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Simulating an Immune Response with a Combined Agent-Based Model of a Triple-Negative Breast Cancer Tumor and Vascular Network

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Introduction

1. Cancer

Research has aimed to categorize a series of biological capabilities essential for the long-term viability of a tumor. Simply recognizing the capabilities is not an end in itself; these hallmark capabilities act as targets for specific therapies with the goal of reducing the threat of the tumor. One cornerstone paper has claimed there are eight hallmarks of cancer that enable tumor growth and metastatic dissemination(Hanahan & Weinberg 2011). These include sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, deregulating cellular energetics, and avoiding immune destruction. In this study, I focus on the last emerging hallmark, the immune destruction of cancer cells in the form of cytotoxic T-cells.

The first of the eight hallmarks is sustaining proliferative signaling (factors that signal for tumor cell reproduction)(Hanahan & Weinberg 2011). Cell signaling refers to the mechanisms used by cells to communicate with each other(Solomon, et al. 112-113). There are several key steps in the process of cell signaling, including the synthesis and release of signaling molecules, transport (of signaling molecules) to the target cell, reception of information by the target cell, and the response of the target cell to the information received(Solomon, et al. 112-113). Tumor expansion can be enabled either

through the sustained production and release of growth promoting signals (Hanahan & Weinberg 2011), or through the stimulation of non-cancerous cells to contribute growth factors(Cheng, et al. 2008; Bhowmick et al. 2004). In addition, the amount of proteins that act as receptors to the growth factor (on the cell surface) can be increased, leading to a response similar to that of increasing the growth factor (without any increase in growth factor levels).

Even with sustained proliferative signaling in effect, the tumor cannot expand continually unless it can evade several different suppressors and programmed cell death. One such growth suppressor is the retinoblastoma protein (Rb), which shuts down the reproductive process if the cell signals to divide while not in the correct reproductive stage(Burkhart & Sage 2008). Another is the transformation-related protein 53 (TP53), which signals for a cell to undergo apoptosis (programmed cell death) if the cell's DNA is irreversibly damaged, or signals for repair if damage is minimal(Hanahan & Weinberg 2011). Certain point mutations affect the genes associated with tumor suppression in these proteins, limiting or eliminating their tumor suppressing qualities, which allows the tumor to evade programmed cell death and continue expansion.

Another cancer hallmark is reprogramming the energy metabolism to fuel increased cell production(Hanahan & Weinberg 2011). Under non-cancerous and normoxic (normal amounts of oxygen present) conditions, cells convert glucose to

pyruvate through a process called glycolysis. This pyruvate is then oxidized in the mitochondria, using oxygen to fuel the process(Hanahan & Weinberg 2011). If there is not enough oxygen present, however, cells will undergo glycolysis through fermentation instead of sending pyruvate to the mitochondria (Warburg 1956). This process can create energy when there is not enough oxygen to create the required energy through respiration, reducing the metabolic processes' dependency on oxygen.

Despite using less oxygen in energy production, tumor cells still need a supply of oxygen and nutrients to survive. While very small tumors in their initial stages can rely on existing vasculature, after the tumor radius reaches ~200 microns, the peripheral cancerous cells cannot be reached by the diffusion of oxygen and become hypoxic (the state of lacking oxygen)(Hanahan & Weinberg 2011). When hypoxia occurs, cancer cells signal for vasculature expansion to these regions in the form of angiogenesis(Ferrara 2009).

To continue to grow, cancer cells must activate invasion, the spreading of cancer cells to outside of the basement membrane, and metastasis, the development of secondary growths at other sites(Hanahan & Weinberg 2011). This process is referred to as the invasion-metastasis cascade, which begins with intravasation, or the invasion of cancerous cells into either blood or lymphatic vessels(Talmadge & Fidler, 2010). Cancer cells then travel through either the blood or lymphatic systems before undergoing extravasation, when the cancer cells leave the vessel. In the new space they

form nodules, which under favorable (or rather detrimental to the host) circumstances can grow into macroscopic growths.

Another hallmark of cancer is enabling replicative immortality, or the ability for cancer cells to divide indefinitely(Hanahan & Weinberg 2011). To reach macroscopic sizes, cancer cells need to be able to reproduce but are limited in that each individual cell can only divide a limited number of times. The limiting factor is the telomeres at the ends of each chromosome. With each cell division, telomeres shorten until there is not enough to protect the ends of the chromosomal DNA(Blasco 2005). At this stage, the cell becomes senescent (no longer able to grow or divide). Cancer stem cells (CSCs) have a key role in replicative immortality, since they can create progenitors with full telomeres (Blasco 2005).

Finally, the last hallmark, evading immune system responses, is the focus of this study(Hanahan & Weinberg 2011). The immune system can send various types of T-cells to the tumor site, with each type fulfilling a unique role. This study looks at such T-cells, specifically the 'killer' (CD8+) variety.

2. Angiogenesis

Angiogenesis is the creation of new blood vessels. When a tumor reaches roughly 200 microns in diameter, cells become hypoxic due to the lack of oxygen caused by the limited range of the diffusion of blood. In response, the so coined 'angiogenic

switch' is activated by cancer cells through growth factor signaling, which recruits blood vessels to supply oxygen to the hypoxic cells (Hanahan & Folkman 1996). This switch is controlled through a balance of chemical factors that bind to endothelial receptors, most notably vasculature endothelial growth factor-A (VEGF) and thrombospondin-1 (TSP-1) (Baeriswyl & Christofori 2009, Bergers & Benjamin 2003). TSP-1 is known to naturally inhibit angiogenesis, while VEGF signals for angiogenesis to occur. In most non-cancerous conditions, the angiogenic switch is maintained in its "off" state by an overabundance of growth suppressing factors (including TSP-1) in comparison to negligible amounts of growth factors (such as VEGF)(Baeriswyl & Christofori 2009). In cancerous conditions however, the angiogenic switch is kept in its "on" position through the presence of various factors, such as VEGF, in order to provide vital oxygen and nutrients to the expanding tumor. In addition to a hypoxic environment, genetic mutations may also cause the VEGF gene to be over-expressed, leading to the release of VEGF from normoxic cells(Ferrara 2009, Gabhann & Popel 2008; Carmeliet 2005).

Interestingly, once angiogenesis occurs in a tumor microenvironment, the rate of vascular growth proves inconsistent. Newly formed vasculature often contains prematurely produced sprouts, abnormally high amounts of branching, enlarged vessels, microhemorrhaging, and leaks(Baeriswyl & Christofori 2009; Bergers & Benjamin 2003). A collection of factors in the stromal microenvironment including hypoxic cells, growth

factors, chemokines, and immune cells, control the amount of VEGF the endothelial cells are exposed to(Hanahan & Weinberg 2011). Variances within the microenvironment result in differing rates of vasculature expansion, resulting in a vastly varying vascular structures(Baeriswyl & Christofori 2009). While non-cancerous vascular networks are well organized and efficient, tumor-induced angiogenesis form irregular and inefficient systems (Baluk, et al. 2005; Nagy, et al. 2010).

Angiogenesis commences with a sprout comprised of tip cells, stalk cells, and phalanx cells, (Norton & Popel 2016) which migrate toward the area with the highest concentration of VEGF(Vempati, et al. 2014). Stalk cells are directly behind the tip cell and can proliferate, extending the sprout and increasing the diameter of the capillary (Norton & Popel 2016). Phalanx cells account for the remaining cells in the chain; once a stalk cell proliferates, the newly formed cell is a stalk cell and the old stalk becomes a phalanx. The newly formed vascular segment continues to grow until it anastomoses, connecting two immature tips, or a tip to a mature capillary, to form a completed vascular circuit through which blood flows.

3. Cancer Stem Cells

Cancer Stem Cells (CSCs) are perhaps the most important subtype of cancer cells and have been described as the foundation of the disease (Clarke, et al. 2006). They are defined functionally by their ability to recapitulate the generation of a continuously

growing tumor (Clarke, et al. 2006). This cornerstone ability stems from the different ways CSCs divide - they can undergo either a symmetric or an asymmetric division. Symmetric divisions result in two daughter cancer stem cells, each of which retain the key ability of self-renewal (dividing to create more stem cells). Conversely, an asymmetric division yields one cancer stem cell and one progenitor cell. Stem cells can divide an essentially "unlimited" amount of times since they are not limited in the same way progenitors are (Clarke, et al. 2006). Telomeres are short, repeating structures found at the ends of chromosomes that are responsible for protecting the ends of the chromosomal DNA from end-to-end fusions(Blasco 2005). Progenitors lose some telomeres with each division until there is not enough to replicate further without risk of genetic damage; this puts them into a state of senescence (where the cell can no longer divide or grow) (Blasco 2005). Telomerase is a specialized DNA polymerase that adds telomere segments to the ends of telomeric DNA(Blasco 2005). Present in CSCs, telomerase allows CSCs to reproduce an (essentially) unlimited number of times. This is the essence of the hallmark "enabling replicative immortality"; if not for the CSC's ability to divide without limit, the tumor could not proliferate after each progenitor had reached their reproductive limit. The CSCs are what create new progenitors with full telomeres enabling tumor expansion (Clarke, et al. 2006).

4. Macrophages

Tumor associated macrophages (TAMs) are an essential cell type found within the tumor stroma that promote tumor growth and lead to a lower overall survival rate among TNBC patients (Shih, et al. 2006). One key function of TAMs is regulating angiogenesis (the creation of new blood vessels) by producing and releasing angiogenetic factors, or by producing proteases that degrade the extracellular matrix and release angiogenetic factors (Condeelis, et al. 2006). TAMs can also modify the paths of existing vasculature to form more coherent blood flow; in normal (non-cancerous) conditions this ability is used for routing and/or expanding vasculature to heal wounds(Condeelis, et al. 2006). In addition to directly affecting tumorigenesis, TAMs promote chronic inflammation, which plays a key role in the infiltration of T-cells to the tumor stroma (Coussens and Werb 2002). They also have been known to increase the movement speed of tumor cells, increasing the rate of invasion (Condeelis, et al. 2006).

Macrophage colony-stimulating factor 1 (CSF1) is a cytokine released by breast cancer cells that is a primary influence on macrophage recruitment (Riabov, et al. 2014). Another factor that promotes macrophage recruitment is vascular endothelial growth factor (VEGF), which is the protein that also signals for angiogenesis to occur (Riabov, et al. 2014). This creates a correlation between areas with a high density of

macrophages and areas where intense angiogenesis occurs(Condeelis, et al. 2006), since VEGF is a factor that signals for both macrophage recruitment and angiogenesis.

5. CCR5+ Progenitors

C-C chemokine receptor 5 (CCR5) is a protein that acts as a receptor for chemokine proteins. Cancer cells secrete interleukin 6 (IL6) which 'educate' lymphatic cells to in turn produce increased amounts of C-C chemokine ligand 5 (CCL5)(Lee 2014). Progenitors then migrate and proliferate at a higher rate due to the information brought by the CCL5 through the cell's CCR5 receptors(Lee 2014). These signals are only received by the progenitor if it has enough CCR5 receptors; A TNBC cell expressing a high amount of CCR5 receptors is considered CCR5+, while a lack of CCR5 receptors makes the cell CCR5-. In CCR5+ progenitors, the CCL5 signals for increased migration and proliferation rates of the progenitor, and about 6% of total progenitors are found to be CCR5+(Norton, et al. 2017).

6. Triple-Negative Breast Cancer

Breast cancer is a particularly common type of cancer, with one in eight women developing it in their lifespans ("Triple-Negative Breast Cancer: Overview, Treatment, and More" 2019). It is the most common cancer in women, and leads to the highest death rate among Hispanic women and second highest among Asian, American Indian, black, and white women ("Triple-Negative Breast Cancer: Overview, Treatment, and

More" 2019). Triple-Negative Breast Cancer (TNBC) is a subtype found in roughly one in six breast cancer patens (Foulkes, et al. 2010). TNBC tumors are defined by a lack of three critical receptors: estrogen receptor (ER), progesterone receptor (PR), and HER2 (Foulkes, et al. 2010). This lack of receptors renders endocrine (hormone) and trastuzumab therapies ineffective (Foulkes, et al. 2010). The accepted common choice for treatment is chemotherapy, but the survival rates for TNBC are lower than that of non-TNBC breast cancers when treated with neoadjuvant chemotherapy (Foulkes, et al. 2010). Surprisingly, this is despite a higher rate of complete pathological responses among TNBC patients (Foulkes, et al. 2010).

7. T-Cells

While many believe in the possibility of a 'magic bullet' that could eliminate the threat of cancer, the scientific community has made great strides in systematically breaking down and understanding the seemingly innumerable mechanisms that are altered in cancer. One hallmark of cancer is avoiding immune destruction, and one of such immune responses to cancer is sending cytotoxic T-lymphocytes (CTLs), also known as killer T-cells, tumor infiltrating lymphocytes (TILs), or CD8+ T-cells, to the tumor(Hanahan & Weinberg 2011). These CTLs differentiate into their specialized form, infiltrate the tumor site, and kill cancerous cells through apoptosis. Studies show a correlation between the amount of CTLs that infiltrate to the tumor site and a better prognosis in colon and ovarian cancers (Pagès, et al. 2005; Zhang, et al. 2013).

The T-cells' process begins outside the tumor area with T-cell differentiation, a transition into a new specialized form through alterations of the genetic code. Under normal conditions, so called naïve (non-differentiated) T-cells flow through the lymphatic vessels from lymph node to lymph node, ready to be dispatched wherever needed. These lymphatic vessels form a system comparable in many aspects to the circulatory system, transporting immune cells in a clear fluid called lymph. The T-cells' natural migration is interrupted when a toxic or foreign substance called an antigen is detected. Antigens are brought to the lymphatic site by dendritic cells so the T-cells can differentiate into different types, one of which being the CD8+ cytotoxic variety(Banchereau, et al. 2000). Along with the antigen, cytokines (a protein produced by cells to impact other cells in different ways) act as differentiation factors, which influence which kind of specialized T-cell the naïve one will become (Banchereau, et al. 2000). One such cytokines is IL-10, which promotes naïve T-cells to become cytotoxic effector T-cells(CTLs) (Banchereau, et al. 2000). With the T-cells differentiated into their specific roles, the next process is relocating them to the site of the antigens.

There are several factors that lead the CTLs to the areas they are needed. One study found that CD8+ T-cells respond to inflammatory cytokines and similarly move toward sites of high inflammation (Weninger, et al. 2001). Cytotoxic T-cells are actually twelve times more efficient at migrating toward inflamed tissue than their memory T-cell counterparts(Weninger, et al. 2001). This is crucial, with tumor-promoting

inflammation being an enabling characteristic of tumor longevity(Hanahan & Weinberg 2011). It has been long known that the body sends immune cells to neoplastic growths, causing inflammation to eradicate the lesion(Dvorak 1986). Although it has been found this acute inflammation actually enhances tumorigenesis and progression(Hanahan & Weinberg 2011), it also plays the reverse role in directing T-cell invasions to tumor sites(Weninger, et al. 2001).

Once inside the tumor, CTLs do not stop moving. A study has shown that although CTLs hone into the problem area, they move randomly within the tumor(Mrass, Paulus, et al. 2006). The speed at which they move varies quite a bit however, with sources claiming anywhere from 6 to 25 microns/minute(Kim and Lee 2012; Boissonnas, et al. 2007). In a straight-line race, this would be up to 60 times as fast as a macrophage. This increased movement speed does not correlate to a large displacement over time however, as the average displacement for a 24-hour period is only 21 microns(Kim and Lee 2012).

Having infiltrated the tumor, the T-cells can kill cells that are dysfunctional or infected in a variety of different ways. CD8+ T-cells specifically utilize the perforin/granzyme system to induce apoptosis in the target cell(Boissonnas, et al. 2007). Granzyme is a protease released by granules in the cytoplasm of the CTLs that trigger apoptosis. Perforin is a protein that acts as a vehicle and is necessary in transferring granzyme to the target cell. During a process called degranulation, perforin and

granzyme are released from the CTL and move together before binding with the target cell's surface(Boissonnas, et al. 2007). Here perforin takes on a secondary role, assisting in internalizing the granzyme in the target by forming a channel in the membrane(Boissonnas, et al. 2007). It is noteworthy that there are five different types of granzyme found in humans, A, B, H, K, and M, but A and B are the most ubiquitous and well-studied(Boissonnas, et al. 2007). Both granzyme A and B kill target cells through DNA alteration, with B ultimately causing DNA fragmentation and A targeting sections associated with DNA repair(Boissonnas, et al. 2007).

8. Computational Modeling

Considering the high costs and lengthy procedures associated with *in vivo* and clinical research, computational modeling offers a cheaper vehicle for scientific research without the risk of harming live subjects. Computational modeling is one method of recreating or simulating real systems and interactions. One advantage of modeling is there is less non-explainable error that occurs in a computer model. Samples cannot be contaminated (aside from human made bugs in the code), and there is no approximation in the counting of things like cells. Models are often more time efficient as well; our model simulates tumor growth over 75 days, but even on non-supercomputer it takes only ~8 hours to run. The computer also takes away some randomness, as the same experiment twice (given the same seed) will produce identical results. A problem with computational modeling, however, is there could be systems *in*

vivo that are not understood yet. When a model is created it could give results that do not account for these unknowns that real trials always do (following the laws of nature).

In constructing a computational model there are numerous ways to simulate an experiment, but these overall approaches can be categorized into two distinct types: discrete and continuous. What separates the two are how the variables (including time) is represented. In a discrete model, the variable is broken up into discrete periods or sections, where a continuous model flows through units continuously(Hall 1986). The most common continuous scientific models are rooted in differential equations, since time is a dimension that is differentiable. These models excel at showing general trends in the data over a given time period, whereas discrete models are better suited for applications where time or the subject can be divided into discrete parts.

An Agent-based model is a type of discrete model and is comprised of a number of individual agents, with time passing in discrete periods. Each agent acts independently, assessing the current environment and making decisions based on a set of rules. The focus of an agent-based model is the interactions between agents, which can highlight key patterns a continuous model cannot represent(Axelrod 1997). They can also show unexpected behaviors or trends of the population, as each individual agent makes its own decisions(Bonabeau 2002). Using an ABM requires a different paradigm of thought as compared to other types of modeling. In the case of the ABM, researchers need not concern themselves with how the system works over time; instead,

the focus is on specific small-scale agent to agent interactions. While ODEs model the macro-interactions, an ABM models the micro-interactions.

As compared to continuous models, ABMs offer several distinct advantages: capturing emergent phenomena; providing a natural description of the system; and being very flexible(Bonabeau 2002). an ABM can capture trends created by individual components but decoupled from the traits of such components. An example of this is how traffic is caused by individual agents (drivers in their cars), but the location where traffic is densest can move in the opposite direction the cars are traveling(Bonabeau 2002). This movement of the system is actually opposite from the movement of each individual agent. While difficult to describe in equations, an ABM can easily show the occurrence of such phenomena, and how simple actions of agents can snowball into large-scale trends. ABMs also provide a more natural description of the system when the agents are 'behavioral' entities(Bonabeau 2002). In the case of cancer cells, each cell acts behaviorally, migrating, proliferating, and taking other actions in relation to the cells around themselves. An ABM is an efficient way to model such complex behaviors and interactions, as equations' complexities increase exponentially with the individual's complexities(Bonabeau 2002). The ABM is also a very flexible method of modeling. Everything from numbers of agents to their specific traits and behaviors can be adjusted. For instance, changing the parameters in the case of a T-cell insertion *in vivo* would mean acquiring new lab mice, preparing them with an insertion of tumor cells,

waiting for the tumor to reach a desired size, and then inserting the desired number of T-cells. If the number of T-cells were to be changed, this process would have to be repeated, where the parameter could be changed within seconds in an *in silico* trial. This effect compounds if the parameter space is multi-dimensional.

In this project I expand upon an agent-based model of a Triple-Negative Breast Cancer (TNBC) tumor by including a therapy that targets one key ability of cancer longevity by injecting cytotoxic T-cells into the system. Within this model I simulate a CTL therapy, to highlight their effect on tumor progression.

Methods

1. TNBC Model:

In this project, I continue development of a combined agent-based model of triple-negative breast cancer and angiogenesis, based on several previous studies (Norton & Popel 2016, Norton, et al. 2017, Norton, et al. 2018). The model includes a vascular network, cancerous progenitor cells, cancer stem cells (CSCs), tumor associated macrophages (TAMs), and CCR5+ cells. I specifically contribute a new module to the model, adding another type of cell to the environment, the CD8+ cytotoxic T-cell..

2. Initial Setup:

This model operates in a simulated 1-millimeter cubed (1000 micron cubed) space, which is represented by two separate grids. The cellular grid is of size 50 x 50 x 50 with each voxel representing the space needed to fit one cell, a 20 x 20 x 20 micron-cubed volume. It initially contains 350 individual cells, 250 of which are stromal TAMs placed randomly throughout the space. The tumor is comprised of the remaining 100 cells, divided into 80 cancerous progenitors and 20 CSCs. They are initially arranged into a small box in one corner, and of these tumor cells, 1 stem and 5 progenitor cells are declared as CCR5+.

A separate grid of size 1000 x 1000 x 1000 contains the vascular network, with each voxel representing a 2 x 2 x 2 micron cubed space. The initial 'host vasculature' is

comprised of 8 capillaries that run vertically along the edges of the space. Each capillary is 10 microns in diameter and remain in a fixed position throughout the simulation. 6 of such capillaries span the entirety of the z axis; 3 lay on the x axis and 3 on the y axis. On each axis (x and y), one capillary is placed toward either side with one roughly bisecting the axis. The remaining two capillaries are simply branches stemming from another capillary.

Each capillary in the host vasculature is comprised of 20 linked endothelial cells which can branch to form new vasculature. All vasculature created during the simulation can be traced back to an initial capillary. We assume the vasculature grid can overlap with the cellular grid due to interstitial space and the fact that cells in a tumor compress, allowing the relatively small vessels to fit in-between cells.

3. Model Iterations

After completing initial setup tasks the model can begin, running for 300 iterations; each iteration is approximately 6 hours in real time. Each iteration begins with the CD8+ T-cell module (if they are present in the space), followed by macrophage migration, angiogenesis module, and tumor cell module. Finally, macrophages can infiltrate, and apoptosis occurs before looping back for another iteration. Each simulation was conducted on MATLAB R2018a.

4. Macrophage Migration (Norton et al.)

Macrophages migrate through the tumor space in differing ways; separated from the tumor, a macrophage migrates with random trajectories at a rate of 3.33 microns an hour. However, if a cancerous progenitor cell is within a 200 micron range of a macrophage, the macrophage will migrate toward the closest progenitor with an increased speed of 26.67 microns per hour. This attraction is due to a factor (CSF-1) released by cancerous cells. Macrophages will migrate until they reach the edge of the cellular grid and can only migrate into an empty space.

5. Angiogenesis Module (Norton et al.)

The angiogenesis module of the model is the most involved section, beginning with the tip cells' actions. They can migrate and if applicable, proliferate. The stalk cells act next, proliferating if able. Anastomosis may occur during either the tip or stalk cells' turns if a viable target is found. Finally, the branching of a new sprout can occur under the correct conditions.

a. Tip Cells

Tip cells undergo two main processes each iteration (if applicable): proliferation and migration. Each tip cell can only proliferate once, and the timing of the proliferation is determined by a cell clock. Once the tip cell has reached the right period of its cell cycle (7 iterations or 42 hours) it proliferates, becoming a new stalk cell in the

same location. A new tip cell is then created in the direction the proliferating tip was facing, but this new tip cell does not reset the cell cycle and allow for another tip proliferation. This new tip cell can migrate toward the highest concentration of vascular endothelial growth factor (VEGF), which is produced in equal amounts by cancerous cells and macrophages in the model. Each progenitor releases 20 nanograms per milliliter of VEGF into the space it occupies. During migration, tip cells check the surrounding 27 spaces in the cell grid and migrate in the direction of the highest VEGF concentration. Tip cells cannot migrate off the grid or in a direction backwards from where they were facing. The amount of VEGF in the space also plays a role in determining the distance the tip cell may migrate, but the tip cell cannot migrate more than 150% of its current length or more than 60 microns. Once migration is completed, the tip checks for a possible target to anastomose with.

b. Stalk Cells

Tip cells are limited to a single proliferation, thus, stalk cell proliferation allows the vasculature to continue growing (stalk cells also do not migrate). Stalk cell proliferation is also based on a cell clock; when the stalk cell is in the right phase of its cell cycle (6 iterations or 36 hours), it proliferates. A new stalk cell is created in the direction the previous one was headed. Its cell clock is reset allowing for multiple rounds of proliferation. The original stalk cell becomes a phalanx cell which no longer proliferates or migrates. Similarly to tip cells, the stalk cells cannot proliferate if the tip

cell will be forced outside the cellular grid. After a successful proliferation, the tip cell checks for a possible anastomosis target.

c. Anastomosis

When a tip cell migrates and is pushed within range of either another tip cell or other endothelial cell anastomosis can occur, fusing the two sections of vasculature together to make one mature capillary. Once anastomosis occurs, tip cells of both joining halves become deactivated and the cells that comprise the new capillary can no longer proliferate or migrate. Mature vasculature has blood flowing through it, bringing nearby cells out of a hypoxic state.

d. Sprouting/Branching

Branching, or the creation of a new sprout, occurs only within the cells comprising mature blood vessels. If the segment is within 250 microns of a hypoxic cell, there is a 20% chance for sprouting to occur each iteration. If triggered, the new sprout will form out of the existing blood vessel, facing toward the nearest hypoxic progenitor. This new sprout is a tip cell, and once it branches from the capillary the two surrounding cells can no longer branch.

6. Tumor Cell Module (Norton et al.)

The tumor cell module encompasses several different cell types, and several different processes for each cells to complete. The tumor is comprised of both progenitors (CCR5- and CCR5+), and CSCs, which both migrate and proliferate. Progenitor cells can become hypoxic (in a state of lacking oxygen) under correct conditions, while CSCs are considered immune.

a. Progenitors

While the simulation begins with only 80 progenitor cells, they very quickly become the most ubiquitously found cell in the simulation. This is mainly because of their relatively quick rate of proliferation, which occurs once every 2 days. Being within a 200 micron range of a macrophage further increases progenitor proliferation rate by 3.5-fold. The limiting factor of progenitors is not the rate at which they can reproduce, it is the number of divisions they can undergo. Each progenitor has only enough telomeres for 12 divisions before it can no longer reproduce, and upon division this number is not reset for its child. For instance, if a new progenitor divides, both children progenitors will have 11 divisions remaining each. During proliferation, each new cell has a 6% chance to be CCR5+. Progenitors migrate relatively slowly (at .83 microns per hour) and migrate randomly. If migration occurs, the progenitor will check the surrounding 26 cell spaces and randomly choose an empty one to migrate into. While

the migration of progenitors is relatively slow, there are several factors that increase their migration rate. If the progenitor is within a 200 micron range of a macrophage it will migrate at 2.5 times as quickly, and if it is CCR5+ it will migrate 10 times as fast as a CCR5- progenitor.

b. Hypoxia

Progenitor cells are affected by hypoxia and will enter a hypoxic state if further than 200 microns from the nearest active blood vessel. Hypoxic progenitor cells migrate at 3 times the normal rate but proliferate at half the normal rate. At the end of each iteration, 10% of hypoxic cells are killed off by apoptosis. CSCs, TAMs, and CTLs are unaffected by hypoxia in this model.

c. CSCs

Cancer stem cells share many properties with their progenitor counterparts, including migration and proliferation; they differ in several key areas, however. A majority of CSC division is asymmetric, resulting in one CSC and one progenitor cell. Newly created progenitors begin with the maximum amount of possible divisions. CSC symmetric division occurs roughly once every 5 days, producing 2 daughter CSCs, and stem cell proliferation is not limited by telomeres. Stem cells are also not killed by hypoxia in this simulation, and the only way they can die is through a CD8+ T-cell killing it off directly.

7. Macrophage Infiltration (Norton et al.)

Macrophages enter the simulation through mature vasculature, and are attracted to factors secreted by tumor cells. If there is a cancer cell within a 200 micron range of a mature blood vessel, there is a 60% chance a macrophage is recruited into the space. First, we check to ensure at least one of the 27 spaces in the cellular grid around the endothelial cell is empty, then the macrophage will choose one randomly to infiltrate into.

8. Apoptosis

At the end of each iteration apoptosis occurs if there are hypoxic or senescent cells. 10% of the total hypoxic and 10% of senescent cells are eliminated.

9. T-cell Module (Ventoso)

This version of the TNBC model is differentiated from previous editions by the introduction of the T-cell module. Initially the model is devoid of any T-cells, allowing the tumor to proliferate. After a predetermined 200 iterations (50 days), a certain number of TILs (either 10 or 25) are inserted into the simulation. The TILs are inserted randomly into the tumor space, and act at the beginning of every iteration. During each iteration we loop through every individual T-cell in a random order and depending on several factors, each can undergo four main processes: migration; killing of nearby cancerous cells; proliferation; and undergoing apoptosis.

Each T-cell migrates through the space randomly. The average total displacement for a T-cell is ~20 microns per day, so each iteration we give each T-cell a 25% chance of migrating one 20-micron space away each 6-hour iteration. If the migration occurs, the cell will check all of the surrounding spaces in the agent matrix and store a list of all which are empty. It then randomly chooses one space from the list of empty spaces to migrate into.

Next, each CTL on average kills one cancer cell per day. In the model, each CTL has a 25% chance every 6-hour iteration to kill a nearby cancerous cell. After checking whether it will kill, the T-cell must check for applicable targets. A function returns a list of any progenitor or cancer stem cell adjacent to the CTL, and if the list is not empty, a random cell is chosen from the list. That cell is then killed through apoptosis.

CTLs can also proliferate at an average rate of one division per 8 hours. Since this equates to an average of three divisions a day, for each 6-hour iteration we give each T-cell a 75% chance of proliferation. If the T-cell will proliferate this iteration, it first checks the surrounding spaces for an empty cell. If an empty space is available, a random point is chosen out of all possible empty spaces, and a new CTL is inserted into the simulation at the chosen coordinates. Care is taken to ensure the newly formed CTL does not take any actions, nor does its cell clock start counting, until the following iteration. Considering the relatively low death rate, CTLs would quickly overwhelm the model if not for a limit on proliferation. The period of proliferation for T-cells is

only 4 days. After those 16 iterations no more T-cell proliferation can occur, and the remaining T-cells simply finish out their existences. This in turn, leads to a very rapid (exponential) growth of CTLs with a more moderate descending taper.

Finally, T-cells have a lifespan of ~2 days, which is represented in the model as 8 six-hour iterations. A timer keeps track of how much time each T-cell has left, with new CTLs starting at 8 and counting down to 0. If a CTL's cell clock reaches 0, it is marked for apoptosis and killed at the end of the T-cell module.

10. Statistical Analysis

First, to ensure all data is normally distributed, I did several Shapiro-Wilk normality tests as shown in Table 2 at the end of the Results. With an alpha of .05 each test suggested normality among the data sets, therefore allowing all other tests to assume normality (data is suggested to be normal when W is greater than the critical value).

To compare how the treatments affected the tumor size (measured in number of cancer cells) at the end of each trial, I performed a one-way ANOVA. Finding significant differences, I performed a Tukey Honestly Significant Difference test as post hoc. In calculating differences between the two treatments, I used a one-tailed independent sample Student's T-tests. For looking at how effective the (random) killing of a CSC is, I performed several Pearson correlations tests. In these tests, the two populations being compared span across the parameter space, as the number of T-cells

is not strictly dictated by the number of CTLs. In plotting the correlation data, I also performed linear regressions.

Results

I developed a T-cell module for an agent-based model of triple-negative breast cancer. To show the effects of increasing the number of T-cells in the tumor, I ran simulations varying the number of T-cells inserted. For one simulation, 10 CTLs were inserted at iteration 200 (the 50th day). In the next, 25 CTLs were inserted at iteration 200. Finally, a control simulation was conducted with no CTL insertion. Each simulation consisted of 6 trials with random seeds.

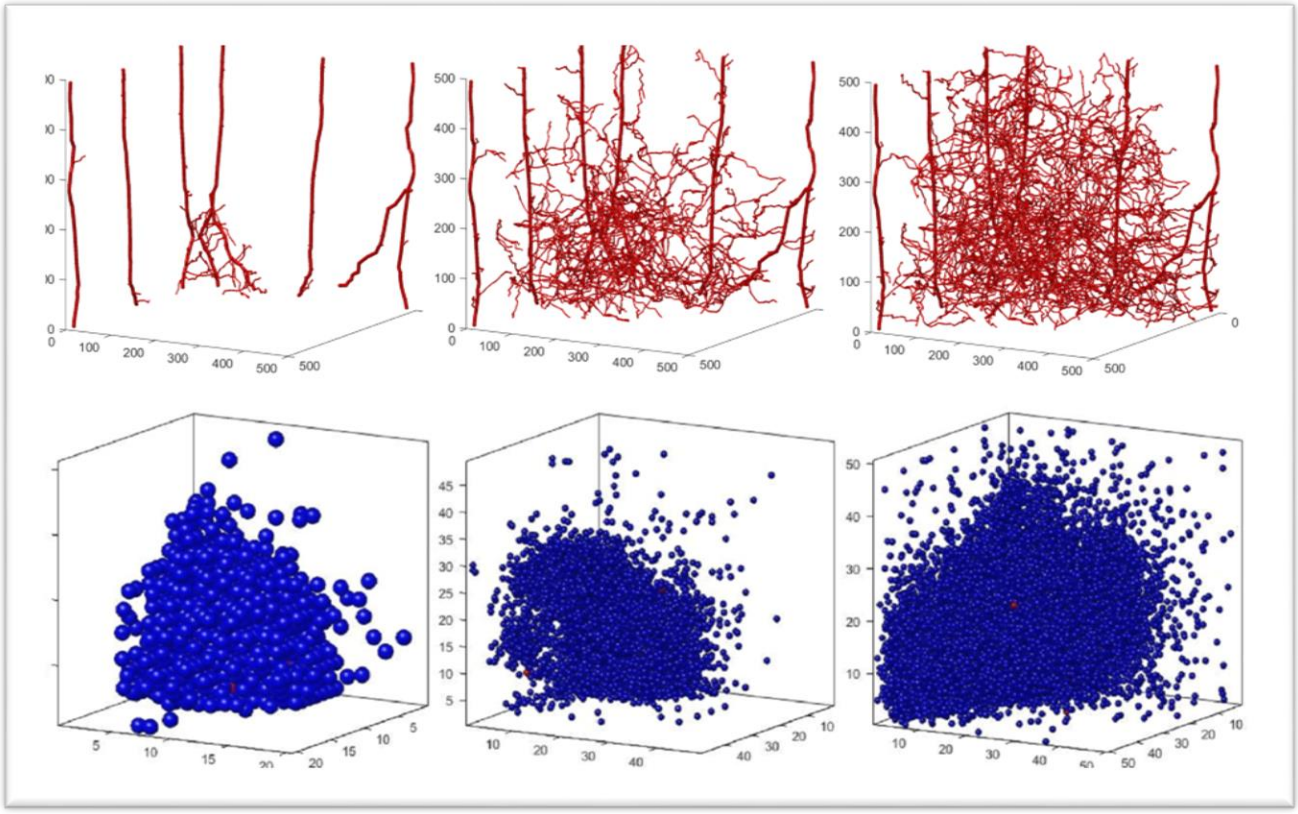


Figure 1. The top row depicts an example of vascular growth over time, while the bottom row shows the tumor's growth over the same time frame. Blue cells are progenitors, while red cells are CSCs. The first column is iteration 10 (2.5 days), the second is iteration 80 (20 days), and the third column is iteration 150 (37.5 days).

In Figure 1 I show examples of both major aspects of the simulated tumor, the vascular and cell grids, at certain intervals to show the tumor's progression. The top row of figures depicts the vascular network, while the bottom row contains the corresponding tumor state, with progenitors in blue, cancer stem cells in red. Until the 50^{th} day, the tumor can proliferate freely with hypoxia being the only factor responsible for the death of cells. Figure 2 shows the effects of the CTLs of the model by depicting an example trial (with 10 initial CTLs) before, during, and after the CTL stage.

A. Increasing initial amounts of CTLs in the tumor space decreases overall tumor size

It has been hypothesized that an invasion of CTLs into the tumor space can reduce tumor size over a relatively short period of time but do not effectively eradicate the entire tumor (leading to the importance of memory T-cells)(Maimela 2018). I performed a one-way independent ANOVA, comparing the number of cells comprising the tumor after the end of 300 iterations (75 days). It shows that there are significant differences among the treatments $F(2,15) = 7.1067$, $p < .05$, $np2 = .47$ (large), MSE = 316,725,565.5 with a large effect size. To follow this up, I performed a Tukey honest significance test with A being no CTL insertion, B being 10 initial CTLs, and C being 25 initial CTLs. With a HSD value of 6884.7, there is significance between A and B with a mean difference of 7444.5 and between A and C with a mean difference of 9507.0,

shown in Table 1. There was no significant difference between B and C however, with a mean difference of only 2062.5.

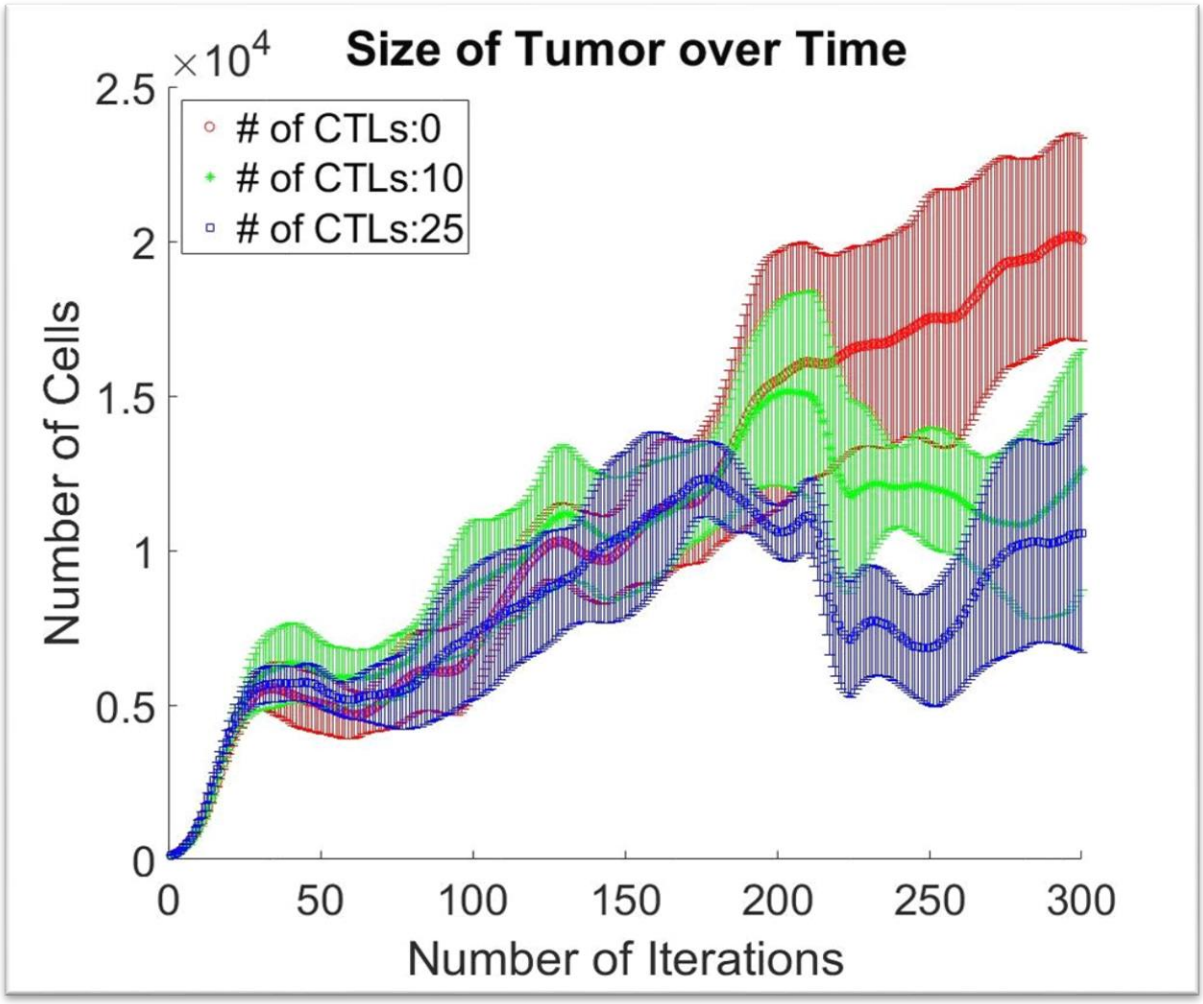


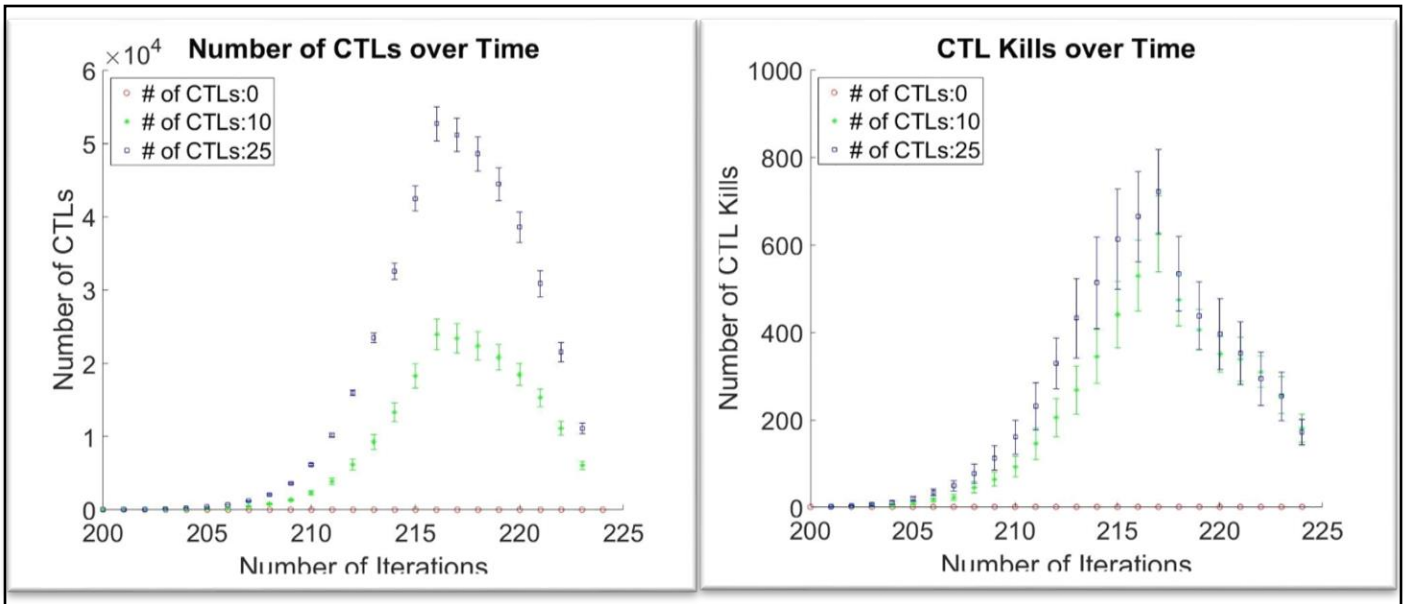
Figure 2. Displayed are the means with a 95% confidence interval for the size of the tumor over time (measured in number of cancer cells). During trials with a T-cell insertion, there is a clear decrease in tumor size during the period the CTLs act (iteration 200-224).

Table 1:

Treatment	Mean (Standard Deviation) # of Cells at Iteration 300 (75 days)
A: Control	20,057.0 (4,082.5)
B: 10 initial CTLs	12,612.5 (4,852.7)
C: 25 initial CTLs	10,550 (4,809.3)

B. Increasing amounts of CTLs increases the number of cancerous cells killed

While the number of CTLs in the space at the peak is more than double when starting with 25 instead of 10, the number of cells killed off is nowhere close to double. A one-tailed independent T-test was performed analyzing the total number of cells killed by CTLs during their time in the simulation. This T-test showed that the total amount of kills when inserting 10 CTLs into the tumor space (Mean = 5136.7, SD = 761.3) is significantly lower than inserting 25 CTLs into the space (Mean = 6421.5, SD = 1186.9), $t(10) = 2.04$, $p < .05$. While there is statistical significance, the difference in means shows that having a tumor environment super-saturated with killer T-cells does not kill a proportionate (to number of T-cells present) number of cells. More than an average 30,000 CTLs at peak lead to an average mean difference of only 1284.83 more kills on average over the 6 days; this is a mere 25% return on having more than 150% additional CTLS.



Figures 4/5. Depicted (left) is the mean number of CTLs in the tumor at a given time, with a 95% confidence interval. On the right we show mean the number of cancer cells killed by the CTLs each iteration and a 95% confidence interval for the given parameters. Note the drastic difference in the number of CTLs versus how many cancer cells they are able to destroy.

c. A higher number of CSCs killed strongly correlates with a smaller tumor size over time and a decreased tumor proliferation rate

One interesting result is that since the number of CSCs in the space greatly affects overall tumor proliferation and size over time, higher amounts of CSCs killed by CTLs will lead to overall lower tumor size. To investigate, I used a Pearson Correlation test to compare the number of CSCs in the space at iteration 225 (directly after all killing is completed and no more CTLs are present) and the overall size of the tumor in number of cells at iteration 300. This test utilizes data from all trials across the parameter space, since we are not looking at CTLs directly. The Pearson Correlation

Test shows a strong positive correlation between number of CSCs present at iteration 225 and total size of the tumor at iteration 300 with R(16) = .8821, a critical value of .590 for alpha < .01, and an effect size R^2 of .7781. Figure 5 shows a plot of the data analyzed with a line of best fit clearly showing the positive correlation.

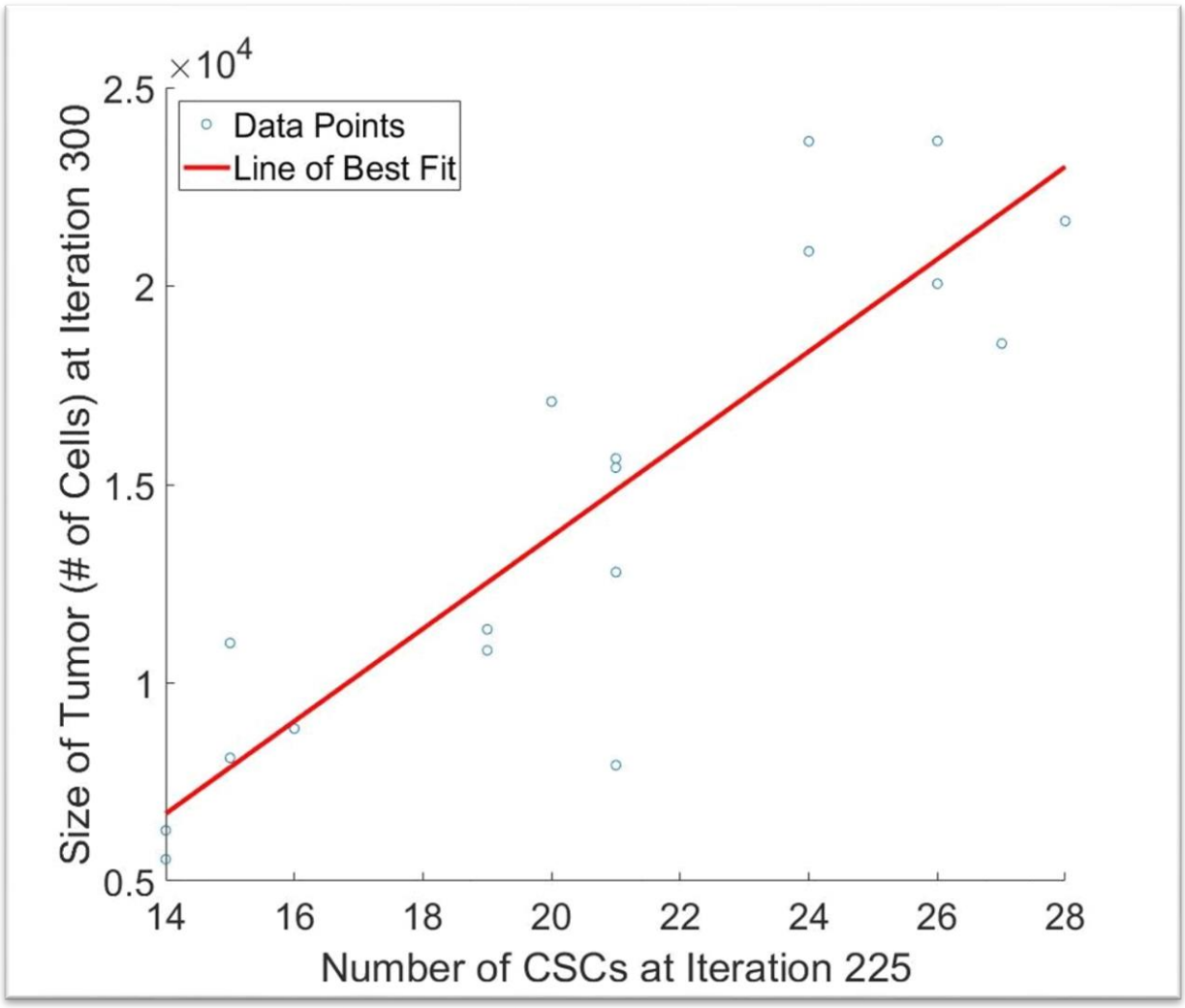


Figure 5. Pearson Correlation between the number of CSCs after the T-cell therapy completes and the size of the tumor at day 75, with a linear regression line.

To further investigate, I did another Pearson Correlation Test between the number of CSCs present at iteration 225 and the net tumor growth between iteration 225 and 300, counted in number of cells at 300 minus number of cells at 225. This time it showed a small but positive correlation of R(16) = .3275.

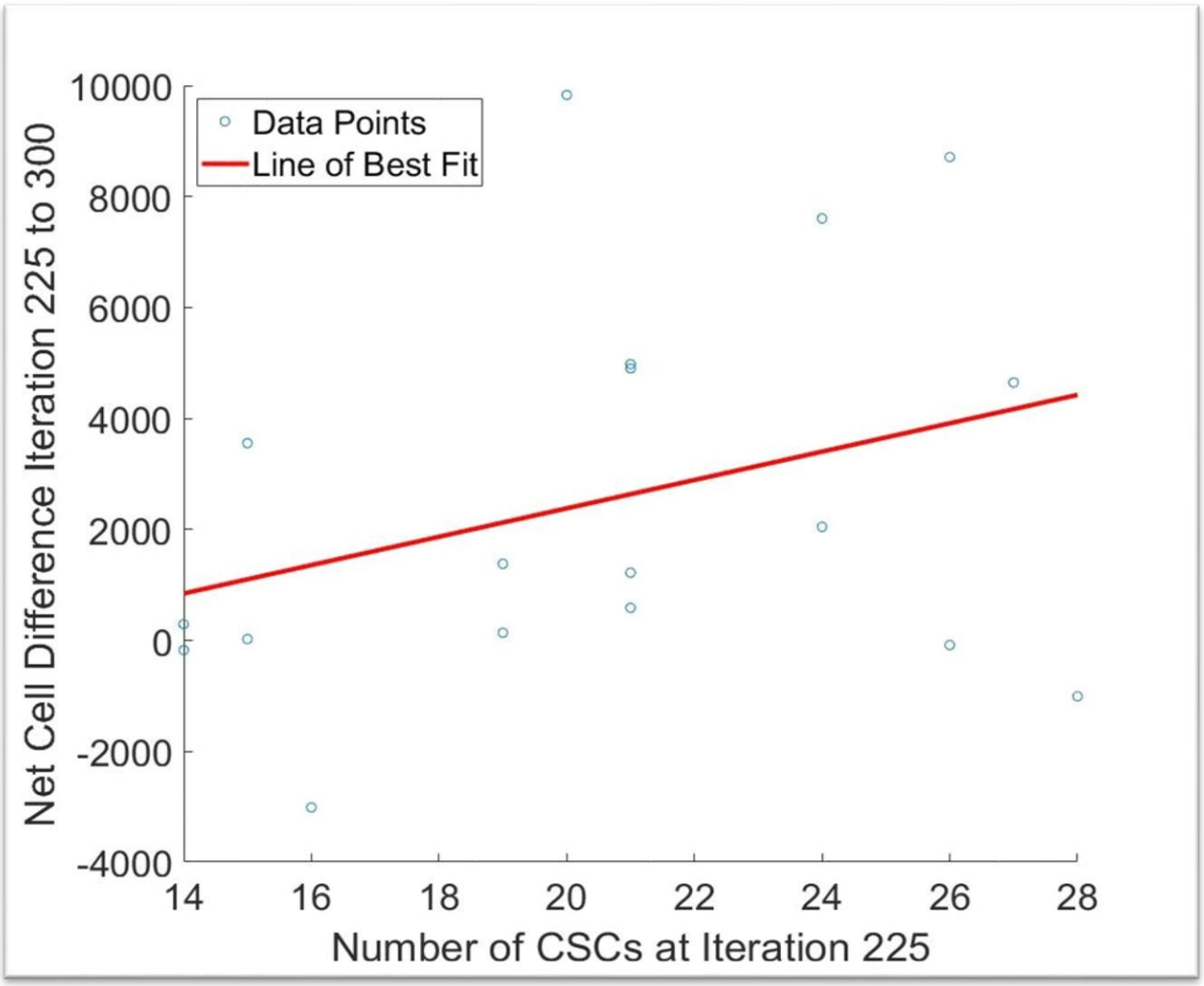


Figure 6. Pearson Correlation between the number of CSCs after the T-cell therapy completes and the net change in cells from directly following the T-cell therapy to the end (iteration 300 minus iteration 225), with a linear regression line.

This does not consider the number of cells that end up dying due to hypoxia however, so I ran one more Pearson Correlation Test between number of CSCs at iteration 225, and the total number of cell proliferations occurring between iteration 225 and 300. This time however, the test showed a very strong positive correlation with R(16) = .9459.

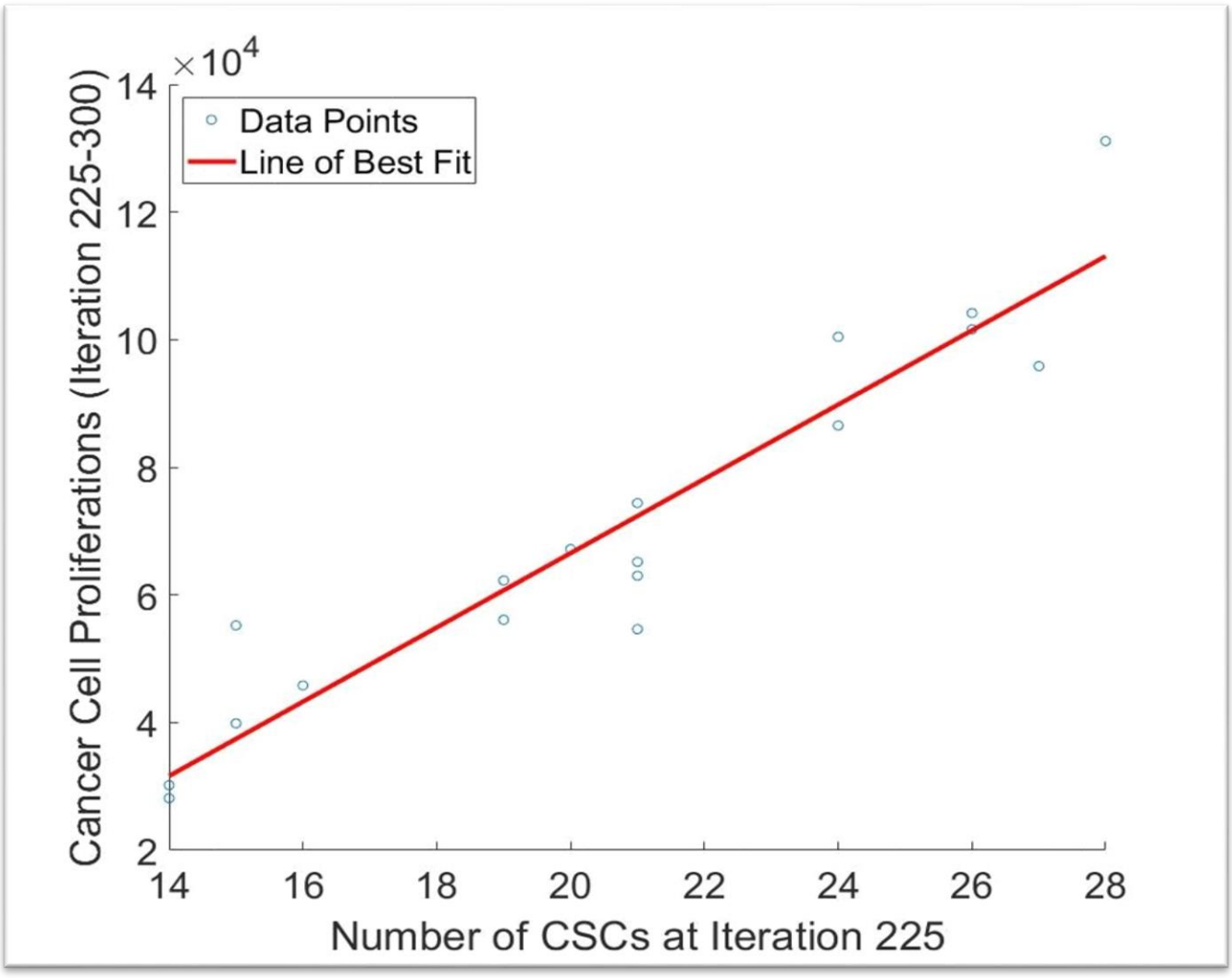


Figure 7. Pearson Correlation between the number of CSCs after the T-cell therapy completes and the total number of tumor proliferations between when the T-cell therapy ends and the end of the simulation (iteration 225-300), with a linear regression line.

Table 2: Shapiro Wilk Tests

Data	n	Critical Value (α=.05)	W
ANOVA Group A	6	.788	.878
ANOVA Group B	6	.788	.895
ANOVA Group C	6	.788	.903
T-Test- 10 CTLs	6	.788	.895
T-Test 25 CTLs	6	.788	.919
Pearson- # of CSCs at day 56	18	.897	.934
Pearson- Size of Tumor	18	.897	.937
Pearson- Net Growth	18	.897	.922
Pearson- Total Tumor Proliferations	18	.897	.958

Discussion

The data shown in terms of T-cell actions matches *in vivo* studies' findings. As shown by Figure 2, there is exponential growth followed by a tapered decay, which follows the know Gomperzian tumor growth curve (Kim 2012; Laird 1964). When starting with 10 CTLs there are ~24,000 CTLs present at the peak, which is a density of about 1536 CTLs per 400 microns cubed, and thus 77 CTLs per 400 microns squared. This is very similar to *in vivo* studies, which show an average of 81 CTLs in 400 microns squared of tumor stromal tissue (Shimizu, et al. 2019). The ~50,000 CTLs that result from 25 initial CTLs is only marginally more than the average ~33,400 that have been found naturally in tissue at the periphery of the tumor(Shimizu, et al. 2019). These CTLs are inserted into the space and allowed to act with the goal of diminishing the size of the tumor as much as possible. They kill, move, and stay in the simulation comparably to other studies(Kim 2012; Yee 2002). While T-cell insertions in very large amounts (~1x10^7) are becoming more popular for *in vivo* studies, it is difficult to specifically analyze the effects of only the CD8+ class of T-cells. While a large majority of the T-cells injected in such studies are CD8+, there are still the other three types in the mix. Additionally, T-cells can differentiate, leading to multiple subtypes with each taking on different roles, which is very difficult to control *in vivo*.

While there was statistical significance in terms of how many total cells the CTLs were able to destroy, the difference (in means) between the two parameters is not very

important in terms of validating a treatment method. In looking at the amount of T-cells in the space versus how effective they are at reducing the size of the tumor, it is apparent that even a super-saturation of CD8+ killer T-cells in the tumor environment is not enough to kill off the tumor completely. If an overestimated amount of CTLs in the tumor space cannot kill off the tumor, perhaps such killer T-cells are not a viable standalone treatment. This could likely be equated to either their rather short window of proliferation, or how they do not linger for very long after the proliferation window closes. Either way, having only a 6 days opportunity to eliminate the tumor is simply not enough time for the CTLs to eradicate the threat, although it is on par with what other studies show.

One interesting result from this study is in the relation of CSCs to the tumor's ability to recover from the CTL stage. Lower levels of CSCs present directly following the CTL stage lead to decreased proliferation and ultimately a smaller tumor size at the end of the simulation. This directly matches previous research (Norton, et al. 2017) and our BSRI research results (paper in progress), but at a slightly different angle; instead of seeking to throttle CSC symmetric division to fight their properties of enabling replicative immortality, I note it is worth attempting to directly target CSCs with a therapy to prevent or inhibit the cancer from recovering. Perhaps there is a method to make CSCs a higher priority target through increased production or overexpression of a factor, which would significantly increase the effectiveness of the CD8+ T-cells.

There are several limitations and opportunities for continued study that could be taken. One limiting factor is with respect to hardware; with trials taking quite a while to finish (~18 hours each), faster computers or a cluster could have allowed for more trials to be run. This in turn could mean either more trials over the same parameter space to increase the accuracy of statistical analysis, or a wider parameter space to analyze (or both). Another factor that limits this study is that the space is considered constant, and the cell densities are fixed. Since the cell grid is a matrix and each voxel can hold one cell, the tumor cannot fill a given space with more cells than there are voxels. This opposes findings that show tumors often have higher cell densities, with many cells packed into a smaller volume than normal. This in turn, also affects how cells become hypoxic, since higher densities mean the same number of cells can fit within a smaller space closer to vasculature. Furthermore, every cell is assumed to be the same size with a diameter of 20 microns. While accurate for macrophages, T-cells have been found to be only roughly 10 microns in diameter. This means that where one macrophage can fit, 4 T-cells could fit. When dealing with such a large amount of T-cells that ratio becomes very important, as 50,000 T-cells would actually only take up the space of 12,500 macrophages. Finally, I only do one T-cell insertion, while many *in vivo* studies do multiple insertions over time(Shimizu, et al. 2019; Yee, et al. 2002). This is because some T-cells differentiate into memory T-cells, which increase the effectiveness of later insertions.

This study leaves several paths for continued study available. One such are to be explored in the model is the interactions between the T-cells and the Tumor Associated Macrophages. It has been known they can act together to create several interesting effects in combination, and since the model now accounts for both separately, it would make sense to explore the direct effects of their interactions. This may also lead to changing the method in which the T-cells enter the space; to control the number of T-cells, a specified (perhaps changing to analyze effects) may be inserted at the beginning of the trial with proliferation turned off to maximize the amount of control over the parameter space. On the other hand, to take a more realistic approach one might simulate the T-cells' invasion through the lymphatic vessels into the tumor region. There could be set lymphatic vessels in the model with which the T-cells infiltrate through instead of simply being inserted into the space; this leads to the infiltration rate being a parameter worth investigating. Another direction possible would be to incorporate the other types of T-cells into the model. Helper T-cells, Natural-Killer T-cells, and Memory T-cells all play separate but related roles in the body, and although a large amount of the T-cells that invade the tumor space *in vivo* are CD8+, the rest of the types are worth including for their unique effects. Another parameter that might be worth instigating is the point at which the T-cell invasion begins or when the injection of T-cells is administered. As I have shown, even an unrealistically high volume of CTLs in the tumor environment is not enough to kill a tumor after it has had months to

progress. It may be worth testing the effects of the CTLs on a smaller tumor to at what point the CTLs are able to overwhelm the tumor and eliminate it completely.

In conclusion, I expanded upon an agent-based model of triple-negative breast cancer, adding a cytotoxic T-cell module and conducting trials to analyze the CTLs effect on a growing tumor. I was able to simulate a realistic amount of CTLs, length of effect, and rate of killing, and my results match other studies, suggesting a killer T-cell invasion alone is not sufficient in eradicating a tumor.

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