Quantifying T cell Mediated Killing for Melanoma Immunotherapy

A Thesis

Presented to
the Faculty of the Department of Chemical and Biomolecular Engineering
University of Houston

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in Chemical Engineering

By
Thai Vu
December 2014

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Abstract

Adoptive cell therapy (ACT), based on the adoptive transfer of tumor-infiltrating lymphocytes (TILs) has well-recognized advantages such as (1) high specificity for target cells; (2) the ability to target even micrometastases; (3) the potential to proliferate *in vivo* within the host thus increasing both surveillance and destruction capabilities, and (4) the feasibility to treat late stage tumors refractory to all other treatment methods with clinical response rates of ~50%. Despite numerous improvements in the last decade, ACT treatments still result in a wide range of outcomes. Functional heterogeneity, at the single-cell level, of cells infused for ACT has not been routinely characterized and consequently their efficacy and persistence *in vivo* following ACT are unpredictable. By using a high-throughput single-cell methodology, we demonstrate here that our assay has been able to quantify and indentify sub-populations within a TILs sample based on their individual killing potentials when matched with different number of targets.

Table of Content

List of Figures

List of Tables

Introduction

1. Introduction to Cancer and Immunotherapy

The human immune system is a complex entity that protects the host from pathogenic organisms and altered cells, and the failure of the immune system to prevent the progression of oncogenic cells results in cancer. The most widely adopted treatment scheme for this disease in the clinic is chemotherapy [1] that utilizes chemical small molecule drugs to non-specifically eradicate rapidly dividing cells including cancerous cells. These drugs, however, are nondiscriminatory by nature and are notorious for their devastatingside effects on patients. The ability to design alternate therapeutic treatments that can induce lasting responses while minimizing undesired side effects is a long standing objective in cancer treatment. One approach is to harness the interactions between cancerous cells and the host. Cancerous cells are believed to arise from genetic defects in normal cells that result in malignant cells effectively competing with and eventually outcompeting healthy cells crucial for the host's vitality [2]. The body however has two defense mechanisms against the genesis of cancerous cells: one relies on programmed self-elimination (also known as apoptosis) of cells with genetic defect, and the other relies on immune cells actively scanning for and eliminating transformed cells [1][2]. The ability of cancer cells to inactivate apoptotic mechanism is well documented [3].The failure of the second mechanism is more complex and has not been completely quantified. Although the early events that promote tumor escape from the immune system are not well-defined, over time it is clear that the cancer cells evolve specific mechanisms to overcome immune mediated elimination and are under a constant process of immunoediting to facilitate survival [2][3]. The field of immunotherapy in the context of cancer treatment aims to assist the immune system to

facilitate the elimination of tumors and has recently emerged as a successful modality for both cancer treatment and management [1] .

Table I: Examples of clinically approved immunotherapeutic treatments for different kinds of cancer

Established therapies	Indication
Monoclonal antibodies	
Rituximab	NHL,CLL
Ibritumomab tiuxetan	NHL
Tositumomab	NHL
Alemtuzumab	CLL
Gemtuzumab	AML
Trastuzumab	Breast cancer
Cetuximab	Colorectal cancer
Panitumumab	Colorectal cancer
Bevacizumab	Colorectal, lung
Immune adjuvants	
BCG	Superficial bladder cancer
Imiquimod	Basal cell carcinoma, VIN, actinic keratosis
Cytokines	
IFN-α	Melanoma, RCC
IL-2	Melanoma, RCC
TNF-α	Soft tissue sarcoma, melanoma

The use of monoclonal antibodies in immunotherapy is an established treatment in the clinic. In the context of cancer treatment, these monoclonal antibodies can selectively bind to molecules expressed on the surface of cancerous cells, The main challenge of this treatment is the identification of unique markers that define cancerous cells since the molecules expressed on cancerous cells, in most cases, are identical to their non-mutated counter parts and thus, monoclonal antibodies lack the ability to distinguish these two resulting in on-target off-tumor responses [4][5][6][7].

Immune adjuvants have also seen effectively used in the clinic. These adjuvants are used to enhance the immune reaction at tumor sites already recognized by the immune system, mostly via inflammatory reactions [1]. Due to the adverse effect of those reactions, immune adjuvants are only used when the tumor sites are strictly localized, as in, early stage tumors. However, it

has showed great results within its limited range of usage. In bladder cancer, for example, after the tumor mass is removed, live bacilli Calmette-Guerin (BCG) are used to induce inflammatory reactions on the bladder walls [8][9]. It has been showed that patients that received BCG had a progression-free survival rate of 61.9% compared to the 37% of those who had surgery without BCG [10].

While immune adjuvants are administrated on a local scale, cytokines are used to regulate the entire immune system. Cytokines are proteins secreted by immune cells to facilitate intercellular communication. Currently, there are three cytokines approved for use in the clinic. Interferon (IFN)-α, mainly used for its antiviral properties, is used in treatment of late stage melanoma prior to metastatis (stage III), the spreading of cancerous cells via blood transport, takes place [11]. Interleukin IL-2 is a growth factor for T cells, an important lymphocyte tasked with eliminating cancerous cells, to be discussed later. IL-2 has showed to work even during metastasis melanoma (stage IV) unlike IFN-α, although both show only low response rates: about 6% complete response and 16% partial response for both [11][12]. Tumor necrosis factor (TNF)-α is an inflammatory inducing cytokine that are administered locally (similar to immune adjuvants) to treat soft tissue sarcoma and melanoma targeting both the tumor and its supporting blood vessels [13][14]. For soft tissue sarcoma, TNF-α can induce up to 85% response rate [13][14].

There are other immune interventions against cancer. Vaccines have been used with great success in cancers caused by microbial infection [1]. Hepatitis B virus (HBV) and human papillomavirus (HPV) vaccines are used to prevent Hepatocellar carcinoma and cervical cancer, respectively with 95% of the individuals producing protective antibodies for HBV and 98% for HPV [15][16]. Bone marrow transplantation is also a form of immunotherapy for blood related diseases such as leukemia that can yield up to 70% complete remission rate [17].

A great deal of research effort has been devoted to improving the current improved treatments and producing new treatment strategies. Notably, a lot of resources are spent to elucidate the pathway of cancer development. From such knowledge, it is feasible to identify key steps and devise antibodies to block the path way of cancer development. For example, Human epidermal growth factor 2 (HER2) proteins are important in the development of breast cancer, and by targeting HER2, investigators hope to halt the progression of the disease [18]. In a similar vein, it is also of great interest to understand the activation pathway of lymphocytes in the immune system, particularly T cells. For example, Cytotoxic T-lymphocyte-associated-antigen 4 (CTLA4) has known to dampen the antitumor response of T cells, and by targeting CTLA4 researchers have been able to elevate the effectiveness of T cells in cancer management. In particular, the use of anti-CTLA4 was approved by the FDA as a treatment for advanced melanoma in 2010 [19]. T cells can also be manipulated indirectly via dendritic cells, an important regulator of the immune system's activities [20].

Adoptive cell therapy (ACT) is another state-of-the-art treatment regimen for cancer. ACT aims to boost the immune system response by infusing a population of T cells that were extracted and cultured *ex vivo* back into the host [21]. This strategy came from the observation that in cancers such as melanoma, there were populations of T cells within the solid tumor mass. These T cells were found to be tumor-specific but yet ineffective in managing the tumor, which is thought to be due to the existence of suppressive mechanisms [22]. By extracting those T cells and expanding them *in vitro*, which was thought to help bypass the suppressive mechanisms, clinicians have been able to treat melanoma patients with up to 50% response rate in clinical trials [21][22]. In liquid tumors, ACT treatment can still be applied to bypass suppressive mechanisms by genetically engineering T cells with chimeric antigen receptors (CAR T cells) designed to target particular cancerous cells [21]. Thus, immunotherapy includes diverse

treatment schemes with a major advantage over conventional chemotherapy: It can achieve high response with limited adverse effects on patients. Plus, it can be used as a last option for late stage cancers, even with relatively modest response rates. The field has been making steady progress with the T cell being one of the central themes in treatment approaches.

2. The Immune System, T Cells and Apoptosis

The immune system

There are two components of the immune system: innate and adaptive. The former provides the first line of defense in the human body. The defense mechanism of the innate components is generic [23]. In most circumstances, it is not tailored against any particular infection but against frequently encountered pathogens, infected cells, and cancerous cells [23]. The innate immune system is not limited to cellular responses, whose effectors constantly patrol the body to identify pathogenic agents. It can encompass factors such as the skin barrier, body temperature, pH etc., all of which can constitute to an unviable medium for a number of pathogens. These physiological differences among species alone can preclude certain ones from particular diseases while making others susceptible to the same diseases. However, among members of the same species, the traits of the innate immune system are fairly uniform [23]. With that being said, cellular members of the innate system play pivotal roles in cancer management, a disease with diverse prognosis and characteristic among different individuals. Dendritic cells (DCs) are the most potent regulators in the immune system as a whole being able to orchestrate activities of cells in both subsystems. These cells constantly sample free-floating molecules as well as cellular matters to detect any deviation [23]. Natural killer (NK) cells are the equivalence of T cells in the adaptive system but differ in terms of target recognition mechanisms. Interestingly, in one methodology for cancerous cells to evade T cells recognition mechanisms, the former have to down-regulate factors which make them more susceptible to NK recognition mechanism. Macrophages, even though not directly involved in cancer elimination process, play a crucial auxiliary role as a cleaner of cellular debris resulting from the said process, without which further actions cannot take place. Neutrophils are in charge of eliminating invading pathogens. One remarkable feature of neutrophils is their persistency in

pursuing their targets. There are dynamic recordings of neutrophils navigating through a matrix of red blood cells going after bacteria. Investigators have seen potential in manipulating neutrophils to target cancerous cells due to the described property [24].

In contrast, the adaptive immune system as the name implies is response specific for the invading pathogen or the transformed tumor. This component of the immune system is activated only when faced with specific immunogenic challenges [23]. A very desirable property of this system is its ability to retain the memory of past challenges and use that "knowledge" to effectively combat the same challenges in future encounters and this property forms the underlying principle of vaccines. There are two main populations that compose the adaptive system: B and T lymphocytes. One of the major differences between them is the mechanism of target recognition. B cells recognize free floating antigens, and upon recognition, can transform into plasma cells which secrete antibodies against the targets [23]. T cells recognize "processed" antigen expressed on cell membranes and can directly eliminate targets Nevertheless, the functionalities of both B and T cells must be looked at with respect to their interactions with other cells within the immune system. While it is true that T cells on their own cannot spot a cancerous cell based on the free floating antigens secreted by the said cell, other cells such as DCs can internalize those antigens and express them peptides on their membrane complexes which then allows T cells to recognize the threat. Similarly, DCs can cannibalize cancerous cellular substances and secrete cytokines to activate B cells although strictly speaking, this route of activation does not constitute B cells recognition capacity but rather a reception to the regulatory function of DCs. It is important to notice that the initial numbers of both B and T cells are low and it usually takes a week before meaningful immune responses are observed [23]. The "memory" of the adaptive system helps accelerate this process. For B cells, a certain percentage of the plasma cells (which last for 2 days) persist to become memory B cells that can quickly

secrete antibodies in the next encounter [23]. For T cells, a population of activated T cells can also persist after a response to become memory T cells to serve the same stated purposes [23].

Even though classified as two different systems, the activities of both are intertwined. Specifically, dendritic cells, a member of the innate system, play an important role in the adaptive system by secreting cytokines governing effector cells' response. The antibodies secreted by B cells, for example, can make cancerous cells targets of macrophages, a member of the innate system in charge of consuming pathogens. Overall, the activity of the immune system exhibits a high degree of complexity.

T cells, maturation and apoptosis

T cells derive their name from the location of their maturation, the thymus. But to define the concept of T cell maturation, it is necessary to understand the interaction of T cells with other cells within the body. In general, there are two classes of molecules expressed on cell's membranes: major histocompatibility complex class I (MHC I) and class II (MHC II). The former is present on virtually all nucleated cells in the body, while the latter is only expressed on antigen presenting cells (APCs) [23]. MHC of both classes are particularly important in T cells functionality, for the latter cannot recognize free floating pathogenic antigens. For recognition, antigens must first be internalized, processed and displayed as peptides on the surface MHC. In general, there are two main classes of T cells, as discussed later, each of which ($CD8^+$ and $CD4^+$, respectively) can interact with peptides on MHC I (pMHC I) or MHC II (pMHC II). The existence of pMHC I on all nucleated cells, however, ensures that those cells are all recognizable by $CD8^+$ T cells. The recognition is done via T cells' receptors (TCRs).

The specificity of T cells can be mapped to the nature of their TCRs [23]. Each TCR is comprised of two protein chains. There are four types of such chains: α, β, γ, δ. The combination

of α and β is most popular among T cells, while the latter two are comparatively rare (95% versus 5%) [23].

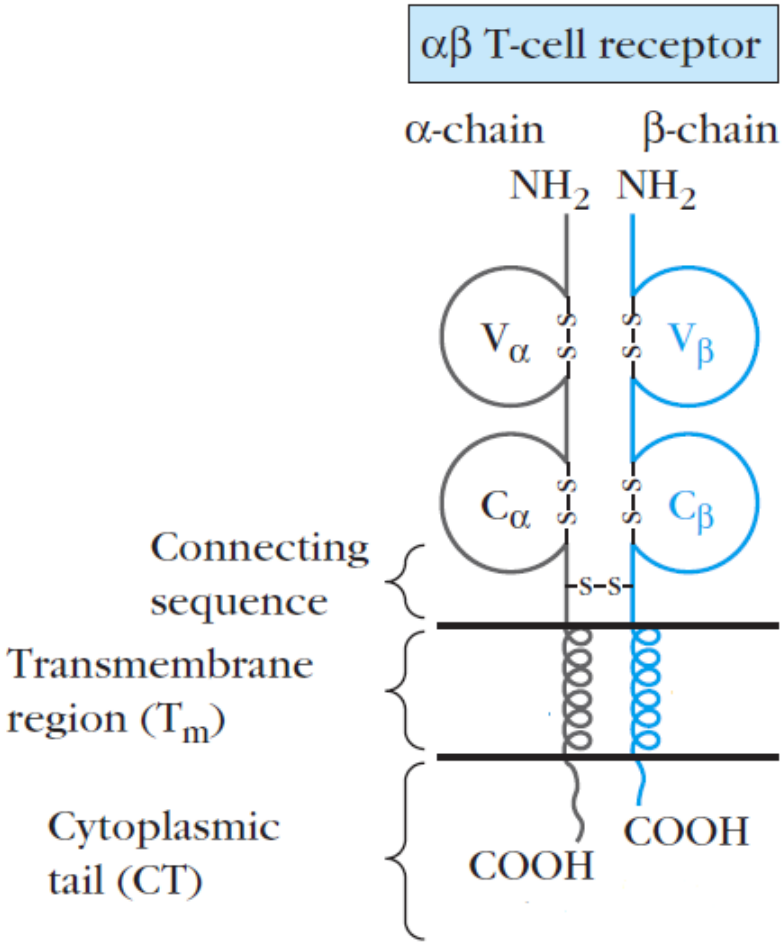

Figure 1: Schematic of a T-cell receptor comprising the α and β chains. The variable regions (V) of the α and β chains make contact with antigenic peptides presented in the context of MHC molecules. The diversity of T cells' receptors arises from the variability in V domain. The constants regions (C), facilitate anchoring the receptors to the membrane and are uniform in a given host. S-S indicates disulfide bonds [23]

When these TCRs bind to and interact with an antigen presented in the context of APCs, they are said to be antigen-specific. Recognition of the cognate antigen via the TCR is only the first step of the process in the differentiation of T cells. For the sake of simplicity, T cells can be categorized into three classes: cytotoxic, helper and regulatory. The first refers to those T cells in charge of eliminating infected cells (T_C). They generally express CD8 on their membranes, hence

are called $CD8^+$ T cells. Helper $CD4^+$ T cells (T_H) mainly secrete cytokines, which are proteins that orchestrates the overall immune response. In reality, $CD8^+$ and $CD4^+$ T cells functions are not strictly restricted by this artificial classification. Some T_c can secrete cytokines and certain classes of T_H can directly eliminate target cells. Regulatory $CD4^+$ T cells (T_{Reg}) that have been discovered recently function mainly to suppress the magnitude of the immune response [25]. This may sound contradictory but it is one of the main themes in the process of T cell maturation, as discussed later. The need for such mechanism arises from the risk of T cells attacking self-cells. The most prolific manifestations of such risk are the autoimmune diseases.

T cells, like all cells, originate from stem cells. This process takes place in the bone marrow. T cell precursors have neither CD4 nor CD8 and leave the bone marrow and migrate into the thymus where their maturation takes place (and thus, the name T cells). There are two criteria for this maturation, which is a selection process, as implied in the above paragraphs. Firstly, the selected T cells must display ability to bind to MHC because that is where the interaction with antigen and thus T cells activation take place. Secondly, the newly emerged T cells must not attack self-cells. These two criteria are enforced by positive and negative selection respectively. $CD4^+$ or $CD8^+$ T cells are resulted. However, as in the cases of all process, there are chances of failure. The consequences are T cells with weak functionality or autoimmune T cells. T_{Reg} also emerge from the selection to safeguard against the latter. The presence of T_{Reg}, however, can undermine the ability of other T cells to eliminate cancerous cells. One of the goals of the *in vitro* expansion of ACT treatment was to bypass this suppressive mechanism [22]. In fact, the effect of T_{reg} is significant enough to warrant a depletion of all circling lymphocytes in a patient prior to T cells' infusion in ACT [22].

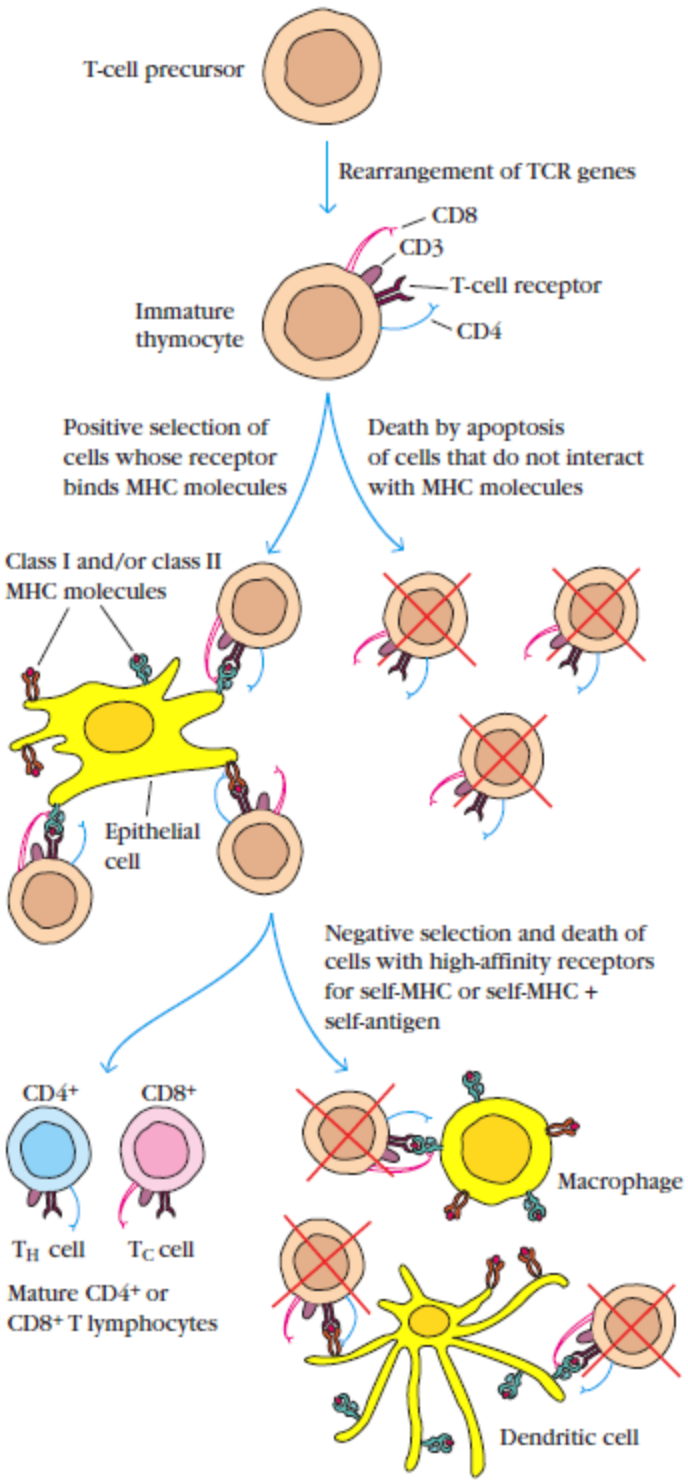

Figure 2: T cells maturation in the thymus. T cell precursors migrate from the bone marrow as double negative cells (CD4-CD8-). Once they exit the thymus, these cells become double positive (CD4+CD8+). First, they undergo positive selection where those not interacting with MHC molecules strongly enough are eliminated. During this process, double $CD4^+CD8^+$ cells become either single $CD4^+$ or single $CD8^+$, depending on the

nature of their interaction with thymus epithelial cells. Next is the negative selection. Those that interact too strongly with MHC molecules are eliminated. Macrophages and dendritic cells serve as executor of those. Immature $CD4^+$ and $CD8^+$ T cells emerge from this process [23]

The schematic above also illustrates a common mechanism in the human body, apoptosis or programmed cell death. This process is important in maintaining a healthy population of cells in the body and to ensure constant turnover, and is also a safeguard against cells that have irreparable genetic defects. However, some of those mutants escape this mechanism and become cancerous. Apoptosis should be distinguished from necrosis, which is cell death by physical factors such as membrane breakage, toxins and dehydration. There are observable differences between apoptosis and necrosis. And in fact, apoptosis can be indentified solely based on those factors.

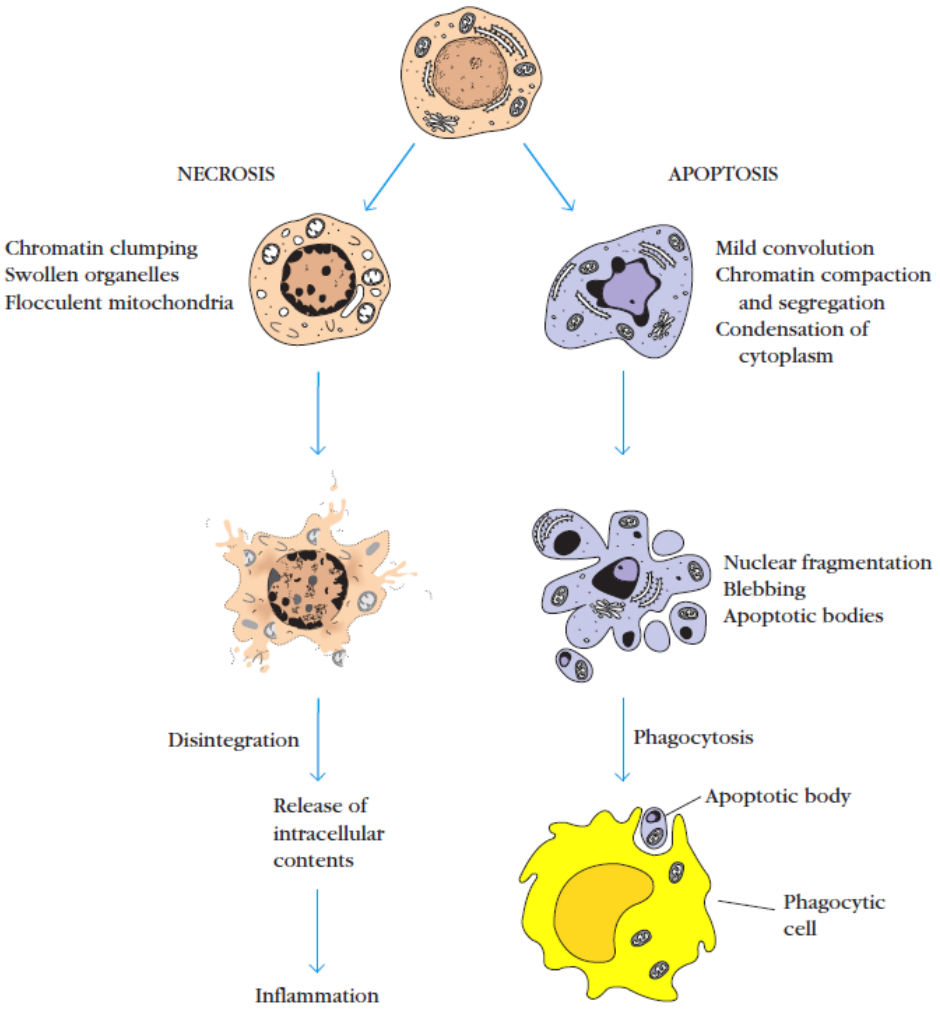

Figure 3: Pathways for cell-death, necrosis and apoptosis. Necrosis can be identified by the disintegration of cells and the release of its contents. The traits of apoptosis are the fragmentation, blebbing and the formation of apoptotic bodies. These bodies can be cleaned up by phagocytic cells. It is important to note that these events are observable and distinguishable using appropriate microscopes [23]

Cytotoxic T cells eliminate their targets by two main mechanisms, both of which are initiated upon successful recognition by TCRs. In the first mechanism, T cells induce apoptosis in targets by crosslinking the apoptosis-inducing receptors found on targets membranes such as apoptosis antigen 1 (APO1/Fas) [26]. In the second mechanism, cytotoxic T cells release perforin and grazyme. The former is a substance served to penetrate target's membrane which allows the latter to permeate and induce apoptosis from within [26].

T cells are of great interest due to their pivotal role in human immune system. They possess two most noticeable properties: specificity toward a certain type of pathogen and retaining the memory of past events [23]. The first property is much more appreciated in the context of cancer treatment. One of the major concerns of cancer treatment is the detrimental effects of the treatment on a patient's body, sometimes more destructive than the cancer itself. T cells specificity can tackle this problem. Even in the event of the cancer being eradicated from the body, there is no guarantee of long term complete remission. T cells once participated in and helped removing the cancer, retain the ability to repeat it and will act as sentinels against the same cancer if it ever comes back. Thus, immunologists aim to take advantage of these properties of T cells in the fight against cancer [27]. The goal is to acquire as much knowledge about T cells as possible and use them to[27] booster the immune system in the fight against cancer.

3. Melanoma, Treatments and Functional Testing

Melanoma and treatments

In the US alone, in 2012 there were approximately 76,000 new cases and 9,200 deaths due to melanoma [28]. It is estimated that in 2012, 1 in 677 males (only rivaled by leukemia) and 1 in 377 females (only breast cancer has higher occurrence in women) below 39 years of age developed invasive melanoma and this probability has been increasing since 2002 [28]. Early stages' mortality rate of the disease is not high and conventional treatments using chemotherapy are possible [28][29]. Unfortunately, late stage melanoma has a death rate above 90% and is refractory to all conventional treatments [29]. There is an urgent need for an effective treatment scheme for late stage melanoma. In the last decade, immunotherapy utilizing T cells, as discussed in the sections above, has emerged as an attractive treatment option for late stage melanoma.

Melanoma is a type of cancer that can elicit an immune response with relative ease due to the fact that melanoma is caused by UV radiation which results in a multitude of different mutations of exposed cells, and thus, should respond well to vaccine treatment [22][30]. Yet, large number of T cells surrounding a melanoma tumor mass are generally ineffective at managing the tumor [22]. As a matter of fact, potent T cells specific for tumor targets are found in patients with advance melanoma [31]. This highlights the role of suppressive mechanisms in the outcome of a treatment using T cells. The permeating effect of these mechanisms, as in whether the suppressing action affects only local T cells or spreads out to circulating T cells and to what degree, is unclear. But *in vivo* circulating T cells in melanoma patients have been found to be potent at both cytotoxic and cytokines secretion activity, but becomes lethargic in the presence of tumor mass. From these observations, a new course of treatment, known as Adoptive Cell Therapy (ACT), was devised to circumvent the suppressive mechanisms of the host

while retaining effectors such as T cells specificity toward certain types of cancer. In ACT, T cells with desired specificity can be obtained in various ways. A small number of those can be found in the blood stream, and larger number can be found in tumor masses. It is also possible to artificially engineer T cells to have appropriate TCR. In the context of melanoma treatment, adopted T cells are found in available tumor masses, and thus those T cells are called tumor infiltrating lymphocytes (TILs). In order to overcome the suppressive mechanism in the host, TILs are expanded *ex vivo*, the goal of which is to generate a population without the *in vivo* suppressed phenotype [22]. Before infusing the generated TILs back, it is also necessary to rid the host of existing lymphocytes to minimize the regulatory effects on new TILs, which is followed by administration of T cells proliferation simulator IL-2. In short, ACT is based on generating tumor specific lymphocytes *ex vivo*, deleting existing suppressive mechanisms prior to infusion and promoting proliferation to maximize the effect of infused cells.

ACT is a personalized treatment scheme. In the context of melanoma treatment, ACT using TILs has shown great potential with up to 50% complete remission rate even among late stage cancer patients in some clinical trials [32]. TILs are those lethargic T cells found in melanoma tumor mass, with properties suitable for *in vitro* expansion. A typical scheme of ACT using TILs is described in the figure below.

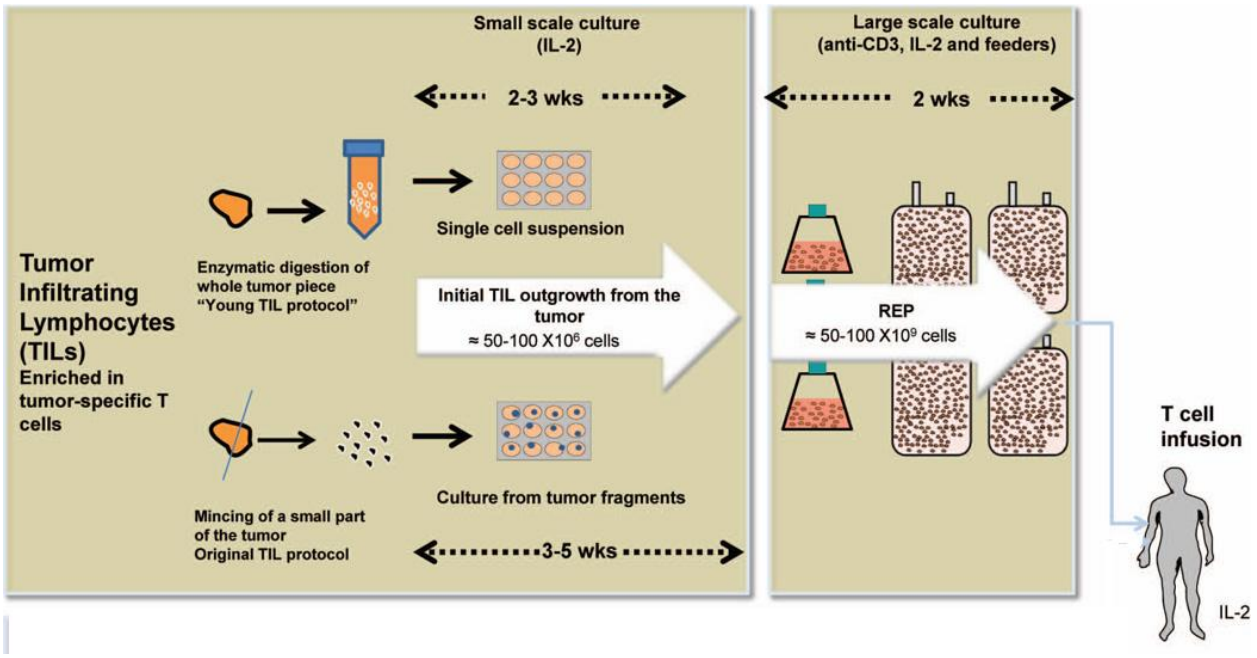

Figure 4: A typical scheme of ACT treatment against melanoma. First, a tumor mass is incised from a patient. In "young TIL protocol," the whole tumor mass are submerged in enzymes to re-suspend TILs in the solution via digestion of connective tissues and epithelial cells. The TILs are then cultured and tested for activity before large scale expansion. In the original TIL protocol, the whole tumor is cut into small pieces from which TILs population can expand. The cytokine IL2 is used to promote T cells growth. In the large scale expansion step, the rapid expansion protocol (REP) is used. TILs are activated through the use of appropriate antigen presenting cells (APCs) and anti-CD3, proteins expressed on T cells surface, to prevent TILs from attacking the feeder APCs and stimulate TIL expansion. IL2 is used concurrently to promote TIL growth. After about 100 billion cells are generated, TILs are infused back into the host patient. To promote the persistence of the TIL population, IL2 is administered after infusion [32].

In the theme of personalized medicine, it is important to study the donor's sample very carefully, down to a single cell. And since everyone is different, there needs to be a metric to evaluate the potential of donor's T cells. In a typical ACT clinical trial, a subset of T cells is first isolated, treated and then tested for potency. The most widely used test is by measuring the amount of INF-γ secreted, which is 200 pg/ml [33]. The test, however, does not correlate to the response of the infusion, and is arbitrary by nature. Those that pass the test are then expanded to reach a required number for infusion. The expansion part can range from three weeks to five

months. After infusion, there is an unspecified amount of time needed for monitoring the result. This lengthy and costly treatment course has a success rate of approximately 50% [32]. The cost, both in terms of time and capital, is one of the major obstacles preventing ACT from being a standard treatment. Had it been a correlation between *in vitro* expansion steps and the resulting *in vivo* response, in other words, the ability to systematically predict the outcome of the treatment before the infusion steps, the cost would be reduced significantly. The long-term goal of developing the method presented here is to reveal that relationship.

There have been numerous attempts to investigate the possible link between *in vitro* expansion of TILs and *in vivo* results. One of the earliest large clinical trials for adoptive cell therapy in 1987 shed light on the elusive correlation. The trial enrolled 55 patients who had failed to respond to other conventional treatments [34]. Overall, 22 out of 55 patients responded to ACT [34]. The partial response criterion at the time of this trial was that there was at least 50% reduction in tumor area as opposed to the 30% standard currently used [35].

In this clinical trial, the clinicians were interested in a correlation between lytic activities of TILs against autologous targets. The killing assay was done via the Chromium 51 release assay. This assay is typically done will a high TIL to targets ratio (10:1 to 100:1). In addition to lytic activities, the investigator also took into account the proliferation states of TILs prior to the killing assay. This arose from the fact that each patient had a different number of TILs in his/her biopsy tumor. There were two associated factors: expansion indexes, which determined the number of TILs grown in culture, and the time (in days) spent to reach the desired population prior to infusion. The latter encompassed the differences in growth rate of TILs. The responses of the patients after infusion were then used to form the graphs showed below.

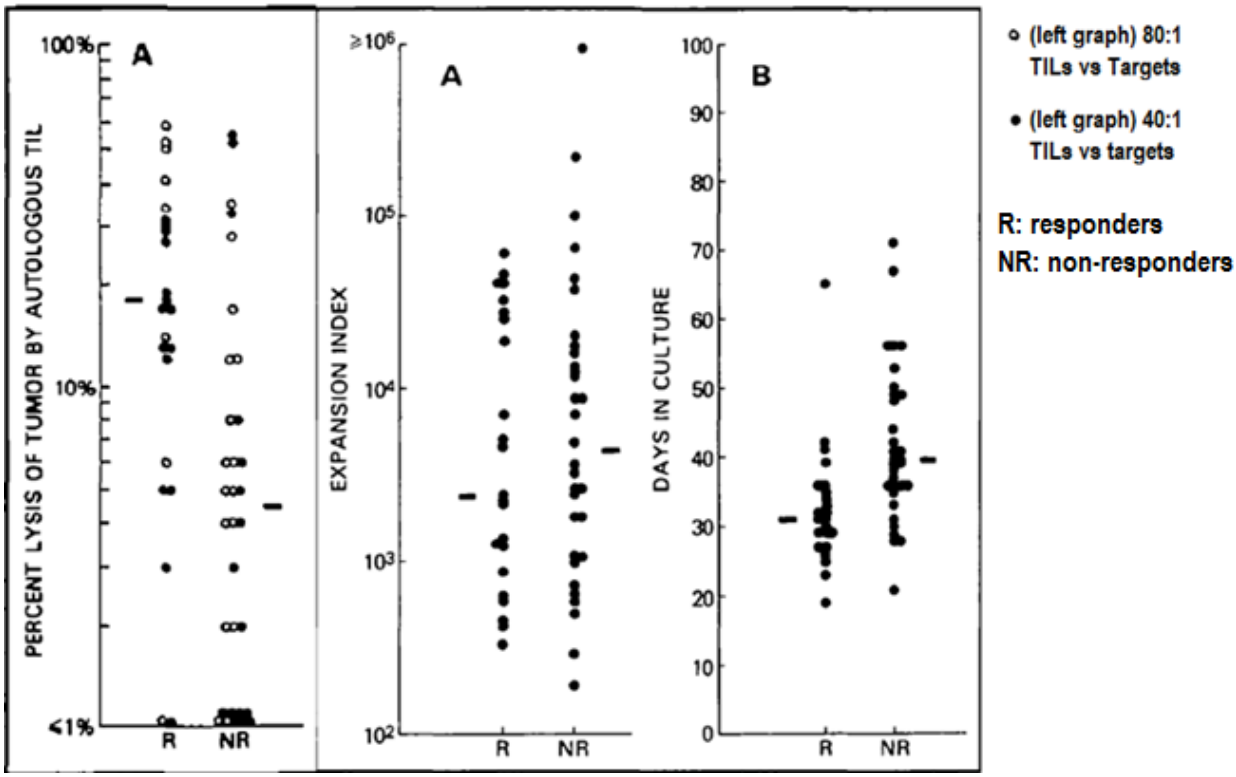

Figure 5: With a lysis percentage above 10%, it has been observed that 62% of the corresponding patients responded to the treatment, whereas with a lysis percentage below 10%, only 20% of the corresponding patients responded to the treatment. Responding patients had higher lysis percentage ($p<0.02$ for 80:1 and $p<0.03$ for 40:1 TILs and target ratio). There was no observable correlation between expansion indexes and response rates. However, it was found that responders' TILs has significantly fewer days in culture than their non-responders counter-parts ($p<0.0008$) [34].

From the graph above, two conclusions can be drawn. First, there is a link between the lysis of autologous targets *in vitro*, and the clinical response showed by the higher response rate of patients whose TILs exhibited more lysis activities. However, the correlation cannot be established. As showed on the graph, there were instances where non-responders' TIL had up to 55% lysis and responders had less than 1% lysis. Furthermore, there were no data showed for the nature of the response in each patient which hinted that there was no observable trend. Second, it appeared that TILs with shorter culturing time gave better responses.

The second observation has inspired recent clinical protocols which concentrated on attaining the required number of TILs as fast as possible for infusion ("young TILs" protocol)

[36]. The first observation, while important, has not generated practical applications. It has been thought that in order to find a correlation between *in vitro* lysis and clinical reponse, lysis data with higher resolution must be attained. As a matter of fact, one of the most detrimental factors in the success of ACT is the suppressive mechanisms of tumors. However, since TILs are a heterogeneous mix, there can be sub-populations of TILs that are more or less resistant to tumor's suppressive mechanisms. Secondly, it is well-known that tumor burdens, or the amount of tumor, have inverse effects on treatments [37]. The practice of generating a large number of TILs before infusion can be thought of as a means to decrease the effect of the burden. It can then be speculated that in order to get a response in treatment, a sufficient number of TILs resistant to suppressing mechanisms is needed. A bulk assay like the one used in the 1987 clinical trial cannot be used to identify those TILs. To that end, assays with single cell level resolution are needed. We theorized that the desired sub-populations can be gauged by their individual lytic activities. Until recently, this was not technologically possible. In the following sections, a currently prominent assay with single cell resolution will be discussed.

Functional testing

The most common method of testing for functionality at single cell level is ELISPOT (enzym link immunospot). This assay is able to monitor killing activity of a T cells at the single cell scale when challenged by its targets. This is achieved by capturing the secretion of a substance called Granzyme-B, which is secreted by T cells to eliminate targets [38]. The working principle of this assay is implied by its name. The assay relies on the interaction of an enzyme and its substrate to emit detectable signals in spot form. The major attraction of ELISPOT is its incredible sensitivities, compared to what is currently offered in the field, as well as the ease of operation. These two factors make it one of the most robust assays in the lab. The assay is typically performed using a 96-well plate. For each of the wells, as few as 10 immune cells are sufficient

to give readable signals [39]. And as one can imagine, a typical diameter of a well in a 96-well plate is about 6.5 mm. Thus, with only ten small spots in a well, the assay in fact yields result at the single-cell level. The high sensitivity of this assay comes from the ability of the captured antibodies to bond with a sufficient number of locally secreted antigens before the said antigens diffuse through the medium or being consumed by any other means. The concentration of cells can be controlled such that it is highly unlikely for more than one cell to be within each other's vicinity, which if held true, will produce single cell spot readout.

Even with those tremendous proven capacities of this assay, it is still plagued with limitations. First, this assay only captures quasi-predetermined events. The scopes of information that can be collected only go as far as the capture antibodies allow. And second, the number of different secreted antigens that can be detected simultaneously is low. For single cell level analysis, it is desirable for a spot to be able to capture multiple types of antigens, which translates into a well's surface area with some degree of organized distribution of capture antibodies. Obviously, this can be overcome with extra effort in the fabrication process, but the practice is not common. And third, in the context of single cell analysis, even though the readouts are at the single-cell level, the stimulation for secretion is essentially of a bulk nature . For certain investigations, it is of great interest to determine the source of cell secretions, especially when it is from a different type of cell. In such cases, the readout results can either come from a single cell with strong secretion by single stimulation or from a single cell with secretion ability but stimulated multiple times. By design, the ELISPOT assay cannot attain this level of resolution. This method relies on bio-markers and does not actually observe the killing event. Thus, questions such as how many targets one particular T cell is able to kill is not answered. It is important because the time consuming part of an ACT clinical trial aims to expand the T cells population to a large enough number before infusing into a patients. And

there is no observable correlation between the killing potency *in intro* and actual result *in vivo*. One of the reasons can be the fact that the population of highly potent T cells that can kill multiple targets vary greatly between successful and failed cases.

Flow cytometry has revolutionized single-cell analysis in many different contexts including T cell immunology. A number of cells flow through a channel designed in such a way that no more than one cell can occupy a radial space concurrently and that the axial spacing between any cells is sufficient to distinguish them. Characteristics of each individual cell such as size, DNA or RNA contents, and membrane-expressed proteins can be readily measured [40]. A typical flow cytometry setup is shown below.

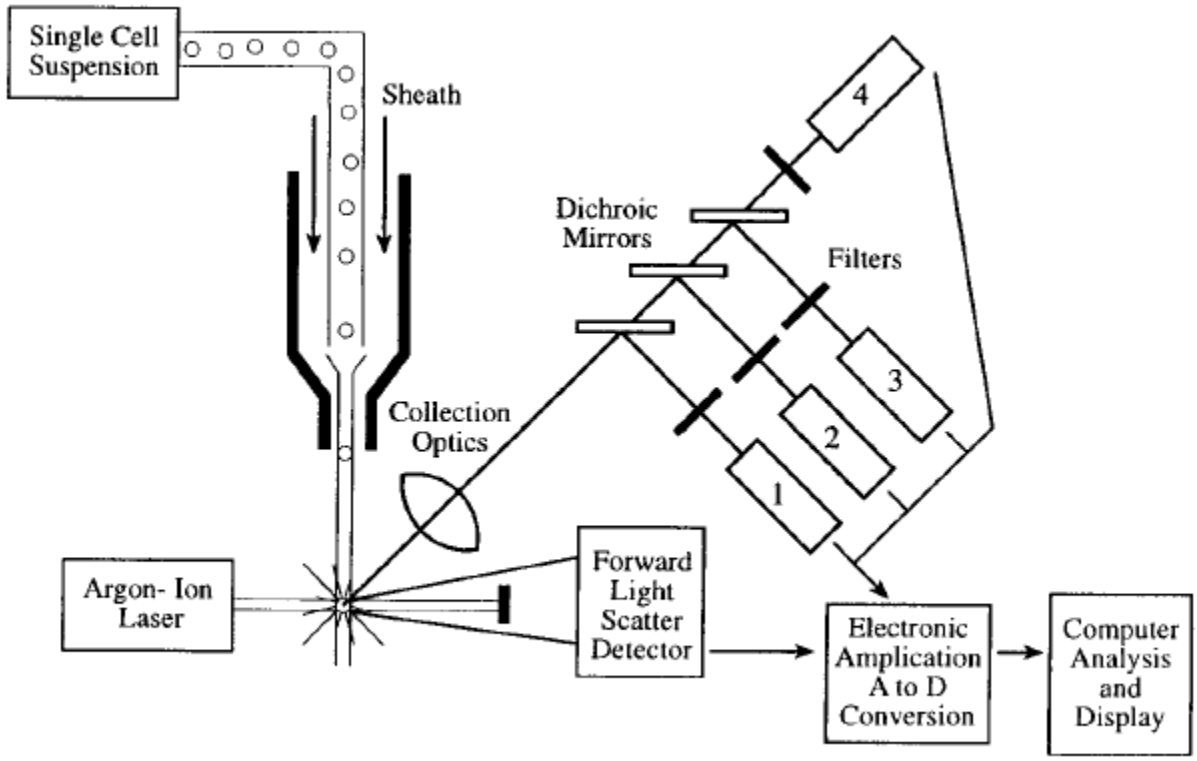

Figure 6: Basic setup of flow cytometry. Cells are suspended in an isotonic fluid and drawn to a narrow channel by a lamellae flow which allows cells to individually pass through the sampling point. At the end of the channel a laser beam, served as the light source, focus on passing single cells. This laser beam serves too function: first it provides the light source for light scattering analysis (size and granularity information are thus

obtained), and second, it excites the fluorescent bio-markers used to label protein contents of passing cells (if intended). The scattered light is collected by a detector (shown at the bottom). The fluorescent light goes through a series of filters. Both are then amplified before being analyzed [40].

Flow cytometry can sort thousands of cells per minute. It is an indispensable tool if any heterogeneity information of a cell population is desired. Cells can differ in physical traits, which can be detected using laser as described in figure 7. When the cells in question have indistinguishable physical properties, for example, individual TILs, it goes back to biomarkers to help sort the cells. A typical flow cytometry setup can accommodate both physical and flourseceence analysis as shown in the figure about. However, as far as single-cell level lytic analysis is concerned, this method has the same limitation as ELISPOT.

4. Goal and hypothesis

From the results of the clinical trials conducted by Aebersold *et al*. [34], it has been found that patients with TILs exhibiting greater than 10% lysis rate had 62% overall response rate. We hypothesize that quantification of integrated functional profile of TILs at single cell level and the ability of single TILs to kill more than one tumor cell (serial killing), can identify correlates of *in vitro* elimination of cancerous cells and *in vivo* outcome of ACT treatments. As far as investigating individual TIL's lytic activities is concerned, no known method has the capacity to achieve that feature. As it has been pointed out, even though Granzyme-B ELISPOT assay could detect responses down to single-cell resolution, the assay would give no information regarding the sources of the stimulations. To assess these activities, an innovative assay is needed. This assay must be able achieve two objectives. First, it must be able to quantify individual TILs. And secondly, it must be able to quantify corresponding stimulations (in this case, the number of targets for each TIL). We have postulated that the lytic activities of single TILs versus a controlled number of targets can be captured and quantified using an experimental setup as described below.

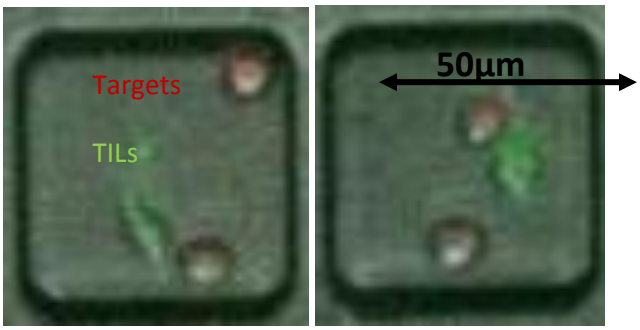

Figure 7: Representative composite micrographs of TIL-tumor cell interactions in nanowell arrays. The dark squares represent wells where TILs (green) and targets (red) are isolated. Each well is a control volume for an experiment on individual TIL's lytic activity. As showed, since each well only contains one TIL and a number of targets, TIL's lysis can be readily quantified.

The details of the assay setup will be discussed in the following section. Regardless, our assay is able to achieve the two stated objectives of controlling the main objects (TILs) and their stimulations (target cells). This allows us to quantify the lytic activities based on the interaction between TILs and their target cells. The setup and the corresponding detection device can also be further improved to acquire a wider range of biomarkers.

Results

1. Experimental Design

a. Subnanoliter well plates

The backbone of the method described here was the use of an array of about 85,000 subnanoliter wells. The entire ensemble is made of Polydimethylsiloxane (PDMS). This material was light-transmissible, inert and bio-compatible [41][42][43][44][45]. It was used in medical applications: contact lenses and breast implant fluids. It was also an established material used in soft lithography. In fact, the fabrication of the nanowell array is done via soft lithography. About 85,000 of cubic nanowells with 50 micrometer in each dimension are imprinted into a PDMS slide. The template was made out of silicon with a Teflon coating to facilitate the removal of the PDMS after the imprinting process.

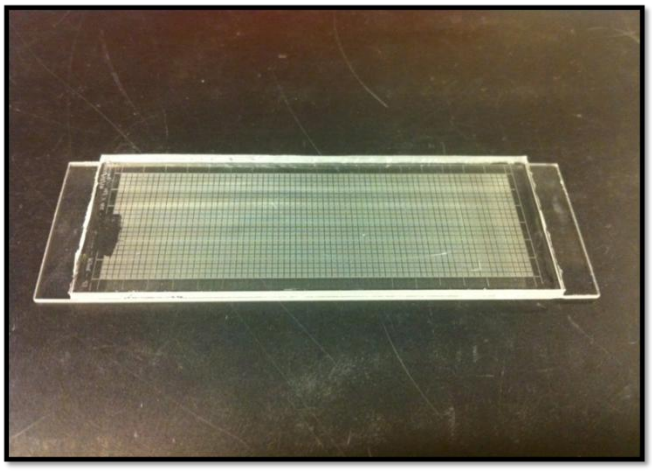

Figure 8: PDMS slide with around 85,000 (504 rows and 168 columns) fifty by fifty micrometer nanowells mounted onto a standard 1” x 3” microscopy glass slide

Each well in this system serves as an isolated control volume, and houses one independent experiment. Observing and analyzing one experiment is relatively simple. Targets and effectors can be visually distinguished based on sizes, and death events can be identified by formations of apoptotic bodies. To utilize these visual clues to analyze tens of thousands of

individual experiments is not practical. Alternatively, each of those three vital elements can be visually marked firstly to make quality control by humans possible, and secondly to analyzed by an image processing program.

b. Fluorescent markers for visual and digital detections

We used fluorescent dyes as markers for detection. For TILs and targets detections, there were several criteria needed to be addressed before certain dyes are chosen. The markers had to be bio-compatible. The commercially avaible dyes used widely in the field addressed this concern. Next, since the experiments were in approximately of the order of ten hours, the markers had to be stable. The fluorescent dyes attached to the markers themselves were stable. However, these bio-markers had to latch on a certain part of the cell to carry the signals. And in order to pick up the signal, the whole cell had to be labeled. The bio-markers could either latch onto cells' DNA or proteins. During a course of the life of cells, DNA chains were constantly broken up and reformed, and the fragments could freely traverse cell membrane. Thus, cell surface membrane bio-markers were chosen. These markers could be lost during cell division; however, it was assumed that unless an experiment was sufficiently long and its condition was favorable for such phenomena, the overall loss would be minimal. And lastly, the signal from fluorescent dyes had to be useful in marking the desired objects or events.

The PKH family of lipophilic dyes was attracted to the lipid layer of cell membranes and was thus ubiquitous for most cells. The structure of the dye contained a lipophilic tail conjugated to a polar fluorescent head group [46]. The affinity of the dye to the cell membranes was due to the tail and prior work has demonstrated that these dyes are both stable over time and not cytotoxic at the concentrations used.

For apoptosis detection via fluorescent signals, we used an early apoptosis marker known as Annexin V conjugated to Alexa Flour 647 dye. Annexin V attached to

phosphatidylserine (PS) displayed on the outer membrane after the initiation of apoptosis, and was internalized by the cells later [47]. These early events eventually led to fully apoptotic cells. The chosen three markers would accomplish the goal of the design: labeling T cells and their targets as well as the "killing" events. The mechanism of the Annexin V staining in the early apoptosis events is showed in the figure below.

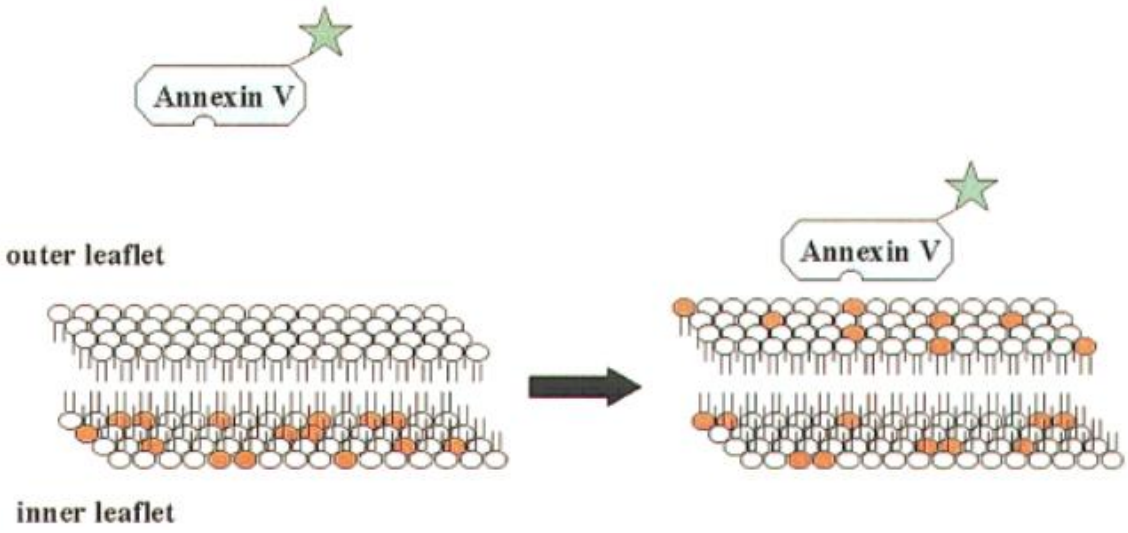

Figure 9: Mechanism of Annexin V labeling of apoptotic cells. Annexin V is protein that has a high affinity for Phosphatidylserine (PS). When a cell is healthy, the PSmolecules, orange circles, are not exposed on the surface membrane. When the apoptosis cascade is initiated, the cell membrane is compromised. PS molecules migrate onto the outer leaflet of the membrane and can bind to Annexin V molecules conjugated to appropriate dyes [48]

c. Interpreting the fluorescent signals

There were some concerns that needed to be addressed when analyzing the data using the proposed system of markers. Eventually, the signals were recorded and a histogram was constructed for each channel to determine the cut-off intensities of positive signals. In the case of PKH dyes which were used for effectors and targets, the histograms provided defined peaks due to the sheer number of definite positive data points (Figure 11, left). For the case of Annexin V, it was more problematic (Figure 11, right). First of all, it was certain that there was a small

number of dead targets at the beginning of the experiment. This number, however, was not enough to produce a visible peak after the negative one. This could be solved by meticulously inspecting the minimal amount of signals visible to the naked eyes in the resulted images, which was arbitrary. And since Annexin V was designed to detect very early apoptotic events and the internalization of Annexin V took place in a diffusive manner [47](with the center of the cell lack of Annexin V), these dead events might not be visible without adjusting background noise. To solve this problem, it was necessary to take advantage of the negative peak. Three standard deviations, which were associated with a 99% confidence level assuming a normal distribution, from the negative means are determined to be a standard for positive events (Figure 11, right). And since these peaks were made of thousands of data points, the confidence in this method was high.

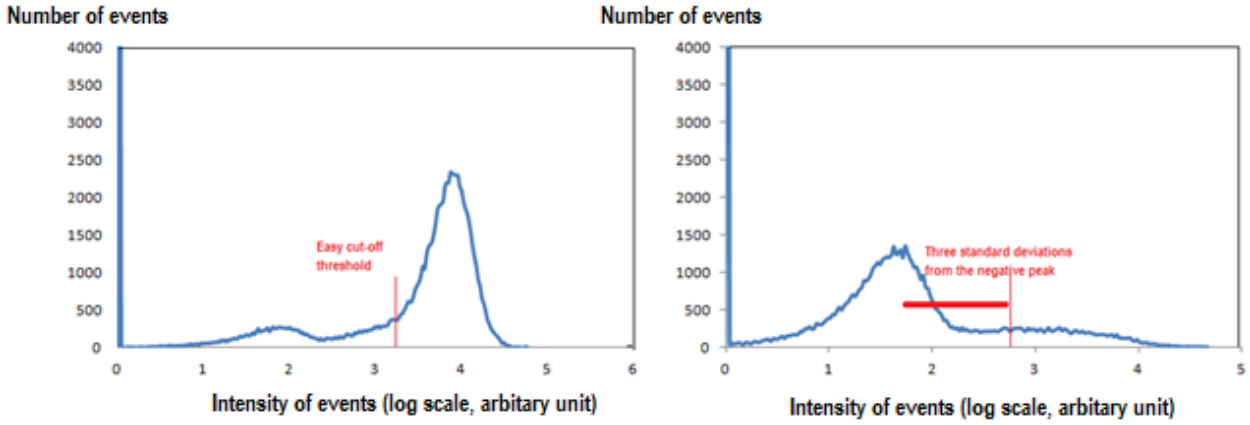

Figure 10: Determining cut-off thresholds for a Annexin V Alexa Fluor 647 channel. On the left is a histogram of PKH26 signal: the positive peak is well defined and threshold can be readily determined. On the right is a histogram of Annexin V signal: there is no visible positive peak. The threshold is determined by calculating three standard deviations away from the center of the negative peak.

2. Result of re-directed killing assays

To eliminate a tumor cell, a T cell firstly has to be able to recognize its target. However, in the context of cancer treatment, recognition is the most challenging aspect of lysis activities. As such, it is more logical to first assay the T cell's ability to eliminate recognized targets before further functionality testing and optimization. The re-directed killing assay was designed to define the potency of T cells when stimulated through their TCR. In this assay, target cell were loaded with an antibody directed against CD3, OKT3. Cross-linking of CD3 receptor with OKT3 antibody stimulates T cells and facilitates target killing [49].

The P815 target cells of this assay were a mouse cancer cell line widely used in clinical study of tumor. This line is used to emulate cancer cells due to its robust growth rate and the presence of receptors for the constant region of OKT3 [49], both of which helped the assessment of T cells functions.

Labeling cells was of most great importance in this assay due to the reliance on fluorescent signal to pick up events. For P815 cells, about 2 million cells were stained with PKH26-red following the protocol provided by the respective vendor. The P815 cells were incubated with 1µg/ml OKT3 to promote uniform capture. Effector TILs were labeled in the same manner with PKH67-green, without the OKT3 incubation step. For each assay, about 1 million cells from each TIL line were needed.

Each effector line was incubated with P815 in the killing assay on a PDMS nanowell chip. To establish a background death rate of targets, a control PDMS chip with only P815 target was run in parallel. Prior to the loading of cells onto the PDMS chips, the chips were plasma oxidized to produce a hydrophilic surface which facilitated loading and retaining cell within each well. Each chip had about 85,000 wells organized in 24x72 clusters. Each cluster was a 7 by 7 matrix, in which there were 3 diamond shape wells among the other 46 square ones. The positioning of

those 3 diamond wells in each cluster served as the ID tag for each cluster. As a result, if a chip was to be images and analyzed in another system, data can be cross-matched.

The desired loading frequency was 1 TIL effector with 1 to 3 P815 targets in each well. Three was the maximum number of targets per well that the analysis program can reliably function. To strive for this specification, effectorTILs were loaded into the chip in 200µl volume at 1 million cells/ml and P815 target cells, with larger diameters, were loaded at 2 million cells/ml. This loading protocol yielded about 9,000 to 14,000 well with 1 effector and fewer than 3 targets wells on a chip. After loading, the chip was secured in a 4-well plate with 5% agarose gel solution, which was a bio-compatible and quick-hardened material. Next, culture medium with 1:60 dilution of stock Annexin V- Alexa Flour 647 submerged the chip labeling any cells undergoing apoptosis during the experiment. The chip was imaged using a fluorescent microscope. The negative control chip with only target P815 cells was processed the same way minus the loading of effector TILs.

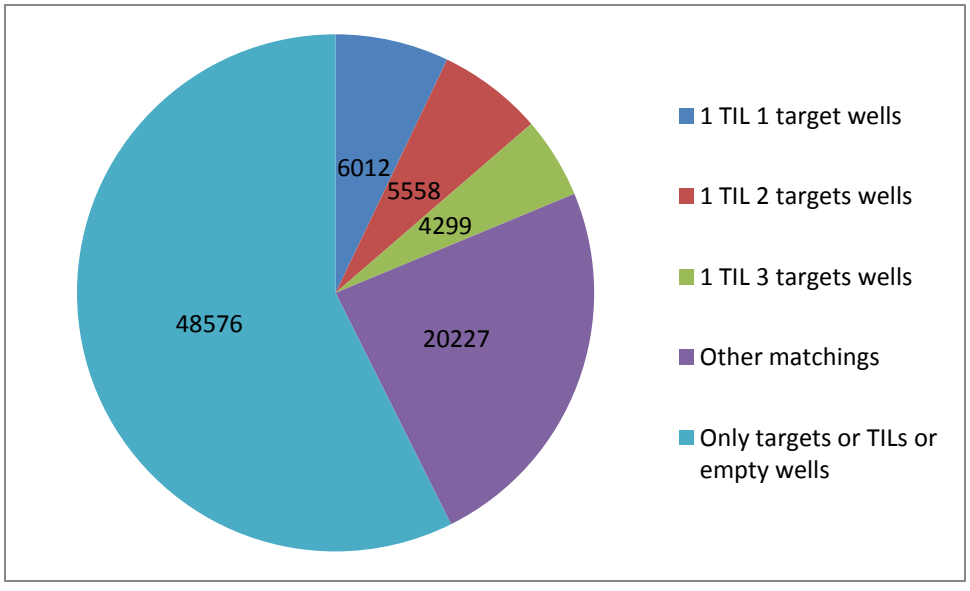

Figure 11: ***Distribution of targets and TILs on a wellplate after loading TILs and target cells.***

There were two imaging rounds for each chip. The first one took place at the first hour, after which the chip is sent back to the incubator at 37 ^{0}C and 5% for 6 hours. After that, the second round of imaging commenced. The six hours incubating period was based on the time frame used by the Granzyme-B ELISA bulk killing assay. This assay allowed T cells four hours with their targets and has proven to yield good results [50]. However, in the bulk assay, T cells were generally more likely to encounter multiple targets and thus had more chance to activate. To compensate for this, T cells in this assay were allowed 50% more time to provide a safe margin for guaranteed cytolysis activities.

The images were segmented so that every fluorescent spot of a size of a cell was detected and computed. The data were then analyzed by successive gating: wells with 1 effector and with 1 to 3 targets, matching wells with those criteria between first hour and sixth hour images. This assumed that meaningful events took place in wells that showed no out of well movement. This safeguarding criterion effectively slashed the number of valid wells by up to 80%. The remaining events were analyzed separately according to the number of target cells in wells. The background control on the target only chip was analyzed in the same manner. Briefly, the analysis kept track of matching wells at the first and the sixth hour images and recorded the number of spontaneous deaths in accordance to the events in effector and target chip: 1 target with 1 or 0 dead, 2 targets with up to 2 deaths, and three targets with up to 3 deaths.

For statistical assessment, confident intervals were calculated based on the magnitude of the frequency and the number of events, assuming a normal distribution. Only the events with the confident interval smaller than the magnitude of the frequency were reported. The result varied greatly among TILs line reflecting the diversities of human TILs activities. The results are summarized here under and illustrated graphically below in Figure 12.

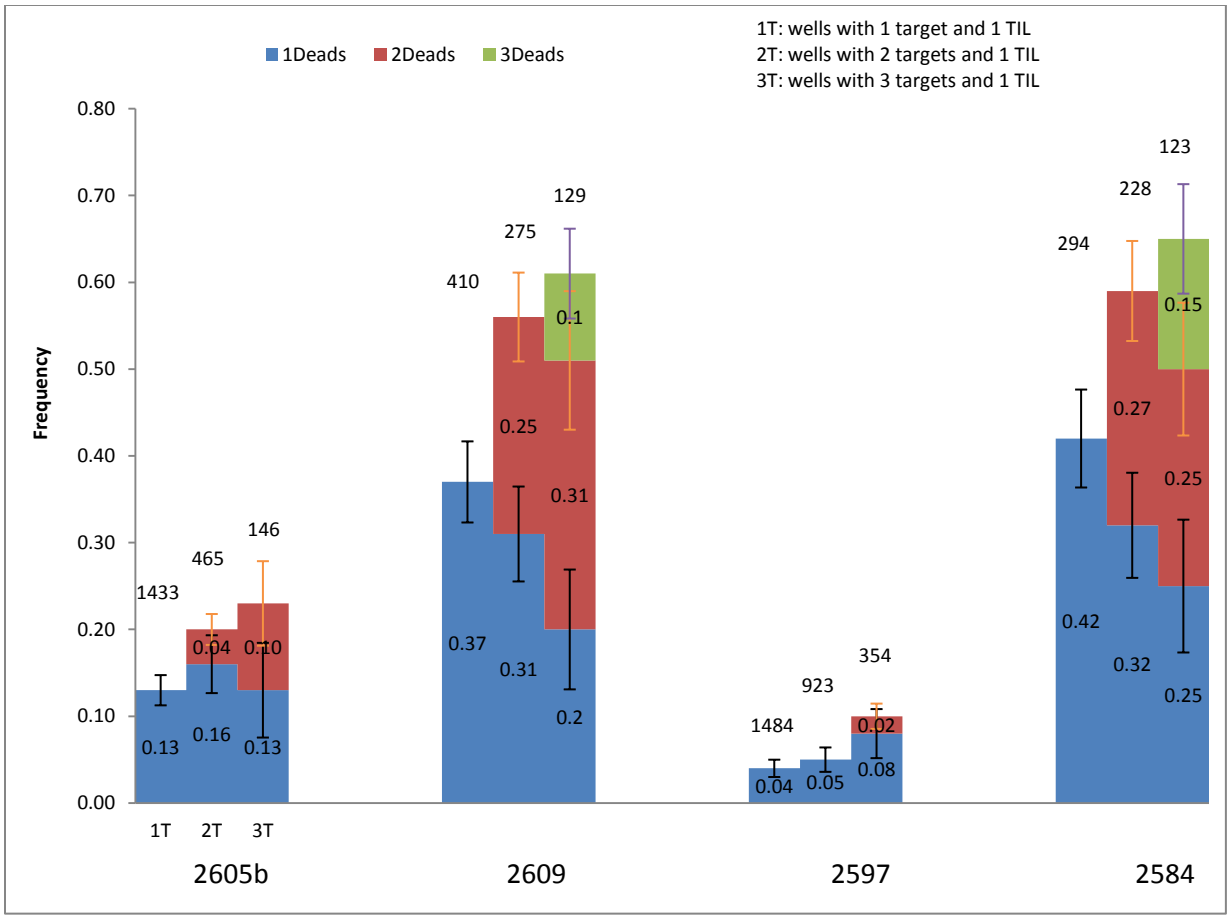

Figure 12: Killing frequency of re-directed killing assays. The patients' numbers are listed under each cluster. The three columns of each cluster represent population of wells with one, two, or three targets as indicated in the legend. The size of each population is indicated by the number hovering on top of each corresponding column. The error bar represents the confident interval of sampling with 95% confidence. From the result, it is clear that for each patient's TIL sample, there are sub-populations of TILs differing in killing potential: those that can kill only one target, those that can kill two targets and those that can kill three targets.

The results reflected the ability of the assay to capture multiple-target killing events. Each resulted killing frequency also had adequate statistical relevance (none of the displayed numbers hadmagnitude lesser than that of the confidence interval). The 1 versus 1 events and 1 versus 2 events were observed across the samples. The 1 versus 2 events were only observed in two patients with significantly higher killing frequency (2609 and 2584). However, the number of events of the other two patients (2605b and 2597) was significantly higher (2044 and 2761

total events compared to 814 and 645 of patients 2609 and 2584 respectively). It has been observed that dead targets would easily float out of wells, which could reasonably account for the discrepancies in the number of events, assuming the execution was consistent.

3. Results of autologous killing assay

After the re-directed killing assay had proven the ability of the setup to establish meaningful statistics of the killing frequency, the next step was to use autologous targets for direct killing assay. In this type of assay, T cells are tested for their ability to kill tumor cells originated from the same host. For each TIL line, there was a corresponding target line.

The T cell lines used in this experiment were harvested from melanoma tumor mass of different patients, each of which will also host the respective TIL lines. Since the TILs were tested for their ability to recognize and eliminate tumor targets, no cytolysis augmentation such as OKT3 incubation was provided. The TIL lines came from two different patients with respective autologous tumor cells. The first patient TILs were prepared by two different protocols labeled PBMC and APC, indicating that the TILs were cultured on a peripheral blood mononuclear cell (PBMC) or on a APC feeder layer. The second patient TIL line was labeled 2576.

The experiment protocols for TILs followed the one described in the previous section. The target preparation was similar without the incubation with OKT3 step. However, melanoma tumor cells were adherent cells and they attached to the wall of the culturing vessel. It was necessary to re-suspend the cell in a medium to facilitate normal processing procedures. This was achieved treatment with Trypsin (a protease used for cleaving peptide chains). In this case, it helped remove the bonding between the targets and the vessel wall [51]. The culturing vessel was drained of medium and then washed with PBS buffer solution. The cells are washed afterward. The protocol then proceeded as described. The loading frequency and criterion remained the same. In short, 1 TIL and 1 to 3 targets per well was desired. Cells were loaded on

a plasma oxidized PDMS chip in 200µl volume with concentration of 1 million TILs/ml and 2 million targets/ml. A negative control chip for each type of respective autologous targets was run in parallel (targets only). Images were taken at first and sixth hours. The events recorded were as described in the previous section.

The analysis detected no statistical meaningful 3 targets killing events and overall, a significant drop in killing frequencies was observed compared to the re-directed experiments. The detailed analysis is summarized here under and illustrated graphically below (Figure 13):

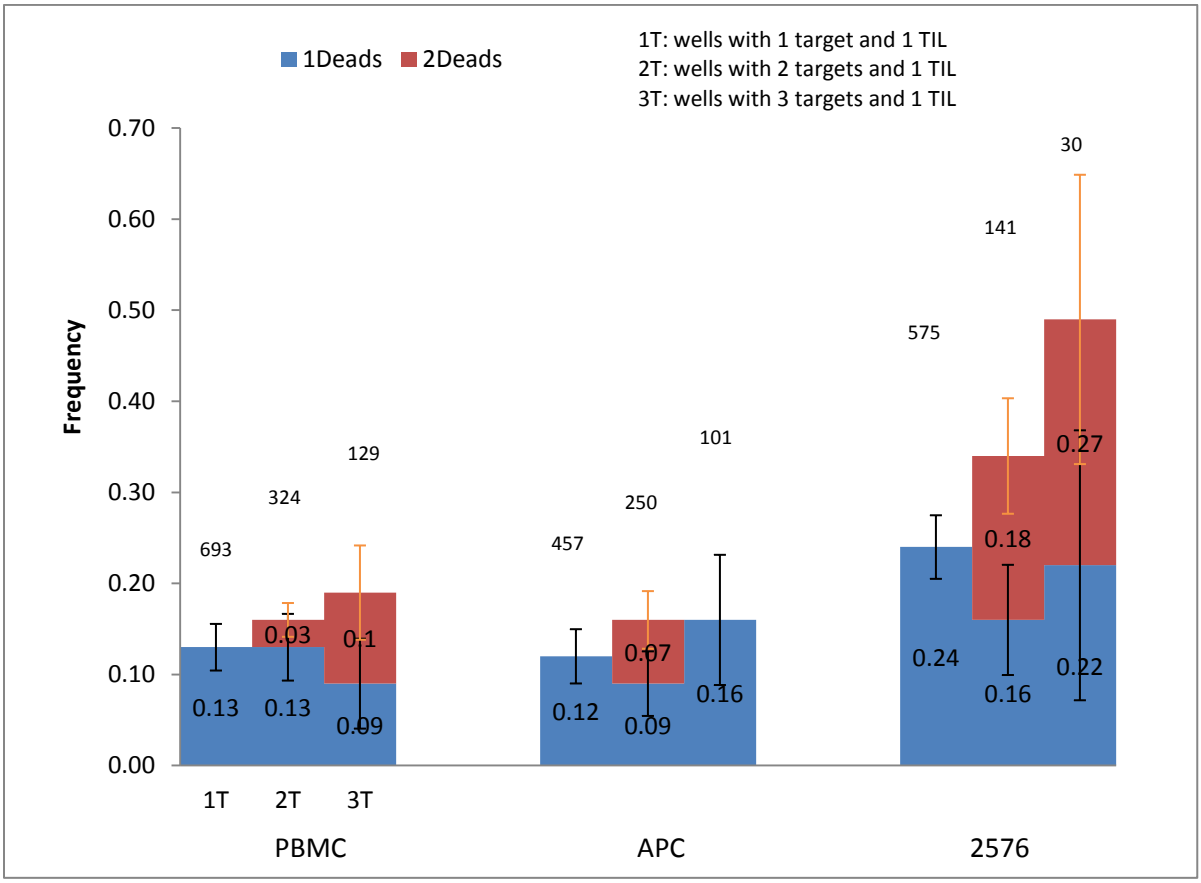

Figure 13: Killing frequency of autologous killing assay. The patients' numbers are listed under each cluster. The three columns of each cluster represent populations of wells with one, two, or three targets as indicated in the legend. The size of each corresponding population is indicated by the hovering number. The error bar represents the confident interval of sampling with 95% confidence. From the result, it is clear that for each patient's TIL sample, there are sub-populations of TILs differing in killing

potential: those that can kill only one target and those that can kill two targets. It is expected that patient 2576 with highest killing potential would yield best clinical responses.

PBMC and APC showed comparable killing frequency. The 2576, which was conventionally cultured and expanded by REP using PBMC, showed higher killing frequency, which was due to natural differences in different individuals' T cells and tumor development. No three target killing event with statistical relevance was reported.

Discussion and Future Directions

The results of re-directed killing assay experiments illuminated the capacity of our killing assay in capturing killing events with a designated ratio of effectors and targets. Patient 2605b and 2597 T cells had relatively low killing potential compared to the other two's. Both of the formers exhibited barely significant killing frequency of more than one target. The other two patients' T cells showed greater killing potential. However, across the board, it was observed that the T cells were more likely to kill at least one target when surrounded by at least two. And likewise, they were likely to kill at least two targets when surrounded by at least three. This was quite surprising given the fact that one of the detrimental factors in cancer treatment is the heavy tumor load, which described a scenario of a proportionally larger number of tumor cells compared to available T cells. The common practice was to reduce the tumor load as much as possible [37]. However, with the given result, it s is possible that there is an optimal level of tumor load that T cell can function best at.

In the direct autologous killing assay, there were fewer events of multiple target killing compared to re-directed assay. The overall killing frequency was also lower compared to the autologous assay (the highest killing frequency of any cases in re-directed assay is 0.42 whereas it is 0.27 in autologous). Nevertheless, the trend previously mentioned was still observed: the TILs were more likely to kill at least one when there were more targets within their domains (wells). Furthermore, the APC and PBMC protocols appeared to produce TILs with similar efficiency as far as single cell cytolysis was concerned. However, with one data point, it was not sufficient to conclude that observation.

From the results, we have demonstrated that our assay has been able to identify sub-populations of TILs based on single cell cytolysis, the stated goal. Instead of conventionally measuring the secretion of Granzyme-B, here the direct killing of target cells was observed via

the employment of Annexin V Alexa Flour 647. With the help of PKH dyes in labeling the participated cells, visual, single-cell level records of the killing were made. Each patient's TILs were classified according to their killing potency when challenged by different numbers of targets. No other known assay is able to achieve this feat. Furthermore, the TILs and targets ratio of our assays was 1:2, much lower than the traditional 40:1 and 80:1. And this is a major advantage as the number of TILs available for expanding and assaying can be very limited. For modern protocols emphasizing short expansion times [21], the low number of TILs required for assaying means that the potential population to be expanded can be identified sooner, which would lead to a reduced overall expansion time.

As far as lytic activities are concerned, there are several areas the assay can be improved upon. First of all, one must pay attention to the morphology of targets, which was seen to elongate in their culturing vessels. The elongated form is of random shape and occupies a large area. In our current protocols, the targets were not given sufficient time to elongate and thus remained round. It would be more desirable to let autologous target elongate first. This calls for a large well size and a more robust segmentation algorithm able to track the randomly shaped targets (the current algorithm only works with round cells). Furthermore, the elongation depends on the interaction between target cells and the surface. Thus, it is important to finely tune the surface chemistry (hydrophilicity) of the PDMS array to closely match that of the culturing vessels, which can be achieved by tweaking the exposure time in the plasma cleaner [52]. And finally, to further improve the requirement for TILs and target cells, a rigorous loading protocol must be adopted. Previously, the cells were loaded by manually dispersing droplets on the PDMS surfaces, which were very prone to human errors. It is also important to look at the big picture regarding the experiment configuration. There are two limitations of conventional experiments: population heterogeneity and the dependence on pre-determined markers. The

design introduced here can overcome the first challenge. For the second, due to the reliance on the tracking program, fluorescent markers were still used in the experiment. However, based on the images acquired, it is feasible to spot the effectors, targets and the dead events just by visual cues in the brightfield images (based on size, granularity, and other visible events related to apoptosis) and thus could get away with the need of expensive florescent bio-markers. Plus, much more information can be obtained if the acquisition is frequent enough to produce a dynamic record of the events happening in each well. Analysis of cells movement and morphology can give clues of the phenotypes of T cells of interest [53], which, if made possible, would be a tremendous break-through in the field. Most importantly, the proposed experiment setup will provide a plethora of information to correlate *in vitro* and *in vivo* results in ACT clinical trials. Overall, there is no current known assay that can potentially provide this scope of information in one setup with high such high throughput (even when compared with the lowest number of events shown above, which is 645 in the case of 2584 TILs with P815 targets in the re-directed killing assay).

The more immediate goal is to simultaneously monitor the other important function of T cells: cytokine secretion. Cytokine is a substance served as the means of communication among T cells. Since, like all immune responses, cancer elimination is a concerted effort, it is not surprising that multiple-functionality T cells are more potent [54]. With the designed experiment where each T cell is isolated in a well, it is not far-fetched to find a reliable way of capturing the cytokines secreted in the assay.

Methodology

Vendors

The individual chemicals, reagents and materials as well as their respective vendors used in the described experiments are listed below.

- Fetal Bovine Serum (FBS) from Bio Master (Middleof, NW)
- RPMI-1640 in 500ml bottles from Cellgro (Manassas, VA)
- HEPES at 10nM from Sigma (St. Louis, MO)
- L-Glutamine at 2nM from Cellgro (Manassas, VA)
- Penicillin-Streptomycin at 50,000 U/50 mg from Cellgro (Manassas, VA)
- OKT3 at 1mg/ml from BioLegend (San Diago, CA)
- PBS buffer solution in 500 ml bottles from Cellgro (Manassas, VA)
- Insulin/Transferrin/Selenium at 100x concentration from Life Technologies (Carlsbad, CA)
- Gentamicin at 10mg/ml from Life Technologies (Carlsbad, CA)
- IL2 at 600,000 IU/ml from Thermo Fisher Scientific (Houston, TX)
- Glass slide 3 inch by 1 inch from Corning (Corning, NY)
- Teflon coat from UCT (Bristol, PA)
- Polydimethylsiloxane (PDMS) from Dow Corning (Midland, MI)
- Sylgard 184 silicon from Dow Corning (Midland, MI)

Cell sources

All cell lines used in this project were provided by M.D Anderson Cancer Research Center. All TILs were cultured and expanded by the REP protocols. Except for one indicated case,

the REP protocol used PBMC as feeder cells. All TILs, P815 cells, and autologous tumor cells were frozen and were thawed and cultured in M.D Anderson prior to handling.

Media

Media for P815 was made by mixing 500ml of RPMI 1640 medium with glutamax with 50ml fetal bovine serum (FBS). Since the medium had no antibiotics, it required more attentions in handling. The P815 was filtered using 200nm pore membrane. The TIL medium was made by mixing 500ml of RPMI 1640 and Glutamax with 50ml human AB serum, 500 µl of 2-mercaptoethanol, 5ml of HEPES, 5ml of Pyruvate, 5 ml of Glutamax and 5ml of PenStrep. The tumor media was made by mixing 500 ml of RPMI 1640 and Gutamax with 50 ml of FBS, 500 µl of gentimicin, 5 ml of HEPES, 5 ml of insulin/transferring/selenium and 5 ml of Penicillin-Streptomycin.

Washing and re-suspending

Washing was done by centrifuging a solution at 1300 rpm for 5 minutes. After that, the vessel was carefully transferred to the bio-safety cabinet to avoid cell perturbation. The residue solution was sucked out using a vacuum line leaving about 50 µl of solution left. The cell platelets were dispersed by shaking the vessel and/or by violently grating the vessel against a rough surface.

Cell counting

Cell counting was done via a hemocytometer. First, 10 µl of suspended cells was diluted by 10 µl of Tryphan Blue stock solution. The cell solution was put on the hemocytometer plate, which contains a grid with four counting areas located at four corners. The number of cells in a volume was determined by counting the number of cells, N, in one of the four counting area. Then the number of cell in a volume (ml) was calculated by

# of cells = N x volume x 20,000.

If the number of cells in a counting area was low (fewer than 20), all cells in four areas were counted. N is then calculated by dividing that number of cells by 4.

PDMS chips

The chip was made from PDMS and Sylgard 184 silicon elastomer, with the mixing ratio of 10: 1 respectively. The template, which was sandwiched by a metal scaffold and a 3 by 1 inch glass slide, used on soft lithography was made of silicone with a Teflon coating to facilitate removal. Before pouring the liquid mixture of PDMS and its elastomer into the template, bubbles and micro-bubbles must be removed. Otherwise, the resulted chips would be filled with air pockets trapped in the hardened PDMS, which made imagining impossible. This can be done by putting the mixture in vacuum for 1 hour. After the mixture became transparent, it was loaded into the mold. 2 hours of curing at 80C was needed to harden the PDMS which can be peeled off. The final result was a PDMS microarray chip on tip of a 3x1 inch glass slide.

TILs culturing

TILs were cultured in 70ml tissue culture flasks (Becton Dickson, Franklin Lakes, NJ) with ventilated caps at 37C and 5%CO2 with flasks aligned vertically. The initial cell concentration was 300,000cells/ml. The cells were fed twice weekly by IL-2. On feeding days, the cell concentration was checked to estimate the growth rate. Splitting was done to keep the concentration at about 300,000cells/ml. Before feeding, cells were washed in TIL medium. IL-2 was added with 1:600 dilution, making the final concentration 1,000IU/ml. The cells used in the experiments were harvested one day after feeding and are younger than a week counting on the day of acquisition.

P815 cells culturing

P815s were cultured in 70ml tissue culture flasks with ventilated caps at 37C and 5%CO2 with flasks aligned veritically. The initial cell concentration was 500,000cells/ml. Due to its robust growth rate, the cells concentration were diluted by 1:10 and cells were fed with new P815 medium three times per week. Because the media had no antibiotics, extra attention must be paid in handling these cells. Cell death rate was proportional to the culture population. Thus, it was of great importance to split the cells culture on a timely fashion. Cells used in the experiment were put in a separate flask with concentration 500,000cells/ml one day prior to the experiment.

Autologous cells culturing

Tumor cells were cultured in 250ml tissue flasks (Becton Dickinson, Franklin Lakes, NJ) with ventilated caps. The larger flasks were used due to the cell adherent nature which required more surface area for good growth. The flasks were aligned horizontally at 37C and 5% CO2. The initial cells concentration was 500,000cells/ml. The cells were fed with new medium twice per week. Cells were separated in another flask at 500,000cells/ml one day prior to the experiment.

Autologous cells detachment

The vessel was drained of liquid and washed with 10 ml of PBS twice. 10 ml of Trysin solution was then added in the vessel which was then incubated at 37 degree C and 5% CO2 for 15 minutes. After that, the suspended cells in the medium were washed in 10 ml of appropriate medium and re-suspended in an appropriate amount of media. For culturing, the concentration was 500,000 cells/ml. For experiments, the total number of cells was 2,000,000.

Suspended targets labeling with PKH26-red

The cells were washed twice in 10 ml RPMI medium to rid of floating proteins. 1 µl of PKH26-red was mixed with 250 µl of Diluent C. 250 µl of Diluent C was added to the cell suspension. Staining was allowed 3 minutes. After that, the staining was stopped by adding 10 ml of tumor media. The cells were then washed and concentrated to around 2,000,000cells/ml.

OKT3 incubation with P815 in re-directed killing assay

After labeling, OKT3 was added to the target solution at final concentration of 1µg/ml. The total volume was around 1 ml. The incubation lasted 30 minutes at 37C and 5% CO2.

TILs labeling with PKH67-green

The cells were washed twice in 10 ml RPMI medium to rid of floating proteins. 1 µl of PKH67-green was mixed with 250 µl of Diluent C. 250 µl of Diluent C was added to the cell suspension. Staining was allowed 3 minutes. After that, the staining was stopped by adding 10 ml of tumor media. The cells were then washed and concentrated to around 2,000,000cells/ml.

Cell loading

The PDMS slide was first oxidized in a plasma chamber to make it hydrophilic. The plasma oxidation process was one minute long and was done with a Harrick plasma cleaner model PDC-32G running at the highest setting. The hydrophilic state was kept by submerging the slide in sterilized PBS. Then the chip was secured in a 4-well plate by 5% agarose and DI water solution. The PBS solution was aspirated prior to loading the cells. TILs were loaded first in each drop of 200 µL with 1,000,000cells/ml. They were then let settled down for about 5 minutes, depending on the how hydrophilic the PDMS chip was (which can be estimated by how easily a droplet spreads). After that, the chip was checked under a microscope to see if it had the desired loading frequency, which was around one TIL for each well. The excessive TILs were

washed away with 1ml of tumor media. When the desired loading frequency was observed, targets are loaded in 200 µl droplets with 2,000,000cells/ml. The chip was let settled for 5 minutes. The desired frequency of targets was about one to two per well. The excessive targets were washed away with tumor media.

Annexin V

A dilution of Annexin V was made by adding 1:60 volume of Annexin V in P815 media for re-direct killing assay and in tumor media for autologous killing assay. For each chip, 3 mL of Annexin V dilution was needed.

Imaging

The chip was imaged by an epifluorescence microscope (ZEISS). Four channels for image acquisition were used: brightfield for general visualization, cy5 for Annexin V- Alexa Flour 647, TxsRed for PKH67-red and FITC for PKH26-green. After the first imaging, the chip went back to the incubator at 37^{o}C and 5% CO_2 for 6 hours, after which the second round of imaging started. The resulted data were analyzed using a computer program.

Data analysis

The basis of data analysis was converting image into digital data (by a custom program) and using those images for quality control. The four-channel microscopy images were used to make three histograms corresponding to three different fluorescent channels. Activities were monitored by the intensities of each fluorescent channel. The results can be constructed into histograms, from which signals cut-off can be determined. Target cells in each well were those with valid TxsRed signal intensities. TILs were those with valid FITC signal intensities. Dead cells were those with valid Cy5 signal intensities. The output of raw data was in form of excel spreadsheets. The main analysis was done in Microsoft Access, which was a data base matching

tool. Each cell in the result had an associated ID, whichdetermined the well it belongs to. The number of targets per well was easily determined by finding the number of valid target cells with the same ID. The limit was set to be lesser than four. Similarly, the number of TIL per well was determined by finding valid TIL with same IDs. The limit number of TILs per well was one. Valid wells were found by matching the ID of single TILs with those of targets. The result was an array containing the ID of wells and the number of targets it contained (all wells have 1 TIL). Dead events were found in the same manner. Dead cells with duplicated IDs were sorted into wells. By matching the IDs of valid wells with those of dead wells, a list of wells with unique well ID and associated number of targets and the number of targets were established. The final step was to count the number of desired events. Finally, a frequency, **q**, calculated by the ratio of death events and the total number of events, was reported. Each death event corresponds to one well. There were three types of events: 1-dead-target events, 2-dead-target events and 3-dead-target events. For example, q, the frequency of TILs that can only kill one target in wells with 1 TIL and 3 targets, were calculated by dividing the number of 1-dead-target events in wells with 3 targets and 1 TIL by the number of wells with 3 targets and 1 TIL. The results of q were then graphed in the fashion showed in the result section.

The statistical significance of q was calculated by assuming a normal distribution of q, with 95% confidence interval associated with a z-score of 1.96. The error bars are then formulated by

$$errors = q \ \pm 1.96 \sqrt{\frac{q(1-q)}{\#of\ events}}.$$

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