Introduction

Cancer has been one of the leading causes of death in the US for decades. As life expectancy increases, the chance of our cells mutation into proliferating cancer cells increases dramatically. Scientists have been working on defeating cancer for hundreds of years and have done incredible work. However, cancer survival rates have stagnated in recent years and cancer is still one of the leading killers in the wealthiest nation in the world (1). In particular, breast cancer survival rates have only increased ~1% over the last 13 years (2).

New immunotherapy techniques show promising results, being able to specifically target locations with tumors and leaving healthy tissue alone. These results are still in their pre-clinical stages but could potentially be a breakthrough in the survival of particularly late-stage cancer prognoses. Of these immunotherapy agents, interleukin-2 shows promising results in being able to regulate the immune system to target and kill cancer cells. However, it has a lot of collateral with healthy tissue, causing incredible damage to the human body over the course of treatment. Gene editing technology could potentially reduce the effect on healthy human tissue while still maintaining interleukin-2 as a professional cancer killer.

Breast Cancer in the US

Breast cancer was first discovered as a fatal disease thousands of years ago. There is evidence the ancient Egyptians cauterized women's chest area who were believed to have tumors, as far back as 1600 BC; rudimentary mastectomies removing all surrounding muscle and bone were performed in the Renaissance area (3). Through all this time breast cancer was treated as a single disease with one course of treatment. In 1904, German physician Steinthal proposed the division of breast cancer into three prognostic stages: small tumors that appeared to be localized to the breast (Stage I), larger tumors that involved the axillary lymph nodes (Stage II), and tumors that had clearly invaded tissues around the breast (Stage III) (4). Staging has two inherent benefits. First, it allows doctors to diagnose and decide on more appropriate treatment methods for the patient on a case by case basis rather than relying on a single course of treatment for all breast cancer patients. Second, it allowed important statistics regarding survival rates for cancers to be stratified by stages as new treatments were discovered and became available.

Breast cancer commonly starts in the milk ducts, although it occasionally begins in the milk glands. It typically progresses throughout the breast tissue and if it spreads enough can reach the lymph nodes located around the chest and collar bone (5). Hereditary breast cancer accounts for around 5% of cases each year, with most coming from mutations in the *BRCA1* and *BRCA2* genes. Women with these mutations have a 63% and 59% chance of being diagnosed with breast cancer at some point in their life, respectively (6). The interconnectivity of the lymphatic system makes cancers that reach these lymph nodes especially deadly, and as a result many treatment options involve removing one or several infected lymph nodes in this area. This is the most common way for breast cancer to metastasize and spread into surrounding organs, increasing the death rate dramatically. Breast cancer survival rates drop dramatically by stages: women today diagnosed with Stage 3 breast cancer have a 72% survival rate, while women with Stage 4 breast cancer have a 5-year survival rate of 19% (4).

The overall 5-year survival rate for breast cancer has risen over the last 4 decades, currently at an all-time high of 85% making it one of the most treatable forms of cancer in the

21st century. However, even with a survival rate this high, tens of thousands of Americans die every year from breast cancer. The National Cancer Institute estimates 276,480 women will be diagnosed with a form of invasive breast cancer in 2020, with an additional 48,530 new cases of non-invasive (*in situ*) breast cancer. Over the same year, 42,170 women are expected to die from breast cancer, making it the second deadliest form of cancer for women in the US, behind only lung cancer (2).

Five-year cancer survival rates in the USA, All races, total
Percentage of cancer patients surviving at least five years since diagnosis, by cancer type. This data is available to view by sex and race.
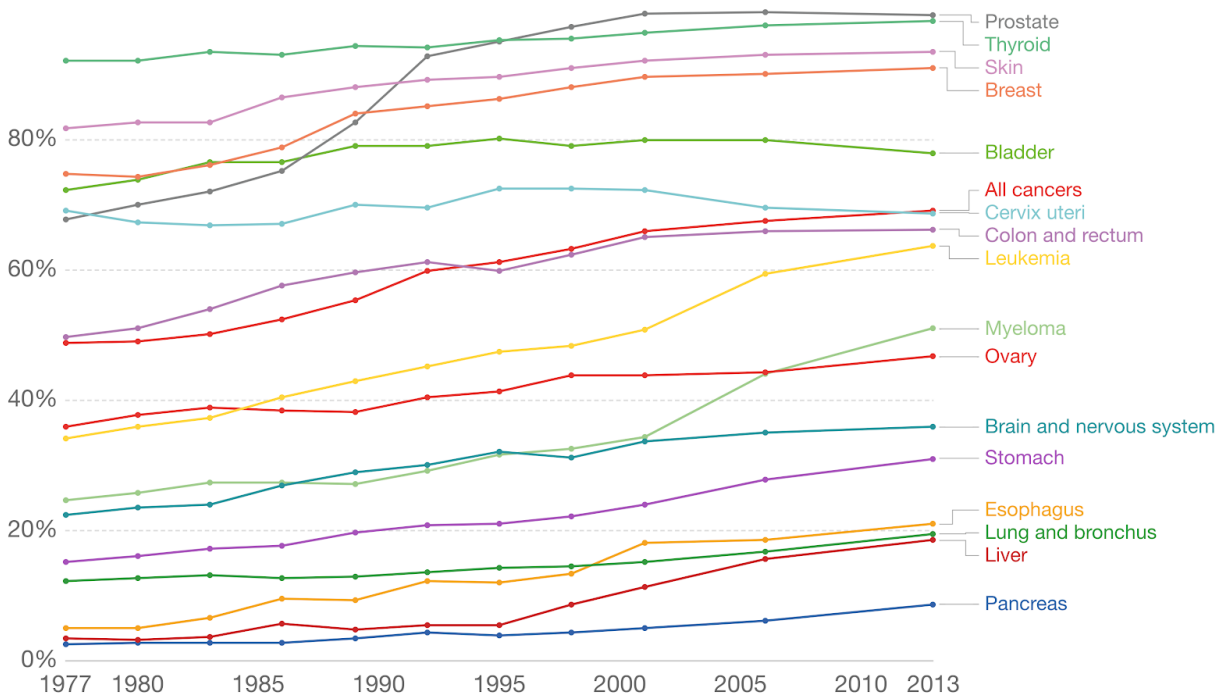

Figure 1: Cancer survival rates in the US have steadily improved over the last 40 years, with notable gains happening at the adoption for cancer screenings (particularly for prostate cancer) and the invention of new cancer treatment options. Increases in recent years have largely been attributed to medical advancements with the use of cytokines and other immunotherapy agents to help kill cancer cells using the patient's immune system instead of toxic radiation (1).

Interleukin-2 and its Role in Our Immune System

Interleukin-2 (IL-2) is a member of the cytokine family of proteins and is responsible for signaling the immune system to attack intruders and cells that are multiplying beyond their programmed life span. Cytokines are small glycoproteins that bind to cell surface receptors and regulate the development, survival, and function of immune cells. IL-2 itself is a 15.5-16 kDa protein that regulates the activity of leukocytes. It is produced primarily by CD4+ and CD8+ T-cells while in turn increases the production of CD4+ and CD25+ T-cells (7).

IL-2 was initially discovered in 1076 as a T-cell growth factor (TCGF), and scientists quickly worked to utilize its effect on our immune response to help fight cancer. In 1992 Proleukin was approved by the FDA as a recombinant version of IL-2 for treatment of metastatic

renal cell carcinoma. Not long after in 1998 Teceleukin was developed as a modified version of human IL-2 and was used to treat metastatic melanoma. Recently, IL-2 was found to be essential for our immune system's self-tolerance, as IL-2 and IL-2 receptor deficient mice exhibit lethal autoimmunity (8).

IL-2 is a member of the γ chain cytokine family, along with IL-4, IL-7, IL-9, IL-15, and IL-21. All these cytokines share the same IL-2 γ receptor (denoted IL-2Rγ), meaning their activity can be regulated by altering a single receptor protein. IL-2's primary function is the regulation and activation of CD8+ T-cells, but it also helps in the homeostasis and survival of natural killer (NK) cells. Both of these processes lead to the upregulation of perforin, granzyme B, and cytokine production; perforin and granzyme B have each been linked to cancer cell apoptosis (7).

IL-2 as a Breast Cancer Treatment Option
  The basic idea behind defeating any cancer is regulating the cell's ability to live and proliferate. Cancer treatment options look first to stopping the proliferation of the tumor, and afterwards to removing the cancerous cell and tissue. Normally, our immune system does an excellent job at regulating intruders and even human cells to stop multiplying after they have reached the end of their life to limit the number of tumors in our system. However, some cells slip under the radar and grow uncontrollably while our immune system sees them as healthy human cells. Immunotherapies such as IL-2 work against this by triggering our immune system to recognize cancer cells as intruders and kill them without harming healthy human cell and tissue.

  When IL-2 was first used as an immunotherapy agent, it was initially fatal at high doses. While IL-2 is naturally occurring in the immune system, at high concentrations it causes NK cells in the immune system to recognize healthy human cells that multiply frequently as cancer cells. This led to tissue damage in the heart, lungs, kidneys, and central nervous system, eventually leading to multi-system organ failure (9). Now, standard practices are in place to ensure the dosage of IL-2 is never enough to cause drastic damage to healthy human tissue; IL-2 is still toxic with these doses and can cause severe side effects. Standard practice for IL-2 treatment is as follows:

   Two-cycle course of high-dose IL-2 administered intravenously (IV) is standard. Each
   course consists of two 5-day cycles (600,000 IU/kg/dose administered IV over 15
   minutes) separated by a minimum of 9 days. If tolerated, IL-2 is given for a maximum of
   14 doses per cycle and 28 doses per course (10).

  Even with this standard practice treatment plan for IL-2, there are still dose-limiting systemic toxicities that vary patient to patient and reduce the effectiveness of IL-2. Recent medical advancements have allowed us to pinpoint where in the body IL-2 should be concentrated to induce the strongest immune response without damaging other parts of the body. However, the inherent toxicity of IL-2 makes it too damaging to local healthy tissue, and the side effects of doses this high are too strong for it to be approved by the FDA. Side effects from high dose IL-2 include fever, chills, malaise, hypotension, organ dysfunction, and cytopenias. The most harmful outcome from IL-2 therapy is called vascular leak syndrome, where lymphocytes

infiltrate surrounding organs and attack healthy organ cells, primarily in the heart and liver. Severe vascular leak syndrome can lead to cardiac failure, one of the primary reasons IL-2 is strictly limited in dosing with current medical treatments (11). In addition, IL-2 has a short half-life of between 15-30 minutes when injected intravenously, as it is cleared out by the renal system quickly. This means injections must be frequent when under treatment to keep IL-2 levels high enough to continue battling the cancer (12). Several projects look to increase the half-life of IL-2 to decrease the number of treatments required, thus improving the quality of life of patients receiving the treatment.
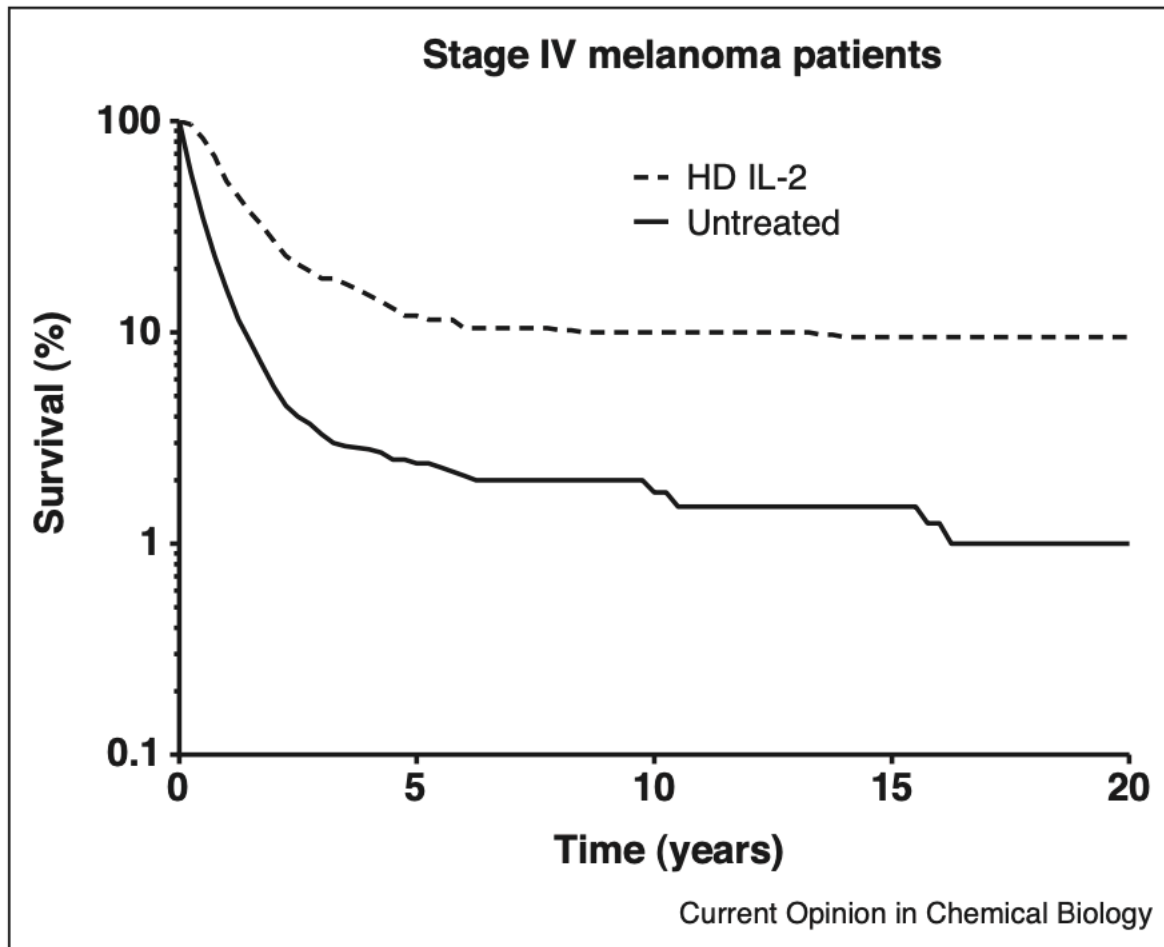

Figure 2: Survival rate for patients with Stage IV melanoma treated with high dose IL-2 versus untreated (13). As seen above, HD IL-2 is incredibly effective at targeting cancer, but the side effects on the patient are often too grave to proceed at the highest concentrations necessary to defeat the most aggressive cancers.

Bioengineering Works on IL-2

To utilize IL-2's immune response to its full extent, several bioengineering projects are under development to minimize the adverse side effects of high doses of IL-2. These projects involve editing the genes responsible for the transcription and translation of proteins in the IL-2 receptor complex. The IL-2 receptor complex (IL-2R) is comprised of three proteins each with

different responsibilities: CD25 (IL-2Rα), CD122 (IL-2R*B*), and CD132 (IL-2Rγ) (9). CD25 is responsible for the development and maintenance of T-*reg* cells; CD122 regulates T*reg* cells and mitogenic activity (proliferation) specifically for NK and memory CD8+ T-cells; CD132 leads to the proliferation of more IL-2. Once the complex is fully formed, several other processes take place. First, Janus Kinase 3 (Jak3) is produced to help proliferate IL-2 (9). Signal Transducer and Activator of Transcription proteins, or STATs, are also produced to regulate the production of mitogenic proteins; cells with low STAT counts have been found to be prone to cancer.
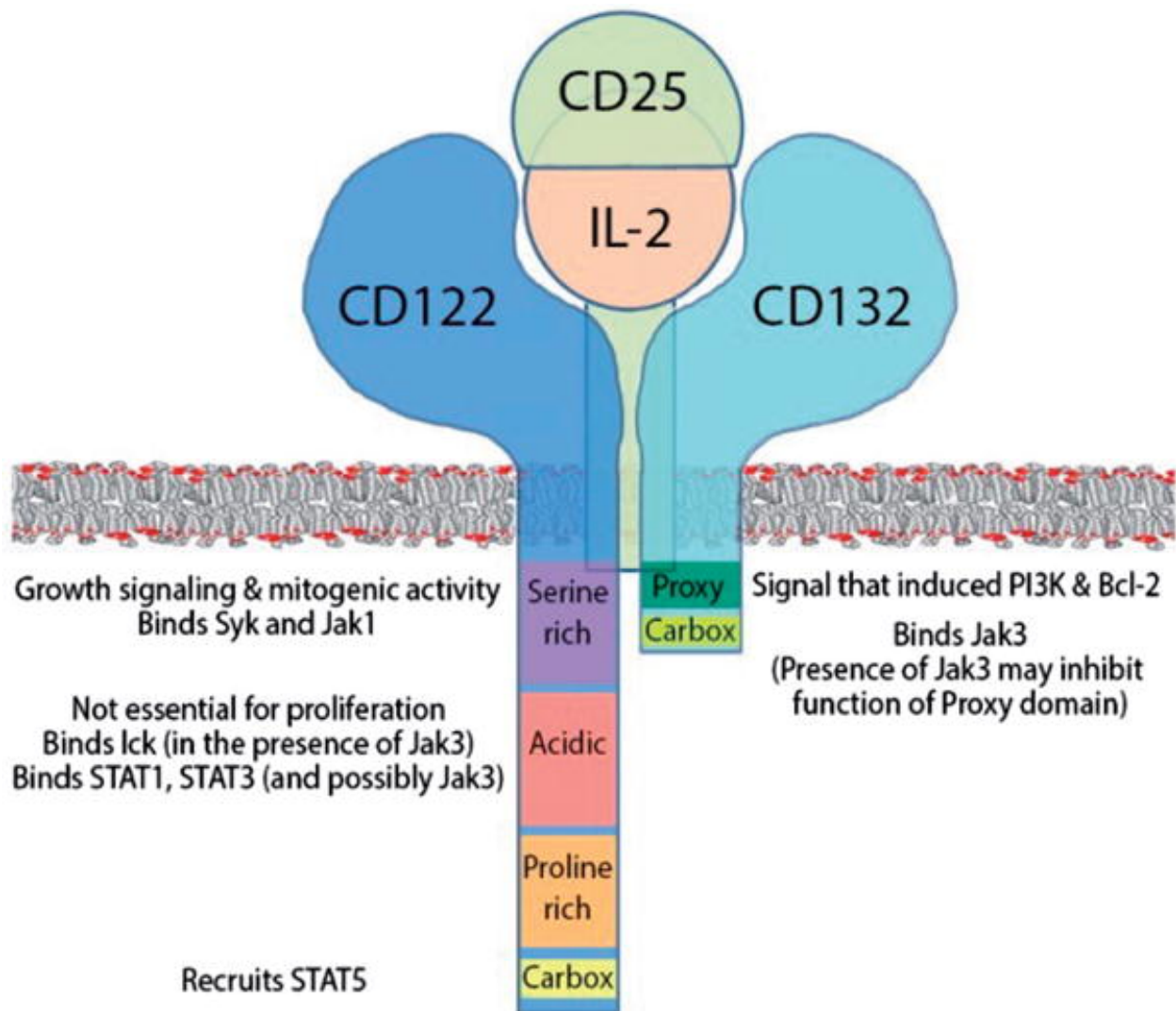

Figure 3: The IL-2 receptor complex is shown in the cell membrane. The complex is not fully formed until IL-2 is present on the surface of the cell; otherwise the three proteins making up the receptor are separated. The downstream effects of CD122 and CD132 can be seen in the figure. The IL-2R is important in the overall status of the body's immune system; mice with low IL-2R counts were found to have abnormally low T-cell counts overall (9).

There are two bioengineering projects focused on changing the IL-2R complex to alter the effects of IL-2. The first deals with increasing the affinity for IL-2 with CD122 to improve the immune system's ability to detect healthy cells from cancer cells. An increased affinity to CD122 does this by increasing the production of NK cells once a cancerous cell has been spotted, as well as regulating the memory T-cells (specifically CD8+) to look specifically for cells proliferating rapidly and beyond control. It makes these immune cells more likely to signal an immune response to a potentially cancerous cell and not for healthy cells (14). The second project tries to maintain the increased accuracy of the immune system without decreasing IL-2 effectiveness against cancerous tumors. Dubbed the "no-α mutein", this mutation intends to reduce IL-2's affinity for the α-receptor (CD25) specifically responsible for the proliferation and maintenance of these T*reg* cells. A high affinity to CD25 produces too many T*reg* cells that are overregulated and are more likely to assume a cancerous cell is a healthy cell (7).

Mutations to IL-2R Complex to Increase and Decrease Affinity

To increase the affinity of IL-2 to the CD122 protein, several mutations were made in the IL-2 gene. This "IL-2 superkine" was generated with several substitutions between amino acid positions 80 and 92. These mutations were made via commercial vector pET28a containing an N-terminal His-tag, with the genes of interest under the lac operator. *E. coli* cells were transformed with the mutant IL2 plasmids under manufacturer protocol, and were allowed to grow in a Lysogenic broth medium for 6 hours. Laboratory mice were given metastatic lung cells and split into three separate groups: one received wild type IL-2, another received the mutant hIL-2, and a third control group was injected with PBS. The injections were given twice a day for 5 days, after which the lungs and livers of each mouse was weighed. Note that the injection given to these mice is four times the dosage used for antitumor treatment (12).

The second project, reducing IL-2's affinity to CD25 without changing its affinity to either of the other receptor proteins, was undergone with a similar methodology. Dubbed the "no-α mutein", this IL-2 mutant would have a decreased affinity for the IL-2Rα receptor to decrease the proliferation of CD8+ T-cells. This mutation was created with four substitutions in the IL-2Rα gene, each replacing the respective amino acid with alanine. Mutations were made at positions 38, 42, 45, and 62, and resulted in an *in vivo* "decrease in affinity for CD25 while maintaining normal binding with IL-2Rβγ" (15).
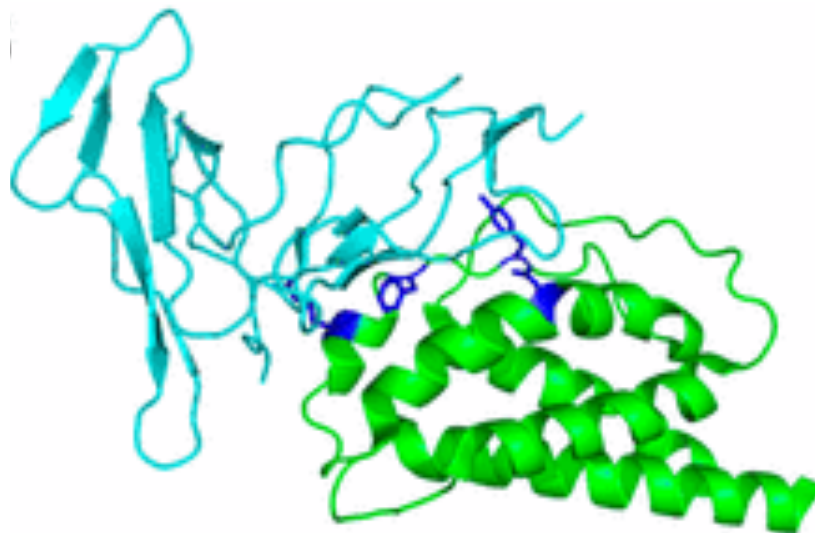
Figure 4: The "no-α mutein" IL-2 and IL-2Rα protein. The green indicates the IL-2 protein, the cyan is the IL-2Rα, and the dark blue region is the mutated region of the IL-2Rα. This mutation reduces the affinity by impeding the connection between IL-2 and IL-2Rα (12).

Clinical Results

The first study showed a stark increase in the mutant IL-2's affinity for the CD122 receptor. From the purification testing, a single change of leucine for valine at position 85 (L85V) resulted in a 5.7-fold increase in affinity for CD122. Four additional mutations (L80F, R81D, I86V, and I92F) led to a 35-fold increase over the original mutations. These five mutations put together result in a 200-fold increase in affinity over wild-type IL-2 (12). This increased affinity lead to an improved immune response to the mice's cancer cells. Mutant IL-2 showed an increased concentration of NK and memory CD8+ T-cells compared to both the control and the wild type IL-2 treated mice. However, this IL-2 superkine was "weaker and could not be significantly improved by repetitive treatment cycles". An upside to this is the superkine reduced IL-2 related pulmonary edema and liver cell damage (15).
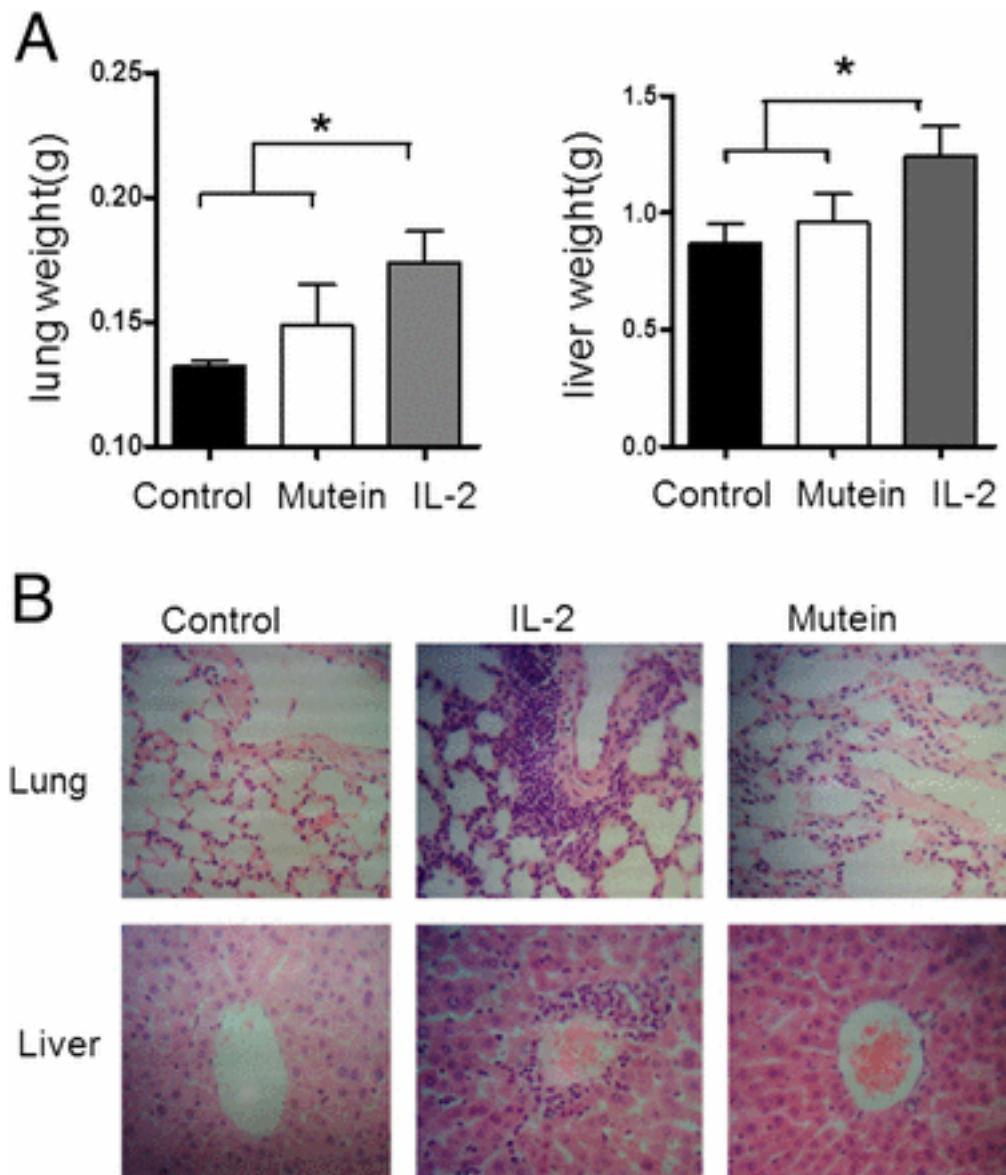
Figure 5: The effects of the IL-2 superkine on mice with metastatic lung cancer show that the superkine is less effective at combating the tumor but show much lower lymphocyte counts in the liver. This indicates the immune system is not attacking healthy tissue nearly as much as with wild type IL-2. Lymphocytes can be seen in purple in the figure, with pink cells showing healthy mouse cells (12).

The "no-α mutein" showed much more promising results. This mutant IL-2 was able to more accurately target cancer cells at roughly the same level as wild type IL-2, with much lower T-cell counts in healthy tissue even in organs with rapidly proliferating cells such as the liver. As with the superkine, NK and memory CD8+ T-cell counts were higher than with wild type IL-2, with a much lower expansion in T*reg* cell counts compared to the superkine. In the mice with metastatic lung cancer, tumor size rapidly decreased with minimal damage to surround healthy tissue (12). In addition, clinical results done on 45 cancer patients with the no-α mutein IL-2 with a variety of cancers found that only 4% of them showed lower effective cancer responses than expected with wild type IL-2, and all patients exhibited healthier surrounding and liver tissue.

Neither mutant IL-2 was found to have an increased half-life compared to the wild type IL-2 of 15-30 minutes, meaning treatment plans for both mutations would be approximately the same frequency as typical (15).

Discussion

IL-2 and other cytokines have been used effectively as immunotherapy agents for a variety of cancers for decades and have saved countless lives in the process. However, recent trends show the survival rates of cancers plateauing, particularly among prominent and deadly cancers such as breast and lung cancer. For women with stage 4 breast cancer, the 5-year survival rate have never reached far above 20%, killing tens of thousands of women every year (1). Newer versions of these immunotherapy agents are needed to continue progressing and to help work towards eradicating cancer all together. These pioneering studies have shown promising results in the ability to reduce the side effects of high dose IL-2, while can help with both increasing the efficacy of IL-2 treatment options for cancer patients as well as increase the maximum dose of IL-2 to administer to patients with aggressive late-stage cancers such as Stage IV breast cancer. Further work needs to be done to verify these results in human patients and to increase the half-life of IV IL-2 to reduce the frequency of treatment.

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