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Understanding T Cell Phenotype and Function to Enable Improved Therapeutics

Introduction

The human immune system is made up of a complex network of cells that provide continuous surveillance for anything that is non-self, such as bacteria or viruses. There are two main branches—innate and adaptive immunity—that serve different functions, but work together to overcome the different challenges the body may face. Innate immune cells are responsible for the initial response, rapidly recognizing the antigen, and generating a proinflammatory response to quickly and efficiently contain it. This includes the recruitment of macrophages, neutrophils, monocytes and dendritic cells, which in turn stimulates cells in the adaptive immunity pathway to multiply and differentiate to overcome the specific infection. This adaptive branch is mainly made up of the thymus-derived lymphocytes (T lymphocytes) and bone-marrow-derived lymphocytes (B lymphocytes)[1], and its roles include the elimination of foreign species, the formation of immunological memory, and ensuring tolerance to self-antigens. Natural killer cells (NK cells) lie in between the two branches, as they possess some qualities of both.

Modulation of T Cells in Novel Therapies

T cells are vital for pathogen elimination and tumor immunosurveillance[2], and the body is capable of producing an array of specialized T cells that provide unique responses to the diverse spectrum of tumor cells and pathogens (viruses, bacteria, and parasites) that are capable of penetrating host defenses. Upon elimination of a tumor or pathogen, the majority of adaptive cells die, and memory cells are formed to provide a rapid response should recurrence or reinfection occur. The immune system therefore relies on a fine balance of pathways to regulate immune activation and immune suppression in order to maintain homeostasis. Too much suppression can reduce host tumor immunosurveillance, allowing oncogenic cells to proliferate uncontrollably, while over-activation of the immune system can lead to autoimmune diseases[2].

The modulation of T cells is an exciting prospect for the development of novel therapies for both immuno-oncology—where increasing T cell function may provide a clinical benefit—and autoimmune applications, where suppressing activation is desired. Additionally, modulating T cell receptors (TCRs) and their associated signaling pathways may be of clinical relevance to many further therapeutic areas, such as adoptive cell therapy or vaccine development.

Immuno-Oncology

In the past, cancer treatment was limited to surgery, chemotherapy and radiotherapy, but there have been a number of recent advances in this field and immunotherapy—using the body's immune system against tumors—has become a promising area for research. Several of these novel cancer treatments target T cell inhibitory receptors, including cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1), both of which are immune checkpoints that help inhibit immune response. In individuals with cancer, overexpression of molecules that bind to these and other inhibitory receptors suppresses the immune system, allowing cancer cells to proliferate without threat. Targeting of these inhibitory receptors with anti-CTLA-4 and anti-PD-1 monoclonal antibodies blocks the interaction between the molecule and its receptor, reducing immunosuppression, and has been shown to be beneficial in a number of cancer types[3].

There are nearly 40 kinase inhibitors (KIs) targeting early signaling events during T cell activation already approved by the FDA for use in oncology applications, plus a further 250 or so in clinical trials[4]. Further to this, other key areas of current research include producing chimeric antigen receptor (CAR) T cells—which are raised against over-expressed receptors on the surface of target cells known as tumor associated antigens (TAAs)—or engineering TCR T cells against neoantigens, which can provide an efficient, patient-specific approach to drive cancer immunity.

Autoimmunity

In contrast to the events seen in tumor cells, increased activation and TCR signaling are both major factors in autoimmune disease. Therapeutic approaches that inhibit T cell activation, proliferation and viability, therefore, offer clinical potential[5]. For example, expression of the transcription factor FOXP3 by regulatory T cells has been observed to suppress immune responses and, in the absence of this transcription factor, autoimmunity arises[2]. Heightened T cell kinase activity is also associated with autoimmunity, and KIs are already used for the treatment of a number of diseases, such as rheumatoid arthritis[6].

Stages in T Cell Biology

Thorough evaluation of T cell phenotype and function is critical to understanding their cell biology and building better therapeutics. There has been huge progress over recent years in the characterization of the T cell family, especially from advances in DNA and RNA sequencing[3]. This characterization and evaluation of different T cell subtypes is ongoing, and is contributing to further discoveries in this field.

Subtypes

'T cell' is an overarching term to describe the function of these immune cells, however, there are multiple subtypes with very distinctive roles. Determining the differences and similarities between these cell populations is vital to understand the roles they could play in therapeutics. The two main types of T cells—cytotoxic (T_C) and helper (T_H)—can be broken down into further subtypes, and there are also additional populations, such as invariant natural killer T (iNKT) cells, and mucosal associated invariant T (MAIT) cells.

T_C cells are programmed to kill infected cells and tumors, while T_H cells produce cytokines and chemokines that recruit and activate other immune cell subsets at areas of infection. T_H cells can be split into numerous subsets that are specialized for different effector functions. The main subsets are T_H1 (respond to virus and intracellular bacteria), T_H2 (parasites), T_H17 (extracellular bacteria and fungi), T_H9, T follicular helper cells (T_{FH}), and regulatory T cells (T_{REG}). T cells recognize pathogens by binding with antigen presenting cells (APCs) that process foreign proteins and display antigens complexed with major histocompatibility complexes (MHCs) on their cell surface, where it can interact with TCRs. The recognition of pathogens by APCs leads to the release of cytokines that cause the genetic differentiation of T cells into specific subsets, allowing them to effectively respond to and eliminate the pathogen. For instance, interferon (IFN)-α/β and IL-12 cytokines are generated in response to many viruses, and induce the expression of the transcription factor T-bet, causing T cells to differentiate into the T_H1 subset[2].

Increased or decreased activity of these T_H cell subsets, and the balance between different subset levels, has been linked with different disease states; T_H1, T_H9 and T_H17 are linked to autoimmunity, while T_H2 is associated with asthma and other allergies. For instance, T_H1 and T_H2 cells cross-regulate one another, and T_H1 | T_H2 imbalance with T_H2 dominance can lead to secondary infections, viral reactivations or an inability to clear the initial infection, and is also seen in sepsis. To help maintain this delicate balance, T_{REG} cells control T cell proliferation and cytokine production. As would be expected, T_{REG} cells reduce the chances of autoimmunity by suppressing inflammatory processes, but abnormal functioning of T_{REG} cells is also associated with some tumors[2]. The modulation of different subtypes of T cells, therefore, has therapeutic potential for a range of disease states, making it crucial to identify the most effective points in T cell biology to target.

Activation

T cells emerge from the thymus in a resting, naïve state (T_N cells), unable to produce any kind of immune response[2]. Activation from this naïve state—principally via the TCR pathway—is therefore critical for their proliferation and programming to the correct subtype. This is achieved through a complex series of interactions between the T cell and an APC.

The TCR cannot bind antigens directly, and must be presented with broken-down antigen peptides by the APC using the CD3 receptor and MHC molecules. There are two types of MHC: class I molecules present antigens to the CD8 co-receptor on T_C cells, while MHC class II interacts with the CD4 co-receptor on T_H cells[2]. There is also co-stimulation through a number of other membrane proteins complexes. Binding of antigen-presenting MHC molecules to CD4 | CD8 allows transduction of the signal into the cytosol of the T cell, inducing IL-2 production and secretion and T cell proliferation, resulting in full T cell activation[3].

Killing

CD8+ T_C cells play a primary role in the cytotoxic elimination of infected or cancerous cells, and can interact with almost every cell in the body. Binding with MHC1 triggers the killer function of these cells, with three major mechanisms to kill infected or malignant cells. The primary method is the calcium-dependent release of cytotoxic granules containing perforin and granzyme B into the cytosol of a target cell[3]. Perforin polymerizes to form transmembrane pores in cell membranes, while granzymes initiate an enzyme cascade, with both actions leading to apoptosis. A second method is the secretion of anti-tumor or anti-viral cytokines—mainly TNF-α and IFN-γ—which are normally delivered into the cytosol of the infected cell. The last method is through FasL molecules on activated T_C cells binding to the Fas receptor on target cells. This activates the caspase pathway, initiating apoptosis of the target cell[7].

Memory

Following the primary response and the termination of a foreign body, an extreme decline in the T cell population occurs. At this point, some T cells differentiate into memory T cells that have an ability to 'remember' the foreign body they played a role in removing, with a half-life of around 8–15 years. These cells are divided into two main subsets—central memory (T_{CM}) and effector memory (T_{EM}) cells[2]—and can be activated more easily, with increased proliferative potential, for a more rapid response.

T_{EM} cells have a rapid effector function—producing granzyme B and IFN-γ—but limited proliferation, and

control initial exposure to the reinfection or reemergence of the foreign body. T_{CM} cells display an increased proliferation potential following antigen reencounter, but require more time to proliferate and induce the production of more effector cells that can eliminate the target. While these two subsets give a broad overview, memory cells show a huge plasticity, and should be seen as a spectrum rather than two distinct classes (Figure 1), offering huge potential for the development of T cell therapies[2].

Exhaustion

One hurdle that T cells can face is exhaustion, a hyporesponsive state first identified in mice with chronic infections, and later witnessed in humans with cancer. It is now understood that the majority of T cells differentiate into exhausted T cells in a hostile tumor microenvironment (TME)—an immunosuppressive network of cancer cells, inflammatory cells, stromal cells and cytokines that restricts T cell activation and induces T cell dysfunction[8]. This hostile microenvironment includes immunosuppressive factors—such as vascular endothelial growth factor, transforming growth factor-β (TGF-β) and indoleamine 2,3-dioxygenase (IDO)—released by tumor cells, and regulatory immunosuppressive cells that are recruited into the tumor, such as T_{REG} cells and myeloid-derived suppressor cells (MDSC)[3].

Exhausted T cells show overexpression of inhibitory receptors, including lymphocyte activation gene 3 protein (LAG-3), PD-1, T cell immunoglobulin domain and mucin domain protein 3 (TIM-3), CTLA-4, band T lymphocyte attenuator (BTLA) and T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT)[8]. They also have reduced production of cytokines, a slower rate of proliferation and lower cytotoxicity, all of which leads to cancer evading the immune system. Therapeutic interventions to reverse this exhausted state by blocking inhibitory receptors, therefore, have the potential to restore anti-tumor mechanisms, and represent a promising strategy to treat cancer, with clinical trials already ongoing[8].

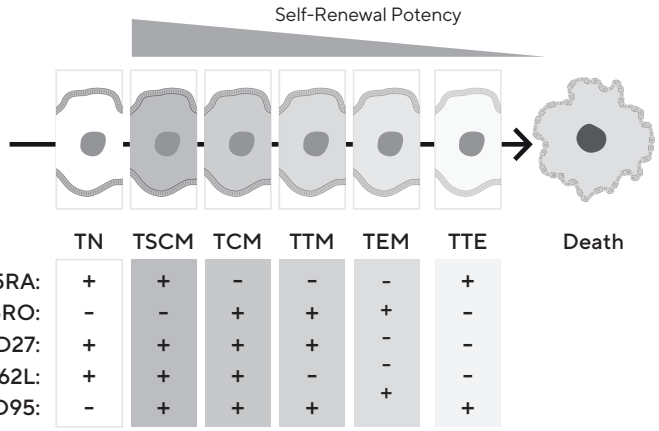


Figure 1: T cell memory development. Schematic showing surface marker expression changes on T cells as they develop into different T memory phenotypes. From naïve (T_N) to stem cell memory (T_{SCM}) to central memory (T_{CM}) to transitional memory (T_{TM}) to effector memory (T_{EM}) to terminal effector (T_{TE}) T cells through to cell death.

A Role for Advanced Flow Cytometry

Understanding the processes controlling the activation, activity, and exhaustion of T cells at the molecular level is fundamental to the identification and validation of novel immunotherapies, creating a need for assays to accurately profile T cell function and health. Immunoassays provide powerful tools to qualify and quantify T cells in both a research and clinical setting, but traditional methods—such as enzyme-linked immunosorbent assays (ELISAs)—only allow the measurement of one parameter or analyte per well[9]. In contrast, flow cytometry can be used to study a wide range of T cell-related applications, from assessing intracellular cytokine production, cellular proliferation and cell viability, to analyzing the cell cycle, rare events, and immunophenotyping. In addition to analyzing intact cells, this approach can be used to measure extracellular analytes of interest, by combining analyte-specific beads with fluorescent detection antibodies. A variety of distinct fluorescent dyes can be used to provide a unique signature for each analyte, allowing multiple parameters to be assessed in a single experiment.

Advanced flow cytometry with the iQue® 3 platform provides a robust, high throughput solution for multiplexed studies of T cells. The iQue® 3 streamlines the flow cytometry workflow, allowing fast sample acquisition in both 96- and 384-well microplate formats, with the ability to connect to automation systems for multiple plate loading—making it ideal for automated, high content applications. Combined with the four of our T cell characterization assay kits (T Cell Activation Cell and Cytokine Profiling Kit (TCA Kit), Human T Cell Mediated Killing Kit, Human T Cell Exhaustion Cell and Cytokine

Profiling Kit, and T Cell Memory Cell and Cytokine Profiling Kit), they collapse the traditional workflow by providing a convenient approach for evaluating cell phenotype, T cell activation markers, cell proliferation, cell viability and secreted cytokines, enabling up to 30 unique analytes to be measured in a single sample[9]. Our T Cell Companion Kits can also be used in combination with the TCA kit and other human T cell immunology kits to measure additional cytokines. This supports a variety of T cell workflows, including antibody screening, functional profiling and cell line development, assessing multiple cell parameters faster, and with fewer cells and less reagents. In addition, it offers the ability to perform high-content phenotypic screening for drug discovery, making it well suited to primary immune cell screening and target identification with siRNA and CRISPR. The case studies discussed here demonstrate the power of using an advanced flow cytometry approach for research on T cell moderation.

Case Study 1: Patient-to-Patient Variability

The dynamics of immune cell activation have been characterized extensively, and events that enable the T cell killing function are well known. However, the balance of factors that result in T cell development, function, and activation is complex, and the significance of these relationships becomes very apparent when comparing blood from different donors, where the same experimental conditions can produce very different T cell behaviors. Here we present work to demonstrate this variability, highlighting the importance of increasing understanding in this area. Peripheral blood mononuclear cells (PBMCs) from two separate donors were isolated and plated at a density of 120 K cells/well, and co-cultured with Nuclight® Green labeled Ramos target cells (effector:target ratio of 3:1). Immune cells were stimulated with increasing concentrations of Dynabeads® Human T-Activator CD3 | CD28 (1.2–360 K beads/well) over a period of 72 hours. Cell and supernatant samples were taken and analyzed on the iQue® 3 using the Human T Cell Mediated Killing Kit.

CD3+ immune cells from both Donor 1 (D1) and Donor 2 (D2) were activated by Dynabeads® in a concentration-dependent manner, where the percentage of CD8 | CD25+ cells increased in response to bead numbers (Figure 2A). The maximal percentage of activated cells was highly comparable (D1 = 96 ±1.5%; D2 = 98 ±0.6%). However, D2's EC_{50} value shifted left by half a log unit when compared to D1 (D1 = 330 K beads; D2 = 136 K beads). Secreted IFN-γ levels were also assessed (Figure 2B), showing a notable difference between the two donors. D1's control levels of IFN-γ were over four-fold higher when compared to D2's. This elevated level of secretion for D1 was also seen in response to low level stimulation with beads. Greater stimulation then caused an increase similar to D2, where the maximal concentration of IFN-γ release was comparable between the two donors (D1 = 38,960 ±301 pg/mL; D2 = 40,380 ±1120 pg/mL).

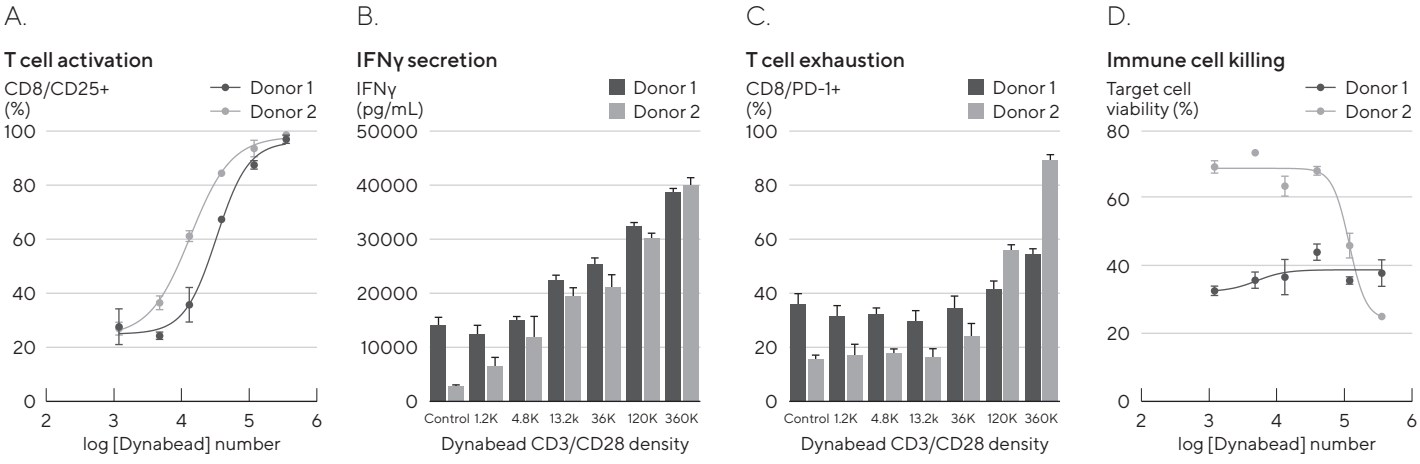


Figure 2: Activation markers and subsequent immune cell killing varies between donors. (A) CD3+ T cell activation showing the % of CD8 | CD25+ cells in response to Dynabead stimulation. (B) Concentration of IFN-γ released into the co-culture assay. (C) Graph showing the % of CD3+ cells expressing exhaustion markers in response to Dynabead activation. (D) Concentration response curves for the % of viable Ramos target cells in relation to the number of Dynabeads® used to activate PBMCs from each donor.

Differences between the two donors were also apparent when comparing the CD3 | CD8 | PD-1+ percentage expression of exhaustion markers (Figure 2C). D1 showed elevated control levels of the exhaustion marker PD-1 that were two-fold greater compared to D2. Levels of expression for D1 did not rise significantly over the control level under stimulation until a bead to immune cell ratio of 3:1 was reached (360 K bead density), and a maximal level of 55 ±1% was observed. D2 CD8 | PD-1+ maximal expression was significantly higher at 90 ±1%. Killing efficacy of the T cells was also assessed where target cell percentage viability was compared between the donors (Figure 2D). D1 displayed elevated levels of immune-mediated target cell death in the presence of low numbers of beads, which resulted in a constant percentage of viable target cells across all bead numbers. Killing was also seen in the control wells containing non-activated PBMCs from D1 (data not shown). In contrast, D2 PBMCs were more sensitive to differences in stimulation, and a concentration-dependent response could be observed.

Overall, Figure 2 illustrates how PBMCs from different donors, stimulated under identical conditions, can result in variations in the levels of activation, killing, and exhaustion. The data here clearly shows how phenotype and cytokine secretion can alter the sensitivity of a particular T cell population to stimulation, which then has a knock-on effect on function.

Figure 3 also demonstrates CD3+ T memory cell development variation between donors under identical stimulation conditions. PBMCs from three separate donors were plated (120 K/well) and activated with increasing concentrations of Dynabeads® CD3 | CD28 (60–480 K/well) over a period of 72 hours. CD3+ T cells were then analyzed on the iQue® using the T Cell Memory Cell and Cytokine Profiling Kit. Upon stimulation with Dynabeads®, T_N cell numbers decreased, as cells were pushed down the development pathway. The Dynabeads® CD3 | CD28 activate through TCRs to produce an effector type function as opposed to memory function, and this was reflected in a concentration-dependent increase in the percentage of T_{EM} cells observed across all donors (Figures 3A to C). However, Donor 2 (D2) had at least 10% greater expression of this subtype when compared to the other donors. This high level appears to be in correlation with IL-10 secretion, which was also significantly higher in D2, and is known to be heavily involved in controlling the immune response. There was also a notable concentration-dependent increase in the T_{TE} cell population across all donors, suggesting this mechanism of activation through the TCR pushes memory T cells towards a more terminally differentiated phenotype, allowing for CD8+ cytotoxic killing.

Throughout the process of therapeutic drug discovery, it is important to be able to build up a picture of the complex factors that are involved in T cell development. Being able to predict using *in vitro* methods which stimuli will result in both successful killing, as well as generating a robust memory response, *in vivo* is very important. Characterization of T cell activation, killing, exhaustion, and memory development are four key areas that provide invaluable data, and the ability to relate changes in phenotype and cytokine secretion to function. This also highlights how small variations in these factors—particularly across donors—can produce discrete but significant results.

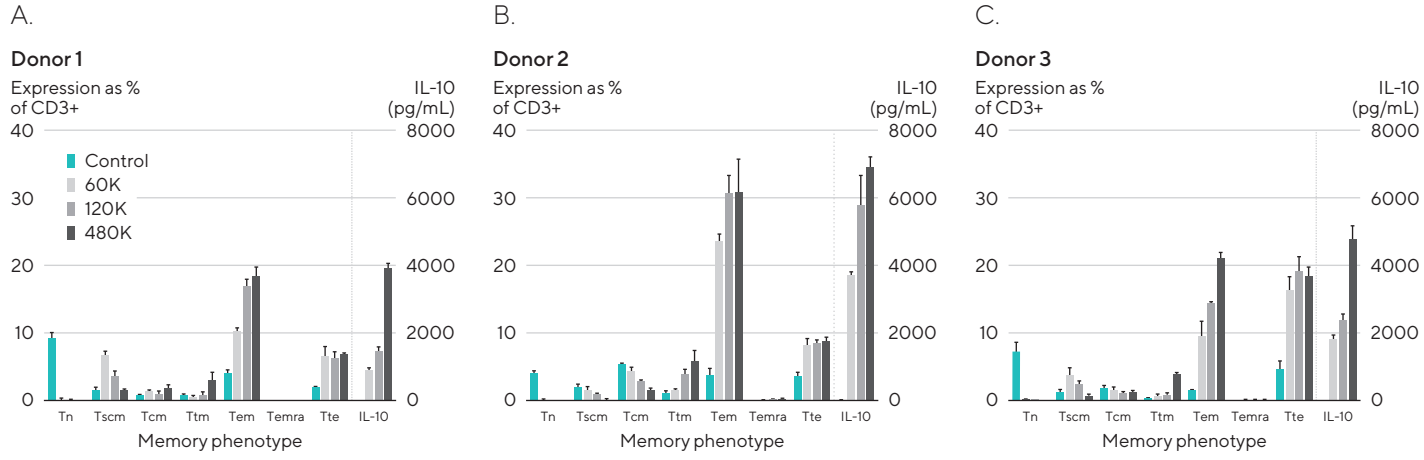
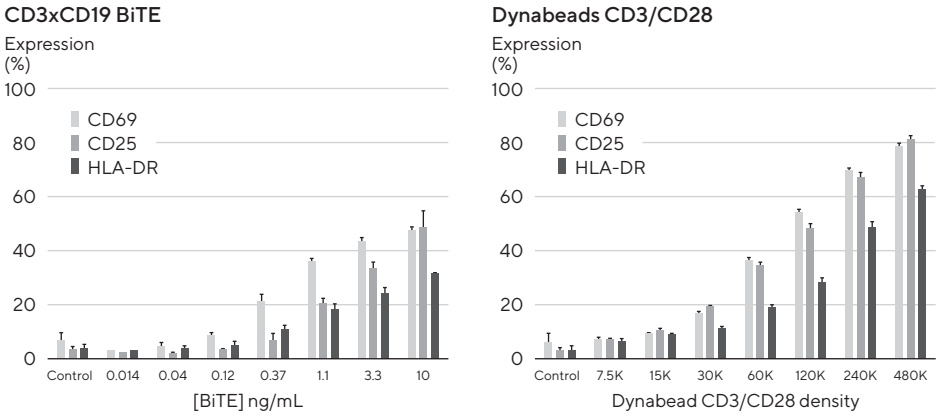


Figure 3: T cell memory development varies between donors. (A) Donor 1, (B) Donor 2, (C) Donor 3 graphs showing % of CD3+ cells expressing proteins from each stage of the T cell memory development. IL-10 release is also shown. Teal bars are controls with no activator. Grey bars represent three ascending concentrations of Dynabeads® (light to dark): 60 K, 120 K and 480 K beads/well.

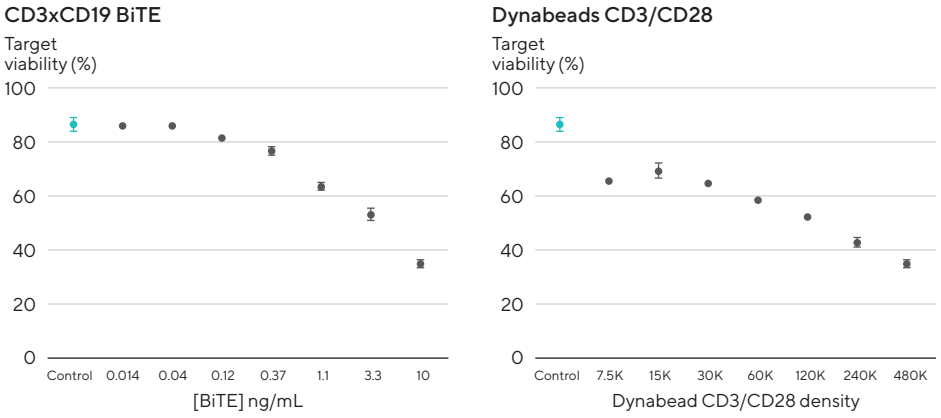
Case Study 2: BiTE® Antibody Evaluation

BiTE® (Bispecific T cell Engager) antibody constructs are a type of fusion protein designed to help fight cancer by harnessing the power of the immune system. There are two arms on the construct, one targets a cell-surface molecule on T cells (commonly CD3), and the other binds to antigens found on malignant tumor cells (e.g., CD19). Upon both arms binding to their targets, a bridge is formed between the T_C cell and the tumor cell, enabling the T_C cell to recognize and attack the tumor cell through the release of cytotoxic molecules. This method of activation is MHC independent, and therefore reduces the issues caused by downregulation of MHCs in many cancer types. The ability to engineer the tumor-binding arm of the molecule to create different antibody constructs allows different types of cancer to be targeted[9]. These cancer-specific activators can reduce overstimulation and the risk of cytokine storms, as well as non-specific tumor cell death when compared to more crude activation types.

Activation



Killing



Exhaustion

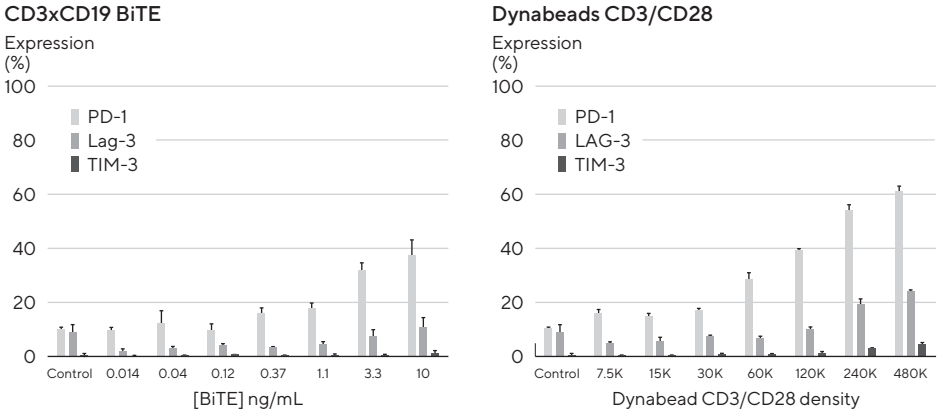


Figure 4: CD3xCD19 BiTE® antibodies induce target cell death, with reduced activation and exhaustion markers compared to Dynabeads® CD3 | CD28 stimulation. PBMCs (120 K/well) were co-cultured with Nuclight® Green labeled Ramos cells (40 K/well). Activation was induced with CD3xCD19 BiTE® antibody or Dynabeads® CD3 | CD28. At 60 h, 10 µL samples were analyzed using the T Cell Activation, Mediated Killing and Exhaustion Kits with the iQue® 3 system.

In this study, the ability of a CD3xCD19 BiTE® to activate PBMCs against a CD19+ B cell lymphoma was compared to the Dynabeads® Human T-Activator CD3 | CD28, which highlighted a multitude of differences in activation, killing, exhaustion, and memory phenotypes. PBMCs (E:T 3:1) were activated in well with activator (7.5–480 K Dynabeads® or 0.014–10 ng/mL BiTE®) and 40 K cell/well Nuclight® Green-labeled Ramos target cells were added. After 60 hours, cell and supernatant samples were taken from the single assay plate and analyzed on the iQue® advanced flow cytometry platform using our T Cell Activation, Killing, Exhaustion, and Memory kits.

Both methods of activation induced concentration-dependent increases in activation and exhaustion marker expression (Figure 4). Maximal marker expression was observed at the highest Dynabead densities (480 K/well), with 64 ±1% CD3+ cells expressing the late activation marker HLA-DR+ and 62 ±2% of cells expressing the early exhaustion marker PD-1+. Activation with CD3xCD19

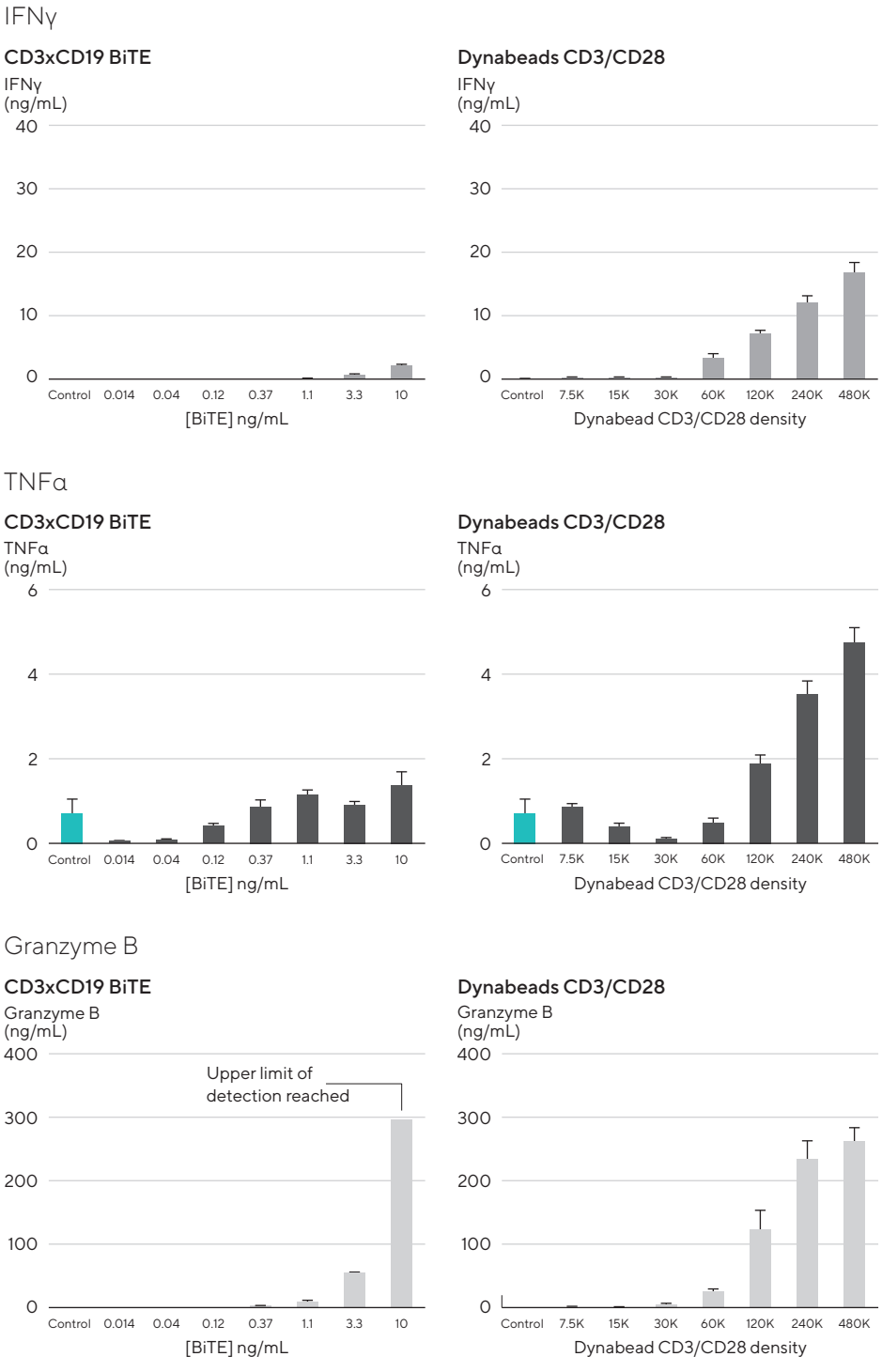


Figure 5: CD3xCD19 BiTE® antibodies induce lower levels of cytokine release. Cytokine release from a co-culture assay containing Nuclight® Green-labeled Ramos cells and PBMCs (3:1 ratio) was measured using Qbeads® from the T Cell Activation, Mediated Killing and Exhaustion Kits. IFN-γ and TNF-α concentrations were measured in multiple kits, and the results from all kits was averaged.

BiTE® also induced concentration-dependent responses in terms of activation and exhaustion, but marker expression was notably less when compared to Dynabeads®. Despite this, a greater increase in target cell killing was seen with the BiTE®, where only 35 ±2% CD19+ Ramos cells were viable after 60 hours. This could also be attributed to the reduced exhaustion marker expression 38 ±5% PD-1+, 11 ±4% LAG-3+ and 2 ±1% TIM-3 on CD3+ cells activated with 10 ng/mL BiTE®.

Cytokine release was also quantified after 60 hours (Figure 5), where a marked increase in proinflammatory (IFN-γ and TNF-α) cytokines was observed when PBMCs were treated with Dynabeads® CD3 | CD28. IFN-γ release was nine-fold lower and TNF-α was four-fold lower in CD3xCD19 BiTE®-treated wells. The ability to measure this *in vitro*, alongside a multitude of protein markers, allows identification of specific activation triggers and the evaluation of overstimulation and cytokine storm risk. Granzyme B is heavily linked with target cell death and is released on interaction of immune cell with target cell, with maximal detectable levels (299 ng/mL) reached using CD3xCD19 BiTE® (10 ng/mL).

Finally, memory phenotypes were assessed (Figure 6). Specific activation using a CD3xCD19 BiTE® leads to increases in stem memory (T_{SCM}), central memory (T_{CM}), and transitional memory (T_{TM}) T cell populations compared to higher levels of Dynabead CD3 | CD28 activation. T_{CM} populations have been shown to increase in a multitude of studies using the clinical grade CD3xCD19 Blinatumomab and, alongside the data presented here, suggests this particular therapeutic can provide long-term memory against CD19+ cancer types[10]. Non-specific Dynabead activation leads to higher proportions of T_{EM} and terminally exhausted (T_{TE}) cells, as described earlier. IL-10 is also produced more readily by cells activated with Dynabeads®.

Comparisons of a specific cancer treatment to a crude activation method highlights the impact these treatment types could have within a patient. This data demonstrates that improvements in killing, reduction in cytokine production, and increase in T cell memory phenotypes can be achieved when using a specific activation method.

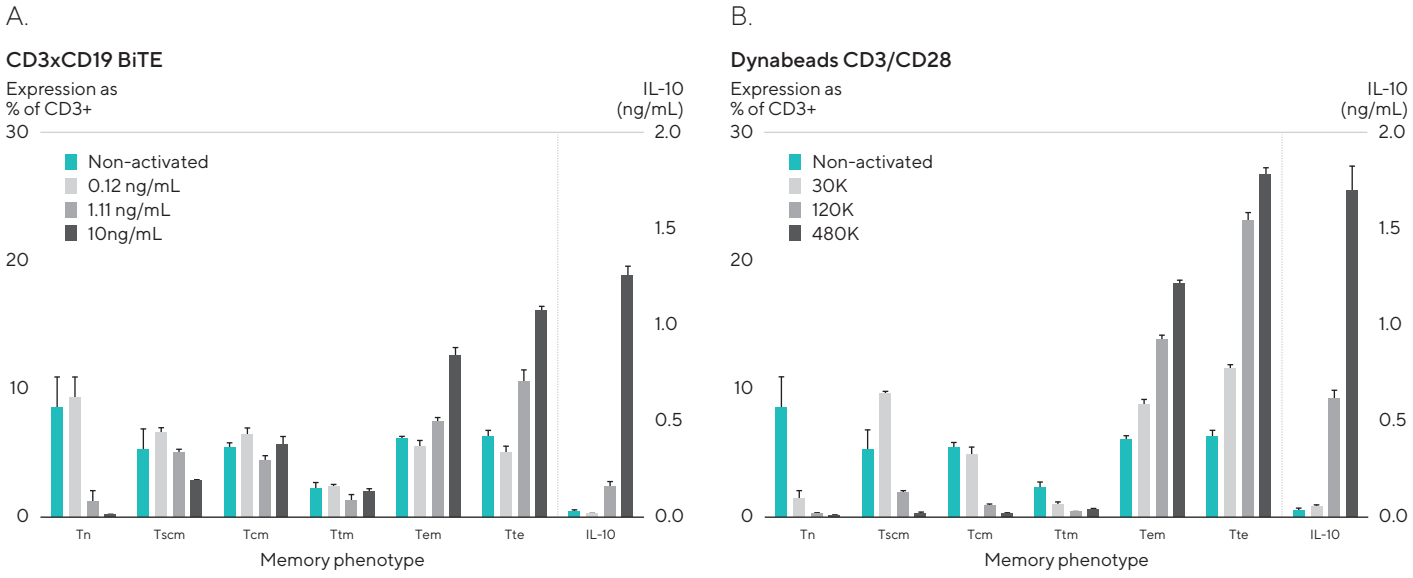


Figure 6: CD3xCD19 BiTE® antibody stimulation induces lower levels of T cell memory transition. Graphs show percentage of CD3+ cells expressing proteins from each stage of the T cell memory development. IL-10 release is also shown. Teal bars are controls with no activator. Grey bars represent three ascending concentrations of each activator (light to dark): (A) CD3xCD19 BiTE® (0.12, 1.1 and 10 ng/mL), (B) CD3 | CD28 Dynabeads® (30 K, 120 K and 480 K beads/well).

Case Study 3: CAR-T Cell Characterization

Immunotherapy has already shown great potential for improving prognosis for cancers. CAR-T therapy is part of a rapidly emerging immunotherapy approach known as adoptive cell transfer (ACT), which uses a patient's own cells to treat their cancer. T_C cells from a patient are transfected with CAR gene constructs, which combine DNA from a number of genes to create a new TCR specific to their tumor type. Upon expressing CARs and reintroduction into the patient, this new receptor binds to antigens found on tumors in an MHC-independent way, activating the T cell, and leading to release of cytolytic granules and proinflammatory cytokines, and the induction of apoptosis[11]. This approach is already in clinical use for several indications, and has shown huge promise[12]. For instance, CAR-T cells that target CD123—found to be overexpressed in most acute myelogenous leukemias and some lymphoid malignancies—has been shown to be effective. However, both primary and adaptive resistance to the therapy can occur[13], requiring an improved understanding of the mechanisms that affect T cell activation and CAR-T cell cytotoxicity. This could offer the potential to modulate pathways using different drugs to help further improve the efficacy of ACT immunotherapies[14].

High throughput flow cytometry is a powerful platform for the analysis of CAR-T therapy, allowing functional characterization of engineered T cells—including the percentage of CAR transduction—alongside simultaneous assessment of T cell-dependent cellular cytotoxicity, T cell phenotype, and T cell function in a single assay. This ability to assess multiple parameters is enabling better *in vitro* evaluation of CAR-T constructs, and platforms such as the iQue® 3 allow rapid sample acquisition and data analysis[11] (Figure 7). For instance, the iQue® 3 has been used to profile the pharmacological effects of over 500 drugs on multiple aspects of T cell function. Using genome-scale CRISPR screens, the removal of genes that impacted CAR-T cells was investigated, with results showing a critical role of death receptor-mediated cytotoxicity through FADD and TNFRSF10B (TRAIL-R2)[14].

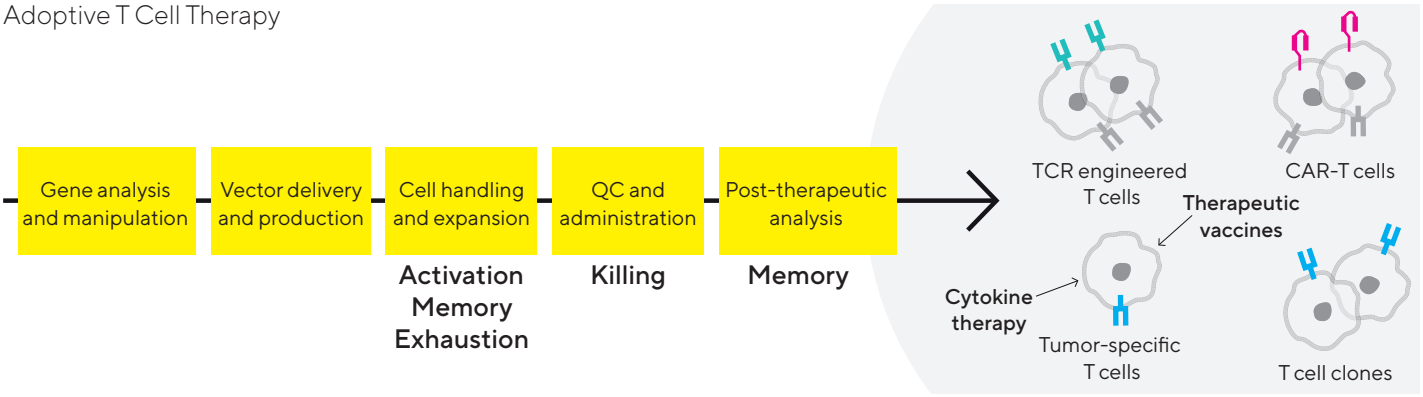


Figure 7: Schematic of the CAR-T workflow. This identifies major decision points in the CAR-T engineering process, and where the T cell characterization kits could be used to enhance immunotherapies.

Future Developments

Understanding the regulation of T cells and their interactions with pathogens or tumor cells is now enabling the development of immunotherapies with high clinical efficacy[3]. The ability to target specific subtypes allows better selectivity or function for the treatment of certain pathologies, and opens up more avenues for engineering immune cells to treat more disease states. For instance, γδ T cells—which have TCRs composed of gamma and delta chains, instead of alpha and beta chains seen in other T cells—have been noted to play a significant role in a number of pathologies, interacting with an array of other immune and non-immune cells to affect host responses to infection, injury or malignancy, as well as having a role in allergic and autoimmune diseases[15]. Controlling human γδ T cell activation has been shown to accelerate pathogen clearance and tissue repair from infections, as well as to improve disease stabilization or promote tumor regression in patients with malignancies, as these cells are able to rapidly produce cytokines such as IFN-γ, TNF-α, IL-17. Unfortunately, the mode of action of the γδ TCR—and its role in the development and triggering of effector functions—is not well understood, especially due to differences displayed between mouse and human cells. This lack of understanding limits translation of research into the clinical setting, and demonstrates the need to increase T cell subtype understanding[12].

Expanding this approach even further to the study of other immune cell types—such as natural killer cells and macrophages—offers additional options for the design of new immunotherapeutic approaches. This is highlighted in the field of oncology, where there is still huge demand for new and improved therapies that can effectively treat a broader range of cancers. Approaches such as tumor-associated APC (tAPC)-reprogramming nanoparticles offer enormous potential, genetically reprogramming cancer cells so that they can act as tAPCs. These particles have already demonstrated cell-mediated cytotoxic immune responses with systemic effects in both *in vitro* and *in vivo* studies, by inducing activation of T_C cells[15].

Monoclonal antibodies are also a popular choice for the development of novel immunotherapies, and T cell characterization could help to improve future treatments (Figure 8). Other cells, such as MAIT cells, have been identified to play a protective role in infections, with cell numbers seen to increase in the lungs and other affected tissues of patients with Mycobacterium tuberculosis (TB). It is thought that these cells actively migrate to the site of inflammation, as MAIT cell numbers are often lower in the blood of patients suffering from inflammatory diseases—such as Crohn's disease, multiple sclerosis and rheumatoid arthritis—but increased in inflamed tissue[16].

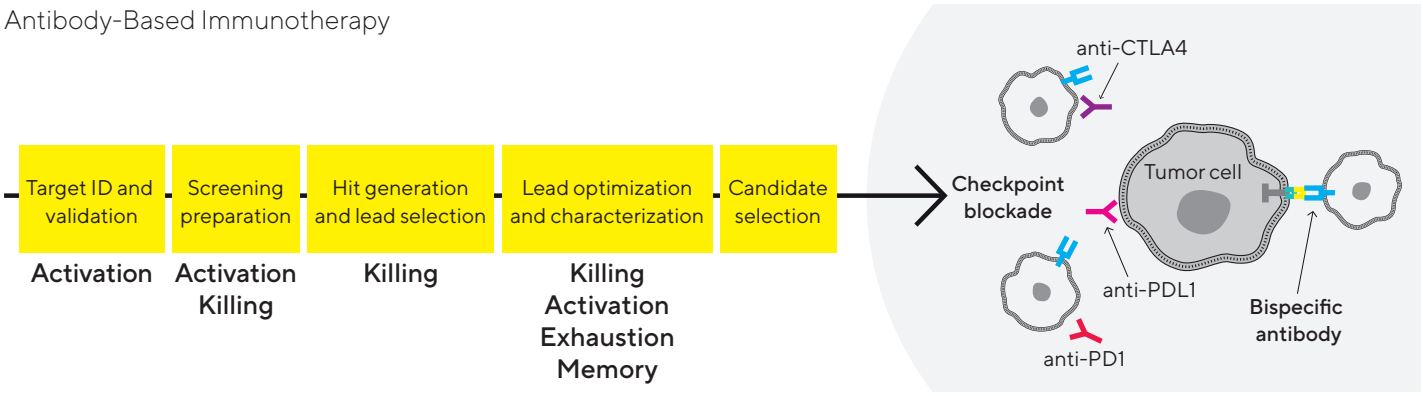


Figure 8: Schematic of the mAbs immunotherapy workflow. This shows major decision points in the therapeutic antibody engineering process and where the T cell characterization kits could be used to progress immunotherapies.

Summary

Furthering our understanding of T cell subtypes, and their phenotypes and functions, is imperative to continue the development of new and improved therapeutic approaches. This will undoubtedly require rapid and reliable profiling of T cell activation and function on a large scale, and advanced flow cytometry offers enormous potential for the characterization, evaluation, and discovery of new modulators for regulating T cell function.

The iQue® 3 advanced flow cytometry platform provides a rapid, high throughput solution to the study or monitoring of T cell function and phenotype, as well as to help identify early biomarkers or perform serological characterizations. This technology is equally relevant to a range of other techniques within T cell immunology, including T cell bioengineering with CRISPR | Cas9. The method offers a rapid, robust, and convenient solution for characterization of cellular responses, and is ideal for assisting in the development of new immunological therapies.

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