Interleukin-1α cytokines with CD8+ target cell selectivity

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Abstract

The T cell pathway is one of the two main branches of the adaptive immune system and is responsible for the detection and destruction of altered host cells or foreign bodies. In particular,CD8+ cytotoxic T cells are responsible for the release of molecules, such as granzymes and perforins, that induce apoptosis and lysis of the targeted cell. In diseases such as cancer, however, upregulation of the protein receptor CTLA4 on CD8+ T cells or of the transmembrane protein PD-L1 on many different types of tumor cells leads to inhibition of CD8+ T cell function and its ability to kill cancer cells. Knowing this, one way in which cancer might be treated could be via drugs that stimulate CD8+ T cells so that they can overcome such inhibitory signals. Molecules that stimulate and enhance T cell function – such as cytokines, small proteins that modulate the immune response - are already present in the human body. A cytokine known as interleukin-1 (IL-1), for example, has been shown to have the ability to significantly increase the proliferation and activity of CD8+ T cells. Therefore, looking at molecules such as IL-1 as potential therapeutic agents against cancer could be very beneficial. The issue with cytokines, including IL-1, however, is that their receptors are often present on many different cell types, which causes a problem of side effect toxicity due to lack of selectivity, especially at higher doses that might be necessary for efficacy. To address this problem and achieve target cell selectivity, Orionis Biosciences has developed attenuated versions of IL-1β cytokines, in a class which is termed Activity-on-Target-cytokines (AcTakines). AcTakines are inactive towards the IL-1 receptor (IL-1R) until first bound to a target-specific cell surface antigen, such as CD8 on cytotoxic T cells (Fig. 1). In this study we sought to determine if it is similarly possible to generate conditionally active, targeted IL-1α cytokines with selective activity for CD8+ target cells. IL-1α and IL-1β both bind to the IL-1R but differ in primary sequence, expression profile and some functional activities. Our findings indicated that two IL-1α AcTakines, constructs 1 and 2, met these criteria, with construct 1 being especially successful; with little to no IL-1R signaling activity in cells that do not express CD8, but with activity equal to or higher than that of wild-type IL-1α in cells that do express CD8.

Introduction

The immune system and cancer

Cancer, a heterogeneous group of diseases categorized by unregulated cell growth with the potential to invade surrounding tissues, is the second highest cause of death each year in the United States, contributing to approximately 600,000 deaths annually. It is the leading cause of death world-wide with 10 million deaths and 19 million new cases reported in the year 2020 (1). Currently, for various types of cancer, there exist various treatments, such as chemotherapy, immune checkpoint inhibitors, and radiation (2). These treatments are not always effective, and most, if not all, of them are associated with a wide range of adverse side effects. Research and discovery of novel anticancer drugs and treatments that are more effective and better tolerated remain a major need and challenge in the field of oncology.

When considering strategies for cancer therapeutics, the adaptive immune system is important to examine since it can play a key role in defense against both emergence and recurrence of cancer. The adaptive immune system has two central pathways: the B cell pathway and the T cell pathway. Adaptive B cell responses play a less relevant and fundamental role compared to T cells in the fight against cancer. T cells, which originate in the bone marrow from hematopoietic stem cells and which finish development in the thymus, are critical for establishment of cell-mediated immunity involving the detection and destruction of altered host cells, such as cancer cells. For T cells to recognize their target cells, they express a specific type of antigen sensing receptor, the T cell receptor (TCR). The TCR recognizes antigens presented on MHC molecules of antigen presenting cells, including cancer cells. There are two types of T cells: CD4+ helper T cells and CD8+ cytotoxic T cells (CTLs). CD4+ and CD8+ T cells are activated after TCR-mediated recognition of an antigen that is being presented on MHC class II or MHC class I molecules on antigen presenting cells. CD4+ T cells generally do not have any phagocytic or cytotoxic abilities, and instead function as support cells to increase the activation and effectiveness of other immune cells such as B cells and CD8+ T cells. CD4+ cells also include T regulatory cells (Tregs) that can suppress CD8+ T cell activity, reflecting yet another mechanism of CD8+ T cell inhibition in established tumor microenvironments. CD8+ T cells function differently from CD4+ T cells in that upon activation they can release molecules such as granzymes, perforin, and interferon gamma (IFN-γ) that induce apoptosis and cell lysis. It is well established that CTL's are important for immune system mediated clearance of cancer cells.

Evolution of novel therapeutic approaches, including T cell stimulation, requires an understanding of fundamental mechanisms of tumor growth control and regulation of the immune system. Tumors evolve largely due to genetic and epigenetic lesions that provide direct and indirect growth advantage to cancer cells by various mechanisms, including tumor cell growth and survival, tumor neovascularization and establishment of an immunosuppressive tumor microenvironment (TME). Genetic lesions have been found to lead to the generation of tumor-specific antigens that are derived from mutant forms of endogenous proteins (e.g., certain oncogenes). These antigens are recognized, processed, and presented to the adaptive immune system by antigen presenting cells (APCs) such as dendritic cells and macrophages. The cDC1 dendritic cells are particularly specialized in presenting tumor antigens to cytotoxic T cells (CD8+ T cells). Antigen uptake by cDC1 cells occurs in the tumor microenvironment, with subsequent migration of these cells to tumor lymph nodes where they present antigen(s) to T cells, promoting their activation (3). Activated T cells then migrate to the tumor microenvironment, where they engage with and kill tumor cells. CD8+ T cells are not only a key cell type of the adaptive immune system for mounting such antigen-specific antitumor responses but are also important in the formation of immunological memory. Activated CD8+ T cells can transition into memory T cells; this is a fundamental mechanism in establishing cancer immunity and preventing recurrence of cancer following effective cancer therapy. IL-1 is a cytokine that is both a potent stimulator of CD8+ T cells as well as a potent inducer of memory T cells. Exploiting such types of biological mechanisms could provide novel approaches to T cell stimulation and effective cancer therapies.

Targeting of CD8+ cells in disease

When looking at the immune system and cancer, it is important to look at the ways in which tumor cells suppress the immune response and related cells. One of the strategies that cancer cells employ to accomplish this is T cell downregulation and suppression (4). As mentioned above, T cells, in particular CD8+ T cells, are a critical component to the adaptive immune response and its ability to regulate and suppress tumorigenesis. CD8+ memory T cells can remain in the host's body for long periods of time and can recognize altered cell types (e.g., cancer cells) that have previously been cleared from the body. Unfortunately, tumors evolve to find multiple mechanisms to suppress activated CD8+ T cells, including rendering them anergic or dysfunctional, reducing their viability, and inhibiting their ability to infiltrate the tumor microenvironment. Prolonged exposure to tumor antigen can also lead to dysfunctional T cells with decreased effector function and capacity to proliferate (5). CTLA4 expression on CD8 T cells is associated with a natural mechanism of downregulation of CD8+ T cell activity during the time course of an adaptive immune response triggered

by antigen presenting cells (6). Expression of PD-L1 on antigen-presenting cells is also a natural mechanism for delayed suppression of CD8 T cell activation. However, PD-L1 expressed on tumor cells (and other tumor infiltrating immune cells, e.g. myeloid derived suppressor cells (MDSCs)) can interact with PD-1 expressed on activated CD8+ T cells, leading to CD8+ T cell suppression (6). In addition to these mechanisms there are other ways in which CD8+ T cell suppression can occur in the TME. Clinical relevance of this is supported
by observations that the immunotherapy drug Keytruda, which binds to PD-1 and prevents its interaction with PD-L1, is not effective in many patients and various cancer Types such as lung, breast, colon, renal, pancreatic cancers. The same is the case for drugs that target CTLA4. Thus, enhancing or boosting the activity of CD8 T cells by mechanisms other than inhibition of PD-L1/PD-1 or CTLA4/B7 interactions would be a promising therapeutic approach.

IL-1 and its role in T cell stimulation

When looking at molecules that have the ability to significantly enhance the activity of CD8+ T cells, cytokines are one of the most important proteins to examine. Cytokines are small proteins that help cells in the immune system, including T cells, communicate with one another; they also regulate cellular activity through signaling. Once bound to cell surface receptors, they can induce a multitude of effects such as inflammatory or anti-inflammatory responses, chemotaxis, and cell proliferation (7). This is important, as cytokines serve as modulators of the immune system and therefore its ability to properly fight diseases. Tight regulation of expression and secretion of these molecules by various cell types is key, since over-activation of cytokine signaling pathways can lead to severe health problems such as autoimmune diseases, cytokine storms, etc (7). One particular cytokine of interest relating to the induction of a stronger T cell response is IL-1.

IL-1 can be found in two forms, IL-1α and IL-1β. Both bind to and activate the receptor IL-1R. Activation of the IL-1R leads to activation of the nuclear factor kappa B (NF-κB) signaling pathway and induction of NF-κB-responsive genes. *In vivo*, its mechanism of action can induce a very strong inflammatory response. Notably, both IL-1 forms promote T cell proliferation, activation, and development into memory T cells (8). IL-1 can also engage with many other cell types in the body, as the IL-1R is present on many different types of cells, including various immune cells, fibroblasts, endothelial cells, keratinocytes and hepatocytes (9). The pleiotropic expression of the IL-1R has posed a major challenge in the use of IL-1 as an anticancer agent, as systemic exposure to IL-1 is associated with significant adverse side effects such as inflammation, fever, nausea and neutrophilia, due to the binding of IL-1 to the cell and

tissue types previously mentioned (10). Potential therapeutic use of IL-1 would therefore require enabling selective targeting of IL-1 activity only to cells of therapeutic interest, such as CD8+ T cells.

To address this problem, Orionis Biosciences has engineered novel IL-1 molecules that have been altered to be conditionally active and selective for CD8+ T cells. This is achieved through the fusion of a mutated, attenuated IL-1 with substantially reduced affinity/activity for the IL-1R with an antibody that can bind to CD8 (Fig. 1). This allows for selective engagement of the fusion protein with CD8-expressing cells such as T cells. Binding of such IL-1 fusion molecules to CD8 promotes subsequent engagement of the attenuated IL-1 with the IL-1R by a mechanism of induced proximity at the cell surface. These types of conditionally active cytokines have been termed activity-on-target-cytokines (AcTakines), and, in the case of IL-1, have been tested for their ability to retain similar levels of activity to wild-type IL-1 when targeted towards CD8+ cells. Successful IL-1 AcTakines can stimulate CD8+ T cells while not engaging with other cell types expressing IL-1R. IL-1β AcTakines have been able to remain inactive *en route* to CD8+ T cells and have resumed full activation upon contact with CD8+ T cells in *in vivo* mouse models. IL-1β AcTakines caused no adverse side effects in mice, were just as effective at generating memory T cells as wild-type IL-1β and were able to be used as a successful influenza vaccine adjuvant (11). Thus, AcTakines could potentially be used as safe and effective adjuvants for other therapeutic treatments, including cancer therapies.

Aside from CD8+ T cells, it should also be mentioned that activation of natural killer cells (NK cells) also play an important role in control of tumor growth. NK cells play an important role in both innate and adaptive immune responses and engage with tumor cells through different mechanisms than activated CD8+ T cells, although they do also release IFN-γ upon activation. NK cell-mediated killing of tumor cells is not dependent on cancer cell antigen presentation by MHC proteins on cancer cells, as it is for CD8+ T cells. A subset of human NK cells, including natural killer T cells, also express CD8. Interestingly, IL-1 promotes the activity of not only CD8+ T cells, as mentioned earlier, but also NK cells (12). These activities of IL-1 underlie, at least in part, its antitumorigenic properties. Thus CD8+ targeted IL-1 cytokines could exert anti-tumor activities via both CD8+ T cells and CD8+ NK cells.

Given the success of the IL-1β AcTakines, conducting similar tests with IL-1α AcTakines would be a logical next step. While both IL-1α and IL-1β bind to and activate the IL-1R, they differ in primary sequence and may exhibit different biological activities and potencies, including in the context of a CD8-targeted AcTakine. The series of experiments conducted in this paper seek to examine different versions of CD8-targeted

IL-1α AcTakines incorporating different mutations in IL-1α, and to compare their ability to selectively activate IL-1R signaling in a CD8+ target-selective manner. Induction of NF-κB-dependent (reporter) gene expression was used as a measure to assess IL-1R activation.

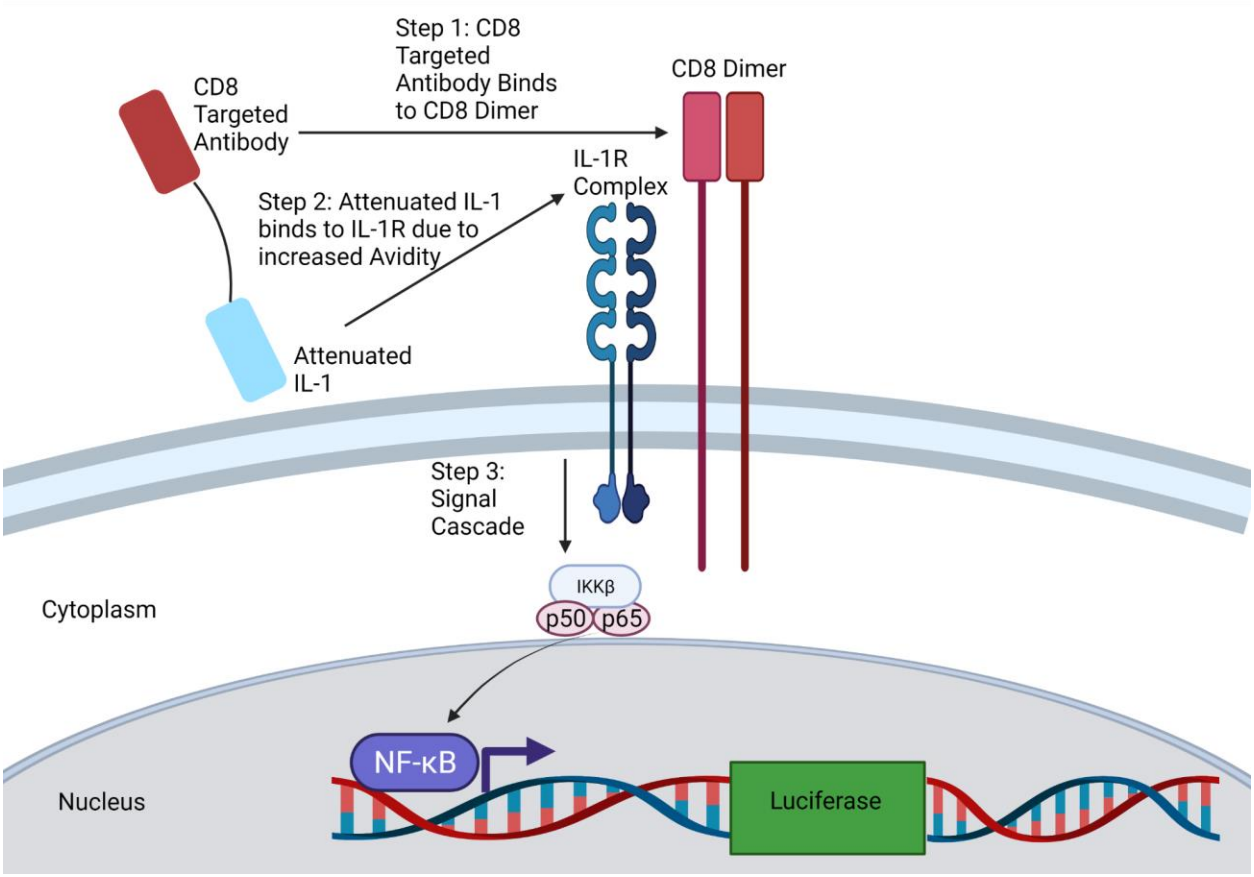


Figure 1. Mechanism of AcTakine binding to IL-1R and CD8 that causes a signal cascade that activates the firefly luciferase gene. The hCD8 targeted antibody of the AcTakine first binds to the CD8 dimer. This enables the attenuated IL-1 to subsequently bind to IL-1R and induce a downstream signal cascade. Signal cascade activates proteins p50 and p65, which activates NF-κB protein complex. Activation of the NF-κB protein complex leads to downstream stimulation of the firefly luciferase reporter gene that harbors a NF-κB -responsive DNA element. Chemiluminescence of the firefly luciferase protein can then be measured and used as an indicator of the activity level of the AcTakine at the IL-1R.

Materials and Methods

Replication of AcTakine DNA for protein synthesis

DNA-containing tubes for four constructs 1-4 (differences between constructs may not be discussed here due to proprietary reasons) were spun down at 13000 rpm for 1 minute (Fig. 2). Biological water was added to a final concentration of .5mg DNA/µl and tubes were spun down again. Tubes containing HB101 competent bacterial cells were thawed. 25µl of HB101 cells and 1mg of prepared construct DNA was added to microcentrifuge tubes and incubated on ice for 30 minutes (repeated individually for all 4 constructs). Tubes were heat shocked for 45 seconds at 42°C and placed on ice for 2 minutes. 1 ml of LB SOC media was added. Tubes were shaken at 225 rpm at 37°C for 1 hour. Transformed bacteria were then spread onto LB agar plates and incubated overnight at 37°C. MidiPrep of transformed bacteria was performed following the Qiagen high speed MidiPrep Protocol to obtain purified bacterial DNA containing the sequence for AcTakines. DNA concentration was measured using a Spectramax nanodrop machine. This purified DNA would be used in later steps to synthesize AcTakine protein.

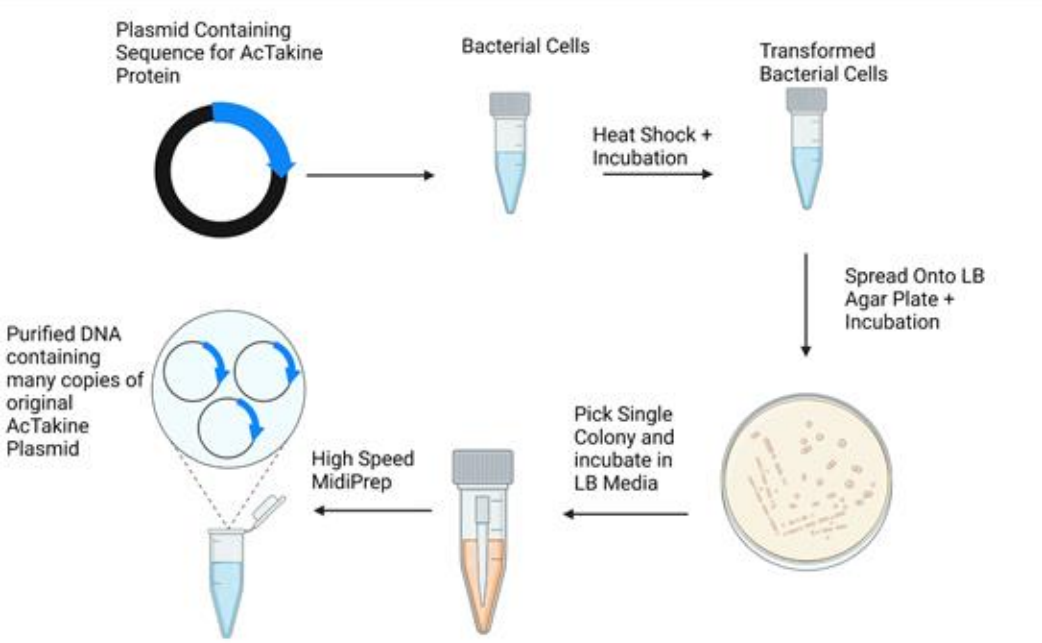


Figure 2. Schematic of replication of plasmids encoding AcTakine protein.

Synthesis and extraction of AcTakine protein

ExpiCHO cells were subcultured and transfected according to ExpiCHO expression system user guide provided by ThermoFisher Scientific (Waltham, MA, USA). ExpiCHO cells were subcultured to a final density of $3x10^6$ - $4x10^6$ and incubated overnight. The following day, cell density and viability were measured using a cellometer (95-99% viability) and diluted to $6x10^6$ cells/ml. Expifectamine CHO/AcTakine DNA complexes were made using DNA, Expifectamine CHO reagent, and OptiPro Medium resulting in a final concentration of 0.5µg/mixture (Fig. 3). CHO/DNA complexes were added to ExpiCHO cells and incubated at 37°C with 8% CO_2 on an orbital shaker**.** After one day, Expifectamine CHO enhancer and ExpiCHO feed were added to cells and returned to the incubator. Seven days after transfection, protein was harvested from cells using the Protein A HP SpinTrap protocol (Chicago, IL, USA) per manufacturer's instructions. Harvested AcTakine protein would further be used in a luciferase assay to measure its activity.

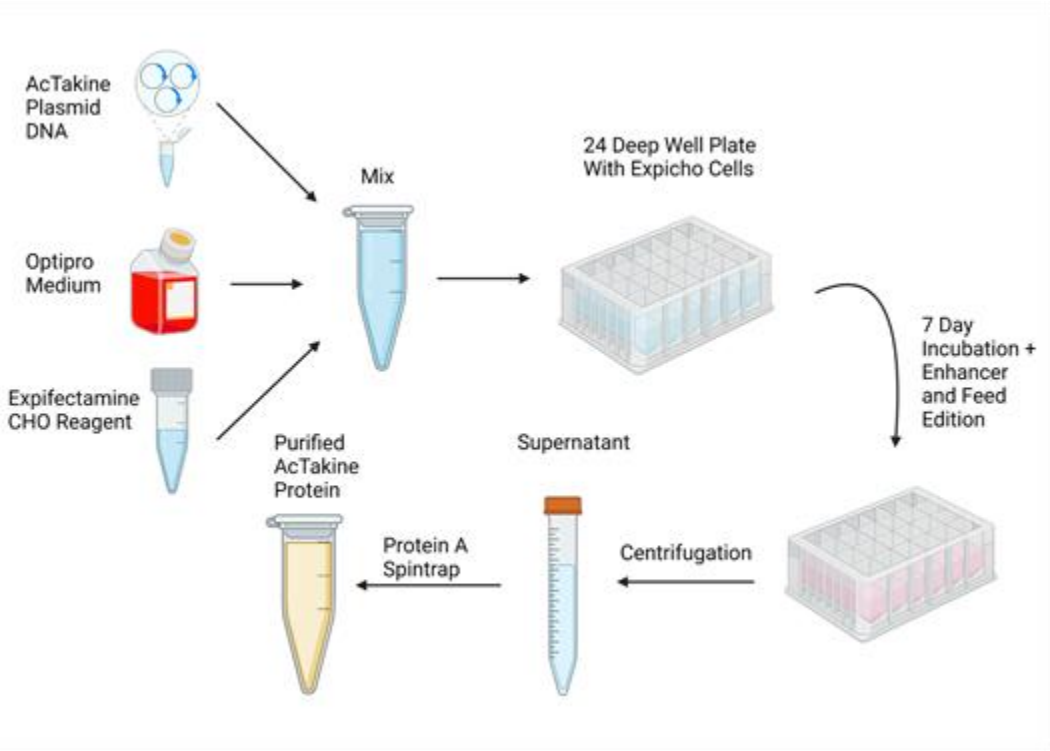


Figure 3. Schematic of ExpiCHO cell transfection and protein purification.

Measuring activity of purified AcTakine protein

293T cells were subcultured using DMEM media**.** Density of 293T cells was measured using Trypan blue and a cellometer and brought to 10,000 cells per 17.5µl media. 2 mixes were prepared: one of water, PLG4.32 (3.08ng), CaCL2, and 2X HBS and one of water, hCD8 P113 (3.01ng), PLG4.32 (3.08ng), and 2X HBS (Fig. 4). Mixes were

added to separate tubes of 293T cells. Tubes were then added to their own 384 well plates and incubated overnight**.** The following day, serial dilutions were made of four AcTakine protein samples (1,2,3 and 4). All samples started at a concentration of 300ng/ml and were diluted 6 times with a 5x dilution series in DMEM media. Serial dilutions were stored overnight at -80C**.** The following day, serial dilutions were thawed and added to 384 well plates and incubated for 5 hours; 5µl of ONE-Glo was added to all wells. Plates were shaken for 5 minutes and spun down at 1000rpm for 1 minute. Protein concentrations were read on a Spectramax microplate reader.

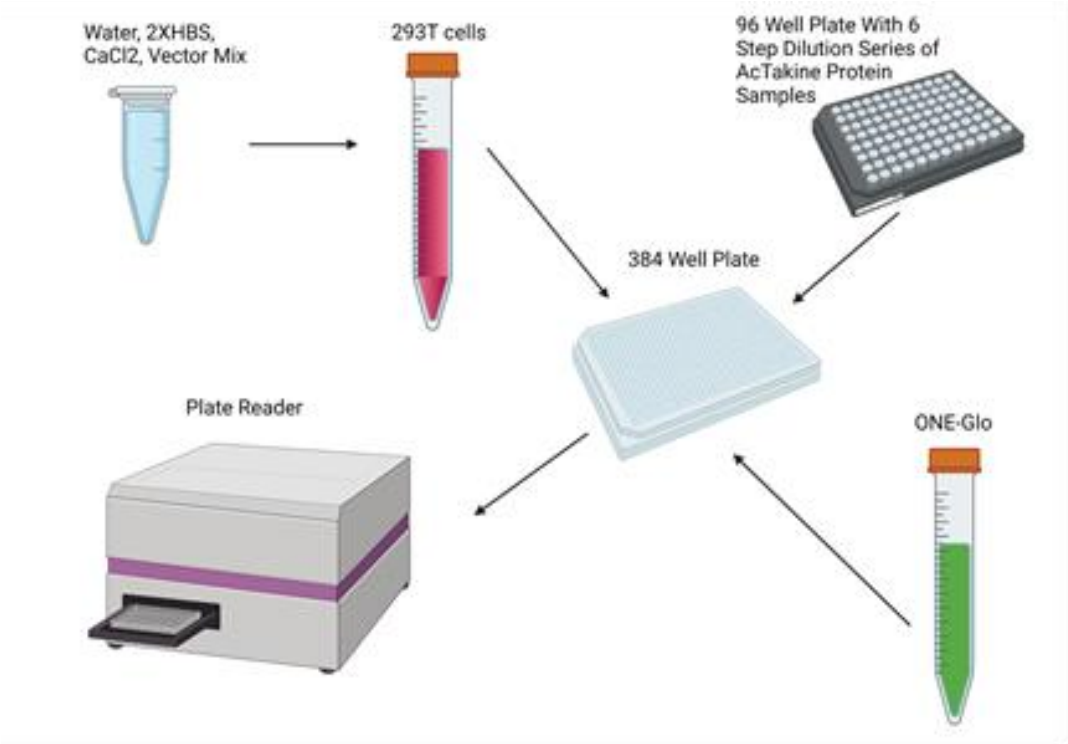


Figure 4. Schematic of ONE-Glo luciferase assay of purified AcTakine protein.

Results

High speed midiprep of transformed bacteria provides significant DNA yield

IL-1β AcTakines have been shown to possess the ability to be inactive *en route* to CD8+ T cells and resume full activation upon contact with CD8+ T cells in *in vivo* mouse models (12). To determine whether IL-1α AcTakines could be generated analogously, AcTakine DNA and protein were synthesized and tested for activity against human CD8 positive (hCD8+) and human CD8 negative (hCD8-) 293T cells. 293T cells naturally express IL-1R. Expression of hCD8 was achieved by transient expression of CD8 via transfection with an hCD8-encoding plasmid.

To accomplish this, the first step was to generate plasmid DNAs encoding various IL-1α AcTakines. This was done by transforming competent bacteria with prepared plasmid DNAs containing the sequence for selected AcTakines incorporating different mutations that lower the affinity and activity of IL-1α for the IL-1R, extracting DNA via high speed MidiPrep, and evaluating DNA concentrations using a Spectramax Microplate reader. Significant amounts of DNA were obtained from the bacterial cell MidiPrep for constructs 1-4 (Table 1), signifying that there was enough purified AcTakine DNA to proceed with protein synthesis.

Construct	**DNA Concentration (ng/µl)**
1	318.4
2	747.6
3	426.4
4	303

Table 1. DNA concentrations of constructs after High Speed Midi Prep.

AcTakine protein synthesis

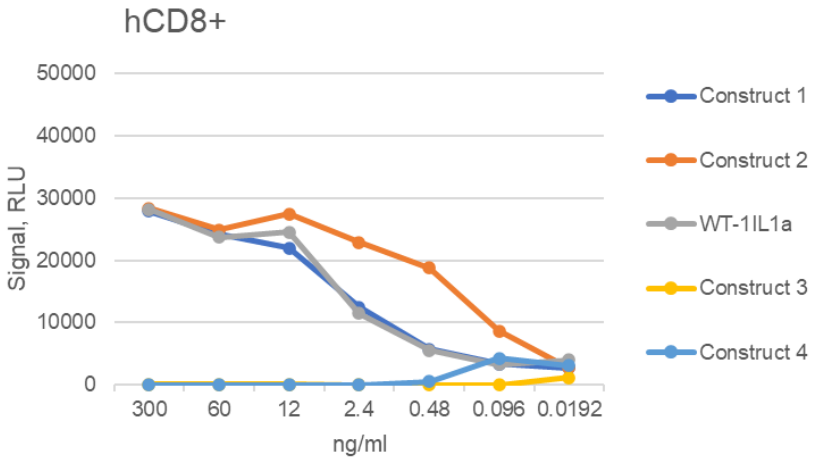
Next, we sought to use the purified AcTakine DNA to synthesize AcTakine proteins, and then to test those AcTakines for their activity levels against hCD8+/- 293T cells. Previously purified DNA was transfected into ExpiCHO cells, a mammalian cell line that is well known for its ability to produce large amounts of proteins. Purification of AcTakine protein was achieved using a Protein A HP SpinTrap protocol. The protein A on the SpinTrap column binds specifically to the Fc region on the AcTakine protein constructs, allowing for targeted purification. Protein levels were measured on a Spectramax microplate reader.

AcTakine activity levels are fully restored on hCD8+ 293T cells

To determine if the IL-1α fusion proteins were able to exhibit CD8+ target-selective, and at activity levels similar to wild type IL-1α (WT-IL-1α), their ability to activate the IL-1R with subsequent induction of NF-κB signaling in CD8+ or CD8- HEK 293T cells was assessed. This was done by measuring induction of an NF-κB -responsive luciferase reporter gene in HEK 293T cells, which do not express CD8 naturally, and HEK 293T cells co-transfected with a plasmid encoding hCD8 . A ONE-Glo luciferase assay was

performed for constructs 1,2,3 and 4 as well as recombinant WT-IL-1α (Figs. 4 and 5). As shown, constructs 1 and 2 exhibited selective and potent activation of the IL-1R in CD8+ HEK 293T cells, with similar or higher levels of activity than recombinant WT-IL-1α (Fig. 5A). At a concentration of 12 ng/ml, for example, constructs 1 and 2 had activity levels of 21979.5 and 27492.75 relative light units, respectively, similar to WT-IL-1α, which had an activity level of 24557 relative light units. This was not seen, however, for hCD8- HEK 293T cells where constructs 1 and 2 had low activity levels of 627.5 and 1422.75 relative light units at a concentration of 12 ng/ml (Fig. 5B). In comparison, WT-IL-1α had an activity level of 47517 relative light units in hCD8- HEK 293T cells. In contrast, no significant activity was seen for constructs 3 and 4 in either the hCD8+ or hCD8- HEK 293T cells, with activity levels of 31 and 15.73 relative light units respectively in the hCD8+ T cells, and 26 and 20.75 relative light units respectively in the hCD8- cells (Figs. 5A and 5B). Taken together, these results indicate that constructs 1 and 2, but not constructs 3 and 4, could induce selective IL-1R activation in CD8+ HEK 293T cells. Induction was dose dependent and quantitative, reaching signal induction levels similar to those observed for WT-IL1α.

A



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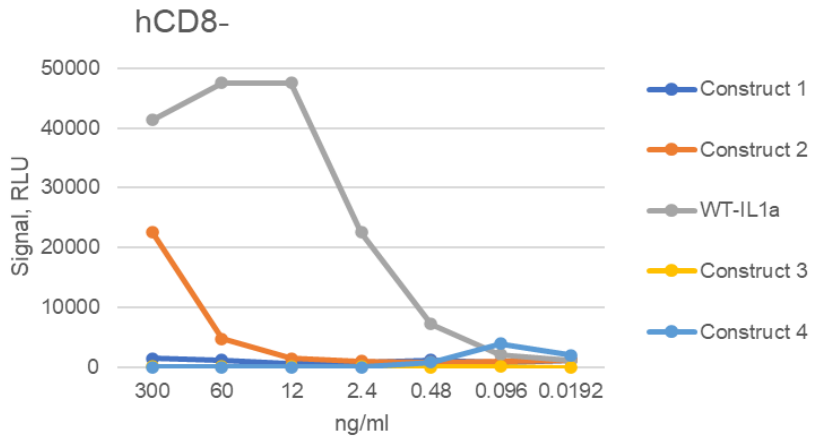

Figure 5. Activity levels of (some) attenuated CD8 targeted IL-1 cytokines remain equal to or above the activity of WT-IL-1 in CD8+ 293T cells. A luciferase assay was performed on human CD8+ (hCD8+, Fig. 5A) or human CD8- (hCD8-, Fig. 5B) 293T cells transfected with a luciferase-encoding NF-κB -responsive plasmid and incubated with various AcTakine protein constructs. Depicted here is the activity of constructs 1,2,3 and 4, as measured in relative light units (RLU) and compared to the activity of WT-IL-1α.

Discussion

The development of IL-1-based therapeutics holds great promise for the development of novel immunotherapies for the treatment of cancer (13). Due to its intrinsic pleiotropic activity profile *in vivo* (11), use of IL-1 as a therapeutic agent requires its activity to be highly controlled and selective for a specific cell type, such as CD8+ T cells. The targeting of IL-1 towards CD8+ T cells is of particular interest since it can potently stimulate T cell activation and proliferation, as well as promote memory T cell formation (14). Prior research has shown that conditionally active forms of CD8-targeted IL-1β, termed IL-1β AcTakines, have been able to remain inactive *en route* to CD8+ T cells and resume full activation upon contact with CD8+ T cells in *in vivo* mouse models, with minimal adverse side effects (11). This led to the question of whether similar results could be achieved with another form of IL-1, IL-1α. In this study we succeeded in the discovery and development of IL-1α variants with substantially reduced activity compared to wild type IL-1, but whose activity could be recovered upon selective targeting to cells expressing the CD8 antigen. The discovery of CD8 target-selective IL-1α AcTakines provides a novel approach in the activation of CD8+ T cells with the potential for eliciting antitumor responses even in the presence of CD8+ T cell repression in tumor microenvironments. The same may apply to activation of CD8+ NK cells.

Targeted activity of IL-1 AcTakines was assessed using HEK293T cells that conditionally express CD8, harbor endogenous IL-1R and naturally respond to IL-1 signaling involving activation of the NF-κB signaling pathway. As shown in Figures 4 and 5, we were able to engineer CD8-targeted AcTakines that exhibited selective IL-1R activation and effective downstream NF-κB signaling (as measured using a NF-κB - responsive luciferase reporter gene) in a manner that is dependent on expression of CD8. Importantly, targeted activity was quantitative and similar to or even more pronounced than that observed for WT- IL-1. Figure 1 describes a model for the mechanism of action of such AcTakines.

Continual experimentation with various IL-1α AcTakines could lead to further optimization of molecules for *in vivo* therapeutic applications. This could include, for example, analysis of molecules for differential activity towards primary CD8+ T cells and/or CD8+ NK cells (not elaborated on in this study), and analysis of molecules with differential half-life and/or potential therapeutic potency *in vivo*. Testing of selected AcTakines with a high degree of CD8+ target selectivity in animal models such as humanized mice would further help to elucidate the potential of such AcTakines as therapeutic agents.

Looking at other types of cytokines that modulate the immune system and determining if they could be manipulated in a similar manner, could also be something to be investigated in the future. Interleukin-2 (IL-2), for example, has been established as a molecule that stimulates cell-mediated immune responses, as well as increasing proliferation and activity of B cells and CD8+ T cells (15). In certain cases, IL-2 has also been seen to have the ability to inhibit the growth of certain types of tumor cells and has seen clinical use against certain cancers such as metastatic renal cell carcinoma and metastatic melanoma (15). Much like IL-1, however, high doses of IL-2 can result in significant adverse side effects such as damage to the heart, lungs (e.g., vascular leak syndrome), kidneys, and central nervous system, due to pleiotropic, non-selective systemic over-activation of IL-2R signaling and subsequent induction of other cytokines by IL-2 (16). Considering that IL-2 has the potential to be such a potent activator of CD8+ T cells, generating successful IL-2 AcTakines using the technologies examined in this paper could be therapeutically strategic. In summary, looking at many different types of cytokines that have the ability to enhance the immune system, and being able to render them conditionally active, could open up many novel ways to treat cancers.

In addition to targeting CD8+ T cells, AcTakines could potentially be targeted towards other cell types as well. MDSCs, for example, are monocytes with potent immunosuppressive activity and are often upregulated in many types of cancer (17). They have been shown to have to ability to suppress a variety of immune cells, including CD8+ T cells, natural killer (NK) cells, dendritic cells, and macrophages, and to contribute to tumor progression via mechanisms such as immune evasion, angiogenesis, pre-metastatic niche formation, and epithelial-mesenchymal transition (18). If AcTakines were able to be developed to selectively target and downregulate MDSCs, for example, the function of the immune cells listed above would likely increase, possibly leading to an antitumor response.

AcTakines, although currently mostly focused around treating cancer, could also be used as therapeutic agents for other diseases. In viral infections, for example, a conditionally active and targeted Tumor Necrosis Factor alpha (TNF-α) could help target foreign molecules without the adverse side effects related to nonselective TNF-α exposure. In autoimmune diseases, targeted AcTakines that downregulate the activity of immune cells could be of interest. In summary, then, the ability to generate conditionally active molecules that reactivate cytokine receptors upon binding to a cell-specific marker/antigen can provide many potential molecules that could be evolved into therapeutic agents for a wide variety of diseases.

The present study lays the foundation for further investigations into the efficacy and tolerability of CD8-targeted IL-1α AcTakines. These investigations would include

assessment of their activity *in vivo*, including in cancer and viral infection models. In cancer, this would also include their ability to synergize with other agents – for example restoring sensitivity to checkpoint inhibitors -- and their ability to induce CD8+ T cell immunological memory and immunity. These studies would further look for selection of the most effective constructs and their possible use and further development as novel immunotherapies.

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