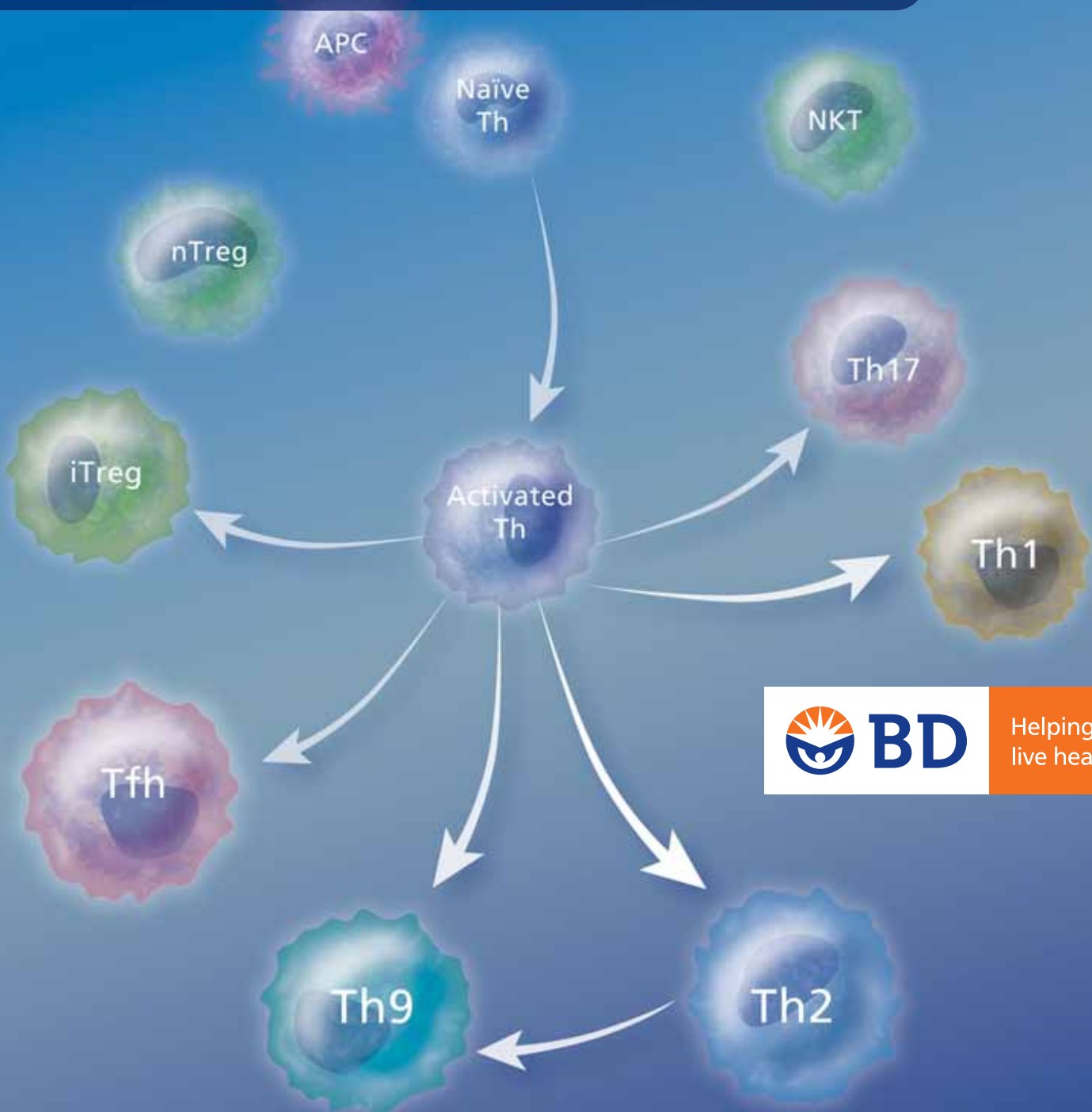
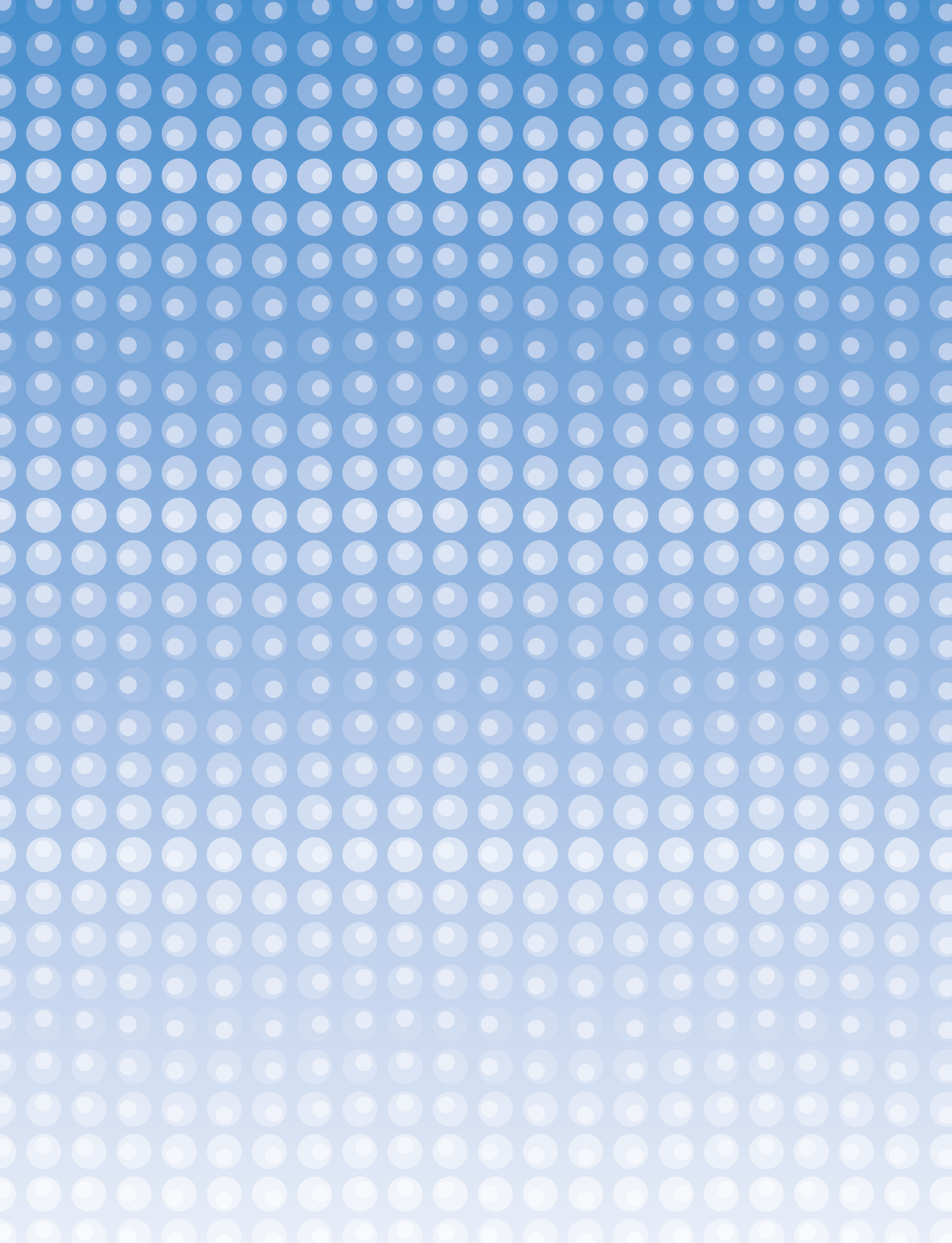


## T-Cell Research

Novel multicolor flow cytometry tools for the study of CD4<sup>+</sup> T-cell differentiation and plasticity



Helping all people  
live healthy lives



# A Solid Commitment to Research: Flexible Ways to Study CD4 T-Cell Differentiation and Plasticity

T cells have become a dynamic area of research. Among the methods used to characterize this major lymphocyte subset, multicolor flow cytometry is preeminent. Additionally, the complexity of the CD3<sup>+</sup> T-cell population—both functionally and phenotypically—makes multiparametric flow cytometry a necessary and powerful platform.

For more than two decades, researchers have made thousands of advances in T-cell study using BD flow cytometry products. And many of today's discoveries involving T cells also involve BD Biosciences platforms, reagents, instruments, and protocols.

BD continues to build on this commitment with new, quality reagents such as the new BD Horizon Brilliant™ Violet and BD Horizon Brilliant™ Ultraviolet fluorochrome conjugated antibodies. They offer improved brightness and support larger panel sizes.

T-cell subtypes can be defined by the combinations of cell surface markers and transcription factors they express and the cytokines they secrete. These proteins are regulated through signaling pathways. For example, the binding of IL-6 to its receptor leads to the phosphorylation of Stat3, which can then lead to the expression of IL-17A.

T-cell plasticity, the ability of a cell to change its phenotype in response to its environment, is of particular interest—especially for Th17 and regulatory T cells. This brochure discusses and demonstrates how the following platforms can be used to study T-cell differentiation:

**Cell Surface Markers** to identify cells from heterogeneous samples

**Intracellular Cytokine Staining (ICS)** to measure cytokines within individual cells

**BD Phosflow™ technology** to measure the phosphorylation of key proteins

**BD™ Cytometric Bead Array (CBA)** to measure secreted cytokines within a sample

BD Biosciences continuously updates our portfolio of products for the analysis and enrichment of T cells. BD Biosciences reagents are backed by a world-class service and support organization to help customers take full advantage of our products to advance their research. Comprehensive services include technical application support and customer assay services provided by experienced scientific and technical experts.

## A dynamic area of research

# T Cells: An Overview

### Summary of T-cell Subsets

T cells can be separated into three major groups based on function: cytotoxic T cells, helper T cells (Th), and regulatory T cells (Tregs). Differential expression of markers on the cell surface, as well as their distinct cytokine secretion profiles, provide valuable clues to the diverse nature and function of T cells.<sup>1</sup>

For example, CD8<sup>+</sup> cytotoxic T cells destroy infected target cells through the release of perforin, granzymes, and granulysin, whereas CD4<sup>+</sup> T helper cells (ie, Th1, Th2, Th9, Th17, and Tfh cells) have little cytotoxic activity and secrete cytokines that act on other leucocytes such as B cells, macrophages, eosinophils, or neutrophils to clear pathogens. Tregs suppress T-cell function by several mechanisms including binding to effector T-cell subsets and preventing secretion of their cytokines.

To support the use of multicolor flow cytometry for the study of T cells, BD offers a deep portfolio of reagents, which are highlighted in red in the table below. BD now also offers more choice. Many of these specificities are available in multiple formats including BD Horizon™ V450 and V500 formats for use with the violet laser.

### Tregs: Essential Regulators of Immunity

Tregs play an important role in maintaining immune homeostasis and have also been implicated in a number of autoimmune diseases.<sup>2</sup> Flow cytometry is a particularly useful application for the sorting and analysis of Tregs.

Two major classes of CD4<sup>+</sup> Tregs have been identified to date: “natural” Tregs (nTregs) that constitutively express CD25 and FoxP3, and adaptive or inducible Tregs (iTregs) in which CD25 and FoxP3 expression is activated.<sup>3</sup> CD25 expression differs between human and mouse Tregs. In mice all CD25<sup>+</sup> cells are considered Tregs, compared to humans, for whom only those cells expressing the highest levels of CD25 are considered to be Tregs.<sup>4</sup>

Type of Cell	Cytotoxic	Th1	Th2	Th9 <sup>5</sup>	Th17	Tfh <sup>6</sup>	Treg
Main Function	Kill virus-infected cells	Activate microbicidal function of infected macrophages, and help B cells to produce antibody	Help B cells and switch antibody isotype production	T cell proliferation and enhanced IgG and IgE production by B cells	Enhance neutrophil response	Regulate development of antigen specific B cell development and antibody production	Immune regulation
Pathogens Targeted	Viruses and some intracellular bacteria	Intracellular pathogens	Parasites	Parasites	Fungi and extracellular bacteria		
Harmful Function	Transplant rejection	Autoimmune disease	Allergy, asthma	Allergy	Organ-specific autoimmune disease	Autoimmune disease	Autoimmune disease, cancer
Extracellular Markers	CD8	CD4 CXCR3	CD4 CCR4, Crth2 (human)	CD4	CD4, CCR6	CD4, CXCR5	CD4, CD25
Differentiation Cytokines		IFN- $\gamma$ , IL-2, IL-12, IL-18, IL-27	IL-4, IL-2, IL-33	IL-4, TGF- $\beta$	TGF- $\beta$ , IL-6, IL-1, IL-21, IL-23	IL-12, IL-6	TGF- $\beta$ , IL-12
Effector Cytokines	IFN- $\gamma$ , TNF, LT- $\alpha$	IFN- $\gamma$ , LT- $\alpha$ , TNF	IL-4, IL-5, IL-6, IL-13	IL-9, IL-10	IL-17A, IL-17F, IL-21, IL-22, IL-26, TNF, CCL20	IL-21	TGF- $\beta$ , IL-10
Transcription Factors		T-bet, Stat1, Stat6	GATA3, Stat5, Stat6	GATA3, Smads, Stat6	ROR $\gamma$ t, ROR $\alpha$ , Stat3	Bcl-6, MAF	FoxP3, Smad3, Stat5

### Summary of T cell Subtypes

To support the use of multicolor flow cytometry for the study of T cells, BD offers a deep portfolio of reagents, which are highlighted in red. BD now also offers more choice. Many of these specificities are available in multiple formats including BD Horizon™ V450 and BD Horizon™ V500 formats for use with the violet laser.

### This table summarizes major known T-cell markers.

Markers can be altered as a result of cellular environment, differentiation state, and other factors. Key cytokines appear in **bold**. BD Biosciences offers reagents for molecules in **red**.

Adaptive or inducible Tregs originate from the thymus as single-positive CD4 cells. They differentiate into CD25 and FoxP3 expressing Tregs following adequate antigenic stimulation in the presence of cognate antigen and specialized immunoregulatory cytokines such as TGF- $\beta$ , IL-10, and IL-2. The iTreg population is also reported to be more plastic, with the ability to convert to other T-cell subtypes such as Th1 and Th17 cells.<sup>7</sup>

FoxP3 is currently the most definitive marker for Tregs, although there have been reports of small populations of FoxP3<sup>-</sup> Tregs. The discovery of the transcription factor FoxP3 as a marker for Tregs has allowed scientists to better define these populations, leading to the discovery of additional Treg markers, including CD127. Several published reports in addition to data generated at BD have demonstrated that CD127 expression is inversely correlated with FoxP3.<sup>6,8</sup> The sorting strategy of collecting CD4<sup>+</sup>, CD25<sup>+</sup>, and CD127<sup>-</sup> cells is useful for obtaining viable, expandable Tregs.

## Enrichment of Tregs

Studies by Miyara<sup>9,10</sup> and Hoffmann<sup>11</sup> have found that CD45RA is a useful marker to identify and isolate naïve Treg subpopulations. CD45RA<sup>+</sup> Tregs may be less plastic, maintaining FoxP3 status, post-expansion. CD45RA antibodies are an optimized drop-in in BD Biosciences new sorting kit.

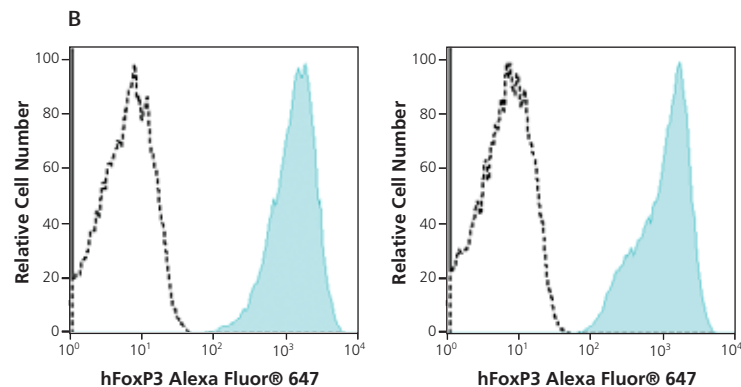
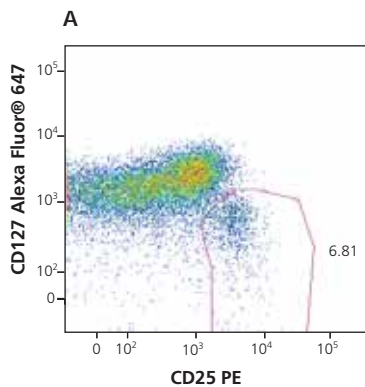
In the experiment below, the CD45RA<sup>+</sup> Treg subpopulation (left histogram, solid blue) showed no tendency to lose its FoxP3 expression. However, unexpectedly, the CD45RA<sup>-</sup> Treg subpopulation (right histogram) did show reduced expression of FoxP3 in some cells. Further research is needed to explore these Treg subsets.

## Four-color analysis of the expression of CD4, CD25, CD127, and CD45RA on sorted peripheral blood mononuclear cells (PBMCs).

PBMCs were stained with the BD Pharmingen Human Regulatory T Cell Sorting Kit (Cat. No. 560753) and then sorted on a BD FACSAria™ cell sorter. Lymphocytes were identified by light scatter profile and CD4<sup>+</sup> expression and sorted for CD4 Treg profile (panel A). The CD45RA negative and positive fractions (data not shown) were sorted, then separately expanded. Fractions were stained with isotype control (Cat. No. 557732) and conjugated anti-human FoxP3 monoclonal antibody (Cat. No. 560045).

**A** Data representing the CD25 and CD127 expression profile of the CD4 positive cells prior to gating on CD45RA populations for sorting.

**B** Data showing hFoxP3 expression on sorted CD25<sup>high</sup>CD127<sup>low</sup> Tregs (blue solid histogram) and isotype control (dashed line) for the CD45RA<sup>+</sup> and CD45RA<sup>-</sup> fractions, respectively. Acquisition and analysis were performed on a BD™ LSR II system.



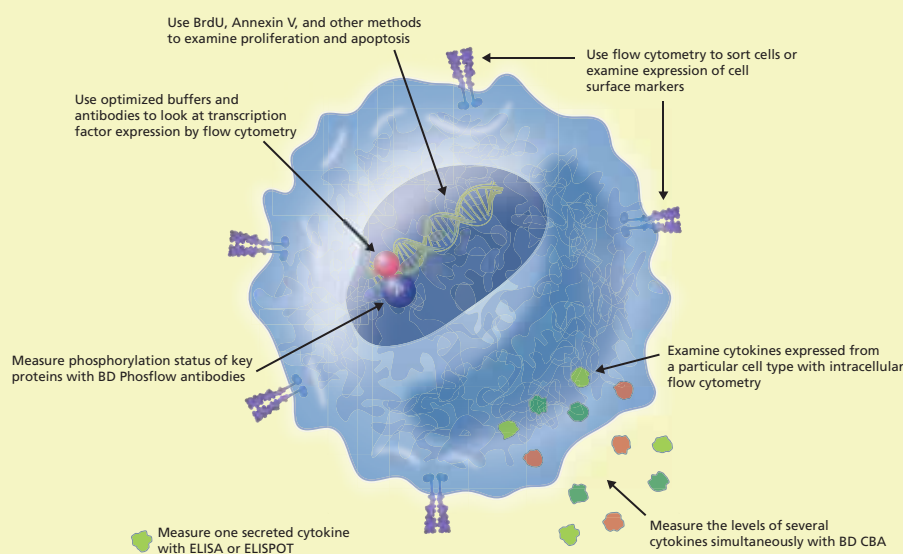
# Leading tools to support and streamline T-cell research

## Tools and Techniques for T-cell Analysis

Donor variability caused by factors such as differences in age or antigen exposure can contribute significantly to heterogeneity in peripheral lymphoid cell populations, including those found in peripheral blood.

BD's comprehensive portfolio of reagents includes products for surface marker analysis for phenotyping cells, and for intracellular flow cytometry for detecting effector molecules (such as cytokines and chemokines) and cell signaling molecules (such as transcription factors and phosphorylated proteins).

BD also provides optimized buffers, fluorescent antibody cocktails, and kits combining surface staining with intracellular flow cytometry to enable researchers to maximize the information obtained from analysis of individual samples.



A variety of tools from BD allow the detailed study of cell populations.

BD products facilitate the detection of cell surface markers, phosphorylated proteins, transcription factors, apoptosis markers, and cytokines. Secreted cytokines can be measured with ELISA or ELISPOT for single cytokines or by CBA for multiplexed assays to measure several cytokines in the same well. Using these techniques, researchers can learn the percentage of a certain type of cell along with its activation status, allowing the effect of minute changes (in protein phosphorylation status, cytokine levels, etc) to be determined within populations of cells.

Tool/Technology	Flow Cytometry/Surface	Flow Cytometry/Intracellular	BD Cytometric Bead Array (CBA)	ELISPOT	ELISA	In Vivo Capture Assay
Molecules detected	Surface	Intracellular and surface	Secreted or intracellular	Secreted (in situ)	Secreted	Secreted (in vivo)
Multiparameter	Yes	Yes	Yes	No	No	No
Single cell/cell subset information	Yes	Yes	No	Frequencies, no subset information	No	No
Antigen specific	Yes	Yes	Yes	Yes	Yes	Yes
Post-assay viability	Yes	No	Yes, for secreted molecules	No	Yes	Yes
Quantitation of protein	Possible*	Possible*	Yes	No	Yes	Yes
Instrumentation	Flow cytometer	Flow cytometer	Flow cytometer	ELISPOT reader	Spectrophotometer	Spectrophotometer

\*With a standard such as BD Quantibrite™ beads

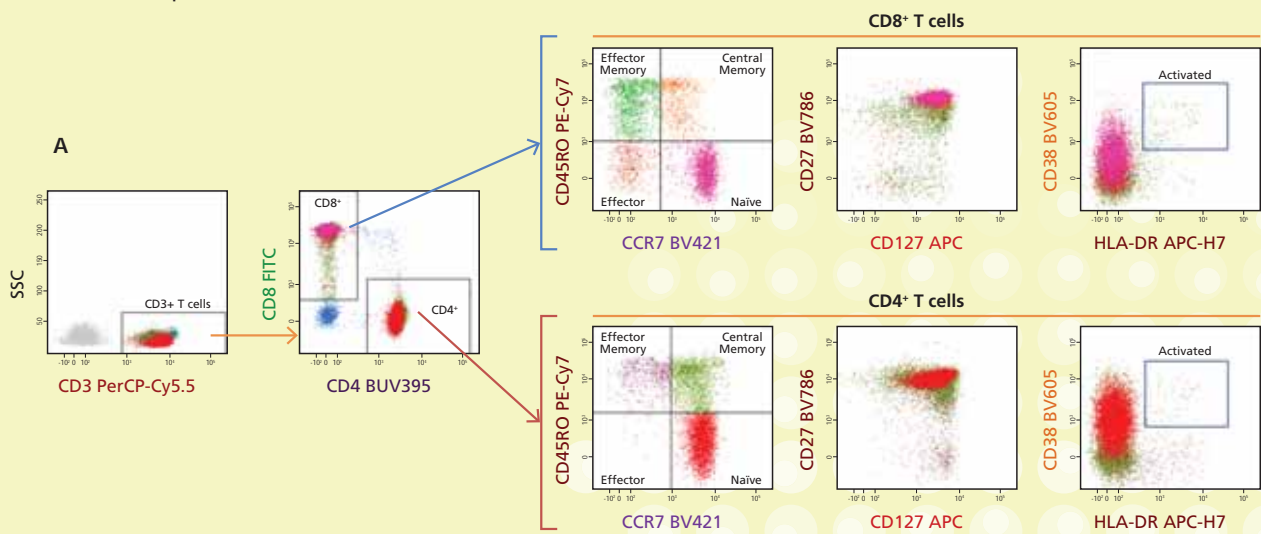
# MULTIPARAMETER

Marker	Fluochrome	Purpose
Viability dye	V500	Viability
CD3	PerCP-Cy5.5	T cell marker
CD4	BUV395	T cell subsetting
CD8	FITC	T cell subsetting
CD127	Alexa Fluor® 647	Regulatory T Cell Marker
HLA-DR	APC-H7	Activation
CD45RO	PE-Cy7	Memory
CD197 (CCR7)	BV421	Naive/Memory
CD38	BV605	Activation
CD27	BV786	Memory
CD25	PE-CF594	Regulatory T Cell Marker/Activation
CD196 (CCR6)	PE	Th17 Cell Marker
CXCR3	Alexa Fluor® 700	Th1 Cell Marker

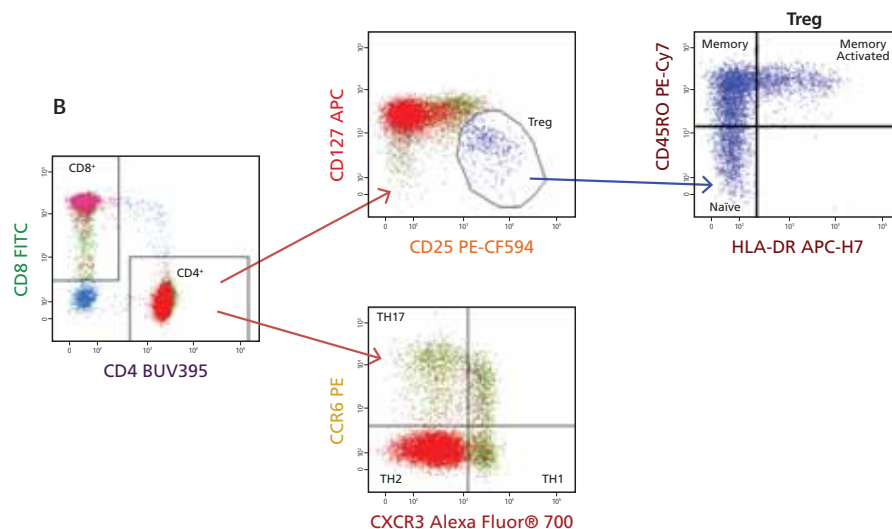
## Phenotyping of Cells with Unique Surface Profiles

T cells and their subsets can be identified by differential expression of cell surface markers including CD3, CD4, CD8, CD25, CD127, and CD196 (CCR6). Adding markers such as CD197 (CCR7), CD62L, CD69, and CD45RO to an analysis provides important information about the potential for cells to home and localize within the body, as well as the activation status of the T-cell subset of interest. This information can also be used to identify different memory subsets.

With the availability of multiple BD Horizon Brilliant Violet and Brilliant Ultraviolet dyes, larger panels can be created that include multiple dim markers. Rich data sets can be obtained from precious samples. The 13-color panel below examines memory and activation status of multiple T-cell subsets.



Data showing whole blood (lysed with BD FACS™ lysing buffer) stained with a 13-color panel to identify various T-cell subsets (A). Tregs and memory cells (B). Acquisition and analysis were performed on a BD LSRFortessa™ system (equipped with 5 lasers).



Obtain the complete picture

# Techniques for the Detection of Secreted Cytokines

Detection of cytokines on an intracellular level provides one useful set of data. To obtain a more complete picture of T-cell cytokine profiles, it is also helpful to quantitate cytokines secreted into the medium.

Cytokines from cell populations can be quantified by techniques such as BD Cytometric Bead Array (CBA) and ELISA. CBA can simultaneously quantify multiple cytokines from the same sample, while ELISA is a useful assay for measuring levels of single cytokines.

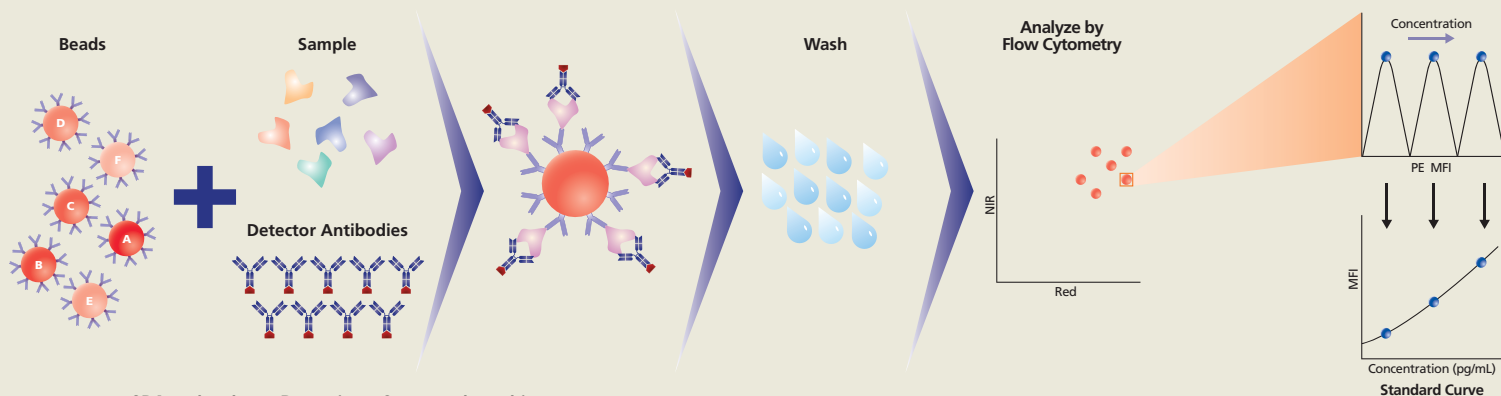
CAPABILITY	CBA	ELISA	ICS
Allows detection of multiple cytokines in same experiment	✓		✓
Can obtain phenotype of specific cells expressing cytokine of interest			✓
Can measure quantity of cytokine secreted	✓	✓	

Comparison of CBA vs ELISA vs intracellular cytokine staining (ICS) for the study of cytokine secretion

CBA is a flow cytometry application that allows users to quantify multiple proteins simultaneously. The CBA system uses the broad dynamic range of fluorescence detection offered by flow cytometry and antibody-coated beads to efficiently capture analytes. Each bead in the array has a unique fluorescence intensity so that beads can be mixed and run simultaneously in a single tube. This method significantly reduces sample requirements and time to results in comparison with traditional ELISA and Western blot techniques.

## Combining CBA and the BD Cytofix/Cytoperm™ System to Determine Th1/Th2/Th17 Cytokine Profiles

Both CBA and intracellular flow cytometry techniques reveal useful information about a sample. The strength of intracellular flow cytometry is its ability to determine the number and phenotype of cells expressing a cytokine from a heterogenous population. The advantage of CBA is the ability to quantitate the levels of multiple cytokines simultaneously. Since CBA detects secreted cytokines in the medium surrounding the cells, the cells can be used for additional experiments. This makes the two methods complementary to one another.



CBA technology: Detection of secreted cytokines.

CBA products, designed for easy and efficient multiplexing, require no assay formulation regardless of plex size. The products deliver quantitative results from a single small-volume sample, and require less time, compared with competitive bead-based immunoassays.



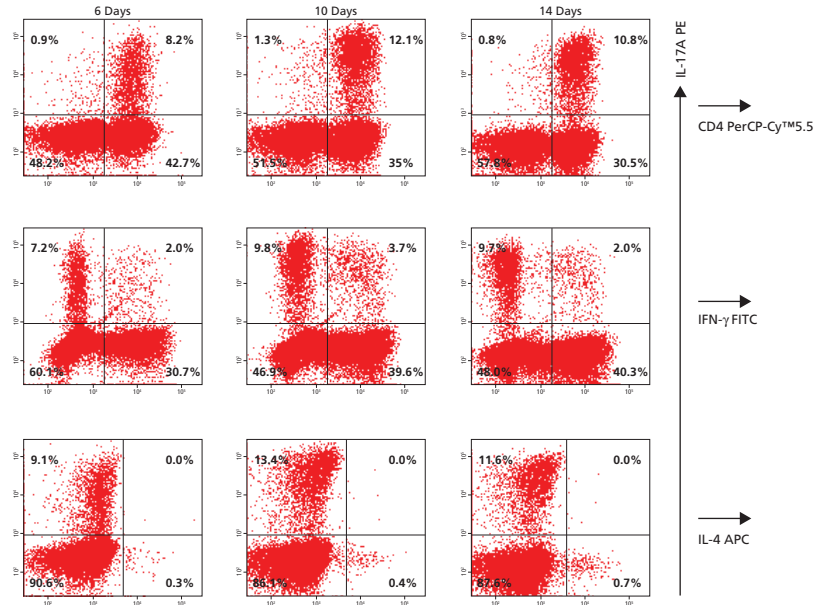
## Combining CBA and Intracellular Flow Cytometry to Examine Th17-cell Differentiation

With both CBA and intracellular cytokine staining (ICS) available, scientists at BD performed an experiment to examine T-cell differentiation, which can be induced by activation and treatment with cytokines. To study Th1/Th2/Th17 cell differentiation, CD4<sup>+</sup>-panned human T cells isolated from normal donors were co-stimulated with CD3/CD28 and:

- IL-2, IL-12, and a neutralizing mAb to IL-4 (Th1 polarization)
- IL-2, IL-4, and a neutralizing mAb to IFN- $\gamma$  (Th2 polarization)
- IL-2, IL-6, IL-1 $\beta$ , TGF- $\beta$ , IL-23, and a neutralizing mAb to IL-4 and IFN- $\gamma$  (also tested with and without IL-2, IL-6, and TGF- $\beta$ ) (Th17 polarization)

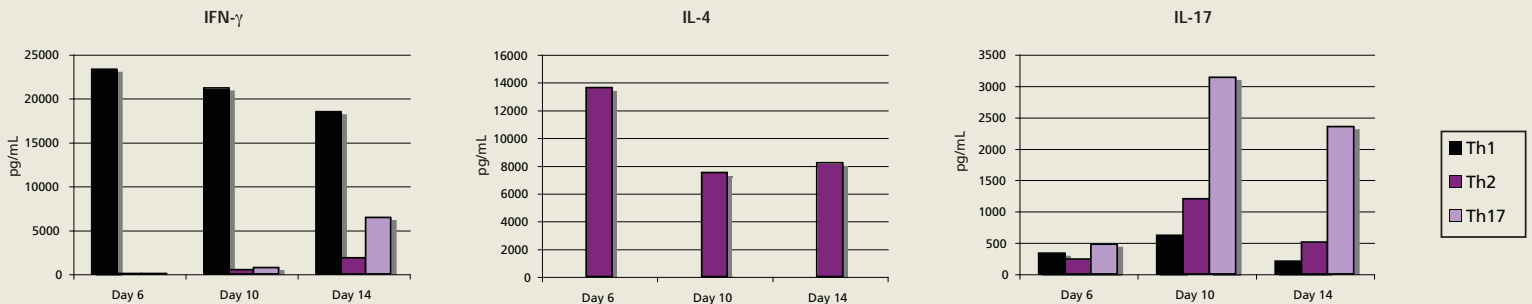
Samples from whole cells and supernatant were collected at the time points indicated, stimulated with PMA/ionomycin, and analyzed by ICS and CBA. Data from the Th17 polarization is shown as an example of ICS, and all three conditions are shown for CBA. Combining these techniques, similar trends were observed when comparing the increase in number of cells expressing the cytokine to the total amount of secreted cytokines.

## Time Course Study of Th17 Cell Population



Representative data from Th17 polarized cell ICS experiments comparing levels of IL-17A with CD4, IFN- $\gamma$ , and IL-4.

Cells were treated under the Th17 polarizing conditions described for the indicated time points. They were treated with BD GolgiStop (monensin) inhibitor, fixed and permeabilized with BD Cytofix/Cytoperm buffer, and then stained with antibodies against the indicated cytokines. At 6 days there were significant numbers of cells expressing IL-17A, with numbers of cells increasing at day 10 and then leveling off.

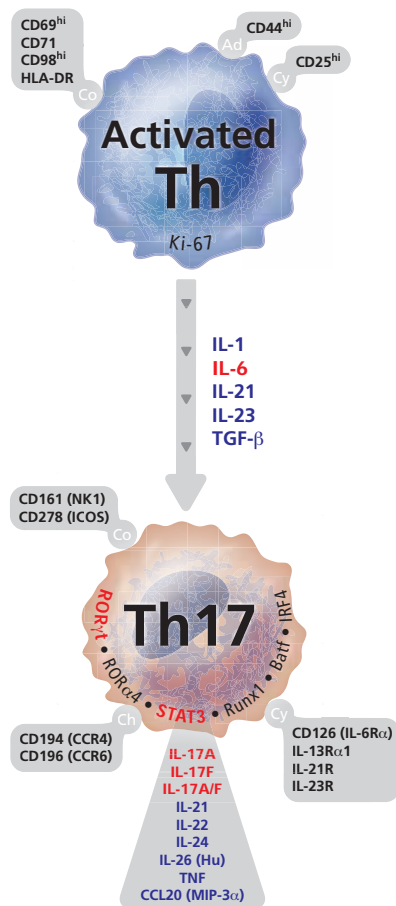


Data comparing cytokine levels as a result of different polarization conditions.

Supernatants from cells were polarized toward a Th1, Th2, or Th17 phenotype and cytokine levels were measured by CBA. As anticipated, each polarized condition resulted in the production of the signature cytokine associated with each Th cell type.

## The importance of phosphoprotein detection

# Tools for Analysis of T-cell Signaling



Signaling pathway for Th17 cell differentiation

IL-6 stimulation leads to the phosphorylation at STAT3. STAT3 in concert with ROR $\gamma$ T induced IL-17, the signature cytokine of Th17 cells.

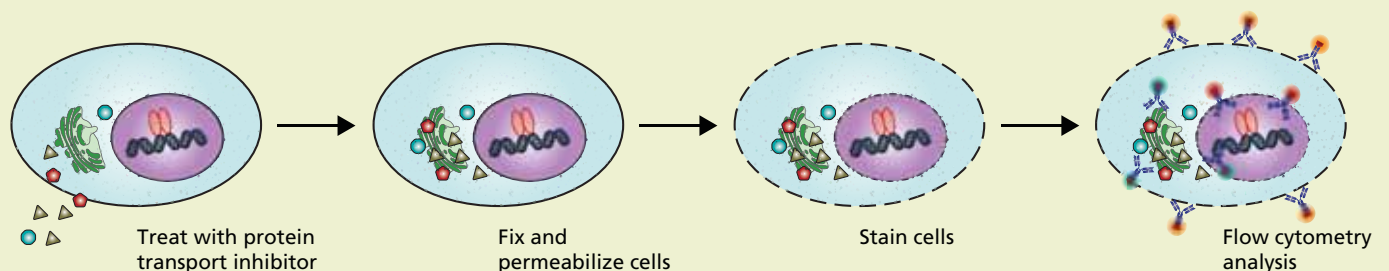
T cells are activated and regulated by complex pathways involving a number of signal transduction molecules, including receptors for antigens and cytokines, kinases, and transcription factors. When foreign antigens enter the body, they are recognized by the innate immune system, which in turn responds with the expression of surface co-stimulatory molecules and the release of cytokines.

These expressed molecules inform the adaptive immune system about the type and strength of the offending pathogen. As a result, naïve CD4<sup>+</sup> T cells differentiate into Th1, Th2, Th9, Th17, Tfh, or Tregs.

Intracellular flow cytometry is a powerful tool for the study of T-cell differentiation. This technique uses small variations to determine the expression of cytokines, transcription factors, and phosphorylated protein. For example, different cytokines bind to their cognate receptors expressed by naïve T cells, which leads to the phosphorylation and dimerization of activating proteins, including Signal Transducers and Activators of Transcription (Stat) proteins.<sup>6,12</sup> Upon phosphorylation and dimerization, activated Stat proteins enter the nucleus and bind to the promoters of many different genes, resulting in the expression of other transcription factors and cytokines specific to a particular T-cell phenotype.

### Basic principles of intracellular staining.

Cells are fixed and permeabilized (symbolized by dashed line membrane), stained, and then analyzed by flow cytometry. For studies of secreted proteins, cells are first treated with a protein transport inhibitor to allow accumulation of the target protein inside the cell.



## BD Kits and Buffer Systems for the Detection of Cytokines, Transcription Factors, and Protein Phosphorylation

Accessing intracellular antigens requires the permeabilization of cells. Methods used to permeabilize cells can lead to the destruction of antigens, particularly on the cell surface. Different antigens require different levels of permeabilization to be accessed. For example, cytokines typically require milder permeabilization than phosphorylated transcription factors that are nuclear and bound to DNA. Optimal results are obtained with the gentlest possible cell permeabilization.

### Detection of Cytokines

BD Cytofix/Cytoperm™ fixation/permeabilization solution (Cat. No. 554722) is suitable for staining most cytokines and cell surface markers. This buffer system can also be used in staining of some transcription factors and other intracellular proteins. This buffer system contains mild detergents along with a formaldehyde-based fixative.

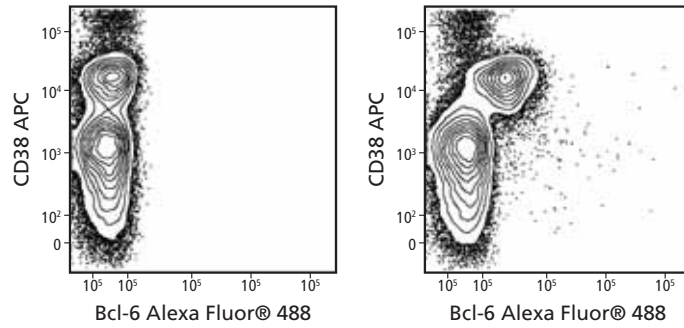
### Transcription Factors

The BD Pharmingen™ transcription factor buffer set (Cat. No. 562574/562725) is designed for the staining of transcription factors alone or in combination with cell surface markers and cytokines. This buffer system contains mild detergents along with a formaldehyde-based fixative.

### Detection of Phosphorylated Protein

BD Phosflow™ perm buffer III (Cat. No. 558050) is the recommended permeabilization buffer for phosphoepitope detection by flow cytometry. Perm buffer III is a harsh alcohol-based buffer. Alternative permeabilization buffers also are available to accommodate particular experimental requirements.

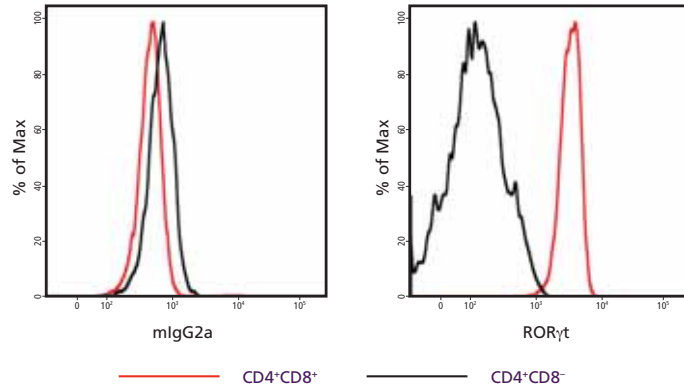
## Human



### Bcl-6 expression in human B lymphocytes

Tonsil cells were stained with BD Horizon™ V450 Anti-Human CD19 (Cat. No. 560353), PE-Cy™7 Anti-Human CD4 (Cat. No. 560649), and APC Anti-Human CD38 (Cat. No. 555462). Contour plots show expression of Bcl-6 vs CD38 by B cells identified as CD4<sup>+</sup>CD19<sup>+</sup> events with the light-scatter characteristics of intact lymphocytes. Bcl-6 staining is observed in CD38<sup>+</sup> germinal center B cells but not in CD38<sup>-</sup> naive B cells or CD38<sup>+</sup> plasma cells.

Data courtesy of Mark Kroenke and Shane Crotty, La Jolla Institute for Allergy & Immunology.



### Buffer comparison for RORγt staining

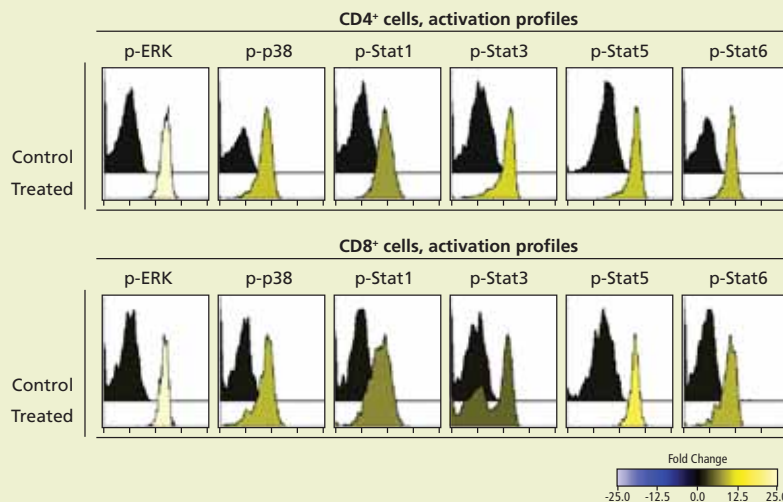
BALB/c mouse thymocytes were fixed/permeabilized with either the BD Cytofix/Cytoperm fixation/permeabilization solution Kit or BD Pharmingen transcription factor buffer set, and stained with RORγt along with CD4 and CD8α antibodies. The overlay histograms show the staining of RORγt in CD4<sup>+</sup>CD8<sup>+</sup> vs CD4<sup>+</sup>CD8<sup>-</sup> thymocytes. Flow cytometry was performed using a BD LSRFortessa system.

Activator (response modifier)	Phosphorylation marker
PMA	ERK 1/2, p38MAPK
hIFN-α	Stat1
hIL-6	Stat3
hIL-2	Stat5
hIL-4	Stat6

### T-cell activation profiles monitored with the BD Phosflow T Cell Activation Kit

The BD Phosflow™ Human T Cell Activation Kit is a comprehensive research system that uses flow cytometry to reliably determine the level of key phosphorylated signaling proteins involved in T-cell activation.

The histogram overlays to the right show CD4<sup>+</sup> and CD8<sup>+</sup> T-cell signaling responses to treatment, monitored using the BD Phosflow Human T Cell Activation Kit. Response modifiers are described in the chart above.



## The importance of differentiation

# Tools for Measuring Treg/Th17 Plasticity

The differentiation of naive T cells into unique subsets was once thought to be irreversible. In the last few years, published reports have demonstrated plasticity among different T-cell subtypes, particularly Tregs and Th17 cells.

Because flow cytometry can look inside the cell, it is well suited to study T-cell plasticity. The data on these two pages shows how ICS, CBA, and BD Phosflow technology together can paint a detailed picture of the mechanisms contributing to Treg/Th17 plasticity.

### Treg and Th17 Differentiation Mechanisms

Both Tregs and Th17 cells require TGF- $\beta$  for induction. Mice lacking TGF- $\beta$  do not have Foxp3<sup>+</sup> Tregs or IL-17 cells, resulting in severe autoimmunity.<sup>13</sup> When antigen activated, naive T cells are exposed to TGF- $\beta$ , and the key transcription factors, Foxp3 for Tregs and ROR $\gamma$ T for Th17, are both expressed. These cells produce less IL-17 compared to cells that do not express Foxp3. One proposed mechanism is that Foxp3 antagonizes IL-17 production induced by ROR $\gamma$ T. Direct intermolecular interactions between a motif on exon 2 of Foxp3 and a conserved domain of both ROR $\alpha$  and ROR $\gamma$ T have been demonstrated.<sup>14</sup>

The amount of TGF- $\beta$  present in combination with other cytokines in the local milieu can influence T-cell fate. High levels of TGF- $\beta$  tend to favor Treg differentiation while lower levels of TGF- $\beta$  in combination with proinflammatory cytokines (eg, IL-1, IL-6) favor Th17 differentiation.<sup>15</sup> These proinflammatory cytokines act through Stat3. Forced expression of the activated form of Stat3 leads to enhanced activation of IL-17. Stat5 is important for Treg development.

### Experimental Design

To illustrate the utility of BD products for the study of Treg/Th17 plasticity, an experiment was performed using intracellular flow cytometry, CBA, and BD Phosflow markers for Th17 and Tregs. CD4<sup>+</sup>-enriched mouse splenocytes were activated with anti-CD3/CD28 and polarized toward a Th17 phenotype by treating them with cytokines as illustrated on the next page. While it is possible to detect Tregs and Th17 cells in the same tube, under these experimental conditions cell polarization toward a Th17 phenotype was observed. Cells co-expressing both Foxp3 and IL-17A were not observed.

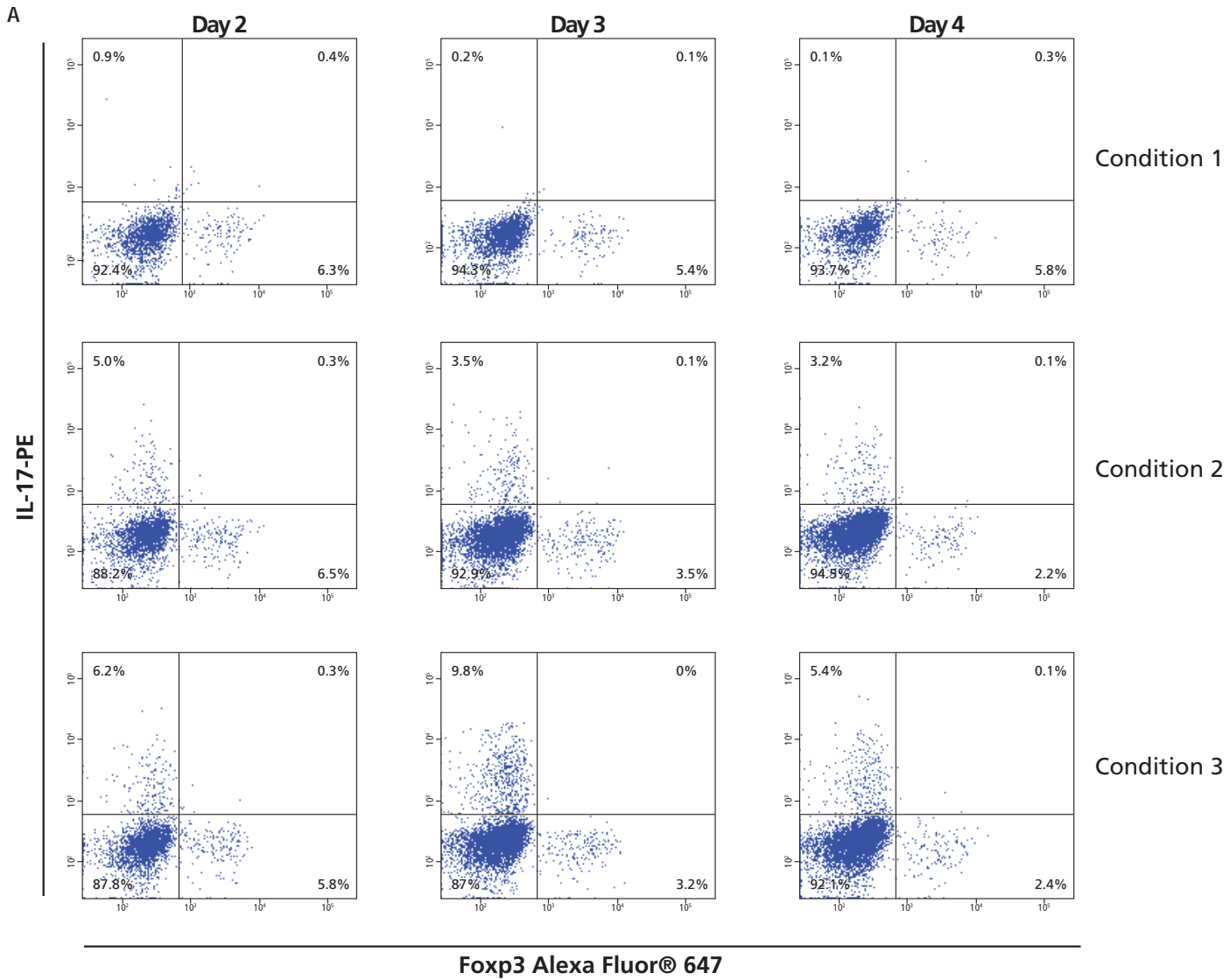
### Comparable studies of Treg/Th17 plasticity

**A** Cells were cultured under the indicated conditions and times, then treated with BD GolgiStop (monensin) inhibitor prior to fixation and permeabilization with mouse Foxp3 buffer. Cells were stained with CD4, IL-17A, and Foxp3. Data is shown starting from day 2.

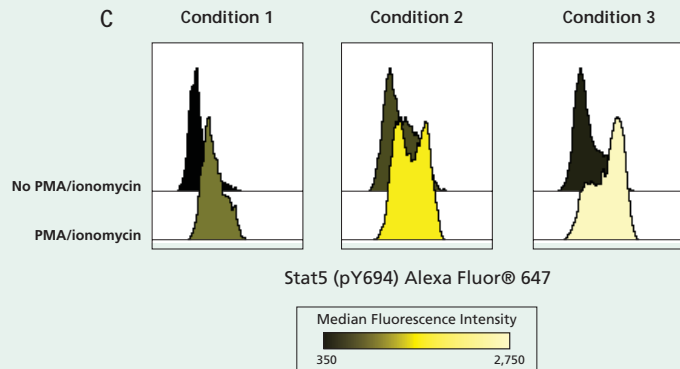
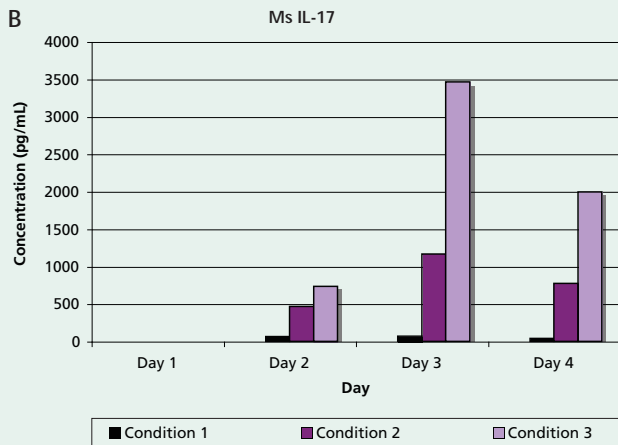
**B** Data from the measurement of IL-17A by CBA from cell supernatants from the indicated culture conditions and time points. No protein transport inhibitor was added to allow secretion of cytokines.

**C** Samples were treated as indicated. On day 4, cells were stimulated with PMA/ionomycin, and phosphorylated Stat5 was measured with BD Phosflow technology using BD Phosflow Permeabilization Buffer III. Flow cytometry was performed on a BD LSR II system. Data was analyzed with Cytobank software, a partner of BD (cytobank.org). No protein transport inhibitor was added to measure the effects of secreted cytokines.

# POLARIZATION



Condition 1: Anti-CD3/CD28 only  
 Condition 2: Anti-CD3/CD28, IL-1 $\beta$ , IL-6, and TGF- $\beta$   
 Condition 3: Anti-CD3/CD28, IL-1 $\beta$ , IL-6, TGF- $\beta$ , and IL-23



## Service and Support

BD Biosciences instruments and reagents are backed by a world-class service and support organization with unmatched flow cytometry experience. For more than 20 years, BD has actively worked with T-cell researchers to develop tools that help improve workflow, ease of use, and performance.

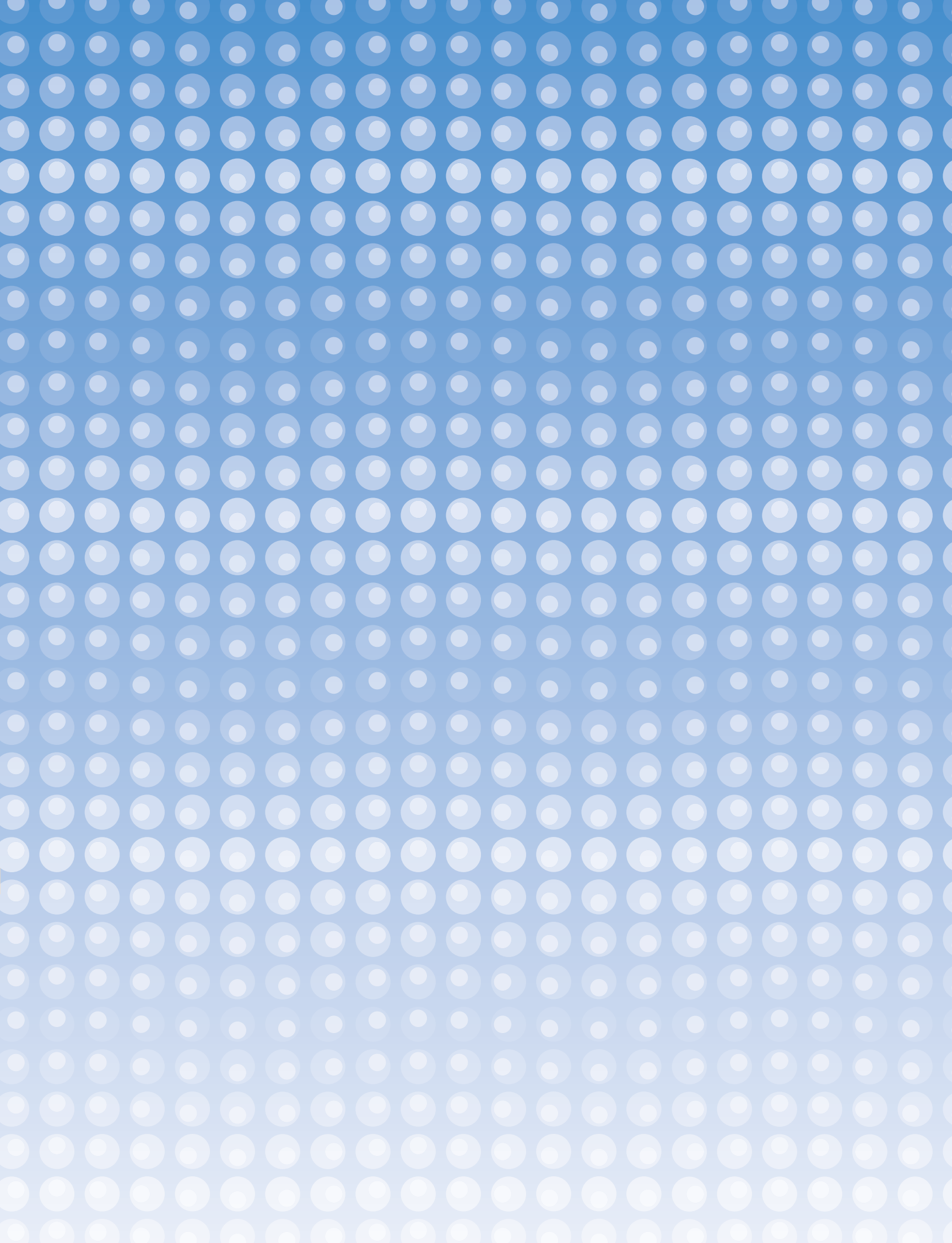
Researchers come to BD Biosciences not only for quality products, but as a trusted lab partner. Our repository of in-depth, up-to-date knowledge and experience is available to customers through comprehensive training, application and technical support, and expert field service.

### Technical Applications Support

BD Biosciences technical applications support specialists are available to provide field- or phone-based assistance and advice. Expert in a diverse array of topics, BD technical application specialists are well equipped to address customer needs in both instrument and application support.

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