Università degli Studi di Napoli "Federico II"

PhD Program in

BIOLOGY

XXXVI Cycle

Development of nanobody-based synthetic modulators to counteract T-cell exhaustion for enhanced T-cell-based immunotherapies

SUPERVISOR
Dr. Velia Siciliano

CANDIDATE
Fabiana Tedeschi

CO-SUPERVISOR
Prof. Caterina Missero

Ph.D. COORDINATOR
Prof. Sergio Esposito

Academic Year 2022-2023

Alla mia famiglia,
che ha sempre creduto in me.

Ad Alessandro,
la scoperta più bella di questo percorso.

A me stessa,
per aver avuto il coraggio
di fare un salto nel vuoto
e lottare per le cose davvero importanti.

Table of contents

Abstract

T-cell exhaustion is a dysfunctional state of $CD8^+$ T cells that occurs during chronic infections or cancer due to chronic antigen stimulation and persistent inflammation. Exhausted T (exT) cells are characterized by progressive loss of effector functions, sustained co-expression of multiple inhibitory receptors (IRs), transcriptional and epigenetic modifications that ultimately result in the inability to mount effective immune responses. Inhibitory receptors, particularly 'Cytotoxic T lymphocyte-associated molecule-4' (CTLA-4) and 'Programmed cell death-1' (PD-1), play a crucial role in the landscape of T-cell exhaustion by negatively regulating T-cell functions. In the past decades, immune checkpoint therapy (ICT) has become the start-of-the-art treatment for T-cell exhaustion and numerous anti CTLA-4 and anti PD-1 monoclonal antibodies (mAbs) have been identified showing promising results in enhancing the anti-tumor immune response and tumor growth control. Nevertheless, only a subset of patients responded to ICT and drug resistance along with immune-related adverse effects have limited the clinical benefits, mostly in solid tumors. Furthermore, mAb-related issues, such as their large size and poor tissue penetration, restricted therapeutic efficacy and applicability of this strategy bringing out the necessity to develop new antibody-based therapeutic agents with improved characteristics for in vivo applications in cancer immunotherapy. Recently, camelid-derived single-domain antibodies (sdAbs), also called nanobodies, have emerged as a promising alternative to mAbs for immunotherapy due to their unique features, such as small size, high solubility, high stability, and excellent tissue penetration in vivo, which make them suitable for therapeutic applications. My PhD project proposes a nanobody-based synthetic biology approach to neutralize T-cell exhaustion and recover full cytotoxic activity for improving T-cell therapies in solid cancers (Figure 1). Specifically, a synthetic system was creating by coupling a sensing module, represented by a synthetic promoter activated by exhaustion-specific transcription factors (exTF), with an actuator module, including genetically encoded anti-exhaustion modulators (Figure 1a). In the detail, my project was focused on the design of actuator modules based on sdAbs targeting CTLA-4 and PD-1 immune checkpoints to restore anti-tumor T-cell activity. T-cell engineering with the synthetic system will allow to achieve a fine regulation of modulator expression and a locally secretion of sdAbs in tumor site avoiding adverse and off-target effects usually due to systemic injection of mAbs (Figure 1b). Our approach for T cell reprogramming has the potential to transform T cell-based immunotherapies since T-cell exhaustion poses a major hurdle in successful anti-tumor treatments limiting clinical efficacy and preventing long-lasting disease control.

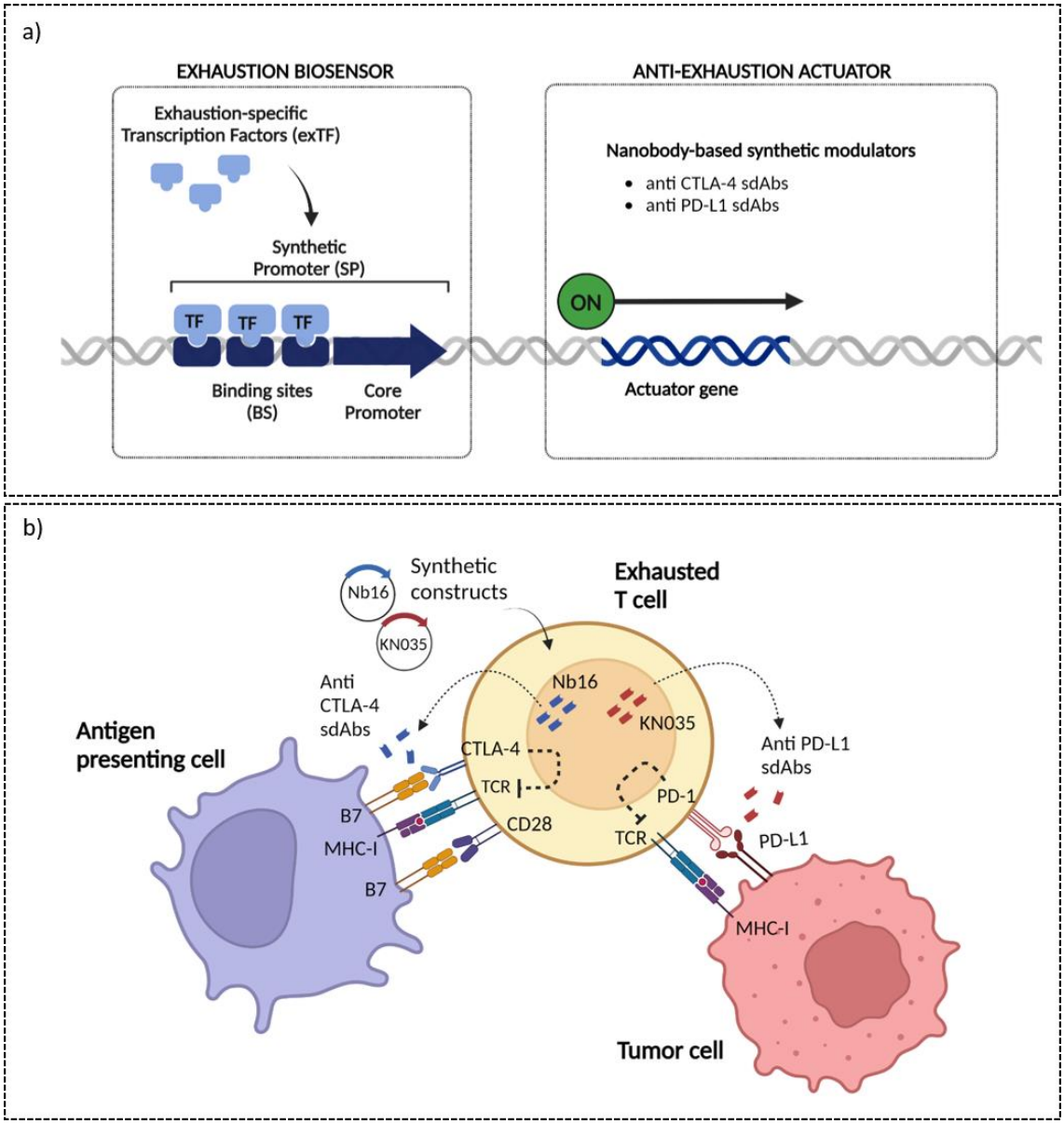


Figure 1 Synthetic biology approach to counteract T-cell exhaustion a) Development of a sensor-actuator synthetic system including a biosensor, based on a synthetic promoter (SP) responsive to exhaustion-specific transcription factors (exTF), and anti-exhaustion activators based on single-domain antibodies (sdAbs). The system will be fine-tuned and activated specifically when exhaustion will onset. **b)** Engineering of T cells with synthetic actuators will enable a local secretion of anti PD-L1 and anti CTLA-4 nanobodies to revert exhaustion and recover T-cell functionality. Created with BioRender.com.

1 Introduction

1.1 Introduction to Synthetic Biology

In 1980, Barbara Hobom introduced the term ‘synthetic biology’ for the first time to describe bacteria genetically engineered using recombinant DNA technology (Hobom 1980). Initially, this term was used to refer to the genetic modification of organisms, which was known as ‘bioengineering’. Synthetic biology was defined as ‘the design and construction of new biological parts, devices, and systems and the redesign of existing, natural biological systems for useful purposes’ (http://syntheticbiology.org). The earliest breakthroughs in synthetic biology have been mainly achieved in prokaryotes and lower eukaryotes, such as *Escherichia coli* and the yeast *Saccharomyces cerevisiae*, due to their more accessibility to precise genetic engineering (Benner and Sismour 2005; Drubin, Way, and Silver 2007; Sprinzak and Elowitz 2005). However, advancements in genomic research and mammalian transcription control technology have led to synthetic biology initiatives in mammalian cells and animals to develop complex multimodal biological circuits for cellular behavior control. Synthetic biology is strongly inspired by electrical engineering, following similar design principles of building complex systems by predictably interconnecting basic modular parts. As electronic circuits, cells function as information-processing systems that integrate and respond to multiple input signals (Ausländer and Fussenegger 2013). In synthetic biology, well-defined and new synthetic biological parts can be rationally reassembled to create complex biological networks to gain a deeper understanding of the fundamental principles of life and to generate novel functionalities in the targeted cells (Hirschi et al. 2022; Menolascina, Siciliano, and Di Bernardo 2012; Benner and Sismour 2005). Over the years, synthetic biology has provided a steadily increasing number of universal genetic and biomolecular tools becoming a powerful and promising platform to understand, control, and create biological systems employable for a variety of applications in industrial processes, environmental preservations, food and agriculture, and biomedicine (Garner 2021; Sargent et al. 2022; Del Valle et al. 2021; Yan et al. 2023; Khalil and Collins 2010).

1.1.1 Synthetic biology in therapeutics and cancer immunotherapy

Through the integration of engineering principles with biological systems, synthetic biology has contributed to the development of sophisticated synthetic biological networks that can improve engineered cell therapies. Current gene therapies are giving promising results in

treating monogenic disorders and hematological malignancies by genetically modifying cells in a therapeutically useful manner (Kohn, Chen, and Spencer 2023; Y. Zhang and Wu 2023). However, these approaches can be used only to overexpress one or a few transgenes, constraining the diseases that can be treated, and do not allow for control of strength, time, and location of the therapeutic effect posing potential risks and uncertainties concerning safety and effectiveness. Synthetic biology methods aim to enable precise control over cellular behavior by fine-tuning gene expression and therapeutic activity in response to biomarkers, thus generating 'programmable' systems for safer and more effective therapies. For therapeutic purposes, sensing and actuating modules are strategically combined to create a responsive biological system capable of generating a desired output based on a given input. Different sensor modules can be used to reshape cellular behavior in response to intracellular or extracellular signals (Manhas et al. 2023), which are then processed and converted to functional outputs by using actuator modules that act on gene expression. To date, several synthetic biology solutions have been developed for the treatment of infectious diseases (Khan et al. 2022) and cancer (Wu, Jusiak, and Lu 2019), as well as approaches in vaccine development (Pfeifer et al. 2023), microbiome engineering (Tan et al. 2021), cell therapy (Kitada et al. 2018; Chakravarti and Wong 2015; Caliendo, Dukhinova, and Siciliano 2019), and rigenerative medicine (Trentesaux et al. 2023). Synthetic biology has found a widespread application in immunotherapy (Chakravarti and Wong 2015), a type of cancer treatment aimed at boosting the immune system to fight cancer, by designing complex synthetic systems that can act synergistically with current therapies to improve them in terms of safety and clinical efficacy. Synthetic circuits have been instrumental in generating novel more powerful engineered T-cell based therapies (Zhao and Cao 2019), including chimeric antigen receptor (CAR) T-cell therapy (Sterner and Sterner 2021), exploiting its ability to create 'programmable' systems by combining several functional modules. For example, innovative CAR designs have been created combining new sensor and actuator modules and implementing genetic circuits with logic gates to achieve precise control of CAR expression and activation to enhance the anti-tumor activity while minimizing side effects (C. Zhang et al. 2022).

1.2 T-cell exhaustion in cancer immunotherapy

1.2.1 Introduction to T-cell exhaustion

T cells play a pivotal role in mounting immune responses and their activation requires two different signals: the T-cell receptor (TCR) signal upon the interaction of the TCR with the MHC-peptide antigen complex expressed on antigen-presenting cells (APCs), and the co-stimulatory signal provided by several T-cell costimulatory receptors after binding to molecules on APCs (von Andrian and Mackay 2000). Both signals are crucial for the activation of naïve T cells and the subsequent anti-tumor responses underlying clinical benefits in cancer immunotherapy. During acute infections, after T-cell activation, $CD8^+$ T cells start to rapidly proliferate and differentiate into effector $CD8^+$ T cells. Effector T cells undergo transcriptional, epigenetic, and metabolic reprogramming becoming capable of migrating to the infection site and producing cytokines and cytotoxic molecules to kill target cells. Following antigen clearance, most activated T lymphocytes die via apoptosis, but a small subset survives and differentiates into long-lived memory $CD8^+$ T cells that confer protective immunity and can persist very long time in the absence of antigen, undergoing slow self-renewal mediated by interleukin-7 (IL-7) and interleukin-15 (IL-15) (Cui and Kaech 2010; Harty and Badovinac 2008; Kaech and Wherry 2007). However, during chronic infections and cancer, $CD8^+$ T cells undergo a dysfunctional state called 'exhaustion' due to persistent antigen stimulation and inflammation (Philip and Schietinger 2022). Exhausted T (exT) cells undergo transcriptional, epigenetic, and metabolic reprogramming and are characterized by a progressive loss of effector functions, low cytokine production, and overexpression of multiple inhibitory receptors, such as PD-1, CTLA-4, TIM-3 and others, which negatively regulate T-cell activity (Yi, Cox, and Zajac 2010; Wherry and Kurachi 2015; Mclane, Abdel-hakeem, and Wherry 2019). T-cell exhaustion was first described in detail in chronic lymphocytic choriomeningitis virus (LCMV) infection model (Zajac et al. 1998), later other studies demonstrated clearly that T-cell exhaustion occurs in many other chronic viral infections (Shankar et al. 2000; Day et al. 2006; Gruener et al. 2001; Ye et al. 2015), as well as autoimmune disorders (McKinney et al. 2015). It is now known that T-cell exhaustion occurs in several human cancers, such as melanoma (Baitsch et al. 2011), ovarian cancer (Matsuzaki et al. 2010), chronic lymphocytic leukemia (Zenz 2013), where dysfunction of tumor-specific T cells contributes to tumor immune escape (Cui and Kaech 2010; Z. Zhang et al. 2020). T-cell exhaustion was discovered and then extensively characterized in $CD8^+$ T cells, however it can also affect $CD4^+$ T cells (Crawford et al. 2014), B cells (Moir and Fauci

2014), natural killer (NK) cells (Bi and Tian 2017), and other immune cells (Catakovic et al. 2017). For the first few years, it was not certain whether T-cell exhaustion was reversible. Then, several studies showed that inhibition of the PD-1 pathway in vivo rejuvenated exT cells leading to enhanced proliferation and effector functions and resulting in an improved control of chronic infections. The capacity of immune checkpoint inhibitors to revitalize exT cells was demonstrated in several chronic infection models (Barber et al. 2006; Kahan, Wherry, and Zajac 2015) as well as in tumor models (Hirano et al. 2005; Curiel et al. 2003; Topalian, Drake, and Pardoll 2012; Iwai, Terawaki, and Honjo 2005; Strome et al. 2003) bringing out the great therapeutic potential of exT cell reinvigoration. Indeed, T-cell exhaustion represents one of the major factors that limit the efficacy of cancer immunotherapy, mainly in the application to solid tumors.

1.2.2 Cellular and functional characterization of exhausted T-cells

ExT cells represent a unique T-cell population that is functionally different from effector and memory T cells with distinct patterns of cytokine receptors, transcription factors, and effector molecules. ExT cells are characterized by peculiar features including hampered cytokine secretion, impaired proliferative potential, reduced tumor-killing activity, and sustained co-expression of multiple inhibitory receptors (IRs) (Blackburn et al. 2009a; McLane, Abdel-Hakeem, and Wherry 2019).

1.2.2.1 Loss of effector functions

ExT cells lose effector functions and other properties in a stepwise manner going through different stages of dysfunction. Interleukin-2 (IL-2) production is one of the first abilities to vanish since it plays a key role in T-cell survival and activation enhancing infection and tumor immune responses. Tumor necrosis factor (TNF) loss also occurs early in the infection process, while the production of interferon-gamma (IFNγ) is more resistant and persists for a more extended period following infection (Yi, Cox, and Zajac 2010). In scenarios involving elevated viral doses or the depletion of $CD4^+$ T cell help, the progressive dysfunction ultimately led to a nearly complete reduction in effector function, culminating in cell death or deletion (Wherry et al. 2003). Due to this physical loss of cells, T-cell exhaustion is also characterized by the inability to generate memory T cells probably due to altered expression of pro- and anti-apoptotic factors as well as an impaired responsiveness to IL-7 and IL-15, which normally regulate T-cell homeostasis (Fuller et al. 2005).

1.2.2.2 Signals contributing to T-cell exhaustion

The development of T-cell exhaustion in chronic infections and cancer is triggered by the presence of different signals:

- **Antigen load**

  Persistent stimulation due to missing antigen clearance is one of the major pro-exhaustion signals leading to hyperstimulation of T-cells. ExT cells gradually lose effector functions and start to overexpress multiple immune checkpoints that prevent the mounting of efficacy immune responses by providing negative regulatory signals on T cells.

- **Soluble mediators**

  In addition to antigen load, virally infected cells or tumor cells contribute to exhaustion by creating a state of inflammation through the production of both proinflammatory and inhibitory cytokines. Among cytokines necessary to achieve an efficient T-cell response, IL-2, a gamma-chain cytokine, augments $CD8^+$ T-cell responses to infections and tumors proving to be essential for T-cell survival and activation, moreover it was the first target used for cancer immunotherapy to recover T-cell functions (Bachmann et al. 2007; Gros et al. 2014; Williams, Tyznik, and Bevan 2006). In the LCMV model, IL-2 delivery increased the number of virus-specific $CD8^+$ T cells and the combination with PD-1 blockade showed a promising synergistic effect (West et al. 2013; Blattman et al. 2003). However, in some persistent infections, IL-2 contributed to the expansion of immunosuppressive T regulatory cells (Tregs) (Boyman, Surh, and Sprent 2006), whereas in HIV infection it enhanced the number of $CD4^+$ T cells but did not significantly affect $CD8^+$ T cells and viral control (Marchetti et al. 2004; Caggiari et al. 2001; Abrams et al. 2009). Therefore, IL-2 has proven to be an interesting target cytokine for immunotherapy, but the controversial results in various infection models point out the need for new strategies to minimize its effects on Tregs and amplify those on exT cells. IL-21, another gamma-chain cytokine like IL-2, emerges as a vital helper factor for the maintenance of $CD8^+$ T-cell responses in chronic infections, as demonstrated by impaired $CD8^+$ T-cell functions and increased exhaustion in lack of IL-21 (Fröhlich et al. 2009; Elsaesser, Sauer, and Brooks 2009). In addition to cytokines sustaining immune response, during chronic infections and cancer, molecules promoting T-cell exhaustion and attenuating T-cell activity are also produced, such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β). IL-10 has been often correlated to exhaustion in chronic infections and cancer (Wilson and Brooks 2011) and several findings demonstrated that IL-10 blockade

prevents T-cell exhaustion facilitating the control and eradication of infections (Brooks et al. 2006; Ejrnaes et al. 2006). In chronic LCMV infection, combining IL-10 and PD-1 blockade has led to persistent and robust effector responses coupled with the recovery of the ability to generate memory T cells and faster antigen clearance (Brooks et al. 2008, 2006). Moreover, combining IL-10 blocking with vaccines has resulted in improved $CD8^+$ and $CD4^+$ T cell responses thereby reducing viral load (Brooks et al. 2008). TGF-β is another cytokine well-known to inhibit T-cell functions and promote T-cell exhaustion in chronic infections through the activation of SMAD-2 signaling that results in increased apoptosis of virus-specific $CD8^+$ T cells due to the upregulation of the pro-apoptotic factor Bim (Derynck and Zhang 2003; Tinoco et al. 2009; M. O. Li et al. 2006). Inhibition of TGF-β signaling led to an increased number and functions of T cells enabling a better control of infection (Tinoco et al. 2009). Given the results achieved by mono- and combined therapies involving cytokines, all these soluble molecules with their respective receptors/signaling represent appealing targets for therapeutic strategies aimed at reversing the effects of the microenvironment on T cell function and exhaustion.

- **$CD4^+$ T cell help**

  $CD4^+$ T cell presence also plays a key role in achieving efficacy $CD8^+$ T cell responses and their absence contributes to exhaustion onset and progression. $CD4^+$ T cells are crucial in orchestrating and optimizing $CD8^+$ T-cell responses during infections by activating antigen-presenting cells, producing supportive cytokines, such as IL-2 and IL-21, and guiding naive and activated T cells to relevant sites (Nakanishi et al. 2009). In chronic infections and cancer, when antigen exposure is persistent, $CD4^+$ T cell help is critical for preventing $CD8^+$ T cells from succumbing to severe exhaustion or deletion (Janssen et al. 2003).

1.2.2.3 Transcriptional profile of exhausted T cells

In addition to impaired effector functions, ExT cells are also characterized by distinct epigenetic and transcriptional patterns compared to effector and memory T cells (Wherry et al. 2007). Transcriptional profiling has shown gene expression alterations associated with exhaustion revealing a distinct biology of transcription factors in T-cell exhaustion. Indeed, exT cells express transcription factors (TFs) commonly used by effector and memory T cells but connect them to different target genes generating unique transcriptional circuits. This may result from the involvement of different cofactors, binding to several gene loci depending on

TF concentration, different TF subcellular localization, or altered gene accessibility due to epigenetic modifications (McLane, Abdel-Hakeem, and Wherry 2019).

Several transcription factors have been identified to have a crucial role in driving T-cell exhaustion, such as T-box expressed in T cells (T-bet), Eomesodermin (Eomes), nuclear factor of activated T cells (NFAT), basic leucine transcription factor, ATF-like (BATF), interferon regulatory factor 4 (IRF4), nuclear receptor subfamily 4 group A (NR4A), and thymocyte selection-associated high mobility group box (TOX).

- **T-bet and Eomes**

  T-bet and Eomes belong to the T-box family of transcription factors and are crucial for T cell differentiation in effector and memory T cells (Zhou et al. 2010; Intlekofer et al. 2005, 2007). In acute infections, these two TFs are upregulated after T cell activation to promote the production of effector molecules, including IFNγ. T-bet is considered a master regulator of cell-mediated immunity and controls the expression of genes encoding effector molecules driving terminal effector cell development (Dominguez et al. 2015), whereas Eomes supports memory T cell generation, quiescence, and homeostasis by enhancing the expression of IL-15 receptor subunit beta (IL-15Rβ). During chronic infections, T-bet and Eomes are upregulated and play different roles compared to effector and memory T cells. High expression of T-bet is typical of intermediate exT cell subset that undergoes low turnover but proliferates in response to antigen, thus generating terminal exT cells that display in turn elevated levels of Eomes. However, several studies showed that the complete elimination of these two TFs does not lead to control of chronic infections highlighting the necessity of a balance between differentiation and renewal, so between T-bet and Eomes expression (McLane, Abdel-Hakeem, and Wherry 2019; Paley et al. 2012).

- **NFAT, BATF and IRF4**

  Physiologically, the NFAT family is involved in the control of T-cell activation and effector cell differentiation by interacting with other transcription factors, such as activator protein-1 (AP-1) (Rao, Luo, and Hogan 1997). NFAT can interact with multiple partners triggering different transcriptional programs based on cell types and signaling contexts. During acute infections, NFAT is activated by TCR-induced calcineurin signaling and, upon translocation into the nucleus, can interact with AP-1 family members forming cooperative NFAT:AP-1 complexes that lead to promoting transcription of effector genes involved in cytokine production and T cell activation. Then, in the late stages of the

immune response, AP-1 expression goes down resulting in an increase of free NFAT that induces suppression of effector and activation signals (Gustavo J. Martinez et al. 2015). During chronic infections, free NFAT accumulates in the nucleus and, binding to the PD-1 promoter, induces the expression of several IRs, such as PD-1, and other genes associated with CD8+ T-cell exhaustion (Gustavo J. Martinez et al. 2015). Conversely, lack of NFAT or inhibition of its nuclear translocation through the calcineurin inhibitor FK506 results in a reduced expression of immune checkpoints associated with T-cell exhaustion, such as PD-1 and LAG-3 (Lin et al. 2023). NFAT, with BATF and IRF4, establishes a transcriptional circuit that promotes T-cell exhaustion during chronic infection by inducing transcription of *Pdcd1* with consequent PD-1 production (Man et al. 2017). BATF belongs to the AP-1 family of transcription factors and, after dimerization with JunB, can inhibit the activity of other AP-1 transcription factors thus promoting T-cell exhaustion. On the contrary, silencing BATF has been found to reverse the function of exhausted T cells in patients with HIV (Quigley et al. 2010). IRF4 is a TCR signaling-sensitive transcription factor and is highly expressed in exT cells inducing IR expression, impaired cytokine secretion, and anabolism (Man et al. 2017, 2013).

- **NR4A and TOX**

  NR4A family and TOX can be activated by NFAT and contribute to T-cell exhaustion by inducing the expression of immune checkpoints and inhibiting effector molecules (Seo et al., 2019). Knockout studies of NR4A and TOX in CD8+ T cells showed promising results in inhibiting T-cell exhaustion and enhancing antitumor responses (X. Liu et al. 2019; Alfei et al. 2019), suggesting the therapeutic potential to block the NFAT-NR4A and NFAT-TOX pathways to reverse exhaustion.

1.2.2.4 Immune checkpoints in T-cell exhaustion

Over-expression of inhibitory receptors (IRs) is considered a hallmark of T-cell exhaustion and represents one of the mechanisms most used by tumor cells to escape the immune system. Physiologically, IRs act as immune checkpoints that fine-tune the degree of immune response for maintaining self-tolerance and preventing autoimmune disease. During T cell activation, they are transiently expressed on functional effector T cells playing a role in the regulation and termination of immune response after antigen elimination to prevent excessive inflammation and tissue damage. However, during chronic infections and cancer, multiple IRs are overexpressed on CD8+ and CD4+ T cells (36–39) and negatively regulate T-cell activity, thus preventing the mounting of powerful immune responses (Odorizzi and Wherry 2012).

Inhibitory pathways have gained ever-increasing interest in the oncology field since their significant impact on the multifaceted landscape of T-cell exhaustion in cancer and over the years numerous immune checkpoint molecules have been identified, showing that the severity of exhaustion is intricately linked to the number of expressed receptors and the level of expression for each receptor (Blackburn et al. 2009b). To date, it is known that IRs can exert their negative regulation of T cell function through several mechanisms (Chemnitz, Riley, et al. 2004). IRs can suppress activation signals originating from TCR and co-stimulatory receptors by directly targeting the TCR or through sequence motifs in their cytoplasmic tails to block the downstream signaling pathways (Chemnitz, Parry, et al. 2004). Additionally, IRs can attenuate T cell activation preventing the co-stimulatory signaling by sequestering ligands of co-stimulatory receptors via direct binding (Kosmaczewska et al. 2001). Lastly, recent genomic analyses showed that IRs can upregulate genes involved in T cell dysfunction, as PD-1 upregulates the expression of the transcription factor basic leucine transcription factor, ATF-like (BATF) impairing T cell proliferation and cytokine secretion (Quigley et al. 2010). Programmed cell death receptor-1 (PD-1) was one of the first IRs identified in T-cell exhaustion, then global transcriptional profiling of exhausted $CD8^+$ T cells enabled the discovery of other inhibitory receptors upregulated on T cells, including but not limited to Cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), Lymphocyte activation gene-3 (LAG-3) (Guy et al. 2022; Maruhashi et al. 2020a), T cell immunoglobulin-3 (TIM-3) (Acharya, Sabatos-Peyton, and Anderson 2020a), and T cell immunoglobulin and ITIM domain (TIGIT) (Chauvin and Zarour 2020a).

- **Programmed cell death 1 (PD-1)**

The inhibitory receptor PD-1 is a surface receptor transiently expressed by functional effector T cells during T cell activation, but in cancer, its sustained overexpression on activated T cells plays a key role in inducing T-cell exhaustion by inhibiting TCR signaling to regulate the effector phase of T cell response. PD-1 is a type I transmembrane glycoprotein and its structure includes a single extracellular IgV domain, a hydrophobic transmembrane domain, and a cytoplasmic tail that contains two tyrosine motifs, an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM), this latter crucial for the immunosuppressive function of the receptor (Ishida et al. 1992). PD-L1 and PD-L2 are the ligands of PD-1, belonging to the protein B7 family (Latchman et al. 2001; Freeman et al. 2000). PD-L1 expression has been reported in tumor microenvironments on tumor cells and other intratumoral cell types, whereas PD-L2 is expressed in APCs. PD-1:PD-

L1 interaction induces the ITSM phosphorylation, which leads to the inhibition of phosphatidylinositol-3-kinase (PI3K) signaling by the recruitment of the Src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP-2) (Yokosuka et al. 2012; Chemnitz, Parry, et al. 2004; Chemnitz, Riley, et al. 2004). This results in the inhibition of T cell biological functions, such as lymphocyte proliferation, inflammatory cytokine secretion, and cytotoxic T lymphocyte (CTL) cytotoxicity, and the promotion of T cell exhaustion and apoptosis, thus enabling tumor immune escape (L. Zhang, Gajewski, and Kline 2009; Blank, Gajewski, and Mackensen 2005).

- **Cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4)**

CTLA-4 is a central immune checkpoint for the regulation of immune response. It acts in the earliest stages of naïve T cell activation, typically in lymph nodes, to prevent the generation of autoreactive T cells and its activity is mainly regulated by different localizations inside the T cells: in resting naïve T cells, the majority of CTLA-4 is expressed intracellularly, whereas TCR and CD28 signaling promote this receptor translocation to the cell surface (Linsley et al. 1996). In cancer, CTLA-4 is overexpressed on the T cell surface due to the persistent antigen stimulation and acts impairing the CD28 signaling by two different mechanisms: competing with CD28 for the binding to the same ligands, B7-1 (CD80) and B7-2 (CD86), thus inhibiting T cell activation (Chambers et al. 2001; Krummel and Allison 1996; Chemnitz, Riley, et al. 2004), and capturing CD80/CD86 from APCs by trans-endocytosis to induce their degradation inside CTLA-4 expressing cells, thus preventing the co-stimulatory signaling via CD28 (Qureshi et al. 2011). Additionally, CTLA-4 is constitutively expressed by regulatory T cells (Tregs) promoting their suppressive functions on effector T cells. The hypothesis is that Tregs exploit surface CTLA-4 expression to downregulate B7 molecules on the APCs to reduce the co-stimulatory signaling, thus reducing activation of naïve T cells (Wing et al. 2008).

- **Lymphocyte-activated gene-3 (LAG-3)**

The cell surface protein Lymphocyte-activated gene-3 (LAG-3) is an additional inhibitory receptor with a key role in the regulation of immune response and subsequently in the context of T-cell exhaustion (Maruhashi et al. 2020b). LAG-3 is notable for its transient expression on $CD8^+$ and $CD4^+$ T cell surface upon T-cell activation, followed by internalization and lysosomal degradation (Bae et al. 2014). LAG-3 expression has been also detected in B cells, NK cells, plasmacytoid dendritic cells, $CD4^+$ type 1 T regulatory (Tr1) cells, and Tregs (C. T. Huang et al. 2004). Some exhaustion-specific transcription factors, including thymocyte selection-associated high mobility group box protein (TOX), nuclear factor of activated T

cells (NFAT), and nuclear receptor subfamily 4, group A (NR4A), have been demonstrated to induce an increase of LAG- 3 when they are overexpressed in T cells. On the contrary, T-box expressed in T cells (T-bet) induces repression of LAG-3 and other inhibitory coreceptors sustaining antigen-specific response of $CD8^+$ T cells (Rudd, Chanthong, and Taylor 2020; Seo et al. 2019a; Kao et al. 2011). Structurally, LAG-3 demonstrates similarities to CD4 and exhibits a heightened affinity for binding to major histocompatibility complex class II (MHCII) compared to CD4. On the T-cell surface, LAG-3 co-localizes with TCR/CD3 complex, binding to MHCII. However, LAG-3 distinctively impacts MHCII downstream signaling through its cytoplasmic KIEELE motif, a highly unique and conserved six amino acid sequence essential for interaction with downstream signaling molecules, without disrupting CD4-MHCII engagement (Huard et al. 1994). This unique modulation of signaling cascades by LAG-3 contributes significantly to the negative regulation of T-cell activation and effector functions. As a result, LAG-3 emerges as a crucial factor in the intricate network of immune regulation, particularly in scenarios of T-cell exhaustion, where immune responses need to be finely tuned for effective control of chronic infections or malignancies.

- **T-cell immunoglobulin and mucin-containing protein 3 (TIM3)**

The cell surface receptor T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) is a critical regulator in the complex landscape of T-cell exhaustion. TIM-3 expression is regulated by the transcription factor T-bet (Anderson et al. 2010) and is present in various T cell populations, including T helper 1 (Th1) cells, $CD8^+$ T cells, and Tregs, as well as on dendritic cells (DCs), macrophages, and monocytes. Unlike other inhibitory receptors such as PD-1 or TIGIT, TIM-3 lacks an ITIM motif in its intracellular domain. TIM-3 interacts with the soluble molecule S-type lectin Galectin-9 (Gal-9), whose expression is induced by interferon-gamma (IFNγ), triggering the recruitment of the Src family tyrosine kinase Fyn and the p85 PI3K adaptor (Sehrawat et al. 2010). This results in impairment of Th1 immune response characterized by decreased IFNγ production and heightened apoptosis in Th1 and cytotoxic CD8+ T cells when examined in vitro (C. Zhu et al. 2005; Asakura et al. 2002). Beyond Gal-9, TIM-3 engages with other ligands, including carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) (Y.-H. Huang et al. 2015), High mobility group box 1 (HMGB1) (Chiba et al. 2012), and phosphatidylserine (Lee et al. 2011). These diverse ligand interactions extend the regulatory impact of TIM-3 across various immune cell types and signaling pathways. A comprehension of the complex network involving TIM-3 signaling and its effects on immune responses provides valuable insights for potential therapeutic strategies,

especially concerning the modulation of Th1-mediated immunity and T-cell functionality (Acharya, Sabatos-Peyton, and Anderson 2020a).

- **T cell immunoreceptor with Ig and ITIM domains (TIGIT)**

Several studies have described TIGIT as a key co-inhibitory receptor in the dysfunction of chronically stimulated $CD8^+$ T cells. TIGIT is a type 1 transmembrane protein presenting an immunoglobulin tail tyrosine (ITT)-like phosphorylation motif and ITIM inside the intracellular domain and is expressed by several T-cell types, such as follicular helper T cells (TFH), Tregs, activated and memory T cells, natural killer (NK) and natural killer T (NKT) cells (Godefroy et al. 2015; Johnston et al. 2014; Stanietsky et al. 2009; S. Liu et al. 2013), under the control of the transcription factor FoxP3. The binding of TIGIT to its ligands induces the phosphorylation of the cytoplasmic tail with subsequent interaction with the cytosolic adaptor growth factor receptor-bound protein 2 (Grb2) and recruitment of Src homology 2 (SH2)-containing inositol phosphate-1 (SHIP-1), that results in the inhibition of PI3K and mitogen-activated protein kinase (MAPK) signaling pathways (S. Liu et al. 2013). Additionally, the interaction of the phosphorylated TIGIT with beta-arrestin 2 prevents the nuclear factor kappa B (NF-κB) activation by suppressing the auto-ubiquitination of the tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF-6) (M. Li et al. 2014; S. Liu et al. 2013). TIGIT has several ligands, including poliovirus receptor (PVR) and CD155 that are expressed on APCs and several tumor cells. Similarly to CTLA-4, it exerts its inhibitory function by blocking co-stimulatory signaling needed for T-cell activity. TIGIT prevents the CD226-mediated activation signal by binding with higher affinity to the same ligands of this co-stimulatory receptor, PVR and nectin-2, and directly disrupting the homodimerization and function of CD226, thus limiting effector functions of chronically stimulated $CD8^+$ T cells (Yeo et al. 2021; Johnston et al. 2014). These results have confirmed TIGIT's role in the regulation of immune response making it a potential target for immunotherapy to restore functional T cells in cancer.

1.2.3 Neutralization of T-cell exhaustion for enhanced immunotherapy

Numerous preclinical and clinical studies have demonstrated the presence of exT cells in several cancers highlighting a close correlation between exhaustion and poor clinical outcomes (Chow et al. 2022; von Andrian and Mackay 2000). Indeed, T-cell exhaustion plays a pivotal role in limiting the therapeutic efficacy of several immunotherapy strategies, mainly T-cell based therapies (X. Zhu, Li, and Zhu 2022; Gumber and Wang 2022).

1.2.3.1 T-cell based immunotherapies

In the realm of cancer treatment, immunotherapy has become a standard approach, alongside surgery, radiotherapy, chemotherapy, and targeted therapy, representing a turning point in the field of oncology. Cancer immunotherapy aims to enhance anti-tumor immunity and minimize off-target effects by activating or supporting the body's immune system, leveraging its ability to recognize and eliminate tumor cells. Immunotherapy strategies under clinical investigation include monoclonal antibodies to help the immune system recognize tumors, immune system modulators reversing cancer-associated immunosuppression, therapeutic vaccines targeting tumors, and adoptive cell therapy (ACT) to boost T-cell ability to fight the tumor (Hamdan and Cerullo 2023). T-cell based therapies include tumor-infiltrating lymphocyte (TIL) therapy and engineered T-cell therapies, both involving *ex vivo* manipulation of patient's lymphocytes and following re-infusion to improve anti-tumor responses. The tumor-infiltrating lymphocyte (TIL) therapy is based on the isolation of tumor-specific T cells from the tumor microenvironment and further expansion *ex vivo* using various regimens to activate cells before re-infusing them into the patients promoting a robust anti-tumor response (Ward 2022). On the other hand, engineered T-cell therapies involve gene transfer of T cells by using lentiviral vectors to express a specific T-cell receptor (TCR) (Shafer, Kelly, and Hoyos 2022) or a chimeric antigen receptor (CAR) (Sterner and Sterner 2021) generating T cell populations more efficient in killing tumor cells. Over the last decade, the groundbreaking achievements in early clinical trials of CAR-modified T cell (CAR-T) therapy have transformed the landscape of anticancer treatments offering a potential cure also for patients resistant to conventional therapies (Wei et al. 2017; Kalos et al. 2011). Typically, a CAR is a recombinant single transmembrane receptor comprising an extracellular antigen-recognizing domain, a flexible hinge, a transmembrane domain, and an intracellular T-cell activating domain. Briefly, the extracellular antigen-recognizing domain, responsible for tumor-antigen recognition and transmission of the activating signal, is traditionally an antibody-derived single-chain variable fragment (scFv). This scFv is linked to a hinge, providing flexibility, and a transmembrane domain, derived from CD3, CD4, CD8, or CD28 and acting as a membrane-anchor signal. The intracellular signaling domain commonly includes the CD3ζ chain, essential for T-cell activation, with costimulatory molecules, such as CD28 or 4-1BB, enhancing T-cell functionality (Davila and Sadelain 2016). The engineered CAR-T cells exhibit potent anti-tumor activity by recognizing and attacking cancer cells expressing the targeted antigen. Notable success has been observed in hematological malignancies, such as

B-cell leukemias and lymphomas, with CAR-T cells showing unprecedented clinical responses and even achieving durable remissions (June et al. 2018). However, challenges persist. Adverse events, including cytokine release syndrome (CRS) and neurotoxicity, pose significant safety concerns (Xu and Tang 2014; Neelapu et al. 2018). The applicability of CAR-T is limited to certain cancers due to several factors such as the lack of a tumor-associated antigen (Chen et al. 2018), tumor immune escape (Majzner and Mackall 2018), and restricted trafficking and infiltration into the tumor (Lanitis et al. 2017). Moreover, in several cancers, particularly in solid tumors, poor CAR efficacy is associated with the exhaustion of CAR-T cells that leads to compromised expansion and persistence of CAR-T cells in vivo resulting in impaired tumor-killing activity (Kouro, Himuro, and Sasada 2022; X. Zhu, Li, and Zhu 2022). For example, anti-mesothelin CAR-T cells displayed an exhaustion phenotype characterized by PD-1 overexpression and reduced cytokine secretion with consequent functional impairment (Cherkassky et al. 2016). Furthermore, other studies demonstrated that CD19 CAR-T cells used to treat patients with chronic lymphocytic leukemia (CLL) or B-cell lymphomas exhibited features of exhaustion and upregulation of transcriptional programs involved in the T cell dysfunction in non-responding patients compared to patients who achieved a complete response (Fraietta et al. 2018; Deng et al. 2020). These results highlight that T-cell exhaustion is a significant hurdle to successful treatment and counteracting this dysfunctional state becomes a central challenge to boost clinical potency and achieve long-lasting disease control in T-cell based immunotherapies, including CAR-T therapy. For this purpose, innovative strategies are necessary to refine the technology, enhance safety profiles, and broaden the spectrum of treatable malignancies through innovative CAR designs and combination strategies.

1.2.3.2 Immune Checkpoint Therapy (ICT)

On the clear evidence that T-cell exhaustion reduces the efficacy of several cancer treatments, mainly T-cell therapies such as CAR-T cell therapy, in recent years, extensive research efforts have focused on understanding and mitigating T-cell dysfunction. Several strategies have been developed to reinvigorate exT cells and improve anti-tumor activity by acting on different aspects of exhaustion, such as inhibitory pathways, inflammatory pathways, expression of exhaustion-related genes, and metabolic impairment (Guan et al. 2023).

Immune checkpoint therapy (ICT) has emerged as a revolutionary approach in cancer immunotherapy enhancing the antitumor immune response through the modulation of key regulatory pathways. The therapeutic success of immune checkpoint inhibitors (ICIs) stems

from their ability to overcome the immunosuppressive microenvironment established by tumors. Immune checkpoint blockade involves the inhibition of key inhibitory receptors, such as PD-1, CTLA-4, LAG-3, and TIM-3, by using monoclonal antibodies (mAb) that prevent the interaction between these receptors and their respective ligands, thus unleashing the cytotoxic potential of T cells and facilitating a robust antitumor immune response (Marin-Acevedo et al. 2018; Sharma et al. 2021). In 2011, the US Food and Drug Administration (FDA) approved the first anti-CTLA-4 mAb 'Ipilimumab' for melanoma treatment showing prolonged overall survival and durable remissions (Hodi et al. 2010; Korman, Garrett-Thomson, and Lonberg 2022). In the following years, ICT yielded impressive clinical results in several types of cancer proving to be a promising approach to revert the dysfunctional state of ExT cells and regain anti-tumor activity in cancer treatment (Ribas and Wolchok 2018), as recognized by the 2018 Nobel Prize for Medicine and Physiology (P. W. Huang and Chang 2019). These first encouraging results have prompted scientists to thoroughly explore immune checkpoint blockade for immunotherapy leading to the discovery of numerous immune checkpoint inhibitors targeting different IRs (Sharma et al. 2023). Ongoing studies involve other anti-CTLA4 monoclonal antibodies like Tremelimumab and Quavonlimab, along with novel forms designed for enhanced safety and efficacy. However, Tremelimumab failed to increase overall survival in multiple late-phase clinical trials, highlighting the complexities involved in developing effective therapeutic antibodies, including considerations of antibody isotype and affinity. As for CTLA-4, anti-PD-1/PD-L1 monotherapies have gained widespread approval across various tumor types. In 2014, the first PD-1 inhibitor 'Nivolumab' was approved for the treatment of advanced melanoma patients presenting long-term efficacy and limited toxicity (Topalian et al. 2014; O'Sullivan Coyne, Madan, and Gulley 2014). To date, numerous monoclonal antibodies targeting PD-1 or its ligand PD-L1 have been developed and several have been FDA-approved for the treatment of several types of cancer, such as melanoma, lung cancer, lymphoma, esophageal carcinoma, gastric cancer, and hepatocellular carcinoma (Kubli et al. 2021; Pan et al. 2020; Sharma et al. 2021). Anti-PD-1 therapies have also given promising clinical outcomes in patients who are diagnosed with tumors exhibiting microsatellite instability-high (MSI-H)/mismatch-repair deficiency (Le et al. 2017) or high tumor mutational burden (Marabelle et al. 2020) and some PD-1/PD-L1 inhibitors have not only proven effective in specific tumor types but have also received FDA approval for maintenance therapy. In addition to CTLA-4 and PD-1/L1 inhibitors, the blockade of other IRs, such as LAG-3, TIGIT, and TIM-3, leads to increased T cell effector functions in different cancers (Acharya, Sabatos-Peyton, and Anderson 2020b; Chauvin and

Zarour 2020b; Maruhashi et al. 2020b). Despite the promising results, ICT monotherapies showed durable responses in only about 20% of patients, encouraging exploration of ICT combinations to enhance efficacy. Combinations of CTLA-4 and PD-1/PD-L1 inhibitors have displayed significantly higher response rates and improved overall survival compared to monotherapies, particularly in cancers such as melanoma (Livingstone et al. 2022; O'Malley et al. 2022). Notably, this combination therapy has demonstrated significant anti-tumor response and tolerability following anti-PD-1/L1 monotherapy failure in advanced melanoma patients (Olson et al. 2021), suggesting its potential to overcome adaptive resistance mechanisms in specific cases.

As the variability of response to CTLA-4 and PD-1/L1 inhibitors, novel combinations involving other IRs have been investigated. Co-blockade of LAG-3 and PD-1 pathways has revealed a synergistic therapeutic effect (Amaria et al. 2022; Tawbi et al. 2022), as well as the concurrent inhibition of TIGIT alongside PD-1 demonstrated a marked enhancement in the functionality of $CD8^+$ tumor-infiltrating lymphocytes (TILs) in vitro (Chauvin et al. 2015). Similarly, the combination of TIGIT and PD-L1 inhibitors led to synergistic effects with heightened proliferation, increased cytokine production, and augmented degranulation of $CD8^+$ T cells (Chu et al. 2023).

1.2.3.3 Limitations and challenges of current ICT

Despite the impressive clinical activity, the efficacy of immune checkpoint blockade is strictly limited by drug resistance and severe toxicities. On one side, resistance to ICT is widespread (Bagchi, Yuan, and Engleman 2021). De-novo resistance to ICT is typical in patients with specific tumor types like glioblastoma and pancreatic cancer, while a subset of patients with ICT-sensitive tumors, such as melanoma, bladder cancer, and renal cancer, can develop adaptive resistance to ICT that can onset after a period of response or after treatment discontinuation (Sharma et al. 2017). On the other hand, the systemic injection of ICT may induce inflammatory or autoimmune reactions against antigens on healty tissues leading to severe side effects, known as 'immune-related adverse events' (irAEs), which can be fatal for a portion of patients (Chow et al. 2022; Bagchi, Yuan, and Engleman 2021). irAEs can affect various tissues and organs leading to severe complications, including skin rashes, pneumonitis, hypothyroidism, pancreatitis, encephalopathy, hepatitis, myocarditis, and immune cytopenias (Ghosh, Barba, and Perales 2020; Gupta et al. 2015; Champiat et al. 2016). Additionally, the therapeutic efficacy of ICIs is restricted only to a subset of patients partly due to the upregulation of other immune checkpoints as a compensatory mechanism (Koyama

et al. 2016; Gao et al. 2017). Furthermore, the limited efficacy and applicability of ICT, mainly in solid tumors, is also due to some issues related to the use of monoclonal antibodies (Wang et al. 2007). Indeed, although the mAbs have provided successful results in several cancer treatments, notable drawbacks limited their efficacy, mainly in solid tumors. Firstly, a relatively large size of mAbs, with a molecular mass of 150 kDa, impairs their penetration into the tumor leading to a suboptimal therapeutic efficiency, with the risk of inducing resistance to the antibody in patients and requiring administration of large doses. Immunogenicity represents another issue with the use of murine antibodies, which can trigger immunogenic responses in patients leading to the generation of human anti-mouse antibodies (HAMA) that recognize and neutralize the administered antibodies (Mosch and Guchelaar 2022; Wang et al. 2007). Furthermore, the low stability of mAbs allows only intravenous or subcutaneous administration, while a long half-life contributes to severe irAEs (Cruz and Kayser 2019). These characteristics together with high production costs and the necessity of a hospital setting for their delivery make ICT potentially prohibitive in terms of affordability, highlighting the necessity to develop novel antibody-based therapeutics to overcome these drawbacks to expand the potentiality and efficacy of ICIs.

1.3 The future of immunotherapy: the nanobodies

Monoclonal antibodies (mAbs) have achieved huge success in cancer immunotherapy in targeting tumor-associated antigens as well as in blocking several pathways involved in the inhibition of T-cell activity in the tumor microenvironment, like the immune checkpoint pathways. Despite the impressive clinical results in various cancers, several patients have not responded to mAb therapies and the characteristics of mAbs, including large size, poor tissue penetration, low stability, and immunogenicity, represent notable drawbacks that have restricted their utility as therapeutics, mainly in solid tumors. These limitations required the development of novel antibody formats that retain the binding specificity of antibodies while exhibiting improved stability and in vivo characteristics.

Nearly 30 years ago, heavy-chain-only antibodies were discovered in the serum of camelids bringing to light the existence of a new type of antibodies called 'single-domain antibodies' (sdAb), also known as 'nanobodies' or 'VHH' (Variable domain of Heavy chain from Heavy-chain only antibodies).

1.3.1 Structural and biochemical properties of nanobodies

Heavy-chain-only antibodies (hcAbs) were first identified in 1989 by Raymond Hamers et al. in the dromedary camel immune system (Hamers-Casterman, Atarchouch et al. 1993). While conventional antibodies consist of two heavy (H) and two light (L) chains and the antigen-binding domain is composed of two variable domains (VH and VL) non-covalently associated, camelid antibodies lack the light chains along with the first constant domain (CH1), thus leading to their size reduction.

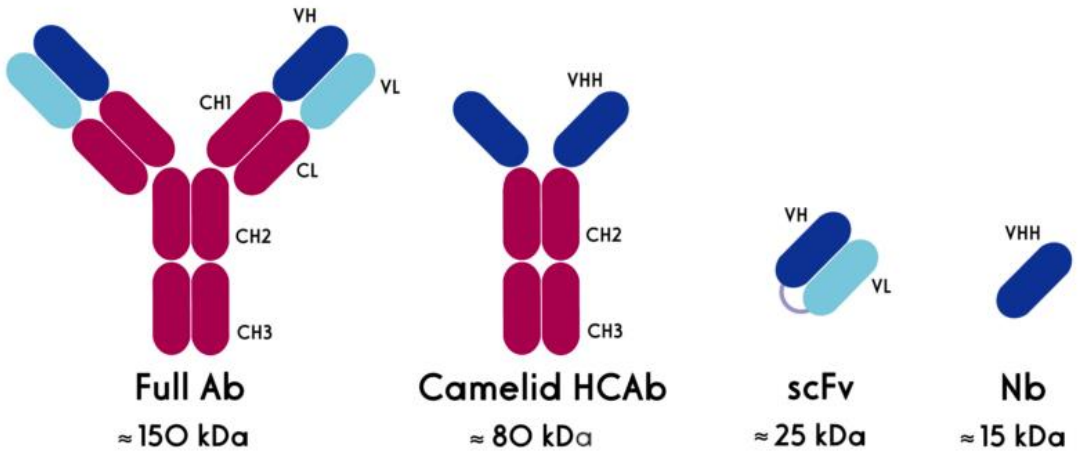


Figure 2 Structures of conventional and camelid antibodies with their derivatives

HCAb: heavy-chain-only antibodies, scFv: single-chain variable fragment, Nb: nanobody (modified from Maali et al. 2023)

Indeed, hcAbs have a molecular mass of 80 kDa rather than 150 kDa for mAbs. The only single variable domain of hcAbs, designated as VHH or nanobody, is responsible for antigen recognition and has a molecular weight of only 12-15 kDa, resulting in a monomeric domain antibody with enhanced tissue penetration (Maali et al. 2023; Jovčevska and Muyldermans 2020). In contrast to conventional antibodies with six complementary determining regions (CDRs), nanobodies possess three CDRs but maintain comparable antigen-binding affinity and effector functions, proving to be the smallest naturally produced fragment with high affinities, in the nanomolar or even picomolar range. The VHH domain comprises four conserved regions, called framework regions (FRs), surrounding the three hypervariable regions (complementary determining regions, CDRs) (Ingram, Schmidt, and Ploegh 2018; Muyldermans 2013). Three CDRs make up the paratope of nanobodies, while FRs regularly play a role in antigen interaction. Specifically, CDR3 plays a primary role in antigen recognition and specificity, while CDR1 and CDR2 contribute to binding strength (Mitchell and Colwell 2018; Zavrtanik et al. 2018). Nanobodies display a longer CDR3 loop providing increased flexibility, and in many cases, CD3R has protruding ends that allow nanobodies to reach deep epitopes hidden in small cavities, such as protein crevices or the active sites of enzymes (Desmyter et al. 1996), thereby making them highly efficient modulators of proteins, receptors, enzyme, and ion channels (Unger et al. 2015; Danquah et al. 2016; Lauwereys et al. 1998).

Unlike mAbs, nanobodies possess higher than 80% homology with the VH domain of human immunoglobulins, demonstrating a low immunogenic profile that makes them very suitable for human applications (Klarenbeek et al. 2015). Notably, VHH domains exhibit remarkable stability, due to the presence of a disulfide bond between CDR1 and CDR3 that makes them more resistant to harsh conditions such as proteolytic degradation, extreme temperature and pH, and chemical denaturants (De Vos et al. 2013), and demonstrate excellent refolding capacity without loss in activity (Harmsen and De Haard 2007; Kunz et al. 2018). Nanobodies are also characterized by a more hydrophilic surface compared to mAbs, due to the presence of four hydrophilic amino acids in the FR2 region that increase their solubility in polar solvents (i.e. water) (Muyldermans 2013). Further appealing features of nanobodies for therapeutic purposes include their ability to penetrate tissues normally inaccessible to conventional antibodies. Several studies demonstrated the ability of nanobodies to cross the blood-brain barrier (BBB) (T. Li et al. 2012) as well as to reach intracellular targets (Wagner and Rothbauer 2020) bringing out their potential application in brain-related diseases (Zheng et al. 2022) and drug delivery. An additional property that makes nanobodies therapeutically

more advantageous than conventional antibodies is the possibility of producing them in microbial systems, such as *Escherichia coli*, *Saccharomyces cerevisiae,* and *Pichia pastoris* (Arbabi-Ghahroudi, Tanha, and MacKenzie 2005), thus allowing large-scale productions with low-cost manufacturing. Lastly, nanobodies can be easily modified or genetically engineered to generate multivalent nanobodies and can be used to create fusion proteins for targeted therapy or probes for imaging (Van Audenhove and Gettemans 2016; Sadeghnezhad et al. 2019; Sheng et al. 2019).

Despite the small size of nanobodies is an attractive property in many aspects, it can represent a drawback in therapy because leads to a rapid renal clearance of administered nanobodies. This results in the reduction of nanobody fraction that will bind to its target, if this is not circulating in the blood. However, multiple approaches have been developed to overcome this limitation, including the PEGylation process (Vugmeyster et al. 2012), nanobody fusion to the IgG Fc region, and fusion to serum albumin or to a nanobody targeting the human serum albumin to increase the half-life (Hoefman et al. 2015; Kontermann 2011).

1.3.2 Nanobodies production

Nanobodies targeting a specific antigen can be easily and rapidly produced in large amounts but with low manufacturing costs in microbial systems, such as bacteria, yeasts, and fungi. For sdAb production, immune, naïve, or synthetic libraries can be used (W. Liu et al. 2018). Immune libraries are the most used and involve an active immunization of camelids, such as camels, dromedaries, llamas, or alpacas, with the target antigen (Romao et al. 2016). Following immunization, peripheral blood lymphocytes are isolated, and the repertoire of variable domains encoding nanobodies is extracted and amplified. Subsequently, phage display technology is used to select nanobodies with high affinity and specificity for the target antigen. Once amplified by PCR, VHH fragments are inserted into a cloning vector to produce a VHH library by infecting the phage expression system. The selection of positive VHH-expressing phages is usually performed by in vitro assays (phage ELISA) through two or three rounds of panning aimed to identify the phages expressing the VHH specific for the cognate antigen (Figure 2). After the identification of lead candidates, putative sdAbs are produced in microbial or mammalian systems, ensuring scalable and cost-effective production (Pardon et al. 2014; W. Liu et al. 2018).

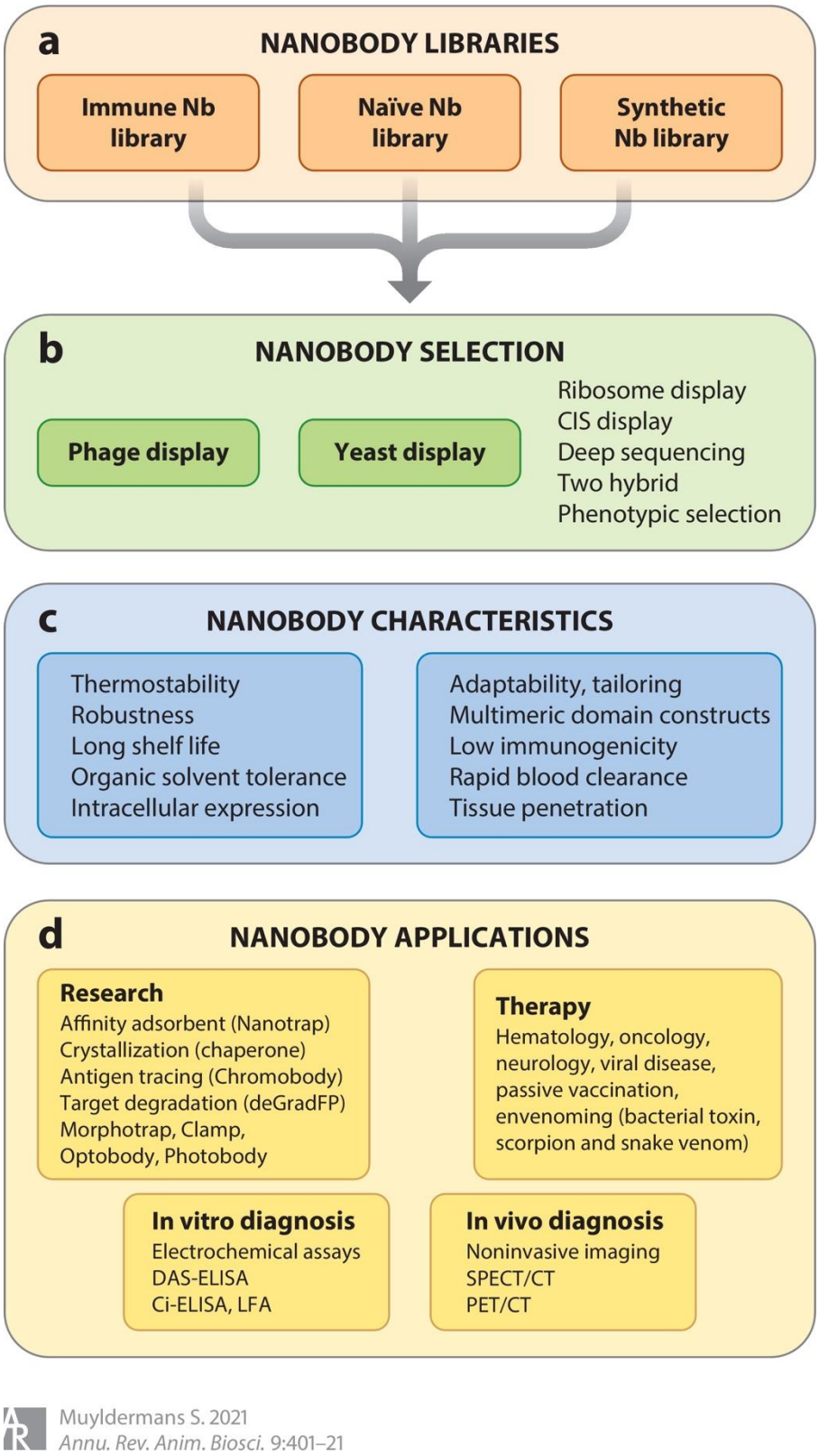


Figure 3 Schematic flow of production, characteristics and applications of nanobodies
Nanobodies can produced by using immune, naïve, or synthetic libraries (a). Then, antigen-specific VHH domains are selected by phage display, or another selection method (b). Their unique properties (c) make nanobodies an excellent tool for several applications in research, therapy and diagnostic fields (d) (from (Muyldermans 2021)).

1.3.3 Applications of nanobodies

The unique properties of nanobodies make them an interesting tool for research and diagnostic applications as well as a promising alternative to mAbs in therapeutics (Figure 2) (Wesolowski et al. 2009).

1.3.3.1 Nanobodies as research tools

As research tools, nanobodies exhibit versatile applications due to their biophysical features. The possibility of easily identifying and producing large amounts of antigen-specific sdAbs together with their structural advantages make sdAbs instrumental in structural biology to assist crystallization processes helping to determine the structure of flexible or aggregating proteins (Koide 2009). Indeed, the interaction with the sdAb stabilizes the antigen in a preferred conformation facilitating both the crystallization of the complex and the design of potential agonists or inhibitors for that antigen (Rasmussen et al. 2011; Wingler et al. 2019; Steyaert and Kobilka 2011). They have been largely employed as probes for antigen capture by direct immobilization of sdAbs on beads via a tag or upon in vivo biotinylation (Saerens et al. 2005), demonstrating high-density coupling on beads and high specificity in cognate antigen capture. Owing to their unique properties such as the capacity to reach intracellular targets and to be easily engineered, sdAb have been exploited to produce powerful fluobodies or chromobodies by fusing the VHH domain to a fluorescent protein enabling real-time monitoring of subcellular localization, dynamics, and interactions of the target protein in living cells (Rothbauer et al. 2006; Mikhaylova et al. 2015; Traenkle and Rothbauer 2017). Moreover, intracellular sdAbs with special signal peptides can be used to redirect and trap the cognate antigen in a specific cellular compartment. For example, a KDEL tag fused to sdAb allows to address and retain the captured antigen inside the endoplasmic reticulum, whereas the fusion to lamin will keep the antigen in the nuclear membrane (Rothbauer et al. 2008; Klooster et al. 2009). Additionally, sdAbs have been employed in designing novel enzymatic devices for example to trigger proteasome degradation (Caussinus, Kanca, and Affolter 2011), gene activation systems by fusion to DNA binding domains (Tang et al. 2013), and monitoring systems to study protein-protein interactions in real-time (Beghein and Gettemans 2017).

1.3.3.2 Nanobodies as diagnostic tools

Nanobodies have emerged also as potent diagnostic tools in various biomedical applications taking advantage of their unique properties (Figure 2). The exceptional antigen-binding affinity and specificity of nanobodies make them ideal candidates for the development of

diagnostic assays, including immunoassays and imaging techniques (De Meyer, Muyldermans, and Depicker 2014; Muyldermans 2021).

For *in vitro* diagnostic tests, nanobodies have been successfully employed to detect and visualize specific targets, ranging from disease biomarkers to cellular structures. They found versatile applications in diagnostic platforms such as enzyme-linked immunosorbent assays (ELISAs) to detect biomarker proteins, viruses, or other pathogens in human sera (D. Li et al. 2020; Morales-Yanez et al. 2019; L. Huang et al. 2005). Notably, the modular nature of nanobodies facilitates the construction of multifunctional diagnostic agents, allowing for the simultaneous targeting of multiple biomarkers.

Nanobodies are also well-suited for *in vivo* imaging applications, enabling the detection of specific molecular targets with high precision. The ability to engineer nanobodies with radionuclides suitable for positron emission tomography/computed tomography (PET/CT) or single-photon emission computed tomography (SPECT) imaging further expands their utility as diagnostic tracers (Rashidian and Ploegh 2020). Indeed, due to their small size nanobodies exhibit rapid clearance from the bloodstream and efficient tissue penetration as well as fast diffusion in tissues after intravenous injection, thus proving to be excellent imaging agents able to enhance imaging contrast and reduce background signal (Oliveira et al. 2013; T. Li et al. 2016). Several radiolabeled sdAbs have been produced to target tumor-associated antigens and biomarkers to monitor or diagnose several diseases in mouse models (Xavier et al. 2016; Keyaerts et al. 2016). This combination of small size, target specificity, and compatibility with various imaging technologies positions nanobodies as valuable tools in advancing noninvasive imaging for enhancing the accuracy and sensitivity of diagnostic methodologies.

1.3.3.3 Nanobodies as therapeutics

Nanobodies, owing to their unique characteristics, have emerged as promising therapeutics with notable applications. Indeed, their small size, robust structure, excellent tissue penetration, and high specificity make them ideal candidates for targeting various disease-associated antigens and for cancer immunotherapy.

"Naked" nanobodies have great potential for targeting tumor antigens in vivo, also outperforming mAbs. Due to their small size and modularity, multivalent or multispecific nanobodies can be developed by genetically fusing several nanobodies via flexible glycine-serine linkers which provide maximal flexibility and improve solubility (Conrath et al. 2001). Bivalent dimers can be obtained by connecting two identical nanobodies in tandem to enhance avidity and reduce off-rate, while biparatopic binders include two different nanobodies

recognizing non-overlapping epitopes of the same antigen. Alternatively, two nanobodies targeting two different proteins can be fused to produce bispecific nanobodies. Nanobodies can be also fused to immunoglobulin G (IgG) fragment crystallizable (Fc) domains to generate sdAb-Fc fusion proteins with a stronger therapeutic potential by specifically blocking a target on tumor cells and at the same time triggering antibody-dependent cell-mediated cytotoxicity (ADCC) through the exposed Fc region (Silva-Pilipich et al. 2023; Awad et al. 2021). Additionally, sdAb fusion to an albumin-specific nanobody can extend the in vivo half-life, thus overcoming the main disadvantage related to sdAb small size (Tijink et al. 2008). For example, the fusion of a biparatopic anti-EGFR nanobody to an anti-albumin nanobody extended the half-life from 1-2 hours to 2-3 days demonstrating an anti-tumor efficacy comparable to that of the respective mAb and higher than the monomeric and bivalent formats (Roovers et al. 2011).

In cancer immunotherapy, nanobodies have been employed as potent inhibitors by blocking key signaling pathways or as vehicles for drug delivery, enhancing the precision of therapeutic interventions (Bathula, Bommadevara, and Hayes 2021; Maali et al. 2023; Jovčevska and Muyldermans 2020). Nanobodies have been pivotal in the development of novel immunotherapeutic strategies, including CAR-T therapies. Nanobodies have been applied in CAR-T cells as antigen-binding domain, as an alternative to single-chain variable fragments (scFvs). This strategy helps overcome challenges associated with conventional CAR-T therapies, such as on-target/off-tumor effects and cytokine release syndrome, improving CAR targeting precision and contributing to enhancing T-cell activation and tumor cell eradication (Safarzadeh Kozani et al. 2022). The use of nanobodies reduces the risk of immunogenicity as they lack linker peptides connecting VH and VL domains of scFvs that can induce immune reactions, thus remarkably restricting the anti-tumor activity of CAR-T cells (Gorovits and Koren 2019; Shah and Fry 2019). Additionally, scFvs have a high tendency to self-aggregate probably due to the presence of exposed hydrophobic residues on variable domains or poor folding stability (Long et al. 2015; Nieba et al. 1997; Gil and Schrum 2013). This CAR aggregation on the surface of CAR-T cells triggers an antigen-independent activation of CAR signaling and effector T-cells which can lead to T-cell exhaustion (Jayaraman et al. 2020). On the contrary, nanobodies exhibit a lower tendency for surface aggregation on CAR-T cells due to their high stability, thereby reducing the risk of T-cell exhaustion. Another advantage of nanobodies relates to their longer CDR3 which allows them to bind epitopes inaccessible to conventional monoclonal antibodies (Vu et al. 1997; Bannas, Hambach, and Koch-Nolte 2017). By leveraging the unique properties of nanobodies, CAR-T cells can be engineered for

improved tumor selectivity and reduced toxicity, thereby enhancing the safety and efficacy of the therapy.

In the field of cancer immunotherapy, nanobodies have also demonstrated significant potential in the realm of immune checkpoint therapy (ICT). Immune checkpoint inhibitors, such as antibodies targeting PD-1/-L1 and CTLA-4 pathways, have revolutionized cancer treatment by unleashing the immune system against tumor cells. The mAb-based immune checkpoint inhibition has provided promising results in inhibiting tumor growth leading to FDA-approval of various mAbs targeting PD-1, PD-L1, and CTLA-4 for several cancers. However, the clinical utility of mAbs has been constrained by some mAb-related limiting factors like high production costs, low tissue penetration, and immune-related side effects. Offering advantages in terms of tissue penetration and pharmacokinetics, nanobodies have emerged as a powerful alternative to mAbs for ICT (Salvador, Vilaplana, and Marco 2019). Several nanobody-based immune checkpoint inhibitors have been developed demonstrating improved therapeutic efficacy and reduced off-target effects and paving the way for the advancement of precision immunotherapies in the fight against cancer (Yu et al. 2021). Currently, the research to identify nanobodies targeting immune checkpoints is mostly focused on the PD-1/PD-L1 pathway. For example, two different anti PD-L1 nanobodies, named KN035 and K2, have been identified to block the interaction between PD-1 and PD-L1 providing interesting results. KN035 binds PD-L1 with high affinity disrupting the interaction with PD-1 and is able to activate PBMCs in vitro (F. Zhang et al. 2017). On the other hand, sdAb K2 improves the capacity of dendritic cells (DCs) to activate T cells and induce cytokine secretion, demonstrating the ability to bind to its target on both immune and non-immune cells (120 from *Nanobodies targeting immune checkpoint molecules*). Nanobodies can also be used as carriers for cytokines to act on the tumor microenvironment (TME). A chemokine-VHH fusion protein was developed by fusing a nanobody targeting PD-L1 with a charge-engineered chemokine CCL21 to restrict chemokine expression in a PD-L1+ environment, thus selectively attracting specific leukocyte populations within TME. Using a microfluidic platform, the fusion protein has been demonstrated to be able to selectively interact with PD-L1+ tumor cells recruiting effector cells to the TME (Fang et al. 2019). In a xenograft mouse model, a unique anti-PD-L1/CXCR4 bispecific nanobody exhibited successful penetration into tumor tissues, displaying superior tumor growth inhibition compared to combination therapy with the monomeric anti-PD-L1 and anti-CXCR4 nanobodies (Hao et al. 2022). Another bispecific antibody was developed by fusing an anti-PD-L1 antibody and an anti-TIGIT nanobody to induce the interaction of PD-L1+ tumor cells with T and NK cells. In this

way, the PD-1/-L1 and TIGIT immune checkpoints have been co-blocked leading to enhanced tumor invasion of cytotoxic T lymphocytes (CTLs) and NK cells and tumor growth inhibition in a mouse model (Zhong et al. 2022). Furthermore, a PD-L1/4-1BB bispecific nanobody was designed to bind PD-L1 on tumor target cells and the activation molecule 4-1BB on effector cells simultaneously. This strategy effectively blocked the PD-1/PD-L1 pathway and induced 4-1BB signaling, demonstrating high anti-tumor activity with minimal toxicity (Zhai et al. 2021). In addition to PD-L1, nanobodies targeting other IRs have been also produced, such as a nanobody targeting CTLA-4 (called Nb16) which has been identified and validated in a melanoma mice model, exhibiting a strong binding affinity to the target and leading to an increase in the mice's survival and a significant inhibition of tumor growth (Wan et al. 2018).

Nanobodies have found diverse applications such as biosensing, protein affinity-capturing, protein crystallization, and targeted drug delivery. In the realm of cancer therapeutics, numerous nanobodies have been discovered to target surface receptors on tumor cells, soluble ligands released in the TME, and inhibitory receptors on immune cells. Notably, about 16 therapeutic nanobodies are under investigation in clinical trials for various diseases (Arbabi-Ghahroudi 2022). In 2019 the first nanobody, a bivalent nanobody targeting von Willebrand factor named Caplacizumab, has been approved by the EMA and FDA for the treatment of adults with acquired thrombotic thrombocytopenic purpura (TTP), showing good efficiency and safety profile (Scully et al. 2019).

As the discovery of new nanobodies and their applications continue to evolve, nanobodies stand at the forefront of innovative and targeted therapeutic interventions, holding immense promise for advancing cancer treatment paradigms. In this context, synthetic biology plays a crucial role in VHH engineering strategies, including dimerization, multimerization, and serum albumin binding, to generate more powerful nanobodies, and in designing complex synthetic circuits to fine-tune nanobodies expression according to specific requirements related to therapeutic purposes to minimize potential toxicities.

2 Aim of the study

T-cell exhaustion has been recognized as a significant challenge in cancer immunotherapy proving to be a limitation of the clinical benefits of T-cell-based therapies, especially in solid tumors. Despite immune checkpoint therapy (ICT) using monoclonal antibodies (mAbs) has shown encouraging results in several cancers leading to tumor growth control and prolonged overall survival, issues like limited patient responsiveness, drug resistance, and adverse effects prompted the exploration of alternative strategies. Camelid-derived single-domain antibodies (sdAbs), also known as nanobodies, have emerged as promising tools to address the shortcomings of mAbs in cancer therapy exhibiting unique features, such as small size, high solubility, stability, and excellent tissue penetration in vivo. In the context of immune checkpoint blockade, nanobodies can target inhibitory receptors like CTLA-4 and PD-1, crucial players in T-cell exhaustion. This study proposes a synthetic biology approach to neutralize T-cell exhaustion for improving T-cell therapies in solid cancers by tackling simultaneously two current limitations of immunotherapy treatments: i) local release of the therapeutics in the tumor microenvironment and ii) control over the expression of therapeutic molecules. The proposed approach involves creating a synthetic system with a sensing module capable of detecting exhaustion conditions and an actuator module incorporating genetically encoded nanobody-based modulators. The goal is to engineer T cells with this synthetic system to enable local secretion of sdAbs targeting inhibitory pathways, aiming to revert T-cell exhaustion in a specific and fine-tuned manner to avoid adverse and off-target effects, thus providing a novel avenue for designing more effective and targeted treatments.

To create this sensor-actuator synthetic system, synthetic actuators were designed based on two promising sdAbs for immune checkpoint blockade in cancer therapy: i) KN035, targeting PD-L1; ii) Nb16, a CTLA-4 binding sdAb. Monomeric sdAbs, bivalent binders, and sdAb-Fc fusion proteins were developed to identify the format with the highest avidity and blockade ability for future T-cell engineering. Several functional assays were performed to evaluate the target specificity and the ability to prevent immune checkpoint activation of the several sdAbs. The sdAb effect was also tested in a CAR-T in vitro model to assess their potentiality to improve CAR-T therapy outcome. Furtherly, sdAb circuits were incorporated into a lentiviral platform with constitutive and exhaustion-responsive promoters, generating engineered cell lines capable of expressing sdAbs constitutively or selectively in presence of exhaustion-specific transcription factors to demonstrate the activation of modulators only in conditions mimicking exhaustion.

3 Materials and Methods

3.1 Vector cloning

3.1.1 Design of nanobody constructs

Sequences encoding for KN035 and Nb16 were taken from their respective publications, as reported in 4.1.1. To generate sdAb-P2A-mcherry plasmids, sequences were codon optimized for mammalian cell expression and were ordered as double-stranded DNA fragments (Invitrogen GeneArt Strings DNA Fragments) adding a motif of six histidine (also known as His-tag) followed by a P2A sequence at C-terminal end. DNA fragments were then inserted in a vector, including the fluorescent reporter mCherry, together with the shEF1α or the PGK promoter by In-Fusion cloning. Furtherly, sdAb sequences were ordered as DNA fragments without P2A peptide and with the secretion signal Igk at N-terminal end. New fragments were inserted by In-Fusion in the destination vector.

Bivalent nanobody plasmids were generated with In-Fusion cloning by linking two identical sequences of monomeric sdAb in tandem separated by a GS linker (GGGGSGGGS), keeping the secretion signal sequence at N-terminal end and the His-tag at C-terminal end.

sdAb-Fc fusion proteins were developed by fusing the sequence of sdAb, inclusive of secretion signal, to a Fc sequence of IgG1 immunoglobulin by In-Fusion recombinase, moving the His-tag at C-terminal end of the Fc sequence. Schemes of all these plasmids were showed in 4.1.2.

3.1.2 Polymerase Chain Reaction (PCR) and gel electrophoresis

PCR reactions of short fragments were performed using 'AccuPrime Pfx SuperMix' (Thermo Fisher Scientific) according to manufacturer's instructions. For the amplification of backbone or long fragments (≥ 4.0 kbp) 'Phusion Flash High-Fidelity PCR Master Mix' (Thermo Fisher Scientific) according to manufacturer's instructions was used instead. Primers annealing temperatures were determined using the NEB Tm Calculator. DNA gels were at different percentages of agarose depending on the fragment size. All gels were stained with SYBR Safe (Thermo Fisher Scientific) and loaded with Purple Loading Dye 6X (Thermo Fisher Scientific). The electrophoresis run was performed at 80-100 V in TAE Buffer with a Life 1 kb Plus DNA Ladder (Thermo Fisher Scientific).

Gel extractions were performed using 'NucleoSpin Gel and PCR Clean-up' (Macherey-Nagel) according to the manufacturer's instructions.

3.1.3 Plasmids assembly

In cut-and-paste cloning, restriction digestions were carried out at 37°C for 2 hours, except for the ones with DpnI (NEB) that were kept for 1 hour. The digested and purified vectors were dephosphorylated to avoid self-ligation. Reactions were set up (20 µL digested vector, 1 µL Antarctic Phosphatase, 2.2 µL Antarctic Phosphatase Buffer) and were incubated at 37°C for 20 minutes and then inactivated for 2 minutes at 80°C in a dry heating block. Ligations were performed using T4 Ligase (NEB) according to manufacturer's instruction for 1 hour on bench with the DNA ratio of backbone to insert at 1:4.

For sdAb plasmid cloning, gene blocks or fragments amplified by PCR from other plasmids were inserted into the backbone by using In-Fusion recombinase (In-Fusion® Snap Assembly Master Mix, Takara) according to manufacturer instructions. For the In-Fusion protocol a ratio of backbone to insert of 1:2 was used.

3.1.4 Lentiviral vector cloning

All nanobody sequences were cloned into a lentiviral vector (named LV.0) by Golden Gate cloning that takes advantage of Type IIS restriction enzymes (in this case Esp3I), which cleave DNA outside their recognition sequences, allowing an ordered assembly of a vector and one or more DNA fragments in a single step. LV.0 vector was obtained modifying the plasmid FUGW (Plasmid #14883, Addgene) by removing the original cassette to generate a dual-promoter vector including two independent transcriptional units (TUs). As first TU, a bacterial cassette encoding for a green fluorescent protein (GFP) was inserted between two Esp3I restriction sites. The second TU was constant in all the LVs and encoded a mutated version of the low-affinity nerve growth factor receptor (LNGFR) driven by the shEF1α promoter. LNGFR sequence was amplified by PCR from pPRIME-CMV-LNGFR-recipient plasmid (Plasmid #11660, Addgene). Promoter and sdAb sequences to insert in the first TU were amplified by PCR adding an Esp3I restriction site at both the ends and then were inserted into LV.0 in place of the bacterial GFP by Golden Gate reaction. The presence of bacterial GFP facilitated the screening of positive colonies that have incorporated the inserts. The obtained plasmids were then used as transfer plasmids for lentivirus production (see 3.4).

Schemes of final LVs were shown in Figure 11 in section 4.4.

3.1.5 Bacterial transformation and culture

Bacterial transformations used Stellar Competent Cells (Clontech). An aliquot of cells was thawed on ice and 25 µL of cells were mixed with roughly 5 µL of each cloned plasmid

(obtained by ligation or In-Fusion as described in 3.1.3) and then were left for 30 minutes on ice. Heat-shock was performed by incubating 45 seconds at 42°C in a dry heat block and then placing for 2 minutes on ice. All transformations underwent an outgrowth step in SOC Medium (Clontech) with incubation in a shaking incubator (37°C, 200 rpm) for 1 hour. Cells were then plated onto warm LB agar plates with antibiotic resistance 100 $mg*mL^{-1}$. Inoculated cultures were incubated at 37°C shaking at 200 rpm in LB broth with the right antibiotic resistance at the above concentration and left overnight. Plasmid purifications were done according to the manufacturer's instructions using E.Z.N.A.® Plasmid Mini Kit I (VWR), QIAGEN Plasmid Plus Kit, and QIAGEN Plasmid Plus Maxi Kit. All plasmids were verified by Sanger sequencing.

3.2 Cell culture

HEK293T-17, SKOV3 and MDA-MB-231 cells (ATCC) used in this study were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS non-USA origin, Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich), 1% L- Glutamine (Sigma-Aldrich) and 1% MEM non-essential amino acids (Sigma-Aldrich). T cells were cultured in RPMI-1640 supplemented with 10% of FBS, 100 mg/mL of P/S, 10 mM Glutamax and 10 mM Hepes. Cells were detached by incubation with Trypsin-EDTA phenol red solution (Sigma-Aldrich) for 2-5 minutes at 37°C. All the cell lines were maintained at 37°C in a 5% CO2-humidifed incubator.

3.3 Cell transfection

HEK293T-17 cell line was transfected in all experiments with PEI® transfection reagent (Polysciences) according to manufacturer's instructions, using a ratio DNA (μg) : PEI (μl) =1:3. Transfections were performed in different plate formats depending of experimental requirements. Transfections to validate sdAb plasmids were performed in 24-well plate by seeding 80.000 cells per well the day before and using 300-500 ng of total DNA per well. Cells were analyzed by flow-cytometry 48 hours post-transfection.

For flow cytometry and qPCR analysis run on the same biological replicates, transfections were carried out in a 12-well plate format by seeding 150.000 cells per well and transfected them one day after using 600 ng total DNA per well. After 48 hours, part of sample was

analyzed by flow cytometry and the remaining part was processed for RNA extraction (see 3.6).

For harvesting of nanobody-rich medium for competition assays, HEK293T-17 cells were transfected in 6-well plate. Cells were seeded at $1x10^6$ cells per well in a final volume of 2 ml and after six hours were transfected by PEI® using 1500 ng of total DNA per well. Transfected cells were analyzed in flow cytometry after 48 hours and the cell medium was harvested to directly perform competition assay or was stored at -80°C for further experiments.

MDA-MB-231 cells were transfected with Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer's instructions. Transfections were performed on adherent cells 24 hours post-seeding at 150.000 cells per well in 12-well plates. DNA, transfection reagents and seeded cells were scaled up or down according to the Lipofectamine 3000 manufacturer's instructions for the desired plate format.

3.4 Lentivirus production

Second-generation lentiviral vectors were produced by transfection of HEK293T-17 with transfer plasmid and the two packaging vectors. Briefly, HEK293T-17 cells were seeded at $1.8x10^6$ in a total volume of 6 mL of DMEM complete medium in a T25 flask. Eighteen hours later, cells were transfected with a mix containing 2.6 µg of transfer plasmid (derived by LV.0 and including the sdAb sequence), 1.3 µg of pMD2.G (Plasmid #12259, Addgene) and 1.3 µg of psPAX2 (Plasmid #12260, Addgene) using PEI® transfection reagent using a ratio DNA : PEI=1:3 (Polysciences). The viral supernatant was harvested at 72h post-transfection, 0.45 µm filtered, divided in aliquots and stored at -80°C until use.

3.5 Cell Transduction

HEK293T-17 cells were engineered with sdAb constructs by lentiviral transduction. Briefly, transductions were performed in 6-well plates seeding 700.000 cells per well in growth medium. About 18 hours later, cells were infected with 0.5 ml of the viral supernatant (dilution 1:3 in final volume) in the presence of Polybrene (Sigma-Aldrich) at a final concentration of 10 µg/ml to enhance transduction efficiency. The day after, LV-containing medium was removed and fresh medium was added. Then, cells were usually split 48 hours after transduction. Transduction efficiency was assessed 7 days post-transduction by LNGFR staining (see 3.7.1).

3.6 mRNA extraction and quantification

RNA extraction was performed with 'E.Z.N.A.® Total RNA I' (VWR). Cells were washed in DPBS and incubated for 2 minutes in lysis buffer before resuspension. The protocol was followed according to manufacturer's instructions and RNA was eluted in 40 µL of RNAse free water to maximize the yield. Extracted RNA was quantified by Multiscan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific) and stored at -80°C.

For reverse transcription to cDNA, 'PrimeScript RT kit with gDNA eraser' (Takara) was used according to manufacturer's instructions. The protocol was performed on ice in an RNAse free environment to avoid RNA degradation. A negative control without Quantiscript Reverse Transcriptase was always prepared to check that samples were not contaminated with genomic DNA. Fast SYBR Green Master Mix (Thermo Fisher Scientific) was used to perform qPCR of cDNAs obtained from 500 ng of RNA and diluted 1:5. Samples were loaded in MicroAmp™ Fast Optical 96-Well Reaction Plate (0.1 mL) and the experiment was carried out with a CFX96 Touch Real-Time PCR Detection System (BioRad) machine. Each well contained a final volume of 10 µL (5 µL SYBR Green Master Mix 2X, 2 µL ddH_2O, 1 µL of each primer 5 µM, 1 µL of cDNA). Nuclease-free water was used as negative control without template (blank). Primers were designed to amplify a region of 60-200 bp (Table 1) and with a temperature of annealing between 50 and 65 °C. Data were analyzed using the Comparative Ct Method according to Applied Biosystems Protocols.

Primer	Sequence (5'-3')
18S FW	TGTGCCGCTAGAGGTGAAATT
18S REV	TGGCAAATGCTTTCGCTTT
KN035 FW	CTGGCAAAGAAAGGGAAAGAGTGG
KN035 REV	GTTGTTCTGGCTGATGGTGAATCTG
Nb16 FW	TGTACCGCCTCTGGCTTCGG
Nb16 REV	TCTGCCCTTCACGCTCTCGC

Table 1: Primers used for qPCR analyses

3.7 Nanobodies characterization

3.7.1 Flow cytometry

Intracellular staining

Staining of transfected cells was performed 48 hours post transfection. For cell permeabilization the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD, 554714) was used. Cells were detached by Trypsin-EDTA (0.25 %), washed in DPBS (Thermo Fisher

Scientific) and permeabilized with the fixation/permeabilization solution for 20 minutes at 4°C. Then, cells were labeled with primary antibody diluted in BD Perm/Wash™ Buffer 1x for 30 minutes at 4°C. Anti His-tag antibody (Product #12698, CST) was used 1:800, whereas the MonoRab™ Rabbit Anti-Camelid VHH Antibody (Product #A01860, Genscript) was used diluted 1:500. After washing with BD Perm/Wash™ Buffer 1x, cells were labeled with F (ab')2-Goat anti-Rabbit IgG (H+L) Secondary Antibody (Product #A10542, Thermo Fisher Scientific) at a dilution of 1:250 for 30 minutes at 4°C. Cells labeled with only the secondary antibody were used as negative control. Afterwards, cells were washed and resuspended in 100 µL PBS for acquisition using a BD FACS Celesta Cell Analyzer.

Extracellular staining

For competition assay, cells transfected or treated in 12-well plates were washed with DPBS (Thermo Fisher), detached with 100 µL of Trypsin-EDTA (0.25 %) and re-suspended in 350 µL of medium. Cell suspension was divided in three for antibody, isotype control and unstained conditions. Cells were washed in DPBS and were labeled with 0.2 µg of anti PD-L1 PE Antibody (Product #12-5983-42, Invitrogen) or 0.2 µg of Mouse IgG1 kappa Isotype Control, PE (Product #12-4714-82, Invitrogen) in a final volume of 50 ul DPBS for 20 minutes at room temperature. For the analysis of $LNGFR^+$ population, seven days after the transduction cells were washed in DPBS and were stained with Pacific Blue™ anti-human CD271 (NGFR) Antibody (Product #345132, Biolegend) or the Pacific Blue™ Mouse IgG1, κ Isotype Ctrl Antibody (Product #400151, Biolegend) for 20 minutes at room temperature. After antibody incubation, cells were washed in DPBS, resuspended in 100 ul of DPBS and analyzed by using a BD FACS Celesta Cell Analyzer.

To detect CAR expression, cell viability was determined using L/D eFluor™ 450 (eBioscience, 65-0863-14) followed by surface antibody staining using goat anti-human IgG-biotin (Jackson ImmunoResearch) followed by streptavidin-PE (Thermo Fisher). Cells were incubated with surface antibodies for 30 min in the dark at room temperature, then they were washed in DPBS and analyzed by a BD FACS Celesta Cell Analyzer.

3.7.2 PD-L1 competition assay

For competition assay in transfected cells, MDA-MB-231 cells were seeded at 150.000 cells per well in 12-well plate and one day after were transfected with Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer's instructions using 600 ng of total DNA per well. After 48 hours, cells were stained with anti PD-L1 mAb.

For competition assay in non-transfected cells, HEK293T-17 cells were seeded at $1x10^6$ cells per well in a final volume of 2 ml in 6-well plate and after six hours cells were transfected by PEI® transfection reagent using a ratio DNA : PEI=1:3 (Polysciences). The same day, MDA-MB-231 or SKOV3 cells were seeded at 150.000 cells per well in a 12-well plate in a final volume of 1 ml. After 48 hours, tumor cells were treated with 1 ml of transfected HEK293T-17 supernatant for 1 hour at 37°C in a 5% CO2-humidifed incubator. The remaining supernatant of transfected cells was harvested and stored at -80°C for further experiments. Cells not treated with HEK293T-17 supernatants were used as untreated (UT) control. Afterwards, tumor cells were stained with anti PD-L1 mAb (as described in section 3.7.1).

3.7.3 PD-1/PD-L1 Blockade Bioassay

The neutralizing activity of the anti-PD-L1 nanobodies was analyzed by a luciferase reporter assay (Product #J1255, PD-1/PD-L1 Blockade Bioassays from Promega) according to the manufacturer's instructions. Briefly, PD-L1 aAPC/CHO-K1 cells were seeded into a white flat-bottom 96-well assay plate and incubated overnight at 37 °C in a 5% CO2 humidified incubator. Cellular medium harvested by transfected HEK293T-17 cells was added to the plates in place of antibody dilutions followed by seeding PD-1 effector cells. After co-culture for 6 h at 37 °C in a 5% CO2 humidified incubator, the Bio-Glo™ reagent was added to the plate and incubated at ambient temperature for 5 min. The luminescence signal was measured using a Glo-max Discover System and reported as relative light units (RLUs), and the data were analysed using GraphPad Prism.

3.7.4 CTLA-4 blockade bioassay

The neutralizing activity of the anti-CTLA-4 nanobodies was analysed by a luciferase reporter assay (Product #JA3005, CTLA-4 Blockade Bioassays from Promega) according to the manufacturer's instructions. Briefly, CTLA-4 effector cells were seeded into a white flat-bottom 96-well assay plate, followed by addition of cellular medium harvested by transfected HEK293T-17 cells that were used in place of the antibody dilutions, without being diluted. Afterwards, aAPC/Raji Cells were added in the plate with CTLA-4 Effector Cells and supernatants. After co-culture for 6 h at 37 °C in a 5% CO2 humidified incubator, the Bio-Glo™ reagent was added to the plate and incubated at ambient temperature for 5 min. The luminescence signal was measured using a Glo-max Discover System and reported as relative light units (RLUs), and the data were analysed using GraphPad Prism.

3.8 Cytotoxic assay with xCelligence

3.8.1 $CD8^+$ T cell isolation and CAR transduction

$CD8^+$ T cells were isolated via negative selection using "CD8+ T cell isolation kit" (Miltenyi Biotec, 130-096-495) from PBMCs previously isolated by buffy coats through density gradient centrifugation via Ficoll Paque Plus (Product #GE17-1440-02, Sigma-Aldrich). Isolated $CD8^+$ T cells were seeded at a final concentration of $0.5x10^6$ cells/ml in 24-well plate in 2ml of RPMI-1640 (Life Technologies, 21875091) supplemented with 10% FBS, 100 mg/mL P/S, 10 mM Glutamax, 10 mM Hepes, and IL-2 (Product #130-097-743, Miltenyi Biotec,) at the final concentration of 30 UI/ml. Cells were stimulated with Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation (Product #11132D, Life Technology) according to the manufacturer's protocol. The following day beads were removed using Dynamag 15 (#12301D, Life technology) and $CD8^+$ T cells were transduced with CAR-encoding lentiviral vector. Briefly, cells were seeded in a 24-well plate at the final concentration of $1*10^6$/ml per well in a final volume of 1 ml of RPMI-1640 medium, supplemented with IL-7 (Product #130-095-362, Miltenyi) and IL-15 (Product #130-095-764, Miltenyi) both at the final concentration of 10 ng/ml. The used ratio between the fresh medium and virus-containing supernatant is 1:3. Protamine sulfate (Product #P3369, Sigma-Aldrich) was added at a final concentration of 10 µg/ml (dilution 1:1000 from the 10 mg/ml stock). Plate was spinned 1000g at 32°C for 1 hour, taken out from the centrifuge and cultured into the incubator. The day after medium was replaced with fresh medium. T cells were counted and fed every two days day and were then expanded until day 10. CAR expression in transduced $CD8^+$ T cells was analyzed in flow cytometry by Fc staining four days after transduction, as described above (see section 3.7.1). On day 8 after CAR transduction, $CD8^+$ T cells were used for the cytotoxicity assay using the xCELLigence Real-Time Cell Analysis (RTCA) system.

3.8.2 In vitro co-culture of CAR-T cells and SKOV3 tumor cells

To perform cytotoxicity assay with CAR-T cells, SKOV3 cells were previously transduced with a lentiviral vector expressing mCherry under a constitutive promoter to generate a fluorescent SKOV3 cell line to monitor its growth. mCherry positive cell population was sorted by flow cytometry to enrich the transduced cells that were then used for the co-culture with CAR-T cells. On day 8 after $CD8^+$ T cells transduction, a 5-days experiment with the xCELLigence Real-Time Cell Analysis (RTCA) system was launched. Briefly, $mCherry^+$

SKOV3 cells were seeded in 96-well plate at 3000 cells per well. After 1 hour, tumor cells were pre-incubated with 50 µl of supernatant previously harvested by HEK293T-17 cells not transfected or transfected with 2xKN035 plasmid. After another hour, CAR-T cells were added in RPMI-1640 medium supplemented with 10% FBS, 100 mg/mL P/S, 10 mM Glutamax, 10 mM Hepes and 300 U/ml IL-2 using effector cells:tumor cells (E:T) ratios 1:1 and 3:1. Each experimental condition was in technical triplicates, for each E:T ratio. Co-culture was monitored overtime by imaging for five days, then the acquired images were exported and analyzed by the RTCA eSight Software to calculate the red total area per image. Graphs showed the normalization of the red total area value of each time-point to the value of the first time-point recorded after the co-culture started to monitor proliferation overtime.

3.9 Statistical analysis

Data were analyzed with Graph Pad Prism version 8.0. Unpaired Student t-test and One-way ANOVA, considering multiple comparison corrections (statistical significance: $P < 0.05$). Statistical significance was determined using the methods described in the results section or in the figure legends.

4 Results

4.1 Design of genetically encoded nanobody-based modulators

The proposed synthetic biology approach involves the design of synthetic circuits to block pathways that have a crucial role in T-cell exhaustion onset, specifically immune checkpoint pathways. Thus, nanobodies targeting inhibitory receptors have been identified and exploited to produce genetically encoded synthetic modulators to locally counteract T-cell exhaustion for improvement of T-cell-based therapies.

4.1.1 Identification of nanobodies for immune checkpoint blockade

Anti-exhaustion synthetic circuits were designed exploiting two sdAbs already identified and described in literature for immune checkpoint blockade in cancer therapy: i) KN035, targeting a ligand of PD-1, the programmed death-ligand 1 (PD-L1); ii) Nb16, which specifically binds to the CTLA-4 inhibitory receptor.

i) Anti PD-L1 nanobody: 'KN035'

PD-1/PD-L1 immune checkpoint is one of the most studied inhibitory pathways involved in T-cell dysfunction. While physiologically this pathway prevents excessive immune response and tissue damage, in tumors its upregulation inhibits T-cell functions and proliferation leading to T-cell exhaustion. Several mAbs against PD-1 have been produced proving to revert exT cell phenotype by preventing the PD-1/PD-L1 interaction. The receptor interaction with the other ligand PD-L2 is also crucial to avoid autoimmunity, therefore targeting the ligand PD-L1 rather than the receptor could be therapeutically more advantageous. *Zhang et al.* have identified a high-affinity anti human PD-L1 sdAb, named KN035, that showed potent anti-tumor activity, comparable to durvalumab, a promising anti PD-L1 mAb in clinical trial (Ibrahim, Stewart, and Shalabi 2015). The identified sdAb is characterized by high thermal stability and no cross-reactivity with PD-L2 or with mouse PD-L1. The fusionbody KN035-Fc, produced by fusing sdAb to the Fc fragment of human IgG1, showed high binding affinity to PD-L1 with a *K*d of 3.0 nM and was able to block the PD-1/PD-L1 interaction with an IC_{50} value of 5.25 nM in a competitive ELISA, competing with PD-1 and disrupting the pre-formed PD-1/PD-L1 complexes also more efficiently than the mAb. KN035-Fc fusion protein induced cytokine secretion in lymphocytes *in vitro* and proved to be

a potent inhibitor of the PD-1 immune checkpoint *in vivo* showing a half-life of 72 hours and a strong anti-tumor activity in an immune xenograft tumor model previously used to characterize durvalumab. Authors have also solved the crystal structure of the complex KN035:PD-L1 demonstrating that sdAb and PD-1 receptor compete with each other for the binding to the same surface of PD-L1, and KN035 has a higher PD-L1 binding affinity compared to PD-1 due to the flexibility of its CDR loops (F. Zhang et al. 2017). Furthermore, the efficacy and safety of KN035-Fc (named Envafolimab) is being studied in clinical trials in multiple tumor types in China, the United States and Japan, including registration/phase III clinical trials in multiple indications. Recently, Envafolimab was approved in China for the treatment of adult patients with previously-treated microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) advanced solid tumors (Papadopoulos et al. 2021; Markham 2022). Given this evidence, I choose KN035 as anti PD-L1 sdAb to design synthetic actuators for blockade of PD-1 immune checkpoint to revert T-cell exhaustion.

ii) Anti CTLA-4 nanobody: 'Nb16'

CTLA-4 is a crucial regulator of immune responses playing a key role in inhibition of T-cell activity in the exhaustion context. In the presence of persistent antigen stimulation, CTLA-4 is upregulated on the T-cell surface and abolishes the CD28 co-stimulatory signal by binding with higher affinity to the same ligands of CD28, CD80 and CD86 that are expressed on antigen presenting cells, thus triggering an inhibitory signal that impairs T-cell activation. Several mAbs against CTLA-4 have been discovered and clinical trials provided promising results in terms of enhancement of anti-tumor response. A CTLA-4-specific sdAb, named Nb16, has been identified from a large immune phage display library and has demonstrated high binding rates for CTLA-4 and anti-tumor activity in a melanoma mice model, leading to reduced tumor growth and prolonged mice survival (Wan et al. 2018). Successively, the same group has deeply explored the anti-tumor function of Nb16 using a model of hepatocellular carcinoma. In vitro, stimulation with Nb16 demonstrated an increase of $CD8^+$ T cell proliferation and IFNγ production in a model based on tumor-specific cytotoxic T lymphocytes. Moreover, findings in murine cancer xenograft models showed that Nb16 is able to significantly inhibit tumor growth and increase apoptosis of cancer cells boosting the anti-tumor

activity of tumor-specific T cells (Hou et al. 2022). Nb16 has been therefore chosen for the design of anti-exhaustion modulators that specifically target the CTLA-4 immune checkpoint.

4.1.2 Development of nanobody-based synthetic circuits

For the development of genetically encoded sdAb-based modulators, KN035 and Nb16 encoding sequences were codon-optimized for expression in mammalian cells and a histidine tag (His-tag) was added at the C-terminal end of the sequence for future detection of sdAb expression. Firstly, monomeric sdAb constructs were designed by fusing the sdAb encoding sequence to a reporter gene to easily verify the functionality of the constructs. Specifically, the sdAb sequence was linked to a sequence encoding for the mCherry fluorescent protein through a 2A self-cleaving peptide (P2A) that allows for the equimolar expression of multiple proteins under the same promoter (Figure 4a). The truncated human elongation factor-1 alpha (shEF1α) promoter and the phosphoglycerate kinase (PGK) promoter were tested to drive sdAb expression because they are among the most used and efficient for primary T-cell engineering (Rad S M et al. 2020) (Figure 4a).

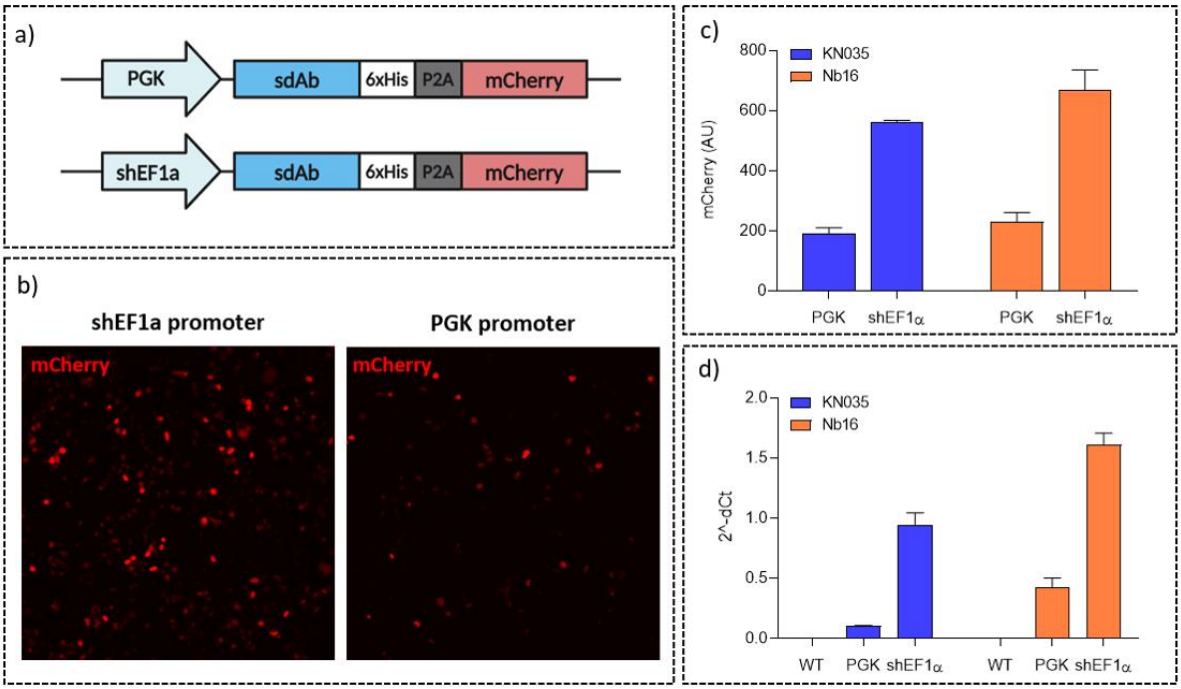


Figure 4 Design and validation of nanobody-based synthetic circuits. a) Scheme of nanobody constructs: sdAb encoding sequence was fused to the fluorescent protein mCherry by a P2A self-cleavage peptide to easily evaluate the functionality of the circuits. A histidine tag (6xHis) was added at the C-terminal end of the sdAb sequence for their further detection. Created with BioRender.com. **b-d)** Validation of sdAb production in HEK293T-17 cells transfected with sdAb plasmids. mCherry protein expression was assessed by microscopy (b) and flow-cytometry (c), meanwhile mRNA levels of nanobodies were analyzed by quantitative PCR (d).

The synthetic circuits were transfected in human embryonic kidney 293T-17 (HEK293T-17) cells to ensure sdAb expression detecting mCherry fluorescence by microscope (Figure 4b) and flow cytometry (Figure 4c) and analyzing gene expression of nanobodies by quantitative PCR (qPCR) (Figure 4d). The results demonstrated the functionality of the constructs, showing strong mCherry expression (Figure 4c) and high sdAb mRNA levels (Figure 4d), majorly with the shEF1α promoter.

Once verified plasmid functionality and sdAb gene expression, new circuits were created to meet the needs for future T-cell engineering. Thus, shEF1a was selected as the constitutive promoter for its strength, the fluorescent reporter was removed and the sdAb sequence was fused to an N-terminal immunoglobulin-derived secretion signal (Igk) to ensure secretion, crucial to block immune checkpoints by extracellular domain binding (Figure 5). In addition to monomeric sdAbs (Figure 5a), multimeric formats were designed to increase the functional affinity and half-life of sdAbs to improve their anti-tumor efficacy in future potential in vivo applications. Bivalent sdAbs were developed by coupling two KN035 or Nb16 molecules by a glycine-serine (GS) linker, referred to as 2xKN035 and 2xNb16, respectively (Figure 5b). Also, sdAb-Fc fusion proteins, called KN035-Fc and Nb16-Fc, were generated by genetically fusing the sdAb KN035 or Nb16 via a hinge region to the sequence of the fragment crystallizable (Fc) region of human IgG1 antibody (Figure 5c). Therefore, the new constructs were validated in vitro by various functional assays to assess the ability of sdAbs to recognize and bind the target, thus blocking the immune checkpoint activation.

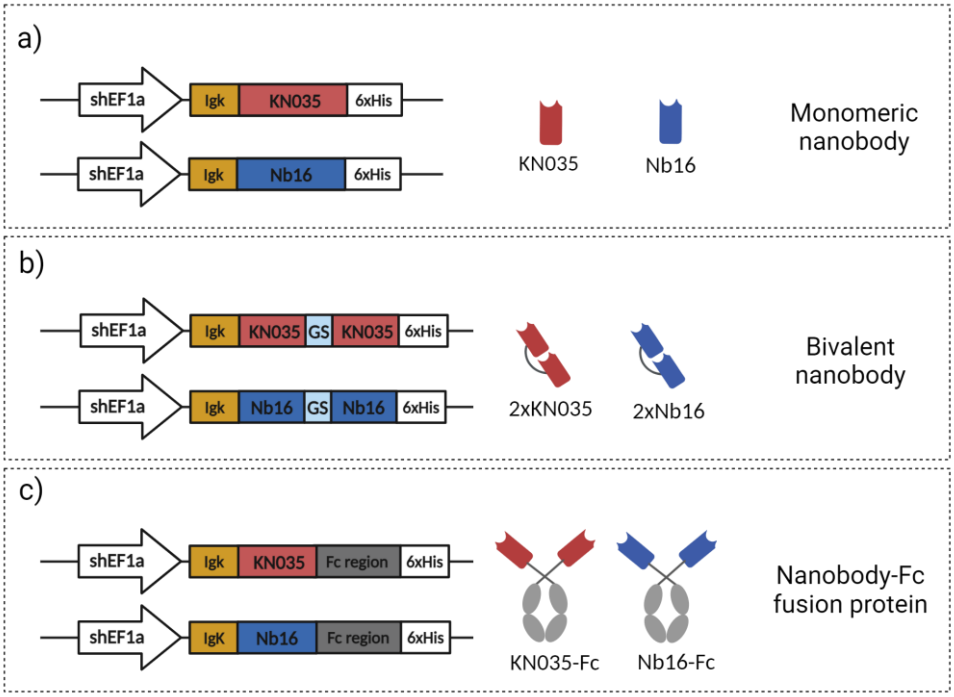


Figure 5 Design of several nanobody-based variants for immune checkpoint blockade. In addition to monomeric sdAbs, multimeric variants were designed. **a)** Schemes of monomeric nanobodies. The constitutive promoter shEF1α was used to drive sdAb expression and the encoding sequence was linked at the N-terminal end to a secretion signal (IgkL) to enable sdAb secretion. **b)** Constructs of bivalent nanobodies in which two KN035 or Nb16 molecules were coupled using a glycine-serine (GS) linker, referred as to 2xKN035 and 2xNb16, respectively. **c)** sdAb-Fc fusion proteins were generated by fusing the sdAb KN035 or Nb16 to the fragment crystallizable (Fc) region of the human IgG1 antibody. Created with BioRender.com.

4.2 Functional characterization of anti PD-L1 nanobodies

Before performing functional assays, the expression of KN035-based synthetic constructs was validated by transfecting HEK293T-17 cells, followed by intracellular His-tag staining and evaluation by flow cytometry after 48 hours. Dot-plot graphs showed the presence of a His-tag$^+$ population in all three conditions proving a successful expression of all the KN035 variants (Figure 6a). sdAb production was also assessed by intracellular staining of transfected cells with an anti-VHH mAb specific for camelid-derived antibodies, highlighting a good expression of KN035-based molecules (Figure 6b). Once verified the anti PD-L1 sdAb expression, several assays were performed to investigate their target specificity and their ability to block the immune checkpoint activation.

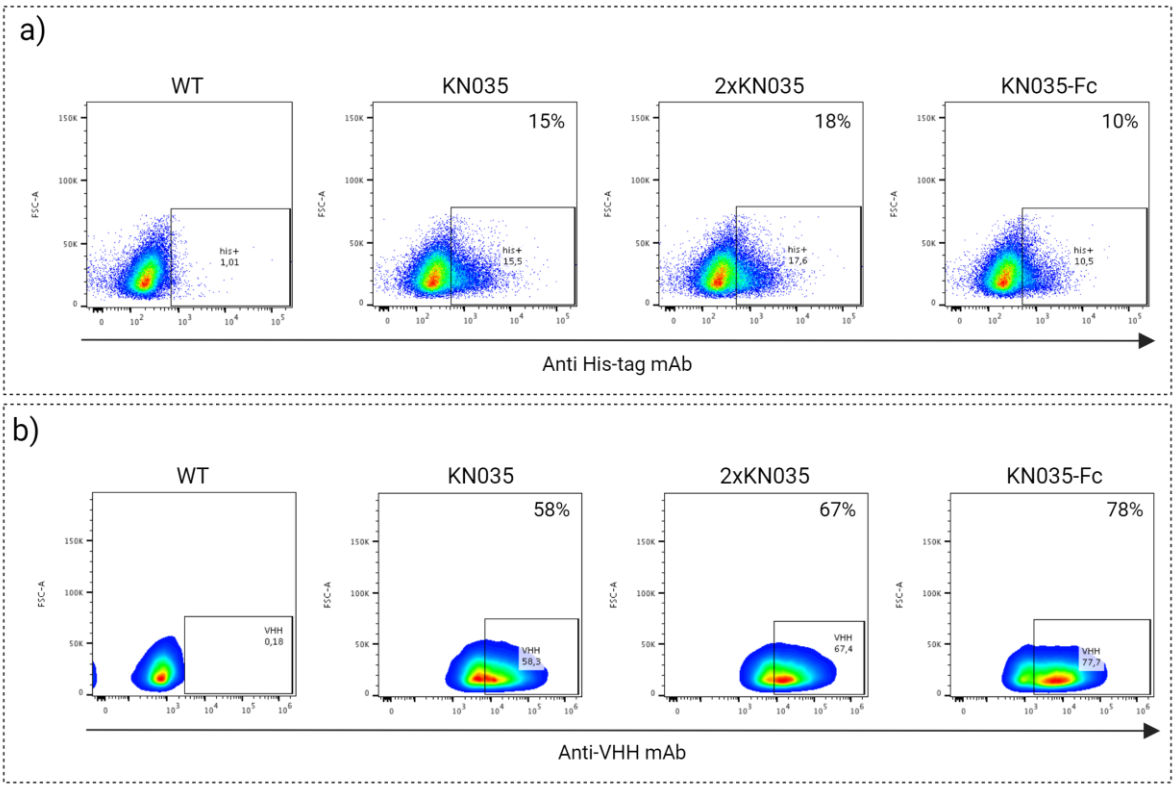


Figure 6 Validation of synthetic constructs encoding for anti PD-L1 nanobodies. Dot-plots graphs showing the percentage of His-tag$^+$ (a) and VHH$^+$ (b) cells upon transfection with the plasmids encoding for the various formats of anti PD-L1 sdAbs. The showed results were derived from two different transfection experiments, but performed following the same transfection protocol.

4.2.1 Competitive anti-PD-L1 binding assay

Target binding specificity of anti PD-L1 sdAbs was initially verified by flow cytometry-based competition assay using cell lines naturally express the PD-L1 target protein.

Firstly, PD-L1 expression was measured in flow cytometry by staining different cell lines, namely the human embryonic kidney HEK293T-17 cells, the breast cancer cells MDA-MB-231 and the ovarian cancer cell line SKOV3. To this end we used an anti PD-L1 conjugated mAb. Results indicated that HEK293T-17 did not express PD-L1, whereas the other two tumor cell lines did. The MDA-MB-231 cells expressed the highest levels of the target protein (Figure 7a).

Therefore, MDA-MB-231 and SKOV3 cells were used to perform competition assay experiments, hypothesizing that if the anti PD-L1 sdAbs efficiently bind the target, the immediate consequence is that the classical fluorophore-conjugated mAb will not be able to bind the same ligand, reducing the emitted signal. Thereby, the nanobody and the mAb will compete for PD-L1 binding resulting in a decrease of the PD-L1 signal in the presence of secreted sdAbs. This first variant of the competitive-binding assay was performed by directly transfecting MDA-MB-231 cells with plasmids encoding for the secreted anti PD-L1 sdAbs and 48 hours post-transfection cells were stained with a PE-conjugated anti PD-L1 mAb to measure blocking of PD-L1 by flow cytometry (Figure 7b).

The results showed no relevant changes in the presence of monomeric nanobody (KN035, blue bar) compared to cells transfected with a control plasmid (GFP, green bar), but a marked decline was observed in the PD-L1 signal in the presence of both the bivalent format 2xKN035 (purple bar) and the KN035-Fc fusion protein (orange bar). Moreover, results highlighted a slight increase of PD-L1 in transfected cells compared to not transfected (WT) cells (Figure 7c). To avoid PD-L1 alterations due to transfection and to evaluate a bystander effect of secreted sdAbs, the competition assay was performed by incubating non-transfected tumor cells with sdAb-including supernatant (SN) to mimic the perspective conditions in which sdAbs secreted by engineered T cells act on surrounding tumor cells. HEK293T-17 cells were transfected with the several sdAb formats and, after 48 hours, the SN was harvested and transferred to the media culturing the tumor cells (Figure 7d). MDA-MB-231 (Figure 7e) and SKOV3 (Figure 7f) cells were treated with sdAb-including SN for 1 hour before staining with the anti PD-L1 mAb. In both cell lines, a significant blockade of PD-L1 was observed when tumor cells were treated with supernatant of cells transfected with multimeric sdAb (2xKN025

and KN035-Fc), whereas incubation with monomeric KN035 SN did not induce any PD-L1 variation in recipient cells displaying levels similar to those of cells treated with WT SN or untreated (UT) (Figure 7e-f).

Overall, the competition assay results demonstrated the capacity of KN035-based sdAbs to block PD-L1 and highlighted a bystander effect of secreted sdAbs on the surrounding non-transfected cells, even though the monomeric format did not induce significant changes in the PD-L1 expression. This suggests that sdAb and mAb may bind simultaneously to the target protein probably due to the small size of nanobody.

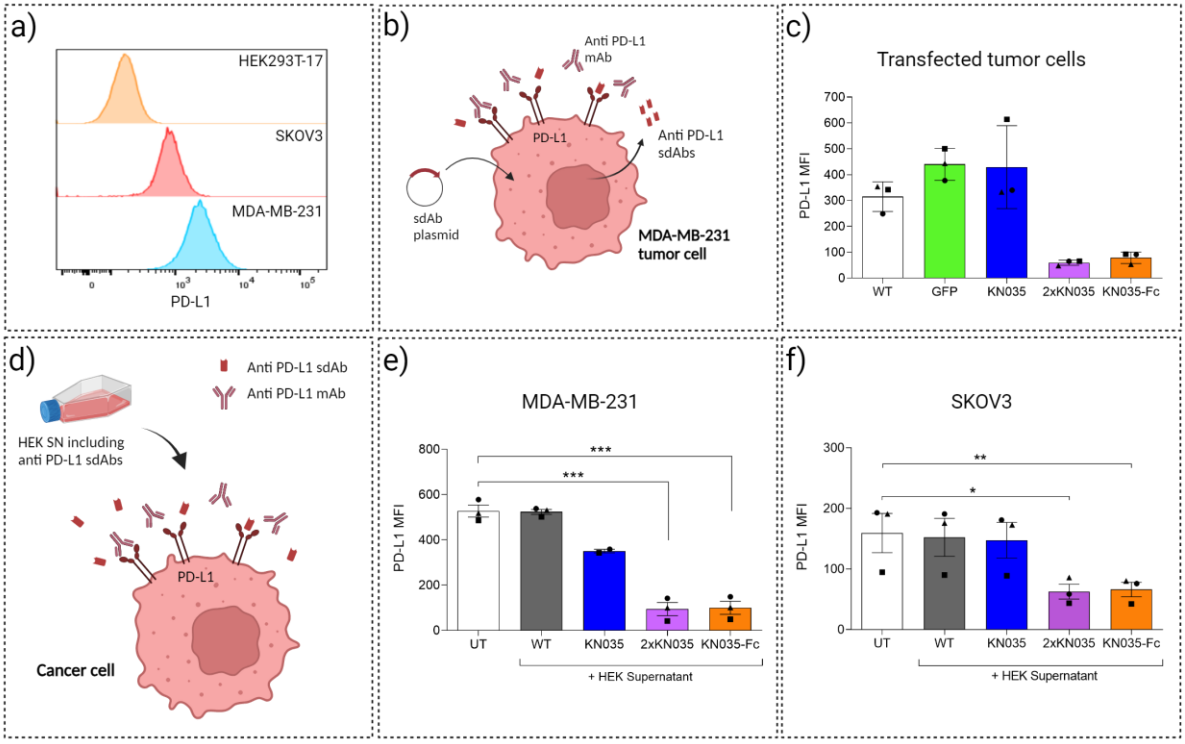


Figure 7 Competition assays to verify the target binding capacity of anti PD-L1 nanobodies. a) PD-L1 expression was measured in different cell lines by flow-cytometry. MDA-MB-231 cells and SKOV3 cells express PD-L1 even though at different levels. **b)** Scheme of competition assay performed by transfecting MDA-MB-231 cells with sdAb plasmids and staining them with anti PD-L1 mAb 48 hours post-transfection for flow-cytometry analysis. Created with BioRender.com. **c)** Flow-cytometry data showed a marked decrease of PD-L1 in cells transfected with the multimeric KN035 variants (2xKN035 and KN035-Fc), but not in cells transfected with the monomeric KN035 that exhibited PD-L1 levels like the transfection control (GFP). Moreover, transfected cells (GFP) were found to have slightly higher PD-L1 expression compared to non-transfected cells (WT) (n=3). **d)** Scheme of competitive binding assay performed by treating tumor cells with supernatant (SN) harvested by HEK293T-17 cells transfected with sdAb plasmids. Created with BioRender.com. **e-f)** Both MDA-MB-231 (e) and SKOV3 (f) cells were treated with HEK SN for 1 hour and then stained with anti PD-L1 mAb. Results showed a significant drop in PD-L1 signal only in cells treated with 2XKN035 and KN035-Fc SN, in both cell lines. Cells not treated (UT) indicated the basal PD-L1 expression, whereas cells treated with SN of not transfected cells (WT) were used as negative controls. **e)** Statistic analysis on MFI values. Mixed-effects model (REML), Dunnett's multiple comparisons test, ***P<0.001 compared to UT (n=3). **f)** Statistic analysis on MFI values. Friedman test, Dunnett's multiple comparisons test, *P<0.05, **P<0.01 compared to UT (n=3).

4.2.2 Bioluminescent PD-1/PD-L1 Blockade Bioassay

Next, the blockade activity of anti PD-L1 sdAbs was investigated performing a bioluminescent cell-based bioassay to evaluate whether the binding of the nanobodies functionally impairs the PD-1/PD-L1 axis. This assay is based on two genetically engineered

cell lines: 1) a PD-1 effector cell line, stably expressing human PD-1 and a luciferase reporter driven by a Nuclear Factor of Activated T cells-response element (NFAT-RE); 2) a PD-L1 positive cell line which stably expresses human PD-L1 along with an engineered cell surface protein designed to activate cognate TCRs in an antigen-independent manner. When the two cell types are co-cultured, the PD-1/PD-L1 interaction inhibits TCR signaling and NFAT-RE-mediated luminescence. The presence of either anti-PD-1 or anti-PD-L1 binders blocking the PD-1/PD-L1 interaction releases the inhibitory signal and results in TCR activation and luciferase expression (Figure 8a). Co-culture of the two cell lines was performed in presence of supernatants (SN) containing secreted nanobodies that were collected from HEK293T-17 cells previously transfected with the several sdAb plasmids (KN035, 2xKN035, KN035-Fc). SN harvested from WT cells and cells transfected with a control plasmid encoding for a secreted red fluorescent protein (Sec-mkate) were used as negative controls. No luminescence was detected in these controls, whereas a significant induction of transcriptional-mediated bioluminescent signal was detected in cells incubated with SN containing the several types of sdAbs (Figure 8b).

These results show that all the KN035-based formats can block the immune checkpoint pathway, including the monovalent KN035 despite it did not induce any PD-L1 decrease in the competition assay. This suggests that the single sdAb and the mAb can bind to PD-L1 simultaneously, probably due to the sdAb small size, while the multivalent formats may prevent the mAb binding for steric hindrance resulting in the PD-L1 decrease.

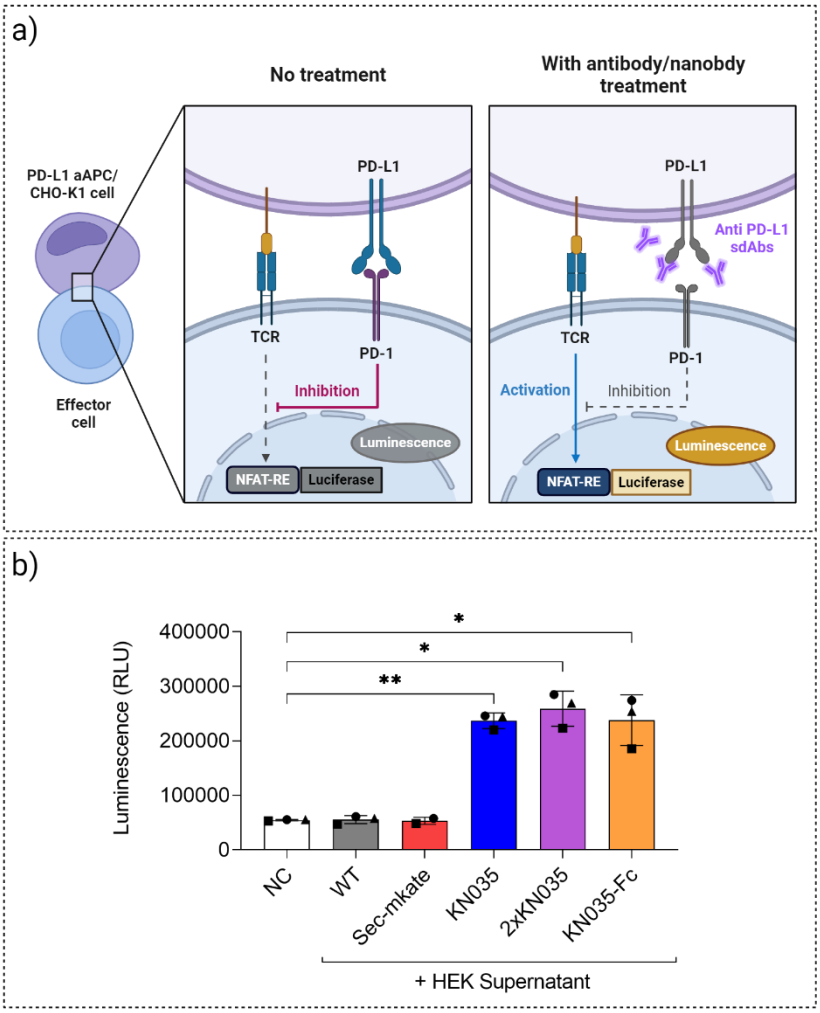
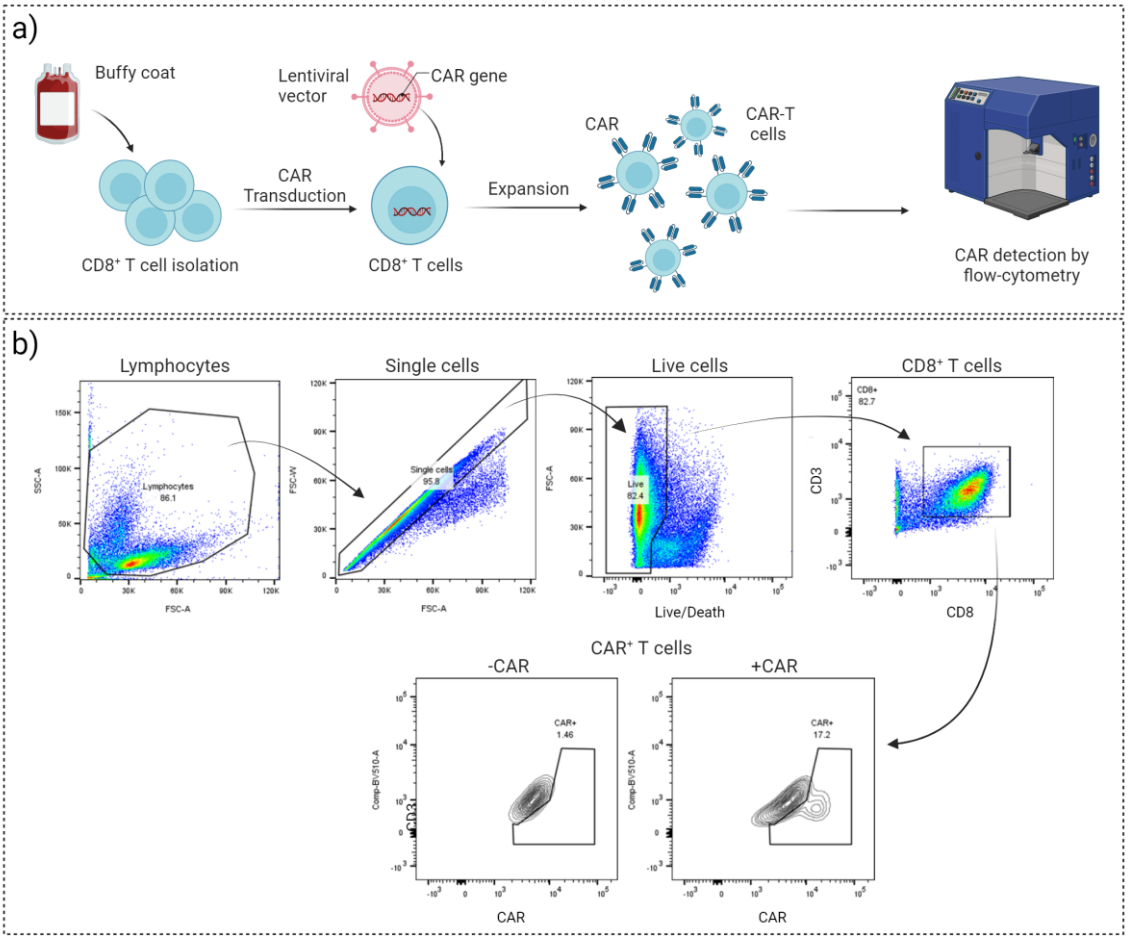


Figure 8 Bioluminescent cell-based PD-1/PD-L1 blockade bioassay. a) The PD-1/PD-L1 blockade bioassay consists of two genetically engineered cell lines, PD-1 Effector Cells and PD-L1 aAPC/CHO-K1 Cells. When co-cultured, the PD-1/PD-L1 interaction inhibits TCR-mediated luminescence. When the PD-1/PD-L1 interaction is disrupted by the presence of PD-1 or PD-L1 binders, TCR activation induces luminescence. Created with BioRender.com. b) PD-L1 aAPC/CHO-K1 Cells were plated and incubated for 16–20 hours prior to the addition of nanobody-including supernatants and PD-1 Effector Cells. SN was harvested by HEK293T-17 cells previously transfected with sdAb plasmids. Co-culture in absence of any SN was used as negative control (NC). All the variants led to a significant induction of luminescence, demonstrating the ability of sdAbs to block the immune checkpoint pathway. Statistical analysis on luminescence values. Mixed-effects model (REML), Dunnett's multiple comparisons test, *P<0.05, **P<0.01 compared to NC (n=3).

4.2.3 Evaluation of anti-tumor activity in a CAR-T model

After proving a strong capacity of anti PD-L1 sdAbs to block PD-1/PD-L1 immune checkpoint, I evaluated if they could improve the outcome of CAR-T-cell therapy. A tumor model based on the ovarian cancer cell line SKOV3 was used and tumor cells were genetically engineered to constitutively express a fluorescent reporter (mCherry) helpful to monitor tumor growth. In parallel, CD28-costimulated CAR-T cells targeting human epidermal growth factor receptor 2 (HER2) were generated. After isolation from buffy coats, $CD8^+$ T cells were transduced with a third-generation LV encoding for an anti-HER2 CAR, including a scFv derived from the humanized mAb 4D5 Herceptin (trastuzumab) and linked to T cell signaling domains derived from CD28 and the CD3ζ, and were then expanded. On the eighth day of T-

cell expansion, the expression levels of surface CAR were assessed by flow cytometry (Figure 9a). CAR$^+$ population was analyzed inside live CD8$^+$ T cells showing a percentage of around



20% in transduced cells (Figure 9b).

Figure 9 CAR-T-cell production a) CD8$^+$ T cells were isolated from buffy coats, transduced to stably express an anti-HER2 CAR and then expanded for 8 days. Before to start the cytotoxicity assay, CAR expression was evaluated by flow cytometry. Created with BioRender.com. b) Gating strategy to verify CAR expression in transduced T cells. CAR$^+$ T cells were selected inside the live CD8$^+$ T cell population identified using the markers CD3 and CD8.

To study tumor-killing activity, HER2 CAR-T cells were co-cultured with mCherry$^+$ SKOV3 tumor cells testing two different effector/target (E:T) ratios (1:1 and 3:1) . SKOV3 cell proliferation was evaluated by using the xCELLigence Real-Time Cell Analysis (RTCA) system that enables to monitor over time the fluorescence provided by mCherry$^+$ tumor cells by imaging. To evaluate the effects of sdAbs on CAR-T cells, SKOV3 cells were seeded and 1 hour later were pre-treated with SN including bivalent anti PD-L1 sdAbs, previously collected by transfected HEK293T-17 cells, before the addition of CAR-T cells. Pre-incubation of tumor cells with SN of WT cells was used as a negative control condition. The decrease of SKOV3 proliferation was evaluated by calculating the red total area at each time point and normalizing it to the value of the first time point upon CAR-T cell addition. After five days of co-culture, red fluorescence was analyzed and a decrease in the red total area is

an index of impaired growth of tumor cells representing thus an indication of CAR-T tumor-killing capacity. Compared to the control condition without CAR-T cells (only SKOV3), SKOV3 cells proliferated much less when co-cultured with CAR-T cells and the extent of red signal decrease correlated with the E:T ratios suggesting a good capacity of CAR-T to recognize and kill cancer cells (Figure 10a). Importantly, a stronger impairment of SKOV3 tumor growth was observed when tumor cells were pre-incubated with 2xKN035-rich supernatant and not with WT SN, resulting in a steady decline of red total area over time. Even though some effects in the presence of sdAbs were observed with both the E:T ratios (1:1 or 3:1), a strong blockade of tumor proliferation was achieved when lymphocytes were three times the number of tumor cells (ratio 3:1), whereas the ratio 1:1 did not induce drastic changes in red total area (Figure 10b-c).

These results suggested that the blockade of PD-L1 due to the presence of sdAbs can positively affect the tumor-killing activity of CAR-T cells, improving their capacity to prevent tumor cell proliferation.

Given these preliminary results, more long-term co-culture experiments will be performed by keeping cell lines in co-culture until 8-10 days and after this step proceeding the experiment by incubating CAR-T cells with fresh tumor cells for evaluating the long-term resistance of CAR-T cells and investigating a potential onset of CAR-T cell exhaustion due to the persistent antigen-dependent T-cell activation. In this context, sdAbs may be useful not only to improve CAR-T killing activity but also to delay or prevent the occurrence of exhaustion by blocking one of the crucial inhibitory pathways involved in the loss of T-cell effector functions. Further experiments will be performed by engineering T cells with both CAR and sdAb circuits to make CAR-T cells able to continuously secrete nanobodies for PD-L1 blockade. This will enable longer cytotoxicity assays using effector cells continuously providing fresh sdAbs, overcoming the potential limitation related to their short half-life and reproducing a context closer to the in vivo situation in tumor site in view of a future in vivo application of sdAb-secreting CAR-T cells.

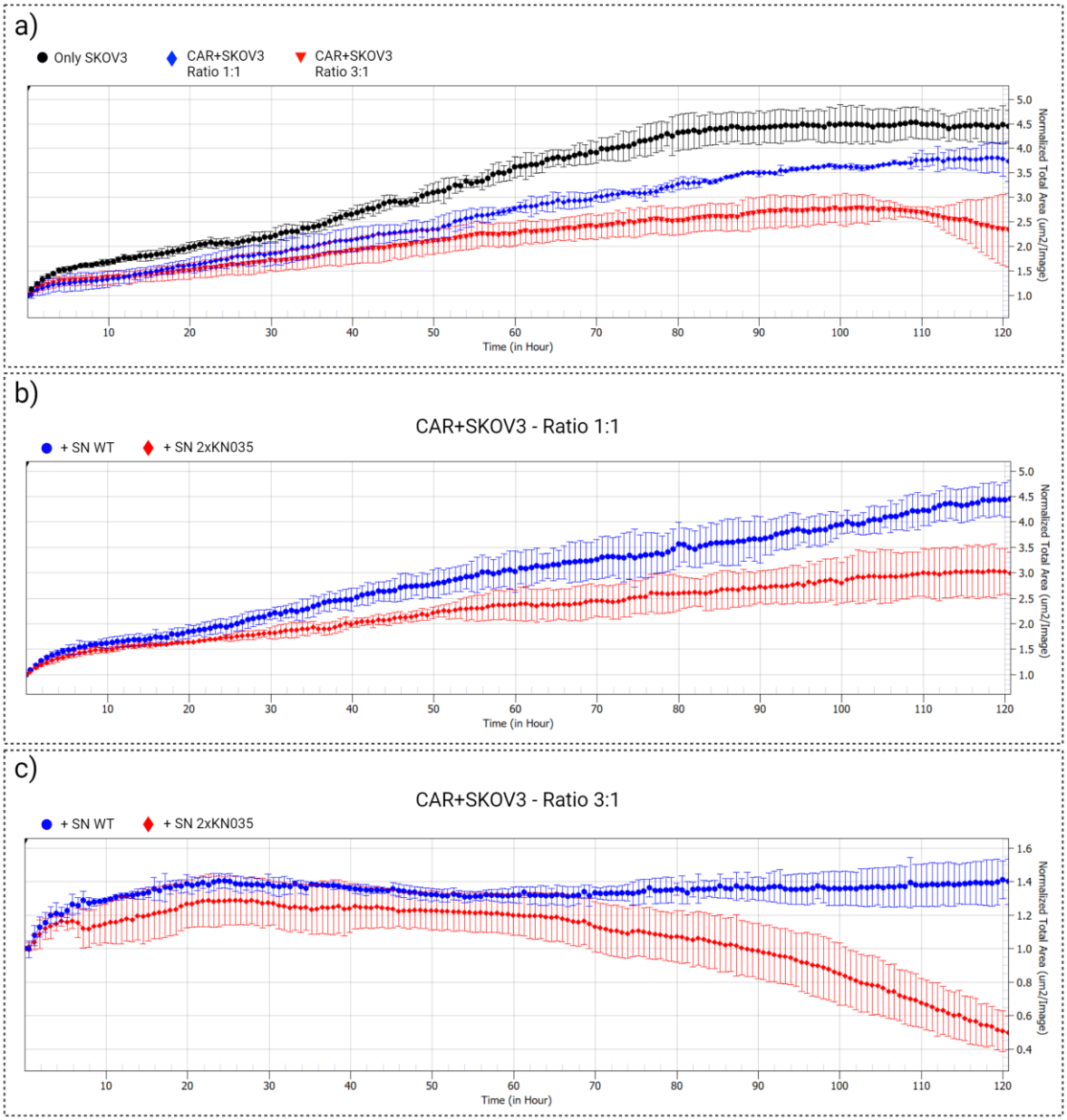


Figure 10 Nanobody validation in a CAR-T cell therapy model. a) Graph showing the effect of CAR-T on SKOV3 proliferation, using E.F ratios 1:1 or 3:1. **b-c)** Graphs showing the red total area provided by mCherry^{+} SKOV3 cells in co-culture experiments. Ratios E:T 1:1 (b) and 3:1 (c) were tested. Synergistic effects of CAR-T and sdAb on tumor cell proliferation was evaluated by adding WT SN or 2xKN035-rich SN in the co-culture. Tumor cell growth was monitored over time for five days by using the xCELLigence Real-Time Cell Analysis (RTCA) system. Red total area values were normalized on the value of the first time-point after starting the co-culture (Technical replicates n=2).

4.3 Functional characterization of anti CTLA-4 nanobodies

As for anti PD-L1 sdAbs, the constructs for the several Nb16-based formats were validated in HEK293T-17 cells that were transfected with sdAb plasmids and after 48 hours were stained with anti His-tag mAb to verify sdAb expression. The dot-plot graphs indicated that all three conditions had a His-tag$^+$ population, confirming the successful expression of all KN035 variants (Figure 11a). In a further transfection with sdAb plasmids, a plasmid encoding for green fluorescent protein (GFP) was used as transfection control and 48 hours later cells were stained with an anti-camelid antibody. Unfortunately, the staining did not detect VHH$^+$ populations, despite the percentage of GFP$^+$ cells was high (Figure 11b), indicating a good transfection efficiency. This could be due to variations in the framework regions of the sdAb sequence compared to anti PD-L1 sdAbs that make the anti-VHH mAb unable to recognize Nb16-based molecules. Since these discordant results, further investigation was needed to understand the functionality of Nb16 constructs. Unfortunately, it was not possible to perform a flow cytometry-based competition assay as done for KN035 sdAbs due to the lack of anti CTLA-4 antibodies that bind the receptor in its extracellular domain. In fact, all the products used in the literature recognize an epitope in the intracellular domain. Therefore, a bioluminescent cell-based bioassay was performed to directly assess the anti CTLA-4 sdAb capacity to disrupt interaction between the inhibitory receptor and its ligands CD80/86.

Similar to the PD-1/PD-L1 functional assay, it consists of two engineered cell lines: 1) a CTLA-4 effector cell line stably expressing human CTLA-4 and a luciferase reporter driven by a native promoter that responds to TCR/CD28 activation; 2) an APC cell line expressing an engineered cell surface protein designed to activate cognate TCRs in an antigen-independent manner and endogenously expressing CTLA-4 ligands, CD80 and CD86. When the two cell types are co-cultured without any treatment, CTLA-4 competes with CD28 for their shared ligands, CD80 and CD86, and thus inhibits TCR/CD28 pathway activation repressing the luciferase transcription. The presence of anti CTLA-4 biologics prevents the interaction of CTLA-4 with CD80/CD86 resulting in TCR activation and expression of luminescence (Figure 11c). The co-culture was performed in the presence of cell supernatant (SN) collected from HEK293T-17 cells transfected with the plasmids encoding the monomeric, the bivalent, and the Fc fusion protein format of the anti CTLA-4 sdAb (referred to as Nb16, 2xNb16, Nb16-Fc, respectively). Unfortunately, results showed no increase in luminescence upon the addition of nanobody-including SN compared to untreated samples or samples treated with SN of WT cells, seeming that no Nb16 format can prevent the CTLA-4

pathway activation (Figure 11d). If the results will be confirmed, two alternative hypotheses may explain the lack of effect: i) it may be that the nanobody is not able to recognize the target or ii) the nanobody binds to CTLA-4 but without preventing the interaction with the ligands CD80/CD86. Further experiments may be necessary to verify these hypotheses and eventually, it will be necessary to look for a new anti CTLA-4 sdAb sequence.

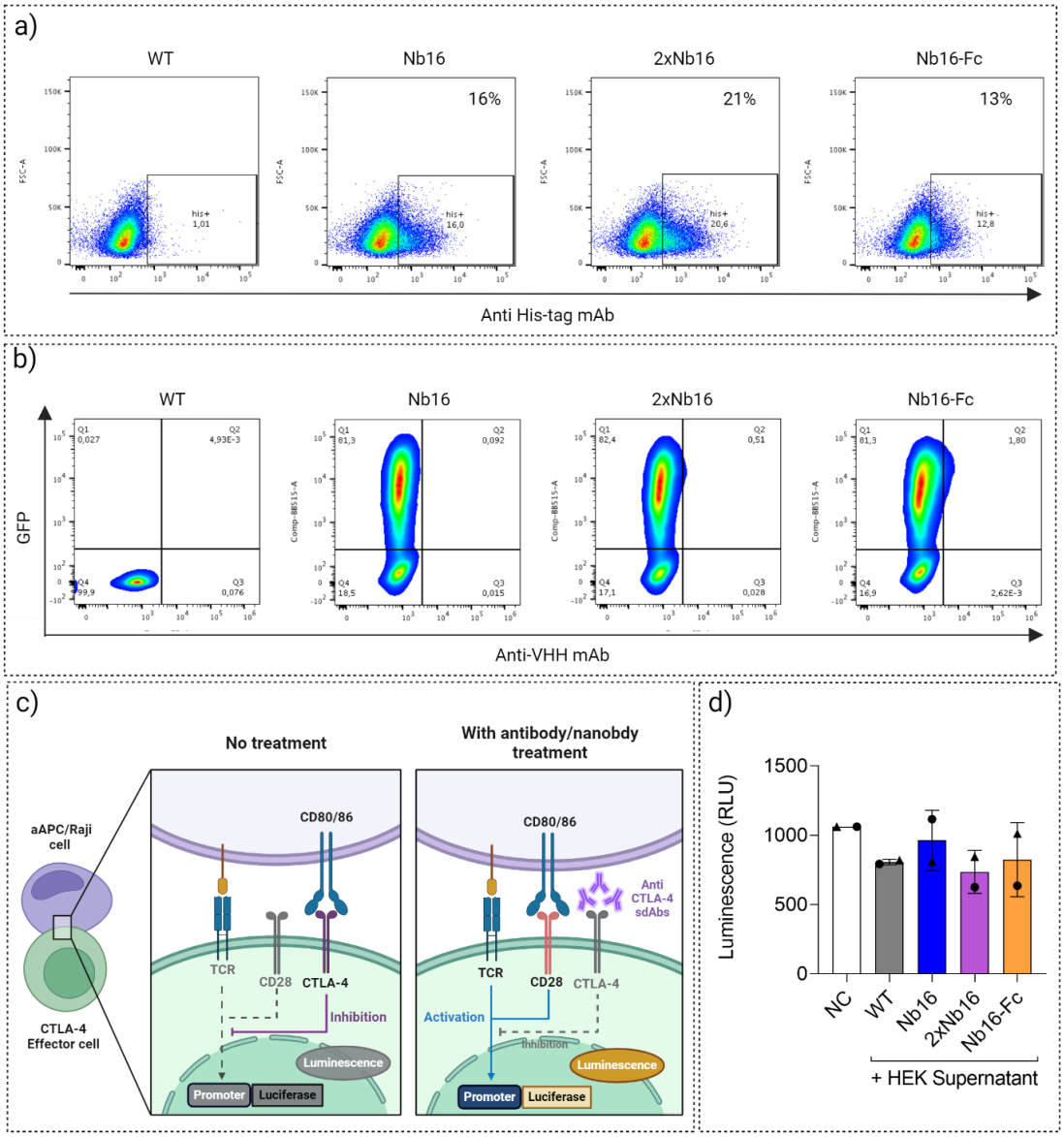


Figure 11 Characterization of anti CTLA-4 nanobodies. a) Dot-plot graphs of HEK293T-17 cells transfected with Nb16 plasmids and then stained with anti His-tag mAb showing a successful expression of all three anti CTLA-4 sdAb variants. **b)** Other experiments were performed by transfecting cells with Nb16 plasmids and a plasmid encoding for a green fluorescent protein (GFP) as transfection control. Then cells were stained using an anti-VHH mAb. Despite the positive results of His-tag staining, no VHH^+ cells were detected with no Nb16 format. **c-d)** The bioluminescent CTLA-4 blockade bioassay was performed to investigate capacity of Nb16-based sdAbs to block CTLA-4 immune checkpoint. **c)** The cell-based assay consists of two genetically engineered cell lines, CTLA-4 Effector Cells and aAPC/Raji Cells endogenously expressing CTLA-4 ligands CD80 and CD86. When co-cultured, the CTLA-4 interaction with CD80/86 prevents the CD28 co-stimulatory pathway activation and inhibits TCR-mediated luminescence. When the CTLA-4 pathway is blocked by anti CTLA-4 nanobodies, CD28 and TCR activation induces luminescence that can be detected by a luminometer. Created with BioRender.com. **d)** CTLA-4 Effector Cells and aAPC/Raji Cells were incubated with nanobody-including supernatant harvested from HEK293T-17 cells transfected with sdAb plasmids. No luciferase induction was observed with no Nb16 format, suggesting that the CTLA-4 immune checkpoint was not blocked (n=3).

4.4 Cell engineering with sensor-actuator synthetic system

According to reported results of the several functional assays, anti-PD-L1 sdAbs have proved to be able to recognize the target PD-L1, disrupt the PD-1:PD-L1 interaction, and importantly improve the tumor-killing activity of CAR-T cells in an in vitro model of human ovarian cancer. In view of T-cell engineering with the best performing circuits, the KN035-based constructs were embedded in a lentiviral platform that is the state-of-the art method for genetic engineering of T cells with therapeutic purposes.

Cell lines stably expressing the anti-PD-L1 sdAbs were generated by transduction with second-generation lentiviral vectors (LVs). To generate sdAb LVs, a common lentiviral backbone was designed by modifying the lentiviral plasmid FUGW provided by Addgene (Plasmid #14883) to create a dual-promoter vector for co-expression of multiple transgenes. The final backbone included two independent transcriptional units (TUs) in the same orientation, a first one encoding for sdAbs under the control of a constitutive or an inducible promoter followed by a second TU including the shEF1α promoter driving the sequence encoding a mutated version of the low-affinity nerve growth factor receptor (LNGFR), lacking the entire cytoplasmic domain, that was used as transduction marker. This vector was used to generate a library of LVs expressing in the first TU the several sdAb formats paired with the human EF1α constitutive promoter (LV.EF), the exhaustion-specific SP responsive to exTF (LV.SP), or a variant of the SP lacking the exTF binding sites (SP^{NC}) as negative control ($LV.SP^{NC}$) (Figure 12).

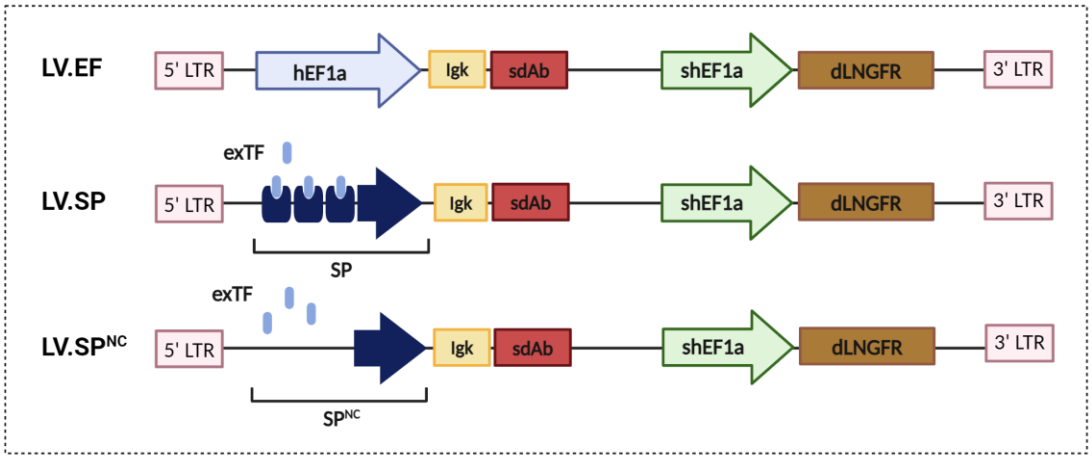


Figure 12 Lentiviral vectors for cell engineering with the sensor-actuator synthetic system. Scheme of the lentiviral vectors (LVs) that were designed to engineer cells with the several KN035 formats. They include two independent transcriptional units (TU) in the same orientation. For each variant, the sdAb encoding sequence was inserted in the first TU coupled to a constitutive promoter (LV.EF), a synthetic promoter (LV.SP) responsive to exhaustion-specific transcription factors (exTF) or the negative control of the SP lacking the binding sites for exTF (LV.SPNC). In all the LVs, the second TU comprises the constitutive shEF1α promoter driving the sequence of a mutated version of the low-affinity nerve growth factor receptor (LNGFR) lacking the entire cytoplasmic domain that was used as transduction marker. Created with BioRender.com.

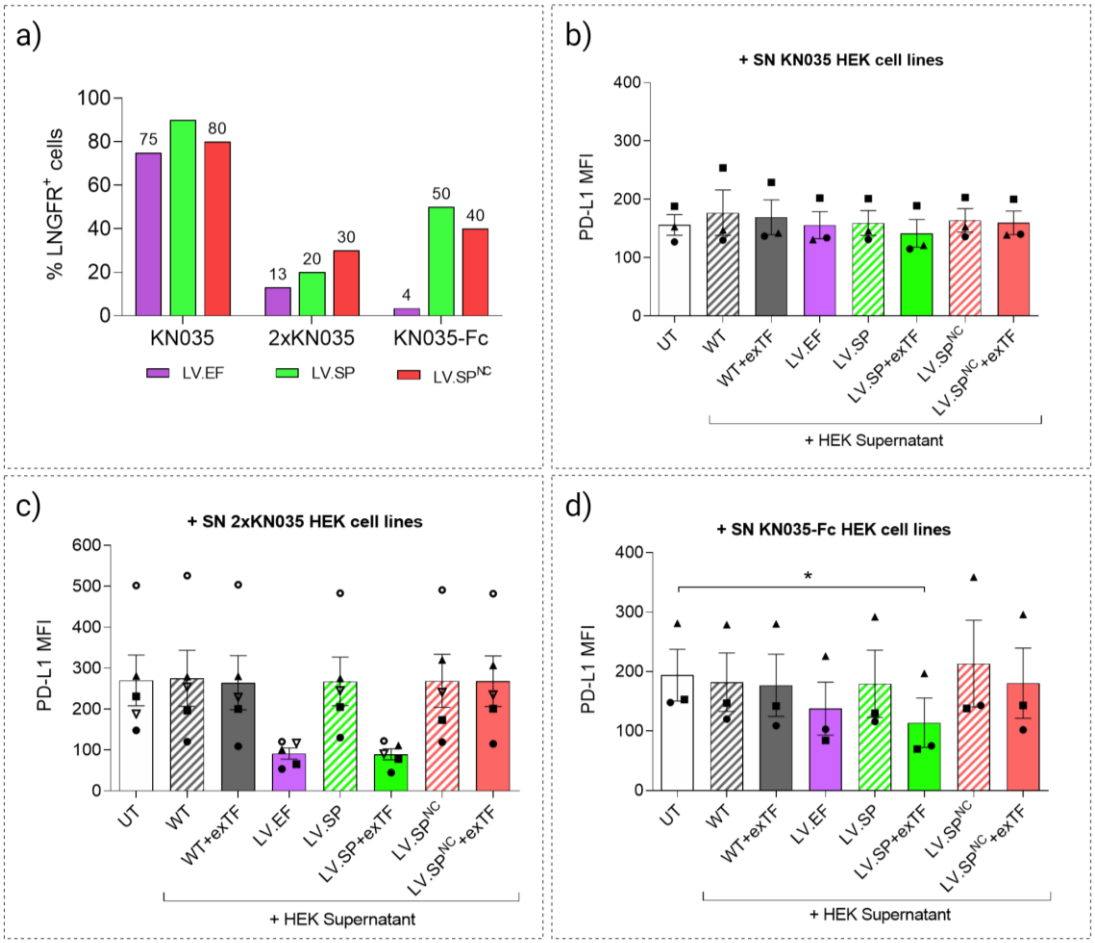
HEK293T-17 cell lines were transduced with the different LVs to stably express the several sdAb formats, each one driven by the three different promoters. Seven days post-transduction, extracellular staining with an anti-LNGFR mAb was performed to assess the transduction efficiency. LNGFR staining results highlighted some variability in the percentages of transduced cells. Cells engineered with the monomeric sdAb vectors showed the highest percentages of $LNGFR^+$ cells (60-70%), while lower efficiencies were achieved in cells integrated with 2xKN035 and KN035-Fc constructs (Figure 13a).

Next, we performed competition assays to verify the SP worked correctly inducing sdAb secretion only in the presence of exTF. Cell lines engineered with the inducible versions of each sdAb format, driven by the SP and the SP without binding sites as negative control (SP^{NC}), were transfected with a plasmid encoding for the exTF that induces sdAb expression. After 48 hours, the SN of transfected (+exTF) and non-transfected cells were transferred onto SKOV3 cells to perform the competitive PD-L1-binding assay.

In specific, SN was harvested from all the engineered cell lines, KN035 (Figure 13b), 2xKN035 (Figure 13c), and KN035-Fc (Figure 13d), with and without exTF in case of the lines engineered with the inducible constructs. The medium of cells integrated with the constitutive sdAb (LV.EF) was used as positive control of sdAb expression, whereas the SN of WT cells transfected with the exTF (WT+exTF) was used as negative control. Untreated SKOV3 cells (UT) represented the basal expression of PD-L1. The incubation with SN of monomeric KN035 cell lines did not induce any changes in PD-L1 signal neither when constitutively expressed (LV.EF) nor when expression was activated by exTF (SP+exTF). On the other hand, exTF-induced expression of both 2xKN035 and KN035-Fc led to a decrease of PD-L1 similarly to that observed upon incubation with the SN of cells constitutively expressing sdAbs. For all the nanobody variants, no PD-L1 changes were observed in cells incubated with both the WT SN and the SN harvested from cells integrated with the negative control of SP and then transfected with exTF. More importantly, the SN of inducible cell lines (SP) lacking the exTF did not block PD-L1 suggesting specific expression of sdAbs in the presence of the transcription factor and poor leakiness of the synthetic promoter (Figure 13b-d). However, the potential leakiness of the system needs to be evaluated also in cell lines with higher percentages of transduced cells to investigate if sdAb residual expression in absence of the exTF may lead an undesired blocking effect. Therefore, $LNGFR^+$ subpopulations will be enriched by cell sorting from integrated cell lines with low transduction efficiencies to repeat competitive binding assays. Moreover, considering the low percentages of $LNGFR^+$ cells in cell lines engineered with multimeric sdAbs, the observed results suggest that low percentages

of transduced cells may be enough to achieve an immune checkpoint blockade. This hypothesis will be further evaluated by performing the bioluminescent cell-based assay using the cell medium harvested from the engineered cell lines, sorted or not, to attempt to understand a sort of threshold to be attained for achieving PD-L1 blockade with recovering of TCR signaling and luciferase expression.

Figure 13 Competition assay in HEK293T-17 cell lines engineered with KN035-based sensor-actuator synthetic systems. a) HEK293T-17 cells were transduced with LVs expressing the several sdAb molecules and after seven days percentages of $LNGFR^+$ cells were analyzed by flow cytometry to evaluate transduction efficiencies. **b-d)** Supernatants of transduced cell lines were used to incubate SKOV3 tumor cells for 1 hour that then were stained with anti PD-L1 mAb. Cell lines engineered with LV.SP and $LV.SP^{NC}$ vectors were first transfected with a plasmid encoding the exTF to induce sdAb



expression. b) SN of cells transduced with monomeric sdAbs did not induce any variations in PD-L1 expression, neither when paired to the constitutive promoter not to the inducible one. c-d) Bar plots indicated that in the presence of exTF, 2xKN035 (c) and KN035-Fc (d) including SN, harvested by inducible cell lines, led to a drop in PD-L1 levels, comparable to that of the same variant driven by the constitutive promoter (LV.EF). Conversely, the inducible sdAbs without the exTF did not induce huge variations in PD-L1 expression. In c), statistical analysis on MFI values. Friedman test, Dunnett's multiple comparisons test, *P<0.05 compared to UT (n=3).

5 Conclusions

Cell-based immunotherapy has emerged as a transformative approach in cancer treatment, harnessing the inherent capabilities of immune cells to combat malignancies. Synthetic biology, with its precision engineering of biological systems, has unearthed several noteworthy insights and advancements in cancer immunotherapy, particularly in cell-based therapy. Synthetic biology enables the design and implementation of intricate synthetic circuits within immune cells, allowing for precise control over their behavior and responses. By incorporating synthetic modules that can sense and respond to specific signals, therapeutic cells can be tailored to respond effectively to the dynamic conditions within the body. Additionally, synthetic biology empowers researchers to engineer immune cells with multiple functionalities, allowing for simultaneous and comprehensive targeting of various aspects of cancer progression.

In this study, a synthetic biology approach was proposed to counteract $CD8^+$ T-cell exhaustion for enhanced T-cell-based immunotherapies. Specifically, a synthetic system was created for T-cell engineering by combining a biosensor, represented by a synthetic promoter responsive to exhaustion-specific transcription factors, with synthetic actuators leveraging single-domain antibodies (sdAbs) for immune checkpoint blockade. The final aim is to generate T cells able to sense exhaustion signals and trigger the transcription of sdAbs targeting PD-L1 and CTLA-4 to revert the T-cell dysfunction. On one side, T-cell engineering with genetically encoded modulators will enable to achieve local sdAb secretion limiting adverse events typical of mAb systemic injections and addressing the issue of poor therapeutic agent distribution within the tumor that impairs the efficacy of current immunotherapies. Moreover, the direct secretion into the tumor microenvironment will allow sdAbs to act also on the other immune cells involved in the anti-tumor immune response, as the $CD4^+$ 'helper' T cells that represent the second arm of adaptive T-cell immunity (Tay, Richardson, and Toh 2021) and are also prone to exhaustion (Miggelbrink et al. 2021). On the other side, transgene delivery via a viral vector will provide a continuous production of therapeutic agents overcoming the limit related to the short half-life of sdAbs. However, a lack of control over transgene expression may have detrimental long-term effects. The combination of the actuator module with the exhaustion biosensor will allow taking over this challenge by selectively inducing the modulator expression in exhaustion conditions, thus making it reversible and providing huge advantages in terms of safety and efficacy for future therapeutic applications. Regarding the design of the actuator module, various sdAb variants were developed and validated, including bivalent

nanobodies and sdAb-Fc fusion proteins, to enhance the avidity of therapeutic molecules and improve anti-tumor activity for future in vivo applications. While limitations surfaced with anti CTLA-4 sdAbs requiring further exploration, anti PD-L1 sdAbs exhibited a robust ability to effectively block PD-L1, thereby demonstrating their potential for immune checkpoint blockade. Importantly, a preliminary test in our CAR-T cell model showed an improved tumor-killing activity of CAR-T cells when co-cultured with SKOV3 tumor cells in a real-time cytotoxic assay, suggesting that the PD-L1 blockade may improve the ability of CAR-T cells to impair tumor cell growth.

These results are in line with those of several studies reporting more effective CAR-T therapy outcomes if associated with immune checkpoint therapy using mAbs, leading to a decrease of T-cell exhaustion and enhancement of CAR-T persistence (Hosseinkhani et al. 2020).

The landscape of combining PD-1/PD-L1 blockade with CAR T-cell therapy is evolving rapidly, particularly in hematological malignancies, and novel strategies involving PD-1 disruption by genome editing have displayed enhanced anti-tumor activity of CAR-T cells (Rupp et al. 2017). Disrupting immune checkpoint through gene editing is considered a safer alternative to systemic injection of mAbs and PD-1 knockout did not affect CAR-T cell activation or cytotoxic specificity (Guo et al. 2018; McGowan et al. 2020). However, disruption of the immune checkpoint by genome editing is irreversible leading to a permanent absence of the inhibitory axis. Conversely, our nanobody-based system will allow reversibly disrupting the PD-1 pathway, selectively inducing the immune checkpoint blockade upon T-cell exhaustion onset and making possible to ‘switch off’ the system when T-cell effector functions will be reverted, thus recovering the physiological PD-1-mediated regulation. Preliminary results obtained performing functional assays using cells engineered with the sensor-actuator system showed a promising sdAb expression only in the presence of the exhaustion-specific transcription factor. Nevertheless, these results will need to be confirmed after cell sorting of transduced cells enriching the population expressing the synthetic circuits to deeper investigate if the system has a leakiness that may improperly activate the actuator expression. Further, T cells engineered with this synthetic system will represent a good model to study in more detail the timing of T-cell exhaustion onset and progression exploiting an in vitro T-cell exhaustion model that was developed in my group. Based on a chronic stimulation of $CD8^+$ T cells, the model tries to mimic the persistent stimulation to which T cells are normally subjected in cancer inducing the typical markers of T-cell dysfunction in terms of phenotype, functionality, and transcriptional profile. Alternatively, the CAR-T cell model could be used to simulate a more natural T-cell exhaustion condition by long-term co-culture

with tumor cells, thus inducing a persistent antigen stimulation, to verify the activation timing of the synthetic modulators driven by the exhaustion-specific synthetic promoter in a co-culture setting.

The innovative synthetic biology approach presented in this study holds promise in advancing cancer immunotherapy offering a nuanced approach to immune checkpoint blockade. Synthetic circuits including modulators that can be selectively activated in exhaustion conditions present a potential strategy to mitigate off-target effects observed in traditional monoclonal antibody therapies. In conclusion, our synthetic biology approach, harnessing the unique features of nanobodies and the capacity of synthetic biology to provide precision engineering in biological systems, marks a significant stride in the quest to neutralize T-cell exhaustion. This work contributes to the evolving landscape of cancer immunotherapy and lays the groundwork for future advancements that may revolutionize the treatment of solid cancers.

References

Abrams, D, Y Lévy, M H Losso, A Babiker, G Collins, D A Cooper, J Darbyshire, et al. 2009. "Interleukin-2 Therapy in Patients with HIV Infection." *The New England Journal of Medicine* 361 (16): 1548–59. https://doi.org/10.1056/NEJMoa0903175.

Acharya, Nandini, Catherine Sabatos-Peyton, and Ana Carrizosa Anderson. 2020a. "Tim-3 Finds Its Place in the Cancer Immunotherapy Landscape." *Journal for ImmunoTherapy of Cancer* 8 (1). https://doi.org/10.1136/jitc-2020-000911.

Alfei, Francesca, Kristiyan Kanev, Maike Hofmann, Ming Wu, Hazem E Ghoneim, Patrick Roelli, Daniel T Utzschneider, et al. 2019. "TOX Reinforces the Phenotype and Longevity of Exhausted T Cells in Chronic Viral Infection." *Nature*. https://doi.org/10.1038/s41586-019-1326-9.

Amaria, Rodabe N, Michael Postow, Elizabeth M Burton, Michael T Tetzlaff, Merrick I Ross, Carlos Torres-Cabala, Isabella C Glitza, et al. 2022. "Neoadjuvant Relatlimab and Nivolumab in Resectable Melanoma." *Nature* 611 (7934): 155–60. https://doi.org/10.1038/s41586-022-05368-8.

Anderson, Ana C, Graham M Lord, Valerie Dardalhon, David H Lee, Catherine A Sabatos-Peyton, Laurie H Glimcher, and Vijay K Kuchroo. 2010. "T-Bet, a Th1 Transcription Factor Regulates the Expression of Tim-3." *European Journal of Immunology* 40 (3): 859–66. https://doi.org/10.1002/eji.200939842.

Andrian, U H von, and C R Mackay. 2000. "T-Cell Function and Migration. Two Sides of the Same Coin." *The New England Journal of Medicine* 343 (14): 1020–34. https://doi.org/10.1056/NEJM200010053431407.

Arbabi-Ghahroudi, Mehdi. 2022. "Camelid Single-Domain Antibodies: Promises and Challenges as Lifesaving Treatments." *International Journal of Molecular Sciences* 23 (9). https://doi.org/10.3390/ijms23095009.

Arbabi-Ghahroudi, Mehdi, Jamshid Tanha, and Roger MacKenzie. 2005. "Prokaryotic Expression of Antibodies." *Cancer and Metastasis Reviews* 24 (4): 501–19. https://doi.org/10.1007/s10555-005-6193-1.

Asakura, Hirofumi, Yumiko Kashio, Kazuhiro Nakamura, Masako Seki, Shuyan Dai, Yukako Shirato, Mohammad J Abedin, et al. 2002. "Selective Eosinophil Adhesion to Fibroblast via IFN-Gamma-Induced Galectin-9." *Journal of Immunology (Baltimore, Md. : 1950)* 169 (10): 5912–18. https://doi.org/10.4049/jimmunol.169.10.5912.

Audenhove, Isabel Van, and Jan Gettemans. 2016. "Nanobodies as Versatile Tools to Understand, Diagnose, Visualize and Treat Cancer." *EBioMedicine* 8: 40–48. https://doi.org/10.1016/j.ebiom.2016.04.028.

Ausländer, Simon, and Martin Fussenegger. 2013. "From Gene Switches to Mammalian Designer Cells: Present and Future Prospects." *Trends in Biotechnology* 31 (3): 155–68. https://doi.org/10.1016/j.tibtech.2012.11.006.

Awad, Robin Maximilian, Quentin Lecocq, Katty Zeven, Thomas Ertveldt, Lien De Beck, Hannelore Ceuppens, Katrijn Broos, et al. 2021. "Formatting and Gene-Based Delivery of a Human PD-L1 Single Domain Antibody for Immune Checkpoint Blockade." *Molecular Therapy - Methods and Clinical Development* 22 (September): 172–82. https://doi.org/10.1016/j.omtm.2021.05.017.

Bachmann, Martin F, Petra Wolint, Senta Walton, Katrin Schwarz, and Annette Oxenius. 2007. "Differential Role of IL-2R Signaling for CD8+ T Cell Responses in Acute and Chronic Viral Infections." *European Journal of Immunology* 37 (6): 1502–12. https://doi.org/10.1002/eji.200637023.

Bae, Joonbeom, Suk Jun Lee, Chung-Gyu Park, Young Sik Lee, and Taehoon Chun. 2014. "Trafficking of LAG-3 to the Surface on Activated T Cells via Its Cytoplasmic Domain

and Protein Kinase C Signaling." *The Journal of Immunology* 193 (6): 3101–12. https://doi.org/10.4049/jimmunol.1401025.

Bagchi, Sreya, Robert Yuan, and Edgar G. Engleman. 2021. "Immune Checkpoint Inhibitors for the Treatment of Cancer: Clinical Impact and Mechanisms of Response and Resistance." *Annual Review of Pathology: Mechanisms of Disease* 16: 223–49. https://doi.org/10.1146/annurev-pathol-042020-042741.

Baitsch, Lukas, Petra Baumgaertner, Estelle Devêvre, Sunil K Raghav, Amandine Legat, Leticia Barba, Sébastien Wieckowski, et al. 2011. "Exhaustion of Tumor-Specific $CD8^+$ T Cells in Metastases from Melanoma Patients." *The Journal of Clinical Investigation* 121 (6): 2350–60. https://doi.org/10.1172/JCI46102.

Bannas, Peter, Julia Hambach, and Friedrich Koch-Nolte. 2017. "Nanobodies and Nanobody-Based Human Heavy Chain Antibodies as Antitumor Therapeutics." *Frontiers in Immunology* 8 (NOV): 1–13. https://doi.org/10.3389/fimmu.2017.01603.

Barber, Daniel L, E John Wherry, David Masopust, Baogong Zhu, James P Allison, Arlene H Sharpe, Gordon J Freeman, and Rafi Ahmed. 2006. "Restoring Function in Exhausted CD8 T Cells during Chronic Viral Infection." *Nature* 439 (7077): 682–87. https://doi.org/10.1038/nature04444.

Bathula, Nuthan V., Hemashree Bommadevara, and Jerrard M. Hayes. 2021. "Nanobodies: The Future of Antibody-Based Immune Therapeutics." *Cancer Biotherapy and Radiopharmaceuticals*. https://doi.org/10.1089/cbr.2020.3941.

Beghein, Els, and Jan Gettemans. 2017. "Nanobody Technology: A Versatile Toolkit for Microscopic Imaging, Protein-Protein Interaction Analysis, and Protein Function Exploration." *Frontiers in Immunology* 8: 771. https://doi.org/10.3389/fimmu.2017.00771.

Benner, Steven A., and A. Michael Sismour. 2005. "Synthetic Biology." *Nature Reviews Genetics* 6 (7): 533–43. https://doi.org/10.1038/nrg1637.

Bi, Jiacheng, and Zhigang Tian. 2017. "NK Cell Exhaustion." *Frontiers in Immunology* 8: 760. https://doi.org/10.3389/fimmu.2017.00760.

Blackburn, Shawn D., Haina Shin, W. Nicholas Haining, Tao Zou, Creg J. Workman, Antonio Polley, Michael R. Betts, Gordon J. Freeman, Dario A.A. Vignali, and E. John Wherry. 2009a. "Coregulation of CD8+ T Cell Exhaustion by Multiple Inhibitory Receptors during Chronic Viral Infection." *Nature Immunology* 10 (1): 29–37. https://doi.org/10.1038/ni.1679.

Blackburn, Shawn D, Haina Shin, W Nicholas Haining, Tao Zou, Creg J Workman, Antonio Polley, Michael R Betts, Gordon J Freeman, Dario A A Vignali, and E John Wherry. 2009b. "Coregulation of CD8+ T Cell Exhaustion by Multiple Inhibitory Receptors during Chronic Viral Infection." *Nature Immunology* 10 (1): 29–37. https://doi.org/10.1038/ni.1679.

Blank, Christian, Thomas F. Gajewski, and Andreas Mackensen. 2005. "Interaction of PD-L1 on Tumor Cells with PD-1 on Tumor-Specific T Cells as a Mechanism of Immune Evasion: Implications for Tumor Immunotherapy." *Cancer Immunology, Immunotherapy* 54 (4): 307–14. https://doi.org/10.1007/s00262-004-0593-x.

Blattman, Joseph N, Jason M Grayson, E John Wherry, Susan M Kaech, Kendall A Smith, and Rafi Ahmed. 2003. "Therapeutic Use of IL-2 to Enhance Antiviral T-Cell Responses in Vivo." *Nature Medicine* 9 (5): 540–47. https://doi.org/10.1038/nm866.

Boyman, Onur, Charles D Surh, and Jonathan Sprent. 2006. "Potential Use of IL-2/Anti-IL-2 Antibody Immune Complexes for the Treatment of Cancer and Autoimmune Disease." *Expert Opinion on Biological Therapy* 6 (12): 1323–31. https://doi.org/10.1517/14712598.6.12.1323.

Brooks, David G, Sang-Jun Ha, Heidi Elsaesser, Arlene H Sharpe, Gordon J Freeman, and

Michael B A Oldstone. 2008. “IL-10 and PD-L1 Operate through Distinct Pathways to Suppress T-Cell Activity during Persistent Viral Infection.” *Proceedings of the National Academy of Sciences of the United States of America* 105 (51): 20428–33. https://doi.org/10.1073/pnas.0811139106.

Brooks, David G, Matthew J Trifilo, Kurt H Edelmann, Luc Teyton, Dorian B McGavern, and Michael B A Oldstone. 2006. “Interleukin-10 Determines Viral Clearance or Persistence in Vivo.” *Nature Medicine* 12 (11): 1301–9. https://doi.org/10.1038/nm1492.

Caggiari, L, S Zanussi, C Crepaldi, M T Bortolin, C Caffau, M D’Andrea, and P De Paoli. 2001. “Different Rates of CD4+ and CD8+ T-Cell Proliferation in Interleukin-2-Treated Human Immunodeficiency Virus-Positive Subjects.” *Cytometry* 46 (4): 233–37. https://doi.org/10.1002/cyto.1132.

Caliendo, Fabio, Marina Dukhinova, and Velia Siciliano. 2019. “Engineered Cell-Based Therapeutics: Synthetic Biology Meets Immunology.” *Frontiers in Bioengineering and Biotechnology* 7: 43. https://doi.org/10.3389/fbioe.2019.00043.

Catakovic, Kemal, Eckhard Klieser, Daniel Neureiter, and Roland Geisberger. 2017. “T Cell Exhaustion: From Pathophysiological Basics to Tumor Immunotherapy.” *Cell Communication and Signaling : CCS*. London. https://doi.org/10.1186/s12964-016-0160-z.

Caussinus, Emmanuel, Oguz Kanca, and Markus Affolter. 2011. “Fluorescent Fusion Protein Knockout Mediated by Anti-GFP Nanobody.” *Nature Structural & Molecular Biology* 19 (1): 117–21. https://doi.org/10.1038/nsmb.2180.

Chakravarti, Deboki, and Wilson W Wong. 2015. “Synthetic Biology in Cell-Based Cancer Immunotherapy.” *Trends in Biotechnology* 33 (8): 449–61. https://doi.org/10.1016/j.tibtech.2015.05.001.

Chambers, C A, M S Kuhns, J G Egen, and J P Allison. 2001. “CTLA-4-Mediated Inhibition in Regulation of T Cell Responses: Mechanisms and Manipulation in Tumor Immunotherapy.” *Annual Review of Immunology* 19: 565–94. https://doi.org/10.1146/annurev.immunol.19.1.565.

Champiat, S, O Lambotte, E Barreau, R Belkhir, A Berdelou, F Carbonnel, C Cauquil, et al. 2016. “Management of Immune Checkpoint Blockade Dysimmune Toxicities: A Collaborative Position Paper.” *Annals of Oncology : Official Journal of the European Society for Medical Oncology* 27 (4): 559–74. https://doi.org/10.1093/annonc/mdv623.

Chauvin, Joe-Marc, Ornella Pagliano, Julien Fourcade, Zhaojun Sun, Hong Wang, Cindy Sander, John M Kirkwood, et al. 2015. “TIGIT and PD-1 Impair Tumor Antigen-Specific CD8$^+$ T Cells in Melanoma Patients.” *The Journal of Clinical Investigation* 125 (5): 2046–58. https://doi.org/10.1172/JCI80445.

Chauvin, Joe-Marc, and Hassane M Zarour. 2020a. “TIGIT in Cancer Immunotherapy.” *Journal for ImmunoTherapy of Cancer* 8 (2). https://doi.org/10.1136/jitc-2020-000957.

Chemnitz, Jens M., James L. Riley, Kenneth A. Frauwirth, Inbal Braunstein, Sumire V. Kobayashi, Peter S. Linsley, Craig B. Thompson, and Richard V. Parry. 2004. “CTLA-4 and PD-1 Receptors Inhibit T-Cell Activation by Distinct Mechanisms.” *Blood* 104 (11): 2657–2657. https://doi.org/10.1182/blood.v104.11.2657.2657.

Chemnitz, Jens M, Richard V Parry, Kim E Nichols, Carl H June, and James L Riley. 2004. “SHP-1 and SHP-2 Associate with Immunoreceptor Tyrosine-Based Switch Motif of Programmed Death 1 upon Primary Human T Cell Stimulation, but Only Receptor Ligation Prevents T Cell Activation.” *Journal of Immunology (Baltimore, Md. : 1950)* 173 (2): 945–54. https://doi.org/10.4049/jimmunol.173.2.945.

Chen, Nan, Xiaoyu Li, Navin K. Chintala, Zachary E. Tano, and Prasad S. Adusumilli. 2018. “Driving CARs on the Uneven Road of Antigen Heterogeneity in Solid Tumors.” *Current Opinion in Immunology* 51: 103–10. https://doi.org/10.1016/j.coi.2018.03.002.

Cherkassky, Leonid, Aurore Morello, Jonathan Villena-Vargas, Yang Feng, Dimiter S Dimitrov, David R Jones, Michel Sadelain, and Prasad S Adusumilli. 2016. "Human CAR T Cells with Cell-Intrinsic PD-1 Checkpoint Blockade Resist Tumor-Mediated Inhibition." *The Journal of Clinical Investigation* 126 (8): 3130–44. https://doi.org/10.1172/JCI83092.

Chiba, Shigeki, Muhammad Baghdadi, Hisaya Akiba, Hironori Yoshiyama, Ichiro Kinoshita, Hirotoshi Dosaka-Akita, Yoichiro Fujioka, et al. 2012. "Tumor-Infiltrating DCs Suppress Nucleic Acid-Mediated Innate Immune Responses through Interactions between the Receptor TIM-3 and the Alarmin HMGB1." *Nature Immunology* 13 (9): 832–42. https://doi.org/10.1038/ni.2376.

Chow, Andrew, Karlo Perica, Christopher A Klebanoff, and Jedd D Wolchok. 2022. "Clinical Implications of T Cell Exhaustion for Cancer Immunotherapy." *Nature Reviews. Clinical Oncology* 19 (12): 775–90. https://doi.org/10.1038/s41571-022-00689-z.

Chu, Xianjing, Wentao Tian, Ziqi Wang, Jing Zhang, and Rongrong Zhou. 2023. "Co-Inhibition of TIGIT and PD-1/PD-L1 in Cancer Immunotherapy: Mechanisms and Clinical Trials." *Molecular Cancer* 22 (1): 93. https://doi.org/10.1186/s12943-023-01800-3.

Conrath, Katja Els, Mark Lauwereys, Lode Wyns, and Serge Muyldermans. 2001. "Camel Single-Domain Antibodies as Modular Building Units in Bispecific and Bivalent Antibody Constructs." *Journal of Biological Chemistry* 276 (10): 7346–50. https://doi.org/10.1074/jbc.M007734200.

Crawford, Alison, Jill M Angelosanto, Charlly Kao, Travis A Doering, Pamela M Odorizzi, Burton E Barnett, and E John Wherry. 2014. "Molecular and Transcriptional Basis of $CD4^+$ T Cell Dysfunction during Chronic Infection." *Immunity* 40 (2): 289–302. https://doi.org/10.1016/j.immuni.2014.01.005.

Cruz, Esteban, and Veysel Kayser. 2019. "Monoclonal Antibody Therapy of Solid Tumors: Clinical Limitations and Novel Strategies to Enhance Treatment Efficac." *Monoclonal Antibody Therapy of Human Cancer*, 103–20. https://doi.org/10.1007/978-1-4613-2627-4_5.

Cui, Weiguo, and Susan M. Kaech. 2010. "Generation of Effector CD8+ T Cells and Their Conversion to Memory T Cells." *Journal of Pediatrics* 236: 151–166. https://doi.org/10.1111/j.1600-065X.2010.00926.x.

Curiel, Tyler J, Shuang Wei, Haidong Dong, Xavier Alvarez, Pui Cheng, Peter Mottram, Roman Krzysiek, et al. 2003. "Blockade of B7-H1 Improves Myeloid Dendritic Cell-Mediated Antitumor Immunity." *Nature Medicine* 9 (5): 562–67. https://doi.org/10.1038/nm863.

Danquah, Welbeck, Catherine Meyer-Schwesinger, Björn Rissiek, Carolina Pinto, Arnau Serracant-Prat, Miriam Amadi, Domenica Iacenda, et al. 2016. "Nanobodies That Block Gating of the P2X7 Ion Channel Ameliorate Inflammation." *Science Translational Medicine* 8 (366): 366ra162. https://doi.org/10.1126/scitranslmed.aaf8463.

Davila, Marco L, and Michel Sadelain. 2016. "Biology and Clinical Application of CAR T Cells for B Cell Malignancies." *International Journal of Hematology* 104 (1): 6–17. https://doi.org/10.1007/s12185-016-2039-6.

Day, Cheryl L, Daniel E Kaufmann, Photini Kiepiela, Julia A Brown, Eshia S Moodley, Sharon Reddy, Elizabeth W Mackey, et al. 2006. "PD-1 Expression on HIV-Specific T Cells Is Associated with T-Cell Exhaustion and Disease Progression." *Nature* 443 (7109): 350–54. https://doi.org/10.1038/nature05115.

Deng, Qing, Guangchun Han, Nahum Puebla-Osorio, Man Chun John Ma, Paolo Strati, Beth Chasen, Enyu Dai, et al. 2020. "Characteristics of Anti-CD19 CAR T Cell Infusion Products Associated with Efficacy and Toxicity in Patients with Large B Cell

Lymphomas." *Nature Medicine* 26 (12): 1878–87. https://doi.org/10.1038/s41591-020-1061-7.

Derynck, Rik, and Ying E Zhang. 2003. "Smad-Dependent and Smad-Independent Pathways in TGF-Beta Family Signalling." *Nature* 425 (6958): 577–84. https://doi.org/10.1038/nature02006.

Desmyter, A, T R Transue, M A Ghahroudi, M H Thi, F Poortmans, R Hamers, S Muyldermans, and L Wyns. 1996. "Crystal Structure of a Camel Single-Domain VH Antibody Fragment in Complex with Lysozyme." *Nature Structural Biology* 3 (9): 803–11. https://doi.org/10.1038/nsb0996-803.

Dominguez, Claudia X, Robert A Amezquita, Tianxia Guan, Heather D Marshall, Nikhil S Joshi, Steven H Kleinstein, and Susan M Kaech. 2015. "The Transcription Factors ZEB2 and T-Bet Cooperate to Program Cytotoxic T Cell Terminal Differentiation in Response to LCMV Viral Infection." *The Journal of Experimental Medicine* 212 (12): 2041–56. https://doi.org/10.1084/jem.20150186.

Drubin, David A., Jeffrey C. Way, and Pamela A. Silver. 2007. "Designing Biological Systems." *Genes and Development* 21 (3): 242–54. https://doi.org/10.1101/gad.1507207.

Ejrnaes, Mette, Christophe M Filippi, Marianne M Martinic, Eleanor M Ling, Lisa M Togher, Shane Crotty, and Matthias G von Herrath. 2006. "Resolution of a Chronic Viral Infection after Interleukin-10 Receptor Blockade." *The Journal of Experimental Medicine* 203 (11): 2461–72. https://doi.org/10.1084/jem.20061462.

Elsaesser, Heidi, Karsten Sauer, and David G Brooks. 2009. "IL-21 Is Required to Control Chronic Viral Infection." *Science (New York, N.Y.)* 324 (5934): 1569–72. https://doi.org/10.1126/science.1174182.

Fang, Tao, Ran Li, Zeyang Li, Julia Cho, Jonathan S. Guzman, Roger D. Kamm, and Hidde L. Ploegh. 2019. "Remodeling of the Tumor Microenvironment by a Chemokine/Anti-PD-L1 Nanobody Fusion Protein." *Molecular Pharmaceutics* 16 (6): 2838–44. https://doi.org/10.1021/acs.molpharmaceut.9b00078.

Fraietta, Joseph A, Simon F Lacey, Elena J Orlando, Iulian Pruteanu-Malinici, Mercy Gohil, Stefan Lundh, Alina C Boesteanu, et al. 2018. "Determinants of Response and Resistance to CD19 Chimeric Antigen Receptor (CAR) T Cell Therapy of Chronic Lymphocytic Leukemia." *Nature Medicine* 24 (5): 563–71. https://doi.org/10.1038/s41591-018-0010-1.

Freeman, G J, A J Long, Y Iwai, K Bourque, T Chernova, H Nishimura, L J Fitz, et al. 2000. "Engagement of the PD-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation." *The Journal of Experimental Medicine* 192 (7): 1027–34. https://doi.org/10.1084/jem.192.7.1027.

Fröhlich, Anja, Jan Kisielow, Iwana Schmitz, Stefan Freigang, Abdijapar T Shamshiev, Jacqueline Weber, Benjamin J Marsland, Annette Oxenius, and Manfred Kopf. 2009. "IL-21R on T Cells Is Critical for Sustained Functionality and Control of Chronic Viral Infection." *Science (New York, N.Y.)* 324 (5934): 1576–80. https://doi.org/10.1126/science.1172815.

Fuller, Michael J, David A Hildeman, Steffanie Sabbaj, Dalia E Gaddis, Anne E Tebo, Liang Shang, Paul A Goepfert, and Allan J Zajac. 2005. "Cutting Edge: Emergence of CD127high Functionally Competent Memory T Cells Is Compromised by High Viral Loads and Inadequate T Cell Help." *Journal of Immunology (Baltimore, Md. : 1950)* 174 (10): 5926–30. https://doi.org/10.4049/jimmunol.174.10.5926.

Gao, Jianjun, John F Ward, Curtis A Pettaway, Lewis Z Shi, Sumit K Subudhi, Luis M Vence, Hao Zhao, et al. 2017. "VISTA Is an Inhibitory Immune Checkpoint That Is Increased after Ipilimumab Therapy in Patients with Prostate Cancer." *Nature Medicine* 23 (5): 551–55. https://doi.org/10.1038/nm.4308.

Garner, Kathryn L. 2021. “Principles of Synthetic Biology.” *Essays in Biochemistry* 65 (5): 791–811. https://doi.org/10.1042/EBC20200059.

Ghosh, Arnab, Pere Barba, and Miguel-Angel Perales. 2020. “Checkpoint Inhibitors in AML: Are We There Yet?” *British Journal of Haematology* 188 (1): 159–67. https://doi.org/10.1111/bjh.16358.

Gil, Diana, and Adam G Schrum. 2013. “Strategies to Stabilize Compact Folding and Minimize Aggregation of Antibody-Based Fragments.” *Advances in Bioscience and Biotechnology (Print)* 4 (4a): 73–84. https://doi.org/10.4236/abb.2013.44A011.

Godefroy, Emmanuelle, Hui Zhong, Petra Pham, David Friedman, and Karina Yazdanbakhsh. 2015. “TIGIT-Positive Circulating Follicular Helper T Cells Display Robust B-Cell Help Functions: Potential Role in Sickle Cell Alloimmunization.” *Haematologica* 100 (11): 1415–25. https://doi.org/10.3324/haematol.2015.132738.

Gorovits, Boris, and Eugen Koren. 2019. “Immunogenicity of Chimeric Antigen Receptor T-Cell Therapeutics.” *BioDrugs : Clinical Immunotherapeutics, Biopharmaceuticals and Gene Therapy* 33 (3): 275–84. https://doi.org/10.1007/s40259-019-00354-5.

Gros, Alena, Paul F Robbins, Xin Yao, Yong F Li, Simon Turcotte, Eric Tran, John R Wunderlich, et al. 2014. “PD-1 Identifies the Patient-Specific $CD8^{+}$ Tumor-Reactive Repertoire Infiltrating Human Tumors.” *The Journal of Clinical Investigation* 124 (5): 2246–59. https://doi.org/10.1172/JCI73639.

Gruener, N H, F Lechner, M C Jung, H Diepolder, T Gerlach, G Lauer, B Walker, et al. 2001. “Sustained Dysfunction of Antiviral CD8+ T Lymphocytes after Infection with Hepatitis C Virus.” *Journal of Virology* 75 (12): 5550–58. https://doi.org/10.1128/JVI.75.12.5550-5558.2001.

Guan, Qianting, Meiwen Han, Qinghao Guo, Fangfei Yan, Ming Wang, Qin Ning, and Dong Xi. 2023. “Strategies to Reinvigorate Exhausted CD8+ T Cells in Tumor Microenvironment.” *Frontiers in Immunology* 14 (June): 1–21. https://doi.org/10.3389/fimmu.2023.1204363.

Gumber, Diana, and Leo D. Wang. 2022. “Improving CAR-T Immunotherapy: Overcoming the Challenges of T Cell Exhaustion.” *EBioMedicine* 77: 103941. https://doi.org/10.1016/j.ebiom.2022.103941.

Guo, Xingliang, Hua Jiang, Bizhi Shi, Min Zhou, Honghong Zhang, Zhimin Shi, Guoxiu Du, et al. 2018. “Disruption of PD-1 Enhanced the Anti-Tumor Activity of Chimeric Antigen Receptor T Cells Against Hepatocellular Carcinoma.” *Frontiers in Pharmacology* 9: 1118. https://doi.org/10.3389/fphar.2018.01118.

Gupta, A, K M De Felice, E V Jr Loftus, and S Khanna. 2015. “Systematic Review: Colitis Associated with Anti-CTLA-4 Therapy.” *Alimentary Pharmacology & Therapeutics* 42 (4): 406–17. https://doi.org/10.1111/apt.13281.

Gustavo J. Martinez, Renata M. Pereira, Tarmo Äijö, Edward Y. Kim, Francesco Marangoni, Matthew E. Pipkin, Susan Togher, et al. 2015. “The Transcription Factor NFAT Promotes Exhaustion of Activated CD8+ T Cells.” *Immunity* 42 (2): 265–78. https://doi.org/10.1016/j.immuni.2015.01.006.

Guy, Clifford, Diana M. Mitrea, Po Chien Chou, Jamshid Temirov, Kate M. Vignali, Xueyan Liu, Hui Zhang, et al. 2022. “LAG3 Associates with TCR–CD3 Complexes and Suppresses Signaling by Driving Co-Receptor–Lck Dissociation.” *Nature Immunology* 23 (5): 757–67. https://doi.org/10.1038/s41590-022-01176-4.

Hamdan, Firas, and Vincenzo Cerullo. 2023. “Cancer Immunotherapies: A Hope for the Uncurable?” *Frontiers in Molecular Medicine* 3 (February): 1–21. https://doi.org/10.3389/fmmed.2023.1140977.

Hamers-Casterman, Atarchouch, T, S Muyldermans, G Robinson, C Hamers, E Bajyana, N Bendahman, and R Hamilton. 1993. “Naturally Occurring Antibodies Devoid of Light

Chains." *Nature* 363 (June): 446–48. https://www.nature.com/articles/363446a0.pdf.
Hao, Shuai, Shuyi Xu, Liangzhu Li, Yaxian Li, Meiqi Zhao, Junsheng Chen, Shunying Zhu, et al. 2022. "Tumour Inhibitory Activity on Pancreatic Cancer by Bispecific Nanobody Targeting PD-L1 and CXCR4." *BMC Cancer* 22 (1): 1–12. https://doi.org/10.1186/s12885-022-10165-7.
Harmsen, M M, and H J De Haard. 2007. "Properties, Production, and Applications of Camelid Single-Domain Antibody Fragments." *Applied Microbiology and Biotechnology* 77 (1): 13–22. https://doi.org/10.1007/s00253-007-1142-2.
Harty, John T, and Vladimir P Badovinac. 2008. "Shaping and Reshaping CD8+ T-Cell Memory." *Nature Reviews. Immunology* 8 (2): 107–19. https://doi.org/10.1038/nri2251.
Hirano, Fumiya, Katsumi Kaneko, Hideto Tamura, Haidong Dong, Shengdian Wang, Masao Ichikawa, Cecilia Rietz, et al. 2005. "Blockade of B7-H1 and PD-1 by Monoclonal Antibodies Potentiates Cancer Therapeutic Immunity." *Cancer Research* 65 (3): 1089–96.
Hirschi, Stephan, Thomas R. Ward, Wolfgang P. Meier, Daniel J. Muller, and Dimitrios Fotiadis. 2022. "Synthetic Biology: Bottom-Up Assembly of Molecular Systems." *Chemical Reviews* 122 (21): 16294–328. https://doi.org/10.1021/acs.chemrev.2c00339.
Hobom, B. 1980. "[Gene surgery: on the threshold of synthetic biology]." *Medizinische Klinik* 75 (24): 834–41.
Hodi, F Stephen, Steven J O'Day, David F McDermott, Robert W Weber, Jeffrey A Sosman, John B Haanen, Rene Gonzalez, et al. 2010. "Improved Survival with Ipilimumab in Patients with Metastatic Melanoma." *The New England Journal of Medicine* 363 (8): 711–23. https://doi.org/10.1056/NEJMoa1003466.
Hoefman, Sven, Ingrid Ottevaere, Judith Baumeister, and Maria Laura Sargentini-Maier. 2015. "Pre-Clinical Intravenous Serum Pharmacokinetics of Albumin Binding and Non-Half-Life Extended Nanobodies®." *Antibodies* 4 (3): 141–56. https://doi.org/10.3390/antib4030141.
Hosseinkhani, Negar, Afshin Derakhshani, Omid Kooshkaki, Mahdi Abdoli Shadbad, Khalil Hajiasgharzadeh, Amir Baghbanzadeh, Hossein Safarpour, et al. 2020. "Immune Checkpoints and Car-t Cells: The Pioneers in Future Cancer Therapies?" *International Journal of Molecular Sciences* 21 (21): 1–28. https://doi.org/10.3390/ijms21218305.
Hou, Xiaoqiong, Shihua Yin, Xiaobing Jiang, Natalia Vasylieva, and Jiexian Dong. 2022. "A Nanobody Against CTLA-4 Increases the Anti-Tumor Effects of Specific CD8+ T Cells" 15 (11): 2229–39. https://doi.org/10.1166/jbn.2019.2859.A.
Huang, Ching Tai, Creg J. Workman, Dallas Flies, Xiaoyu Pan, Aimee L. Marson, Gang Zhou, Edward L. Hipkiss, et al. 2004. "Role of LAG-3 in Regulatory T Cells." *Immunity* 21 (4): 503–13. https://doi.org/10.1016/j.immuni.2004.08.010.
Huang, Lieven, Gunter Reekmans, Dirk Saerens, Jean-Michel Friedt, Filip Frederix, Laurent Francis, Serge Muyldermans, Andrew Campitelli, and Chris Van Hoof. 2005. "Prostate-Specific Antigen Immunosensing Based on Mixed Self-Assembled Monolayers, Camel Antibodies and Colloidal Gold Enhanced Sandwich Assays." *Biosensors & Bioelectronics* 21 (3): 483–90. https://doi.org/10.1016/j.bios.2004.11.016.
Huang, Pei Wei, and John Wen Cheng Chang. 2019. "Immune Checkpoint Inhibitors Win the 2018 Nobel Prize." *Biomedical Journal* 42 (5): 299–306. https://doi.org/10.1016/j.bj.2019.09.002.
Huang, Yu-Hwa, Chen Zhu, Yasuyuki Kondo, Ana C Anderson, Amit Gandhi, Andrew Russell, Stephanie K Dougan, et al. 2015. "CEACAM1 Regulates TIM-3-Mediated Tolerance and Exhaustion." *Nature* 517 (7534): 386–90. https://doi.org/10.1038/nature13848.
Huard, B, M Tournier, T Hercend, F Triebel, and F Faure. 1994. "Lymphocyte-Activation

Gene 3/Major Histocompatibility Complex Class II Interaction Modulates the Antigenic Response of CD4+ T Lymphocytes." *European Journal of Immunology* 24 (12): 3216–21. https://doi.org/10.1002/eji.1830241246.

Ibrahim, Ramy, Ross Stewart, and Aiman Shalabi. 2015. "PD-L1 Blockade for Cancer Treatment: MEDI4736." *Seminars in Oncology* 42 (3): 474–83. https://doi.org/10.1053/j.seminoncol.2015.02.007.

Ingram, Jessica R., Florian I. Schmidt, and Hidde L. Ploegh. 2018. "Exploiting Nanobodies' Singular Traits." *Annual Review of Immunology* 36: 695–715. https://doi.org/10.1146/annurev-immunol-042617-053327.

Intlekofer, Andrew M, Naofumi Takemoto, Charlly Kao, Arnob Banerjee, Felix Schambach, John K Northrop, Hao Shen, E John Wherry, and Steven L Reiner. 2007. "Requirement for T-Bet in the Aberrant Differentiation of Unhelped Memory CD8+ T Cells." *The Journal of Experimental Medicine* 204 (9): 2015–21. https://doi.org/10.1084/jem.20070841.

Intlekofer, Andrew M, Naofumi Takemoto, E John Wherry, Sarah A Longworth, John T Northrup, Vikram R Palanivel, Alan C Mullen, et al. 2005. "Effector and Memory CD8+ T Cell Fate Coupled by T-Bet and Eomesodermin." *Nature Immunology* 6 (12): 1236–44. https://doi.org/10.1038/ni1268.

Ishida, Y, Y Agata, K Shibahara, and T Honjo. 1992. "Induced Expression of PD-1, a Novel Member of the Immunoglobulin Gene Superfamily, upon Programmed Cell Death." *The EMBO Journal* 11 (11): 3887–95. https://doi.org/10.1002/j.1460-2075.1992.tb05481.x.

Iwai, Yoshiko, Seigo Terawaki, and Tasuku Honjo. 2005. "PD-1 Blockade Inhibits Hematogenous Spread of Poorly Immunogenic Tumor Cells by Enhanced Recruitment of Effector T Cells." *International Immunology* 17 (2): 133–44. https://doi.org/10.1093/intimm/dxh194.

Janssen, Edith M, Edward E Lemmens, Tom Wolfe, Urs Christen, Matthias G von Herrath, and Stephen P Schoenberger. 2003. "CD4+ T Cells Are Required for Secondary Expansion and Memory in CD8+ T Lymphocytes." *Nature* 421 (6925): 852–56. https://doi.org/10.1038/nature01441.

Jayaraman, Jayapriya, Michael P. Mellody, Andrew J. Hou, Ruchi P. Desai, Audrey W. Fung, An Huynh Thuy Pham, Yvonne Y. Chen, and Weian Zhao. 2020. "CAR-T Design: Elements and Their Synergistic Function." *EBioMedicine* 58. https://doi.org/10.1016/j.ebiom.2020.102931.

Johnston, Robert J., Laetitia Comps-Agrar, Jason Hackney, Xin Yu, Mahrukh Huseni, Yagai Yang, Summer Park, et al. 2014. "The Immunoreceptor TIGIT Regulates Antitumor and Antiviral CD8+ T Cell Effector Function." *Cancer Cell* 26 (6): 923–37. https://doi.org/10.1016/j.ccell.2014.10.018.

Jovčevska, Ivana, and Serge Muyldermans. 2020. "The Therapeutic Potential of Nanobodies." *BioDrugs* 34 (1): 11–26. https://doi.org/10.1007/s40259-019-00392-z.

June, Carl H., Roddy S. O'Connor, Omkar U. Kawalekar, Saba Ghassemi, and Michael C. Milone. 2018. "CAR T Cell Immunotherapy for Human Cancer." *Science* 359 (6382): 1361–65. https://doi.org/10.1126/science.aar6711.

Kaech, Susan M., and E. John Wherry. 2007. "Heterogeneity and Cell-Fate Decisions in Effector and Memory CD8+ T Cell Differentiation during Viral Infection." *Immunity* 27 (3): 393–405. https://doi.org/10.1016/j.immuni.2007.08.007.

Kahan, Shannon M, E John Wherry, and Allan J Zajac. 2015. "T Cell Exhaustion during Persistent Viral Infections." *Virology* 479–480 (May): 180–93. https://doi.org/10.1016/j.virol.2014.12.033.

Kalos, Michael, Bruce L Levine, David L Porter, Sharyn Katz, Stephan A Grupp, Adam Bagg, and Carl H June. 2011. "T Cells with Chimeric Antigen Receptors Have Potent

Antitumor Effects and Can Establish Memory in Patients with Advanced Leukemia." *Science Translational Medicine* 3 (95): 95ra73. https://doi.org/10.1126/scitranslmed.3002842.

Kao, Charlly, Kenneth J. Oestreich, Michael A. Paley, Alison Crawford, Jill M. Angelosanto, Mohammed Alkhatim A. Ali, Andrew M. Intlekofer, et al. 2011. "Transcription Factor T-Bet Represses Expression of the Inhibitory Receptor PD-1 and Sustains Virus-Specific CD8+ T Cell Responses during Chronic Infection." *Nature Immunology* 12 (7): 663–71. https://doi.org/10.1038/ni.2046.

Keyaerts, Marleen, Catarina Xavier, Johannes Heemskerk, Nick Devoogdt, Hendrik Everaert, Chloé Ackaert, Marian Vanhoeij, et al. 2016. "Phase I Study of 68Ga-HER2-Nanobody for PET/CT Assessment of HER2 Expression in Breast Carcinoma." *Journal of Nuclear Medicine : Official Publication, Society of Nuclear Medicine* 57 (1): 27–33. https://doi.org/10.2967/jnumed.115.162024.

Khalil, Ahmad S, and James J Collins. 2010. "Synthetic Biology: Applications Come of Age." *Nature Reviews. Genetics* 11 (5): 367–79. https://doi.org/10.1038/nrg2775.

Khan, Anooshay, Julian Ostaku, Ebru Aras, and Urartu Ozgur Safak Seker. 2022. "Combating Infectious Diseases with Synthetic Biology." *ACS Synthetic Biology* 11 (2): 528–37. https://doi.org/10.1021/acssynbio.1c00576.

Kitada, Tasuku, Breanna DiAndreth, Brian Teague, and Ron Weiss. 2018. "Programming Gene and Engineered-Cell Therapies with Synthetic Biology." *Science*. https://doi.org/10.1126/science.aad1067.

Klarenbeek, Alex, Khalil El Mazouari, Aline Desmyter, Christophe Blanchetot, Anna Hultberg, Natalie de Jonge, Rob C Roovers, et al. 2015. "Camelid Ig V Genes Reveal Significant Human Homology Not Seen in Therapeutic Target Genes, Providing for a Powerful Therapeutic Antibody Platform." *MAbs* 7 (4): 693–706. https://doi.org/10.1080/19420862.2015.1046648.

Klooster, Rinse, Michael R Eman, Quint le Duc, Peter Verheesen, C Theo Verrips, Rob C Roovers, and Jan A Post. 2009. "Selection and Characterization of KDEL-Specific VHH Antibody Fragments and Their Application in the Study of ER Resident Protein Expression." *Journal of Immunological Methods* 342 (1–2): 1–12. https://doi.org/10.1016/j.jim.2008.10.009.

Kohn, Donald B., Yvonne Y. Chen, and Melissa J. Spencer. 2023. "Successes and Challenges in Clinical Gene Therapy." *Gene Therapy*, no. December 2022. https://doi.org/10.1038/s41434-023-00390-5.

Koide, Shohei. 2009. "Engineering of Recombinant Crystallization Chaperones." *Current Opinion in Structural Biology* 19 (4): 449–57. https://doi.org/10.1016/j.sbi.2009.04.008.

Kontermann, Roland E. 2011. "Strategies for Extended Serum Half-Life of Protein Therapeutics." *Current Opinion in Biotechnology* 22 (6): 868–76. https://doi.org/10.1016/j.copbio.2011.06.012.

Korman, Alan J, Sarah C Garrett-Thomson, and Nils Lonberg. 2022. "The Foundations of Immune Checkpoint Blockade and the Ipilimumab Approval Decennial." *Nature Reviews. Drug Discovery* 21 (7): 509–28. https://doi.org/10.1038/s41573-021-00345-8.

Kosmaczewska, A, L Ciszak, D Boćko, and I Frydecka. 2001. "Expression and Functional Significance of CTLA-4, a Negative Regulator of T Cell Activation." *Archivum Immunologiae et Therapiae Experimentalis* 49 (1): 39–46.

Kouro, Taku, Hidetomo Himuro, and Tetsuro Sasada. 2022. "Exhaustion of CAR T Cells: Potential Causes and Solutions." *Journal of Translational Medicine* 20 (1): 1–10. https://doi.org/10.1186/s12967-022-03442-3.

Koyama, Shohei, Esra A Akbay, Yvonne Y Li, Grit S Herter-Sprie, Kevin A Buczkowski, William G Richards, Leena Gandhi, et al. 2016. "Adaptive Resistance to Therapeutic

PD-1 Blockade Is Associated with Upregulation of Alternative Immune Checkpoints." *Nature Communications* 7 (February): 10501. https://doi.org/10.1038/ncomms10501.

Krummel, M F, and J P Allison. 1996. "CTLA-4 Engagement Inhibits IL-2 Accumulation and Cell Cycle Progression upon Activation of Resting T Cells." *The Journal of Experimental Medicine* 183 (6): 2533–40. https://doi.org/10.1084/jem.183.6.2533.

Kubli, Shawn P., Thorsten Berger, Daniel V. Araujo, Lillian L. Siu, and Tak W. Mak. 2021. "Beyond Immune Checkpoint Blockade: Emerging Immunological Strategies." *Nature Reviews Drug Discovery* 20 (12): 899–919. https://doi.org/10.1038/s41573-021-00155-y.

Kunz, Patrick, Katinka Zinner, Norbert Mücke, Tanja Bartoschik, Serge Muyldermans, and Jörg D Hoheisel. 2018. "The Structural Basis of Nanobody Unfolding Reversibility and Thermoresistance." *Scientific Reports* 8 (1): 7934. https://doi.org/10.1038/s41598-018-26338-z.

Lanitis, E., D. Dangaj, M. Irving, and George Coukos. 2017. "Mechanisms Regulating T-Cell Infiltration and Activity in Solid Tumors." *Annals of Oncology* 28 (September): xii18-xii32. https://doi.org/10.1093/annonc/mdx238.

Latchman, Y, C R Wood, T Chernova, D Chaudhary, M Borde, I Chernova, Y Iwai, et al. 2001. "PD-L2 Is a Second Ligand for PD-1 and Inhibits T Cell Activation." *Nature Immunology* 2 (3): 261–68. https://doi.org/10.1038/85330.

Lauwereys, M, M Arbabi Ghahroudi, A Desmyter, J Kinne, W Hölzer, E De Genst, L Wyns, and S Muyldermans. 1998. "Potent Enzyme Inhibitors Derived from Dromedary Heavy-Chain Antibodies." *The EMBO Journal* 17 (13): 3512–20. https://doi.org/10.1093/emboj/17.13.3512.

Le, Dung T., Jennifer N. Durham, Kellie N. Smith, Hao Wang, Bjarne R. Bartlett, Laveet K. Aulakh, Steve Lu, et al. 2017. "Mismatch Repair Deficiency Predicts Response of Solid Tumors to PD-1 Blockade." *Science* 357 (6349): 409–13. https://doi.org/10.1126/science.aan6733.

Lee, Judong, Ee Wern Su, Chen Zhu, Sarah Hainline, Jiayao Phuah, Jamie A Moroco, Thomas E Smithgall, Vijay K Kuchroo, and Lawrence P Kane. 2011. "Phosphotyrosine-Dependent Coupling of Tim-3 to T-Cell Receptor Signaling Pathways." *Molecular and Cellular Biology* 31 (19): 3963–74. https://doi.org/10.1128/MCB.05297-11.

Li, Dongyang, Christophe Morisseau, Cindy B McReynolds, Thomas Duflot, Jérémy Bellien, Rashed M Nagra, Ameer Y Taha, and Bruce D Hammock. 2020. "Development of Improved Double-Nanobody Sandwich ELISAs for Human Soluble Epoxide Hydrolase Detection in Peripheral Blood Mononuclear Cells of Diabetic Patients and the Prefrontal Cortex of Multiple Sclerosis Patients." *Analytical Chemistry* 92 (10): 7334–42. https://doi.org/10.1021/acs.analchem.0c01115.

Li, Man, Pengyan Xia, Ying Du, Shengwu Liu, Guanling Huang, Jun Chen, Honglian Zhang, et al. 2014. "T-Cell Immunoglobulin and ITIM Domain (TIGIT) Receptor/Poliovirus Receptor (PVR) Ligand Engagement Suppresses Interferon-γ Production of Natural Killer Cells via β-Arrestin 2-Mediated Negative Signaling." *Journal of Biological Chemistry* 289 (25): 17647–57. https://doi.org/10.1074/jbc.M114.572420.

Li, Ming O, Yisong Y Wan, Shomyseh Sanjabi, Anna-Karin L Robertson, and Richard A Flavell. 2006. "Transforming Growth Factor-Beta Regulation of Immune Responses." *Annual Review of Immunology* 24: 99–146. https://doi.org/10.1146/annurev.immunol.24.021605.090737.

Li, Tengfei, Jean-Pierre Bourgeois, Susanna Celli, Fabienne Glacial, Anne-Marie Le Sourd, Salah Mecheri, Babette Weksler, et al. 2012. "Cell-Penetrating Anti-GFAP VHH and Corresponding Fluorescent Fusion Protein VHH-GFP Spontaneously Cross the Blood-Brain Barrier and Specifically Recognize Astrocytes: Application to Brain Imaging."

FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 26 (10): 3969–79. https://doi.org/10.1096/fj.11-201384.

Li, Tengfei, Matthias Vandesquille, Fani Koukouli, Clémence Dudeffant, Ihsen Youssef, Pascal Lenormand, Christelle Ganneau, et al. 2016. "Camelid Single-Domain Antibodies: A Versatile Tool for in Vivo Imaging of Extracellular and Intracellular Brain Targets." *Journal of Controlled Release : Official Journal of the Controlled Release Society* 243 (December): 1–10. https://doi.org/10.1016/j.jconrel.2016.09.019.

Lin, Yibin, Yifu Song, Yaochuan Zhang, Mengwu Shi, Ana Hou, and Sheng Han. 2023. "NFAT Signaling Dysregulation in Cancer: Emerging Roles in Cancer Stem Cells." *Biomedicine and Pharmacotherapy* 165 (May): 115167. https://doi.org/10.1016/j.biopha.2023.115167.

Linsley, P S, J Bradshaw, J Greene, R Peach, K L Bennett, and R S Mittler. 1996. "Intracellular Trafficking of CTLA-4 and Focal Localization towards Sites of TCR Engagement." *Immunity* 4 (6): 535–43. https://doi.org/10.1016/s1074-7613(00)80480-x.

Liu, S., H. Zhang, M. Li, D. Hu, C. Li, B. Ge, B. Jin, and Z. Fan. 2013. "Recruitment of Grb2 and SHIP1 by the ITT-like Motif of TIGIT Suppresses Granule Polarization and Cytotoxicity of NK Cells." *Cell Death and Differentiation* 20 (3): 456–64. https://doi.org/10.1038/cdd.2012.141.

Liu, Wenshuai, Haipeng Song, Quan Chen, Jianli Yu, Mo Xian, Rui Nian, and Dongxiao Feng. 2018. "Recent Advances in the Selection and Identification of Antigen-Specific Nanobodies." *Molecular Immunology* 96 (April): 37–47. https://doi.org/10.1016/j.molimm.2018.02.012.

Liu, Xindong, Yun Wang, Huiping Lu, Jing Li, Xiaowei Yan, Minglu Xiao, Jing Hao, et al. 2019. "Genome-Wide Analysis Identifies NR4A1 as a Key Mediator of T Cell Dysfunction." *Nature* 567 (7749): 525–29. https://doi.org/10.1038/s41586-019-0979-8.

Livingstone, Elisabeth, Lisa Zimmer, Jessica C Hassel, Michael Fluck, Thomas K Eigentler, Carmen Loquai, Sebastian Haferkamp, et al. 2022. "Adjuvant Nivolumab plus Ipilimumab or Nivolumab Alone versus Placebo in Patients with Resected Stage IV Melanoma with No Evidence of Disease (IMMUNED): Final Results of a Randomised, Double-Blind, Phase 2 Trial." *Lancet (London, England)* 400 (10358): 1117–29. https://doi.org/10.1016/S0140-6736(22)01654-3.

Long, Adrienne H, Waleed M Haso, Jack F Shern, Kelsey M Wanhainen, Meera Murgai, Maria Ingaramo, Jillian P Smith, et al. 2015. "4-1BB Costimulation Ameliorates T Cell Exhaustion Induced by Tonic Signaling of Chimeric Antigen Receptors." *Nature Medicine* 21 (6): 581–90. https://doi.org/10.1038/nm.3838.

Maali, Amirhosein, Monireh Gholizadeh, Saba Feghhi-Najafabadi, Ahmad Noei, Seyedeh Sheila Seyed-Motahari, Shafieeh Mansoori, and Zahra Sharifzadeh. 2023. "Nanobodies in Cell-Mediated Immunotherapy: On the Road to Fight Cancer." *Frontiers in Immunology* 14 (January): 1–19. https://doi.org/10.3389/fimmu.2023.1012841.

Majzner, Robbie G., and Crystal L. Mackall. 2018. "Tumor Antigen Escape from Car T-Cell Therapy." *Cancer Discovery* 8 (10): 1219–26. https://doi.org/10.1158/2159-8290.CD-18-0442.

Man, Kevin, Sarah S Gabriel, Yang Liao, Renee Gloury, Simon Preston, Darren C Henstridge, Marc Pellegrini, et al. 2017. "Transcription Factor IRF4 Promotes CD8 + T Cell Exhaustion and Limits the Development of Memory-like T Cells during Chronic Infection." *Immunity* 47 (6): 1129–1141.e5. https://doi.org/10.1016/j.immuni.2017.11.021.

Man, Kevin, Maria Miasari, Wei Shi, Annie Xin, Darren C Henstridge, Simon Preston, Marc Pellegrini, et al. 2013. "The Transcription Factor IRF4 Is Essential for TCR Affinity-Mediated Metabolic Programming and Clonal Expansion of T Cells." *Nature*

Immunology 14 (11): 1155–65. https://doi.org/10.1038/ni.2710.

Manhas, Janvie, Hailey I Edelstein, Joshua N Leonard, Leonardo Morsut, Los Angeles, United States, Biological Engineering, et al. 2023. "The Evolution of Synthetic Receptor Systems" 18 (3): 244–55. https://doi.org/10.1038/s41589-021-00926-z.The.

Marabelle, Aurélien, Marwan Fakih, Juanita Lopez, Manisha Shah, Ronnie Shapira-Frommer, Kazuhiko Nakagawa, Hyun Cheol Chung, et al. 2020. "Association of Tumour Mutational Burden with Outcomes in Patients with Advanced Solid Tumours Treated with Pembrolizumab: Prospective Biomarker Analysis of the Multicohort, Open-Label, Phase 2 KEYNOTE-158 Study." *The Lancet. Oncology* 21 (10): 1353–65. https://doi.org/10.1016/S1470-2045(20)30445-9.

Marchetti, Giulia, Luca Meroni, Chiara Molteni, Alessandra Bandera, Fabio Franzetti, Massimo Galli, Mauro Moroni, Mario Clerici, and Andrea Gori. 2004. "Interleukin-2 Immunotherapy Exerts a Differential Effect on CD4 and CD8 T Cell Dynamics." *AIDS (London, England)* 18 (2): 211–16. https://doi.org/10.1097/00002030-200401230-00010.

Marin-Acevedo, Julian A., Bhagirathbhai Dholaria, Aixa E. Soyano, Keith L. Knutson, Saranya Chumsri, and Yanyan Lou. 2018. "Next Generation of Immune Checkpoint Therapy in Cancer: New Developments and Challenges." *Journal of Hematology and Oncology* 11 (1): 1–20. https://doi.org/10.1186/s13045-018-0582-8.

Markham, Anthony. 2022. "Envafolimab: First Approval." *Drugs* 82 (2): 235–40. https://doi.org/10.1007/s40265-022-01671-w.

Maruhashi, Takumi, Daisuke Sugiura, Il-mi Okazaki, and Taku Okazaki. 2020a. "LAG-3: From Molecular Functions to Clinical Applications." *Journal for ImmunoTherapy of Cancer* 8 (2). https://doi.org/10.1136/jitc-2020-001014.

———. 2020b. "LAG-3: From Molecular Functions to Clinical Applications." *Journal for ImmunoTherapy of Cancer* 8 (2). https://doi.org/10.1136/jitc-2020-001014.

Matsuzaki, Junko, Sacha Gnjatic, Paulette Mhawech-Fauceglia, Amy Beck, Austin Miller, Takemasa Tsuji, Cheryl Eppolito, et al. 2010. "Tumor-Infiltrating NY-ESO-1-Specific CD8+ T Cells Are Negatively Regulated by LAG-3 and PD-1 in Human Ovarian Cancer." *Proceedings of the National Academy of Sciences of the United States of America* 107 (17): 7875–80. https://doi.org/10.1073/pnas.1003345107.

McGowan, Eileen, Qimou Lin, Guocai Ma, Haibin Yin, Size Chen, and Yiguang Lin. 2020. "PD-1 Disrupted CAR-T Cells in the Treatment of Solid Tumors: Promises and Challenges." *Biomedicine & Pharmacotherapy* 121: 109625. https://doi.org/https://doi.org/10.1016/j.biopha.2019.109625.

McKinney, Eoin F, James C Lee, David R W Jayne, Paul A Lyons, and Kenneth G C Smith. 2015. "T-Cell Exhaustion, Co-Stimulation and Clinical Outcome in Autoimmunity and Infection." *Nature* 523 (7562): 612–16. https://doi.org/10.1038/nature14468.

McLane, Laura M., Mohamed S. Abdel-Hakeem, and E. John Wherry. 2019. "CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer." *Annual Review of Immunology* 37: 457–95. https://doi.org/10.1146/annurev-immunol-041015-055318.

Mclane, Laura M, Mohamed S Abdel-hakeem, and E John Wherry. 2019. "CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer," 457–95.

Menolascina, Filippo, Velia Siciliano, and Diego Di Bernardo. 2012. "Engineering and Control of Biological Systems: A New Way to Tackle Complex Diseases." *FEBS Letters* 586 (15): 2122–28. https://doi.org/10.1016/j.febslet.2012.04.050.

Meyer, Thomas De, Serge Muyldermans, and Ann Depicker. 2014. "Nanobody-Based Products as Research and Diagnostic Tools." *Trends in Biotechnology* 32 (5): 263–70. https://doi.org/10.1016/j.tibtech.2014.03.001.

Miggelbrink, Alexandra M, Joshua D Jackson, Selena J Lorrey, Ethan S Srinivasan, Jessica

Waibl-Polania, Daniel S Wilkinson, and Peter E Fecci. 2021. “CD4 T-Cell Exhaustion: Does It Exist and What Are Its Roles in Cancer?” *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 27 (21): 5742–52. https://doi.org/10.1158/1078-0432.CCR-21-0206.

Mikhaylova, Marina, Bas M C Cloin, Kieran Finan, Robert van den Berg, Jalmar Teeuw, Marta M Kijanka, Mikolaj Sokolowski, et al. 2015. “Resolving Bundled Microtubules Using Anti-Tubulin Nanobodies.” *Nature Communications* 6 (August): 7933. https://doi.org/10.1038/ncomms8933.

Mitchell, Laura S, and Lucy J Colwell. 2018. “Analysis of Nanobody Paratopes Reveals Greater Diversity than Classical Antibodies.” *Protein Engineering, Design & Selection : PEDS* 31 (7–8): 267–75. https://doi.org/10.1093/protein/gzy017.

Moir, Susan, and Anthony S Fauci. 2014. “B-Cell Exhaustion in HIV Infection: The Role of Immune Activation.” *Current Opinion in HIV and AIDS* 9 (5): 472–77. https://doi.org/10.1097/COH.0000000000000092.

Morales-Yanez, Francisco J, Idalia Sariego, Cécile Vincke, Gholamreza Hassanzadeh-Ghassabeh, Katja Polman, and Serge Muyldermans. 2019. “An Innovative Approach in the Detection of Toxocara Canis Excretory/Secretory Antigens Using Specific Nanobodies.” *International Journal for Parasitology* 49 (8): 635–45. https://doi.org/10.1016/j.ijpara.2019.03.004.

Mosch, Romy, and Henk-Jan Guchelaar. 2022. “Immunogenicity of Monoclonal Antibodies and the Potential Use of HLA Haplotypes to Predict Vulnerable Patients.” *Frontiers in Immunology* 13: 885672. https://doi.org/10.3389/fimmu.2022.885672.

Muyldermans, Serge. 2013. “Nanobodies: Natural Single-Domain Antibodies.” *Annual Review of Biochemistry* 82: 775–97. https://doi.org/10.1146/annurev-biochem-063011-092449.

———. 2021. “Applications of Nanobodies.” *Annual Review of Animal Biosciences* 9: 401–21. https://doi.org/https://doi.org/10.1146/annurev-animal-021419- 083831.

Nakanishi, Yusuke, Bao Lu, Craig Gerard, and Akiko Iwasaki. 2009. “CD8(+) T Lymphocyte Mobilization to Virus-Infected Tissue Requires CD4(+) T-Cell Help.” *Nature* 462 (7272): 510–13. https://doi.org/10.1038/nature08511.

Neelapu, Sattva S., Sudhakar Tummala, Partow Kebriaei, William Wierda, Cristina Gutierrez, Frederick L. Locke, Krishna V. Komanduri, et al. 2018. “Chimeric Antigen Receptor T-Cell Therapy-Assessment and Management of Toxicities.” *Nature Reviews Clinical Oncology* 15 (1): 47–62. https://doi.org/10.1038/nrclinonc.2017.148.

Nieba, L, A Honegger, C Krebber, and A Plückthun. 1997. “Disrupting the Hydrophobic Patches at the Antibody Variable/Constant Domain Interface: Improved in Vivo Folding and Physical Characterization of an Engineered ScFv Fragment.” *Protein Engineering* 10 (4): 435–44. https://doi.org/10.1093/protein/10.4.435.

O’Malley, David M, Maryna Neffa, Bradley J Monk, Tamar Melkadze, Marilyn Huang, Anna Kryzhanivska, Iurie Bulat, et al. 2022. “Dual PD-1 and CTLA-4 Checkpoint Blockade Using Balstilimab and Zalifrelimab Combination as Second-Line Treatment for Advanced Cervical Cancer: An Open-Label Phase II Study.” *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 40 (7): 762–71. https://doi.org/10.1200/JCO.21.02067.

O’Sullivan Coyne, Geraldine, Ravi A Madan, and James L Gulley. 2014. “Nivolumab: Promising Survival Signal Coupled with Limited Toxicity Raises Expectations.” *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*. United States. https://doi.org/10.1200/JCO.2013.54.5996.

Odorizzi, Pamela, and E. John Wherry. 2012. “Inhibitory Receptors on Lymphocytes: Insights from Infections.” *J Immunol.* 188 (7): 2957–65.

https://doi.org/10.4049/jimmunol.1100038.
Oliveira, Sabrina, Raimond Heukers, Jirawas Sornkom, Robbert J Kok, and Paul M P van Bergen En Henegouwen. 2013. “Targeting Tumors with Nanobodies for Cancer Imaging and Therapy.” *Journal of Controlled Release : Official Journal of the Controlled Release Society* 172 (3): 607–17. https://doi.org/10.1016/j.jconrel.2013.08.298.
Olson, Daniel J, Zeynep Eroglu, Bruce Brockstein, Andrew S Poklepovic, Madhuri Bajaj, Sunil Babu, Sigrun Hallmeyer, et al. 2021. “Pembrolizumab Plus Ipilimumab Following Anti-PD-1/L1 Failure in Melanoma.” *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 39 (24): 2647–55. https://doi.org/10.1200/JCO.21.00079.
Paley, Michael A., Daniela C. Kroy, Pamela M. Odorizzi1, Jonathan Johnnidis, Douglas V. Dolfi, Burton E. Barnett, Elizabeth K. Bikoff, et al. 2012. “Progenitor and Terminal Subsets of CD8+T Cells Cooperate to Contain Chronic Viral Infection.” *Science*. https://doi.org/10.1126/science.1229620.
Pan, Chongxian, Hongtao Liu, Elizabeth Robins, Wenru Song, Delong Liu, Zihai Li, and Lei Zheng. 2020. “Next-Generation Immuno-Oncology Agents: Current Momentum Shifts in Cancer Immunotherapy.” *Journal of Hematology and Oncology* 13 (1): 1–15. https://doi.org/10.1186/s13045-020-00862-w.
Papadopoulos, Kyriakos P, Wael Harb, Cody J Peer, Qiong Hua, Siying Xu, Haolan Lu, Ni Lu, et al. 2021. “First-in-Human Phase I Study of Envafolimab, a Novel Subcutaneous Single-Domain Anti-PD-L1 Antibody, in Patients with Advanced Solid Tumors.” *The Oncologist* 26 (9): e1514–25. https://doi.org/10.1002/onco.13817.
Pardon, Els, Toon Laeremans, Sarah Triest, Søren G.F. Rasmussen, Alexandre Wohlkönig, Armin Ruf, Serge Muyldermans, Wim G.J. Hol, Brian K. Kobilka, and Jan Steyaert. 2014. “A General Protocol for the Generation of Nanobodies for Structural Biology.” *Nature Protocols* 9 (3): 674–93. https://doi.org/10.1038/nprot.2014.039.
Pfeifer, Blaine A., Marie Beitelshees, Andrew Hill, Justin Bassett, and Charles H. Jones. 2023. “Harnessing Synthetic Biology for Advancing RNA Therapeutics and Vaccine Design.” *Npj Systems Biology and Applications* 9 (1): 1–10. https://doi.org/10.1038/s41540-023-00323-3.
Philip, Mary, and Andrea Schietinger. 2022. “CD8+ T Cell Differentiation and Dysfunction in Cancer.” *Nat Rev Immunol*. 22 (4): 209–23. https://doi.org/10.1038/s41577-021-00574-3.CD8.
Quigley, Michael, Florencia Pereyra, Björn Nilsson, Filippos Porichis, Catia Fonseca, Quentin Eichbaum, Boris Julg, et al. 2010. “Transcriptional Analysis of HIV-Specific CD8+ T Cells Shows That PD-1 Inhibits T Cell Function by Upregulating BATF.” *Nature Medicine* 16 (10): 1147–51. https://doi.org/10.1038/nm.2232.
Qureshi, Omar S, Yong Zheng, Kyoko Nakamura, Kesley Attridge, Claire Manzotti, Emily M Schmidt, Jennifer Baker, et al. 2011. “Trans-Endocytosis of CD80 and CD86: A Molecular Basis for the Cell-Extrinsic Function of CTLA-4.” *Science (New York, N.Y.)* 332 (6029): 600–603. https://doi.org/10.1126/science.1202947.
Rad S M, Ali Hosseini, Aarati Poudel, Grace Min Yi Tan, and Alexander D McLellan. 2020. “Promoter Choice: Who Should Drive the CAR in T Cells?” *PloS One* 15 (7): e0232915. https://doi.org/10.1371/journal.pone.0232915.
Rao, A, C Luo, and P G Hogan. 1997. “Transcription Factors of the NFAT Family: Regulation and Function.” *Annual Review of Immunology* 15: 707–47. https://doi.org/10.1146/annurev.immunol.15.1.707.
Rashidian, M, and H Ploegh. 2020. “Nanobodies as Non-Invasive Imaging Tools.” *Immuno-Oncology Technology* 7 (September): 2–14. https://doi.org/10.1016/j.iotech.2020.07.001.

Rasmussen, Søren G F, Brian T DeVree, Yaozhong Zou, Andrew C Kruse, Ka Young Chung, Tong Sun Kobilka, Foon Sun Thian, et al. 2011. "Crystal Structure of the B2 Adrenergic Receptor-Gs Protein Complex." *Nature* 477 (7366): 549–55. https://doi.org/10.1038/nature10361.

Ribas, Antoni, and Jedd D. Wolchok. 2018. "Cancer Immunotherapy Using Checkpoint Blockade." *Science* 359: 1350–55. https://doi.org/10.1126/science.aar4060.

Romao, Ema, Francisco Morales-Yanez, Yaozhong Hu, Maxine Crauwels, Pieter De Pauw, Gholamreza Ghassanzadeh Hassanzadeh, Nick Devoogdt, Chloe Ackaert, Cecile Vincke, and Serge Muyldermans. 2016. "Identification of Useful Nanobodies by Phage Display of Immune Single Domain Libraries Derived from Camelid Heavy Chain Antibodies." *Current Pharmaceutical Design* 22 (43): 6500–6518. https://doi.org/10.2174/1381612822666160923114417.

Roovers, Rob C, Maria J W D Vosjan, Toon Laeremans, Rachid el Khoulati, Renée C G de Bruin, Kathryn M Ferguson, Arie J Verkleij, Guus A M S van Dongen, and Paul M P van Bergen en Henegouwen. 2011. "A Biparatopic Anti-EGFR Nanobody Efficiently Inhibits Solid Tumour Growth." *International Journal of Cancer* 129 (8): 2013–24. https://doi.org/10.1002/ijc.26145.

Rothbauer, Ulrich, Kourosh Zolghadr, Serge Muyldermans, Aloys Schepers, M Cristina Cardoso, and Heinrich Leonhardt. 2008. "A Versatile Nanotrap for Biochemical and Functional Studies with Fluorescent Fusion Proteins." *Molecular & Cellular Proteomics : MCP* 7 (2): 282–89. https://doi.org/10.1074/mcp.M700342-MCP200.

Rothbauer, Ulrich, Kourosh Zolghadr, Sergei Tillib, Danny Nowak, Lothar Schermelleh, Anja Gahl, Natalija Backmann, et al. 2006. "Targeting and Tracing Antigens in Live Cells with Fluorescent Nanobodies." *Nature Methods* 3 (11): 887–89. https://doi.org/10.1038/nmeth953.

Rudd, Christopher E., Kittiphat Chanthong, and Alison Taylor. 2020. "Small Molecule Inhibition of GSK-3 Specifically Inhibits the Transcription of Inhibitory Co-Receptor LAG-3 for Enhanced Anti-Tumor Immunity." *Cell Reports* 30 (7): 2075–2082.e4. https://doi.org/10.1016/j.celrep.2020.01.076.

Rupp, Levi J, Kathrin Schumann, Kole T Roybal, Rachel E Gate, Chun J Ye, Wendell A Lim, and Alexander Marson. 2017. "CRISPR/Cas9-Mediated PD-1 Disruption Enhances Anti-Tumor Efficacy of Human Chimeric Antigen Receptor T Cells." *Scientific Reports* 7 (1): 737. https://doi.org/10.1038/s41598-017-00462-8.

Sadeghnezhad, Golnaz, Ema Romão, Robert Bernedo-Navarro, Sam Massa, Khosro Khajeh, Serge Muyldermans, and Sadegh Hassania. 2019. "Identification of New DR5 Agonistic Nanobodies and Generation of Multivalent Nanobody Constructs for Cancer Treatment." *International Journal of Molecular Sciences* 20 (19). https://doi.org/10.3390/ijms20194818.

Saerens, Dirk, Filip Frederix, Gunter Reekmans, Katja Conrath, Karolien Jans, Lea Brys, Lieven Huang, et al. 2005. "Engineering Camel Single-Domain Antibodies and Immobilization Chemistry for Human Prostate-Specific Antigen Sensing." *Analytical Chemistry* 77 (23): 7547–55. https://doi.org/10.1021/ac051092j.

Safarzadeh Kozani, Pouya, Abdolhossein Naseri, Seyed Mohamad Javad Mirarefin, Faeze Salem, Mojtaba Nikbakht, Sahar Evazi Bakhshi, and Pooria Safarzadeh Kozani. 2022. "Nanobody-Based CAR-T Cells for Cancer Immunotherapy." *Biomarker Research* 10 (1): 1–18. https://doi.org/10.1186/s40364-022-00371-7.

Salvador, J. Pablo, Lluïsa Vilaplana, and M. Pilar Marco. 2019. "Nanobody: Outstanding Features for Diagnostic and Therapeutic Applications." *Analytical and Bioanalytical Chemistry* 411 (9): 1703–13. https://doi.org/10.1007/s00216-019-01633-4.

Sargent, Demi, Warren C. Conaty, David T. Tissue, and Robert E. Sharwood. 2022.

"Synthetic Biology and Opportunities within Agricultural Crops." *Journal of Sustainable Agriculture and Environment* 1 (2): 89–107. https://doi.org/10.1002/sae2.12014.

Scully, Marie, Spero R Cataland, Flora Peyvandi, Paul Coppo, Paul Knöbl, Johanna A Kremer Hovinga, Ara Metjian, et al. 2019. "Caplacizumab Treatment for Acquired Thrombotic Thrombocytopenic Purpura." *The New England Journal of Medicine* 380 (4): 335–46. https://doi.org/10.1056/NEJMoa1806311.

Sehrawat, Sharvan, Pradeep B J Reddy, Naveen Rajasagi, Amol Suryawanshi, Mitsuomi Hirashima, and Barry T Rouse. 2010. "Galectin-9/TIM-3 Interaction Regulates Virus-Specific Primary and Memory CD8 T Cell Response." *PLoS Pathogens* 6 (5): e1000882. https://doi.org/10.1371/journal.ppat.1000882.

Seo, Hyungseok, Joyce Chen, Edahí González-Avalos, Daniela Samaniego-Castruita, Arundhoti Das, Yueqiang H. Wang, Isaac F. López-Moyado, et al. 2019a. "TOX and TOX2 Transcription Factors Cooperate with NR4A Transcription Factors to Impose CD8+ T Cell Exhaustion." *Proceedings of the National Academy of Sciences of the United States of America* 116 (25): 12410–15. https://doi.org/10.1073/pnas.1905675116.

Seo, Hyungseok, Joyce Chen, Edahí González-Avalos, Daniela Samaniego-Castruita, Arundhoti Das, Yueqiang H Wang, Isaac F López-Moyado, et al. 2019b. "TOX and TOX2 Transcription Factors Cooperate with NR4A Transcription Factors to Impose CD8(+) T Cell Exhaustion." *Proceedings of the National Academy of Sciences of the United States of America* 116 (25): 12410–15. https://doi.org/10.1073/pnas.1905675116.

Shafer, Paul, Lauren M. Kelly, and Valentina Hoyos. 2022. "Cancer Therapy With TCR-Engineered T Cells: Current Strategies, Challenges, and Prospects." *Frontiers in Immunology* 13 (March): 1–24. https://doi.org/10.3389/fimmu.2022.835762.

Shah, Nirali N., and Terry J. Fry. 2019. "Mechanisms of Resistance to CAR T Cell Therapy." *Nat Rev Clin Oncol.* 16 (6): 372–85. https://doi.org/10.1038/s41571-019-0184-6.

Shankar, P, M Russo, B Harnisch, M Patterson, P Skolnik, and J Lieberman. 2000. "Impaired Function of Circulating HIV-Specific CD8(+) T Cells in Chronic Human Immunodeficiency Virus Infection." *Blood* 96 (9): 3094–3101.

Sharma, Padmanee, Sangeeta Goswami, Deblina Raychaudhuri, Bilal A. Siddiqui, Pratishtha Singh, Ashwat Nagarajan, Jielin Liu, et al. 2023. "Immune Checkpoint Therapy—Current Perspectives and Future Directions." *Cell* 186 (8): 1652–69. https://doi.org/10.1016/j.cell.2023.03.006.

Sharma, Padmanee, Siwen Hu-Lieskovan, Jennifer A Wargo, and Antoni Ribas. 2017. "Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy." *Cell* 168 (4): 707–23. https://doi.org/10.1016/j.cell.2017.01.017.

Sharma, Padmanee, Bilal A. Siddiqui, Swetha Anandhan, Shalini S. Yadav, Sumit K. Subudhi, Jianjun Gao, Sangeeta Goswami, and James P. Allison. 2021. "The next Decade of Immune Checkpoint Therapy." *Cancer Discovery* 11 (4): 838–57. https://doi.org/10.1158/2159-8290.CD-20-1680.

Sheng, Yamin, Kun Wang, Qizhong Lu, Pinpin Ji, Baoyuan Liu, Jiahong Zhu, Qingyuan Liu, et al. 2019. "Nanobody-Horseradish Peroxidase Fusion Protein as an Ultrasensitive Probe to Detect Antibodies against Newcastle Disease Virus in the Immunoassay." *Journal of Nanobiotechnology* 17 (1): 35. https://doi.org/10.1186/s12951-019-0468-0.

Silva-Pilipich, Noelia, Ester Blanco, Teresa Lozano, Eva Martisova, Ana Igea, Guillermo Herrador-Cañete, María Cristina Ballesteros-Briones, et al. 2023. "Local Delivery of Optimized Nanobodies Targeting the PD-1/PD-L1 Axis with a Self-Amplifying RNA Viral Vector Induces Potent Antitumor Responses." *Cancer Letters* 561 (March). https://doi.org/10.1016/j.canlet.2023.216139.

Sprinzak, David, and Michael B Elowitz. 2005. "Reconstruction of Genetic Circuits." *Nature* 438 (7067): 443–48. https://doi.org/10.1038/nature04335.

Stanietsky, Noa, Hrvoje Simic, Jurica Arapovic, Amir Toporik, Ofer Levy, Amit Novik, Zurit Levine, et al. 2009. “The Interaction of TIGIT with PVR and PVRL2 Inhibits Human NK Cell Cytotoxicity.” *Proceedings of the National Academy of Sciences of the United States of America* 106 (42): 17858–63. https://doi.org/10.1073/pnas.0903474106.

Sterner, Robert C., and Rosalie M. Sterner. 2021. “CAR-T Cell Therapy: Current Limitations and Potential Strategies.” *Blood Cancer Journal* 11 (4). https://doi.org/10.1038/s41408-021-00459-7.

Steyaert, Jan, and Brian K Kobilka. 2011. “Nanobody Stabilization of G Protein-Coupled Receptor Conformational States.” *Current Opinion in Structural Biology* 21 (4): 567–72. https://doi.org/10.1016/j.sbi.2011.06.011.

Strome, Scott E, Haidong Dong, Hideto Tamura, Stephen G Voss, Dallas B Flies, Koji Tamada, Diva Salomao, et al. 2003. “B7-H1 Blockade Augments Adoptive T-Cell Immunotherapy for Squamous Cell Carcinoma.” *Cancer Research* 63 (19): 6501–5.

Tan, Xiao, Justin H. Letendre, James J. Collins, and Wilson W. Wong. 2021. “Synthetic Biology in the Clinic: Engineering Vaccines, Diagnostics, and Therapeutics.” *Cell* 184 (4): 881–98. https://doi.org/10.1016/j.cell.2021.01.017.

Tang, Jonathan C Y, Tamas Szikra, Yevgenia Kozorovitskiy, Miguel Teixiera, Bernardo L Sabatini, Botond Roska, and Constance L Cepko. 2013. “A Nanobody-Based System Using Fluorescent Proteins as Scaffolds for Cell-Specific Gene Manipulation.” *Cell* 154 (4): 928–39. https://doi.org/10.1016/j.cell.2013.07.021.

Tawbi, Hussein A, Dirk Schadendorf, Evan J Lipson, Paolo A Ascierto, Luis Matamala, Erika Castillo Gutiérrez, Piotr Rutkowski, et al. 2022. “Relatlimab and Nivolumab versus Nivolumab in Untreated Advanced Melanoma.” *The New England Journal of Medicine* 386 (1): 24–34. https://doi.org/10.1056/NEJMoa2109970.

Tay, Rong En, Emma K Richardson, and Han Chong Toh. 2021. “Revisiting the Role of CD4+ T Cells in Cancer Immunotherapy—New Insights into Old Paradigms.” *Cancer Gene Therapy* 28 (1): 5–17. https://doi.org/10.1038/s41417-020-0183-x.

Tijink, Bernard M, Toon Laeremans, Marianne Budde, Marijke Stigter-van Walsum, Torsten Dreier, Hans J de Haard, C René Leemans, and Guus A M S van Dongen. 2008. “Improved Tumor Targeting of Anti-Epidermal Growth Factor Receptor Nanobodies through Albumin Binding: Taking Advantage of Modular Nanobody Technology.” *Molecular Cancer Therapeutics* 7 (8): 2288–97. https://doi.org/10.1158/1535-7163.MCT-07-2384.

Tinoco, Roberto, Victor Alcalde, Yating Yang, Karsten Sauer, and Elina I Zuniga. 2009. “Cell-Intrinsic Transforming Growth Factor-Beta Signaling Mediates Virus-Specific CD8+ T Cell Deletion and Viral Persistence in Vivo.” *Immunity* 31 (1): 145–57. https://doi.org/10.1016/j.immuni.2009.06.015.

Topalian, Suzanne L, Charles G Drake, and Drew M Pardoll. 2012. “Targeting the PD-1/B7-H1(PD-L1) Pathway to Activate Anti-Tumor Immunity.” *Current Opinion in Immunology* 24 (2): 207–12. https://doi.org/10.1016/j.coi.2011.12.009.

Topalian, Suzanne L, Mario Sznol, David F McDermott, Harriet M Kluger, Richard D Carvajal, William H Sharfman, Julie R Brahmer, et al. 2014. “Survival, Durable Tumor Remission, and Long-Term Safety in Patients with Advanced Melanoma Receiving Nivolumab.” *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 32 (10): 1020–30. https://doi.org/10.1200/JCO.2013.53.0105.

Traenkle, Bjoern, and Ulrich Rothbauer. 2017. “Under the Microscope: Single-Domain Antibodies for Live-Cell Imaging and Super-Resolution Microscopy.” *Frontiers in Immunology* 8: 1030. https://doi.org/10.3389/fimmu.2017.01030.

Trentesaux, Coralie, Toshimichi Yamada, Ophir D. Klein, and Wendell A. Lim. 2023. “Harnessing Synthetic Biology to Engineer Organoids and Tissues.” *Cell Stem Cell* 30

(1): 10–19. https://doi.org/10.1016/j.stem.2022.12.013.
Unger, Mandy, Anna Marei Eichhoff, Lucas Schumacher, Moritz Strysio, Stephan Menzel, Carsten Schwan, Vanina Alzogaray, et al. 2015. "Selection of Nanobodies That Block the Enzymatic and Cytotoxic Activities of the Binary Clostridium Difficile Toxin CDT." *Scientific Reports* 5 (January): 7850. https://doi.org/10.1038/srep07850.
Valle, Ilenne Del, Emily M. Fulk, Prashant Kalvapalle, Jonathan J. Silberg, Caroline A. Masiello, and Lauren B. Stadler. 2021. "Translating New Synthetic Biology Advances for Biosensing Into the Earth and Environmental Sciences." *Frontiers in Microbiology* 11 (February): 1–29. https://doi.org/10.3389/fmicb.2020.618373.
Vos, Jens De, Nick Devoogdt, Tony Lahoutte, and Serge Muyldermans. 2013. "Camelid Single-Domain Antibody-Fragment Engineering for (Pre)Clinical in Vivo Molecular Imaging Applications: Adjusting the Bullet to Its Target." *Expert Opinion on Biological Therapy* 13 (8): 1149–60. https://doi.org/10.1517/14712598.2013.800478.
Vu, K B, M A Ghahroudi, L Wyns, and S Muyldermans. 1997. "Comparison of Llama VH Sequences from Conventional and Heavy Chain Antibodies." *Molecular Immunology* 34 (16–17): 1121–31. https://doi.org/10.1016/s0161-5890(97)00146-6.
Vugmeyster, Yulia, Clifford A Entrican, Alison P Joyce, Rosemary F Lawrence-Henderson, Beth A Leary, Christopher S Mahoney, Himakshi K Patel, et al. 2012. "Pharmacokinetic, Biodistribution, and Biophysical Profiles of TNF Nanobodies Conjugated to Linear or Branched Poly(Ethylene Glycol)." *Bioconjugate Chemistry* 23 (7): 1452–62. https://doi.org/10.1021/bc300066a.
Wagner, Teresa R., and Ulrich Rothbauer. 2020. "Nanobodies Right in the MIDDLE: INTRABODIES as Toolbox to Visualize and Modulate Antigens in the Living Cell." *Biomolecules* 10 (12): 1–19. https://doi.org/10.3390/biom10121701.
Wan, Ruirong, Aiqun Liu, Xiaoqiong Hou, Zongqiang Lai, Jieping Li, Nuo Yang, Juntao Tan, et al. 2018. "Screening and Antitumor Effect of an Anti-CTLA-4 Nanobody." *Oncology Reports* 39 (2): 511–18. https://doi.org/10.3892/or.2017.6131.
Wang, Wei, Satish Singh, David L Zeng, Kevin King, and Sandeep Nema. 2007. "Antibody Structure, Instability, and Formulation." *Journal of Pharmaceutical Sciences* 96 (1): 1–26. https://doi.org/10.1002/jps.20727.
Ward, Jeffrey P. 2022. "Tumor Infiltrating Lymphocytes (TIL): From Bench to Bedside." In *Gene and Cellular Immunotherapy for Cancer*, edited by Armin Ghobadi and John F DiPersio, 223–48. Cham: Springer International Publishing. https://doi.org/10.1007/978-3-030-87849-8_13.
Wei, Guoqing, Lijuan Ding, Jiasheng Wang, Yongxian Hu, and He Huang. 2017. "Advances of CD19-Directed Chimeric Antigen Receptor-Modified T Cells in Refractory/Relapsed Acute Lymphoblastic Leukemia." *Experimental Hematology & Oncology* 6: 10. https://doi.org/10.1186/s40164-017-0070-9.
Wesolowski, Janusz, Vanina Alzogaray, Jan Reyelt, Mandy Unger, Karla Juarez, Mariela Urrutia, Ana Cauerhff, et al. 2009. "Single Domain Antibodies: Promising Experimental and Therapeutic Tools in Infection and Immunity." *Medical Microbiology and Immunology* 198 (3): 157–74. https://doi.org/10.1007/s00430-009-0116-7.
West, Erin E, Hyun-Tak Jin, Ata-Ur Rasheed, Pablo Penaloza-Macmaster, Sang-Jun Ha, Wendy G Tan, Ben Youngblood, Gordon J Freeman, Kendall A Smith, and Rafi Ahmed. 2013. "PD-L1 Blockade Synergizes with IL-2 Therapy in Reinvigorating Exhausted T Cells." *The Journal of Clinical Investigation* 123 (6): 2604–15. https://doi.org/10.1172/JCI67008.
Wherry, E. John, Joseph N. Blattman, Kaja Murali-Krishna, Robbert van der Most, and Rafi Ahmed. 2003. "Viral Persistence Alters CD8 T-Cell Immunodominance and Tissue Distribution and Results in Distinct Stages of Functional Impairment." *Journal of*

Virology 77 (8): 4911–27. https://doi.org/10.1128/jvi.77.8.4911-4927.2003.
Wherry, E. John, Sang Jun Ha, Susan M. Kaech, W. Nicholas Haining, Surojit Sarkar, Vandana Kalia, Shruti Subramaniam, Joseph N. Blattman, Daniel L. Barber, and Rafi Ahmed. 2007. "Molecular Signature of CD8+ T Cell Exhaustion during Chronic Viral Infection." *Immunity* 27 (4): 670–84. https://doi.org/10.1016/j.immuni.2007.09.006.
Wherry, E. John, and Makoto Kurachi. 2015. "Molecular and Cellular Insights into T Cell Exhaustion." *Nature Reviews Immunology*. https://doi.org/10.1038/nri3862.
Williams, Matthew A, Aaron J Tyznik, and Michael J Bevan. 2006. "Interleukin-2 Signals during Priming Are Required for Secondary Expansion of CD8+ Memory T Cells." *Nature* 441 (7095): 890–93. https://doi.org/10.1038/nature04790.
Wilson, Elizabeth B, and David G Brooks. 2011. "The Role of IL-10 in Regulating Immunity to Persistent Viral Infections." *Current Topics in Microbiology and Immunology* 350: 39–65. https://doi.org/10.1007/82_2010_96.
Wing, Kajsa, Yasushi Onishi, Paz Prieto-Martin, Tomoyuki Yamaguchi, Makoto Miyara, Zoltan Fehervari, Takashi Nomura, and Shimon Sakaguchi. 2008. "CTLA-4 Control over Foxp3+ Regulatory T Cell Function." *Science (New York, N.Y.)* 322 (5899): 271–75. https://doi.org/10.1126/science.1160062.
Wingler, Laura M, Conor McMahon, Dean P Staus, Robert J Lefkowitz, and Andrew C Kruse. 2019. "Distinctive Activation Mechanism for Angiotensin Receptor Revealed by a Synthetic Nanobody." *Cell* 176 (3): 479–490.e12. https://doi.org/10.1016/j.cell.2018.12.006.
Wu, Ming Ru, Barbara Jusiak, and Timothy K. Lu. 2019. "Engineering Advanced Cancer Therapies with Synthetic Biology." *Nature Reviews Cancer* 19 (4): 187–95. https://doi.org/10.1038/s41568-019-0121-0.
Xavier, Catarina, Anneleen Blykers, Ilse Vaneycken, Matthias D'Huyvetter, Jan Heemskerk, Tony Lahoutte, Nick Devoogdt, and Vicky Caveliers. 2016. "(18)F-Nanobody for PET Imaging of HER2 Overexpressing Tumors." *Nuclear Medicine and Biology* 43 (4): 247–52. https://doi.org/10.1016/j.nucmedbio.2016.01.002.
Xu, Xiao-Jun, and Yong-Min Tang. 2014. "Cytokine Release Syndrome in Cancer Immunotherapy with Chimeric Antigen Receptor Engineered T Cells." *Cancer Letters* 343 (2): 172–78. https://doi.org/10.1016/j.canlet.2013.10.004.
Yan, Xu, Xu Liu, Cuihuan Zhao, and Guo Qiang Chen. 2023. "Applications of Synthetic Biology in Medical and Pharmaceutical Fields." *Signal Transduction and Targeted Therapy* 8 (1): 1–33. https://doi.org/10.1038/s41392-023-01440-5.
Ye, B, X Liu, X Li, H Kong, L Tian, and Y Chen. 2015. "T-Cell Exhaustion in Chronic Hepatitis B Infection: Current Knowledge and Clinical Significance." *Cell Death & Disease* 6 (3): e1694. https://doi.org/10.1038/cddis.2015.42.
Yeo, Jinah, Minkyung Ko, Dong Hee Lee, Yoon Park, and Hyung Seung Jin. 2021. "Tigit/Cd226 Axis Regulates Anti-Tumor Immunity." *Pharmaceuticals* 14 (3): 1–20. https://doi.org/10.3390/ph14030200.
Yi, John S., Maureen A. Cox, and Allan J. Zajac. 2010. "T-Cell Exhaustion: Characteristics, Causes and Conversion." *Immunology*. https://doi.org/10.1111/j.1365-2567.2010.03255.x.
Yokosuka, Tadashi, Masako Takamatsu, Wakana Kobayashi-Imanishi, Akiko Hashimoto-Tane, Miyuki Azuma, and Takashi Saito. 2012. "Programmed Cell Death 1 Forms Negative Costimulatory Microclusters That Directly Inhibit T Cell Receptor Signaling by Recruiting Phosphatase SHP2." *Journal of Experimental Medicine* 209 (6): 1201–17. https://doi.org/10.1084/jem.20112741.
Yu, Sheng, Gui Xiong, Shimei Zhao, Yanbo Tang, Hua Tang, Kaili Wang, Hongjing Liu, Ke Lan, Xiongjie Bi, and Siliang Duan. 2021. "Nanobodies Targeting Immune Checkpoint

Molecules for Tumor Immunotherapy and Immunoimaging (Review)." *International Journal of Molecular Medicine* 47 (2): 444–54. https://doi.org/10.3892/ijmm.2020.4817.

Zajac, A J, J N Blattman, K Murali-Krishna, D J Sourdive, M Suresh, J D Altman, and R Ahmed. 1998. "Viral Immune Evasion Due to Persistence of Activated T Cells without Effector Function." *The Journal of Experimental Medicine* 188 (12): 2205–13. https://doi.org/10.1084/jem.188.12.2205.

Zavrtanik, Uroš, Junoš Lukan, Remy Loris, Jurij Lah, and San Hadži. 2018. "Structural Basis of Epitope Recognition by Heavy-Chain Camelid Antibodies." *Journal of Molecular Biology* 430 (21): 4369–86. https://doi.org/10.1016/j.jmb.2018.09.002.

Zenz, Thorsten. 2013. "Exhausting T Cells in CLL." *Blood* 121 (9): 1485–86. https://doi.org/10.1182/blood-2013-01-475939.

Zhai, Tianhang, Chao Wang, Yifeng Xu, Weifeng Huang, Zhijun Yuan, Tao Wang, Shuang Dai, et al. 2021. "Generation of a Safe and Efficacious Llama Single-Domain Antibody Fragment (VHH) Targeting the Membrane-Proximal Region of 4-1BB for Engineering Therapeutic Bispecific Antibodies for Cancer." *Journal for Immunotherapy of Cancer* 9 (6). https://doi.org/10.1136/jitc-2020-002131.

Zhang, Cuilin, Qiuyu Zhuang, Jingfeng Liu, and Xiaolong Liu. 2022. "Synthetic Biology in Chimeric Antigen Receptor T (CAR T) Cell Engineering." *ACS Synthetic Biology* 11 (1): 1–15. https://doi.org/10.1021/acssynbio.1c00256.

Zhang, Fei, Hudie Wei, Xiaoxiao Wang, Yu Bai, Pilin Wang, Jiawei Wu, Xiaoyong Jiang, et al. 2017. "Structural Basis of a Novel PD-L1 Nanobody for Immune Checkpoint Blockade." *Cell Discovery* 3: 1–12. https://doi.org/10.1038/celldisc.2017.4.

Zhang, Long, Thomas F Gajewski, and Justin Kline. 2009. "PD-1/PD-L1 Interactions Inhibit Antitumor Immune Responses in a Murine Acute Myeloid Leukemia Model." *Blood* 114 (8): 1545–52. https://doi.org/10.1182/blood-2009-03-206672.

Zhang, Yi, and Zhi-Ying Wu. 2023. "Gene Therapy for Monogenic Disorders: Challenges, Strategies, and Perspectives." *Journal of Genetics and Genomics = Yi Chuan Xue Bao*, August. https://doi.org/10.1016/j.jgg.2023.08.001.

Zhang, Zhen, Shasha Liu, Bin Zhang, Liang Qiao, Yi Zhang, and Yi Zhang. 2020. "T Cell Dysfunction and Exhaustion in Cancer." *Frontiers in Cell and Developmental Biology* 8 (February). https://doi.org/10.3389/fcell.2020.00017.

Zhao, Lijun, and Yu J. Cao. 2019. "Engineered T Cell Therapy for Cancer in the Clinic." *Frontiers in Immunology* 10 (October). https://doi.org/10.3389/fimmu.2019.02250.

Zheng, Fang, Yucheng Pang, Luyao Li, Yuxing Pang, Jiaxin Zhang, Xinyi Wang, and Geert Raes. 2022. "Applications of Nanobodies in Brain Diseases." *Frontiers in Immunology* 13: 978513. https://doi.org/10.3389/fimmu.2022.978513.

Zhong, Zhenwei, Mengyao Zhang, Yanan Ning, Guanchao Mao, Xiaopei Li, Qi Deng, Xiaorui Chen, et al. 2022. "Development of a Bispecific Antibody Targeting PD-L1 and TIGIT with Optimal Cytotoxicity." *Scientific Reports* 12 (1): 18011. https://doi.org/10.1038/s41598-022-22975-7.

Zhou, Xinyuan, Shuyang Yu, Dong-Mei Zhao, John T Harty, Vladimir P Badovinac, and Hai-Hui Xue. 2010. "Differentiation and Persistence of Memory CD8(+) T Cells Depend on T Cell Factor 1." *Immunity* 33 (2): 229–40. https://doi.org/10.1016/j.immuni.2010.08.002.

Zhu, Chen, Ana C Anderson, Anna Schubart, Huabao Xiong, Jaime Imitola, Samia J Khoury, Xin Xiao Zheng, Terry B Strom, and Vijay K Kuchroo. 2005. "The Tim-3 Ligand Galectin-9 Negatively Regulates T Helper Type 1 Immunity." *Nature Immunology* 6 (12): 1245–52. https://doi.org/10.1038/ni1271.

Zhu, Xiaoying, Qing Li, and Xiaojian Zhu. 2022. "Mechanisms of CAR T Cell Exhaustion and Current Counteraction Strategies." *Frontiers in Cell and Developmental Biology* 10

(December): 1–11. https://doi.org/10.3389/fcell.2022.1034257.