and Control (NIBSC) has produced a panel of lyophilized antibody controls for use in various CRA platforms, designed to harmonize results across laboratories and assay methodology. A set of three different positive control antibodies which are known to induce dose-dependent CRS in patients include anti-CD52, anti-CD3, and anti-CD28. These antibodies can be used as positive reference controls with their appropriate isotype negative controls to set baseline activation for each type of monoclonal antibody.

# TOP 5 ASPECTS TO CONSIDER BEFORE RUNNING A CRA

- ☐ Does my drug have the potential to trigger an excessive immune response or cytokine release based on its mechanism of action?
- ☐ Did I observe any cytokine elevation or clinical immune-related events in animal studies that require *in vitro* confirmation?
- ☐ Am I choosing the right assay format (PBMC, whole blood, solid/aqueous phase) based on my drug's biology?
- ☐ Have I selected the appropriate cytokines, concentrations of TA, and timepoints that reflect both my drug's MoA and real-world exposure?
- ☐ Do I have the right controls and donor diversity to ensure my CRA results are reliable and translatable?

# ALTASCIENCES' CASE RESULTS

## Reference Controls

In this example, we share results obtained from reference controls used in our CRAs that match the biological activity of the investigational drug.

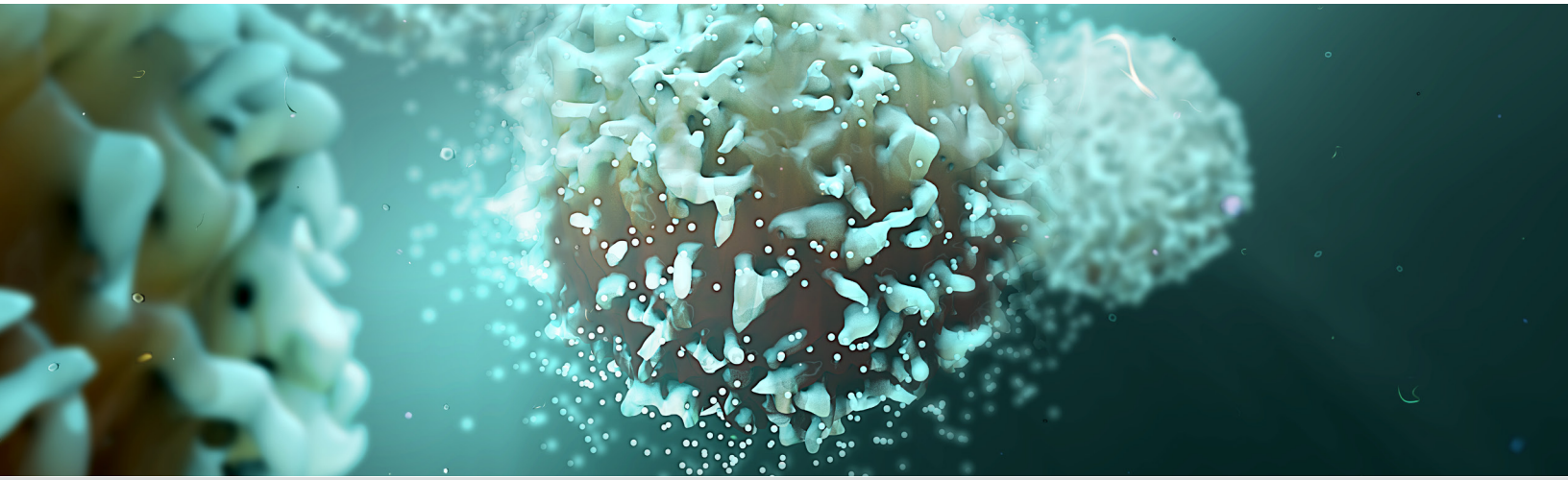
### Controls Used

A positive reference control that has not received FDA approval because of reported immune toxicity data is indicated here as Drug A. Drug B (negative control), a licensed product shown to be safe in humans, served as a baseline for acceptable cytokine stimulation threshold compared to blank. We also used pokeweed mitogen (PWM), known to activate T and B cells, leading to a broad panel of cytokine and chemokine production. Since PWM activation is not antigen-driven, it acts as a non-specific stimulator for all donors' PBMCs and is an excellent positive control for the stimulations.

### Process/Methods

PBMCs were isolated from the whole blood of six healthy donors and stimulated with either blank, Drug A, Drug B, or the investigational drug (not included in Figure 1) for six and 24 hours. Based on the nature of the investigational drug, the stimulation was performed using an AQ format.

Cytokines IL-10, IFN-γ, IL-1β, IL-6, IL-8, TNF-α, IL-12/IL23p40, MCP-1, and MIP-1β were selected based on the known immune stimulation profile reported for Drug A (reference control). Following the incubation period, the supernatant was collected, and cytokine/chemokine concentrations were determined using a qualified MSD Kit. During the assay set-up, several concentrations of the investigational drug, reference, and negative controls, were previously tested on three healthy donors to narrow down the optimum concentrations to use.



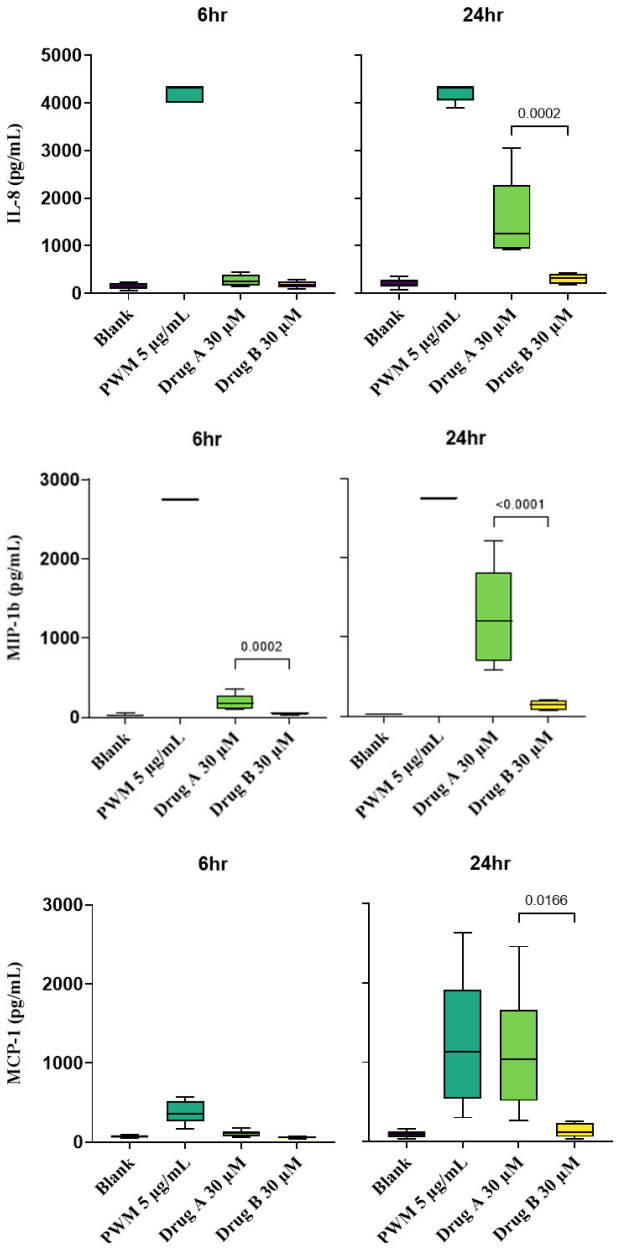
## Results

Figure 1 shows the cytokine and chemokines with significant differences between Drug A (reference control) and Drug B (negative control). Those include IL8, MIP-1 β, and MCP-1, showing a significant increase in Drug A compared to Drug B, mostly apparent at 24 hours for IL8 and MCP-1, and at six hours and 24 hours for MIP-1 β.

These results were then compared to the investigational drug, at two concentrations, to provide a comparator of cytokines with significant increases in the magnitude and prevalence across the six donors tested.

It is important to note that responses across the six donors can be variable due to genetic and immunological differences. In some cases, a larger pool of donors is tested to evaluate whether the immune responses observed are statistically significant.

**Figure 1.** Cytokine release following healthy human PBMC acqueous phase stimulation after 6 and 24 hours.




## Conclusion

While the use of reference reagents can help to identify CRS risk of investigational new drugs, care should be taken to avoid a sub-optimal CRA platform that might fail to identify CRS potential. Most importantly, the predicted mechanism of action of a therapeutic, whether via its Fc region and/or its hypothesized action on antigen-expressing cells, must match the biology of the assay. Thus, although differences in the mechanisms of action between the test therapeutic and reference reagents may present some potential limitations, the assay still serves as a robust and reliable platform for hazard identification. To achieve optimal confidence in the safety evaluation data, results from multiple CRA formats encompassing various mechanisms of action and immune cell subsets may be required.

# HOW ALTASCIENCES CAN HELP

Altasciences' bioanalytical laboratories offer vast expertise and scientific acumen to support your drug development projects, from discovery to Phase IV. We provide custom services to develop, optimize, and validate sponsor-specific assays, in addition to the frequently requested assays below.

## Complement and Cytokine Monitoring

Relying on a simple blood draw, complement and cytokine testing has the advantage of being a non-invasive, low-cost solution. Altasciences will select the appropriate technology combination to meet your needs and help you obtain reliable, quality data.

**Flow cytometry**—Flow cytometry is a highly sensitive fluorescent labeling and detection system used to measure biomarkers. Flow cytometry can detect multiple intracellular cytokines simultaneously. It can also distinguish cytokine-secreting cell subgroups based on cellular immune phenotype. No tissue culture is required, and whole blood analysis is possible.

**ELISA**—Enzyme-linked immunosorbent assay (ELISA) is a commonly used, well-established method for screening a wide variety of cytokines and complement factors.

**Multiplexed assays**—Multiplexing systems, such as the Luminex® and Meso Scale Discovery platforms, can detect multiple biomarkers simultaneously, significantly reducing the time required to generate data for multiple biomarkers.

# ALTASCIENCES' RESOURCES

## Webinars

Automation in the Bioanalytical Laboratory

Bioanalytical Project Management

Critical Sample Handling Processes

## Videos

Three Labs, One Vision

Our Bioanalytical Experience

Bioanalytical Services Overview

## Webpages

Comprehensive Immunogenicity Assays and Testing Solutions

Flow Cytometry Services

Expert Solutions for Oligonucleotide Discovery, Development, and Analysis

## Assay List

Assay List

## Scientific Journal

*The Altascientist*—Immunomodulation Assessments for Clinical Trials—Sophisticated Bioanalytical Approaches to Support Complex Modalities

## Scientific Posters

Implementation of a Semi-Automated Bead Extraction Procedure During Analyte Purification for High-Throughput Immunogenicity Assays

Strategy to Increase Throughput and Method Sensitivity for Clinical Immunogenicity Studies

Determination of Normal: Flow Cytometry Analysis of Major Immune Cell Populations in Peripheral Blood of Naïve Cynomolgus Monkeys

## Fact Sheet/eBook

Key Biomarkers of Immunomodulation: Complement Factors and Cytokines



# ABOUT ALTASCIENCES

Altasciences is an integrated drug development solution company offering pharmaceutical and biotechnology companies a proven, flexible approach to preclinical and clinical pharmacology studies, including formulation, manufacturing, and analytical services. For over 30 years, Altasciences has been partnering with sponsors to help support educated, faster, and more complete early drug development decisions. Altasciences' integrated, full-service solutions include preclinical safety testing, clinical pharmacology and proof of concept, bioanalysis, program management, medical writing, biostatistics, clinical monitoring, and data management, all customizable to specific sponsor requirements. Altasciences helps sponsors get better drugs to the people who need them, faster.

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