FUNDING AIDS CURE RESEARCH AND A DECENTRALIZED DATABASE FOR CLINICAL DATA FROM HUMAN TRIALS

INNOVATIVE BIORESEARCH WHITEPAPER V1.0

CONTENT

INTRODUCTION AND BACKGROUND

Innovative Bioresearch is a privately held biotech company based in Italy (http://www.innovativebioresearch.com). Owned and founded by research scientist Jonathan Fior with the goal of bringing innovation to the field, with a focus on HIV, cancer and regeneration research. We are launching an ICO (with a pre-ICO first) to raise funds for our AIDS cure research as well as to develop an application providing a decentralized database for clinical data generated by our future human trials, to overcome the limitations of the current ancient centraliazed databases. Let's first focus on the first goal of this ICO, which is to fund the development of our AIDS cure research, with an overview of the current state of SupT1 cell infusion therapy, the novel cell-based therapy for HIV conceived by Jonathan Fior, owner and chief scientific officer of Innovative Bioresearch.

SUPT1 CELL INFUSION THERAPY FOR HIV, AN OVERVIEW

HIV infection usually leads to a progressive decline in number and functionality of CD4+ T lymphocytes, resulting in AIDS development. As explained in Jonathan Fior's scientific publications [1–3], the HIV virus has a higher tropism for SupT1 cells than for primary CD4+ T cells. Several hypotheses have been proposed as an explanation, most notably the higher surface expression of CD4 and CXCR4 receptors in SupT1 cells. In addition, in vitro studies of HIV evolution show that persistent growth in the SupT1 cell line results in a less cytopathic virus with a reduced capacity for syncytium formation, a higher sensitivity to neutralization, improved replication in SupT1 cells and impaired infection of primary CD4+ T cells [4–6]. Accordingly, Jonathan Fior proposed the infusion of irradiated SupT1 cells as a cell-based HIV therapy to exploit the therapeutic potential of these phenomena [1–3]. The rationale behind this approach is that moving infection toward the inoculated cells should prevent infection and depletion of the patient's own CD4+ T cells and, therefore, AIDS. In such a strategy, SupT1 cells would act as a "decoy target" for the HIV virus to prevent CD4+ T cell depletion as well as to render the virus less cytopathic. As previously mentioned, in vitro studies of HIV evolution show that prolonged replication in SupT1 cells renders the virus less cytopathic and more sensitive to neutralization. Accordingly, replication of the virus in the inoculated SupT1 cells should also have a vaccination effect; that is, the therapy should also induce the virus to become progressively less aggressive and harmful for the patient. The use of SupT1 cells as a decoy target for HIV has been investigated in vitro and in vivo, with interesting results [1,3]. In vitro data showed that, when primary CD4+ T cells are infected with HIV in the presence of SupT1 cells, the preferential infection of SupT1 cells can spare primary CD4+ T cells from infection and depletion. In vivo data in humanized mice showed that significantly lower viral replication (~10-fold) and potentially preserved CD4+ T cell frequency at Week 1 was scored in animals treated with SupT1 cell infusion. Of note, one animal exhibited a sustained decrease in HIV replication and CD4+ T cell depletion (no virus detected anymore at Weeks 3 and 4), a result that may hold the key to future HIV treatments. Given the urgent and global need for a cost effective cure for HIV, we believe that the millions of people infected by this terrible disease deserve highly innovative HIV cure research strategies, such as SupT1 cell infusion therapy.

In summary, these are some of the potential therapeutic benefits of this cell-based treatment that go beyond what can be achievable with traditional drug based therapies such as cART:

1)The vaccination effect. As previously mentioned, SupT1 cells have been shown to have a very powerful vaccination effect in vitro [4–6]. In this regard, in vitro studies of HIV evolution showed that upon prolonged replication in SupT1 cells, the X4 HIV-1 LAI virus evolves toward a less virulent phenotype with a reduced capacity for syncytium formation, thus losing the main cytopathic feature characterizing X4 strains, and most notably the virus adaptation to replicate in SupT1 cells results in gradually losing the ability to replicate in primary CD4+ T cells [4]. In addition, the variation to neutralization sensitivity after viral growth in tumor T cell lines has also been examined. Interestingly, one study reported that primary isolates that were initially resistant to neutralization acquired sensitivity to neutralization after continuous growth in tumor T cell lines, and that the sensitivity to neutralization progressively increased during the days of culturing [5]. Specifically, it was shown that after 14 days in continuous culture, 100 micrograms/mL of rsCD4 (recombinant soluble CD4) were needed to neutralize 1 TCID of primary isolate, while only 0.3 micrograms/mL of rsCD4 were needed to neutralize 1 TCID of the virus after 75 days in continuous culture. This means that there was a 300 fold increase in virus sensitivity to neutralization after prolonged replication in a tumor T cell line, which is really something remarkable. All these phenomena could therefore harbor a significant therapeutic potential that could be exploited with SupT1 cell infusion therapy to induce HIV infection to evolve into a more tractable state for therapy.

2)Potentially no organ toxicity; cART is a drug based treatment and as such is associated with organ toxicity because the drugs are metabolized by various organs. By contrast, SupT1 cell infusion is a cell-based treatment and there is no chemical substance injected into the body that needs to be metabolized, which could significantly improve the quality of the patient's life.

3)Be effective in patients in a terminal state of disease that developed drug resistant and very aggressive HIV strains. When a patient is treated with cART, the virus fights back because it strives to survive, which can result in the development of very aggressive and drug resistant HIV strains, especially in the terminal stage of the disease and in such cases cART becomes ineffective. By contrast, SupT1 cell infusion therapy provides the virus with a permissive cell-line in which it can preferentially replicate, so that a peaceful coexistence between virus and host becomes possible, which could dramatically improve the patient's health as the virus infection progressively moves toward the inoculated SupT1 cells and the virus becomes increasingly less pathogenic for its host.

4)Possible association of the treatment with novel molecular compounds such as a Vif-inhibitor to act on HIV reservoirs. The HIV-1 Vif protein is essential for viral replication in primary CD4+ T cells but not in SupT1 cells [1]. Accordingly, pharmacologic inhibition of Vif could be combined with SupT1 cell infusion to further restrict viral replication to the inoculated SupT1 cells. Considering that APOBEC3G is expressed by different cell types, such as neuronal cells, astrocytes, and macrophages [2], pharmacologic inhibition of Vif may also have the benefit of acting on HIV reservoirs in the brain and other body areas. There are several molecules with promising anti-Vif activity currently being tested [2]. Similarly, other HIV-1 accessory proteins that are not essential for replication in SupT1 cells (e.g., Vpr, Vpu, and Nef [3]) may also be the target of pharmacologic inhibition. It is important to point out

that these drugs would not affect virus replication in the inoculated SupT1 cells, and therefore in combination with SupT1 cell infusion therapy, there should not be development of drug resistance normally associated with drug based treatments.

Below some considerations with regard to potential issues:

1)Safety. We take this issue very seriously and are committed to performing very rigorous preclinical research to ensure there is enough data on safety to obtain approval from regulatory agencies for human experimentation. In this regard, injection of irradiated tumor cells as a therapy is already performed in cancer vaccination. In such cases, irradiating the cells prior to inoculation has been shown to ensure treatment safety both in animal and clinical studies [7]. We used the same protocol used in cancer vaccination studies (i.e., 30 Gy of radiation dose for the cells), which resulted in safe in vivo inoculation in our animal study as well [3]. Specifically, all animals successfully survived the treatment and presence of SupT1 cells was almost undetectable at late time points, which means that irradiating the cells prior to inoculation efficiently prevented SupT1 cell replication. Furthermore, we infused high doses of cells (40 million SupT1 cells were infused weekly), which in a highly immunodeficient mouse strain would rapidly lead to animal death in case of tumor development. Therefore, based on the clinical data we already have from cancer vaccination studies, and from the results of our first animal study, we believe that meeting the safety standards required for human trials is something feasible.

2)Rejection issues. Tumors can develop because tumor cells are able to evade immune recognition. For example, SupT1 cells do not express HLA-DR, which is an antigen highly associated with immune recognition [8]. Accordingly, given the tumoral nature of SupT1 cells, they should be significantly less immunogenic than normal cells and as such should survive in the patient long enough to provide a therapeutic effect. However, it is possible that the HIV virus will eradicate the cells faster and more efficiently than the immune system itself in any case.

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THE CHALLENGE OF HAVING TO ACCESS CLINICAL DATA FROM THE CURRENT ANCIENT CENTRALIZED DATABASES

Traditionally, all data generated by scientific research is collected in the form of a scientific article, which is published in a peer reviewed scientific journal, and it is usually made available on a public database such as the NIH's pubmed database (https://www.ncbi.nlm.nih.gov/pubmed). When a novel therapeutic strategy, such as SupT1 cell infusion therapy, goes through the several stages of clinical research, different research teams all over the world may perform the research. And once the therapy is finally approved for human treatment, different clinicians all over the worlds may administer the treatment to patients. This means that when a research team wants to perform clinical research, they have to go through the tedious and time consuming process of searching for all the published data from previous trials, read all the papers, select the useful data, and make the best possible interpretation of the data to create a protocol for their trial, personalizing it for their typology of patients. In fact, every patient may have different individual characteristics (e.g., age, ethnicity, HIV tropism) and thus may require personalized treatment protocols. And the same issue is present when clinicians begin to administer an approved treatment to patients; they have to go through all the papers and make a best guess on what treatment protocol should be used. As mentioned, this process of accessing clinical data can be quite tedious and time consuming, but it can also introduce human error.

A Possible Solution: A Decentralized Database Using the Distributed Processing Power of the Blockchain to Process Clinical Data

A possible solution to the challenge posed by having to access clinical data from the current ancient system could be provided by the creation of a parallel decentralized database for clinical data that takes advantage of the distributed processing power of the blockchain technology. This is what we are proposing with the creation of the "You're not alone" application. First, let's focus on the blockchain aspect of the app, we will explain why it has such a name later. The "You're not alone" application would feature an interface allowing any clinician running a clinical investigation on SupT1 cell infusion therapy to enter the clinical data produced by their trials. The application would allow the input of a broad range of individual parameters for each treated patient, such as age, ethnicity, disease progression stage, viral tropism of HIV virus carried, along with the clinical protocol used (e.g., dosage of SupT1 cells infused each week) and the outcome of the treatment (e.g., viral load, CD4+ T cell count). The distributed computational power of the blockchain would then be used to elaborate all the data; statistical analysis (means, standard deviations, correlations, power) and even the elaboration of suggested treatment profiles that could work best for each type of patient could be performed. This would take away the burden of having to manually search and process all the data as in the current centralized databases. And such an application would have an infinite potential for growing. For instance, an amazing feature could be to input genotypic information of the HIV virus carried by each patient, allowing to keep track of all mutations and genetic modifications caused by the treatment as well as the resulting viral phenotypic changes. Therefore, such a database would be constantly growing and evolving as new parameters are added to it, and it would be always available, anytime, anywhere, and to anyone connected. Another important aspect is that usually in a scientific publication the raw data is not published but instead a graphical representation is used to summarize the data; another amazing feature of a decentralized database is that due to not having the constraints of a centralized database, the whole raw data generated by any research could be included in the database. To preserve data integrity, each clinician would be required to register with us to obtain a special version of the app reserved for approved professionals, so that a unique key would be included in the data entered in the database; therefore, only approved clinicians with genuine keys would be able to get the data approved and added to the blockchain, similarly to what happens with cryptocurrecy transactions. Such an app would help speed up tremendously the clinical development research of SupT1 cell infusion therapy; it could be a game changer.

Now, let's explain why we named the application "You're not alone". The application will also document the progress of our AIDS cure research project, featuring periodic updates such as articles, vlogs. This will create a community were HIV seropositive people can stay up to the date, comment, share, interact with us working on the cure, and with each others. Something that creates a bridge between HIV seropositive people and us research scientists working on the cure. Something that allows the HIV seropositive community and anyone interested to be part of this journey. Something that can send the message that they are not alone in their battle. That we are fighting for them. That we care about them. A message of hope. It will also provide information about all the ongoing clinical trials, how to participate, and once the treatment is approved, it will provide information about the clinics and hospitals offering the treatment, and how to make appointments with legit clinicians that are officially collaborating with us.

OUR MISSION: DEVELOPING A CELL BASED LOW COST HIV CURE SOLUTION AND A DECENTRALIZED CLINICAL DATABASE

Our mission is to provide a cost effective cure solution for AIDS. In contrast with traditional cell-based and gene-based therapies that make use of modified autologous cells and are therefore very expensive and often unpractical for a large scale application, using a standardized T cell line such as the SupT1 cell line should significantly reduce the treatment costs associated with SupT1 cell infusion therapy, allowing access to the therapy where access to traditional HIV therapies are restricted by economic and social limitations. The social and economical impacts of a low cost HIV cure solution would be enormous. Our mission is also to provide clinicians with a novel tool that allows access to all data produced during the clinical development of SupT1 cell infusion therapy, through an application that uses the distributed computational power of the blockchain technology to store and processing clinical data, which could tremendously speed up the clinical research process. We would still publish the data through the traditional peer reviewed system, but once reviewed and published, the data would also be made immediately available for easy access through the application. We are planning to create this application specifically for our research, to overcome the limitations of current centralized databases. However, if we see there is enough interest we also have plans to release a commercial version of the app for any company or institution interested in using it for their own clinical data. After all, it would be an application designed by a research scientist for research scientists. This could create a very profitable business.

With regard to the development of our novel cell-based HIV therapy, SupT1 cell infusion therapy, we are currently in the phase of preclinical animal research. We will use the funds we raise from the pre-ICO and ICO to continue funding the preclinical development research of SupT1 cell infusion therapy, with the goal to generate data that will allow us to obtain approval from regulatory agencies for proceeding to the successive phase of human trials. Our next studies will be performed using in vitro and in vivo models of HIV infection. Specifically, these are the goals of our next preclinical in vivo investigations;

Aim 1: Generating more in vitro data on the dynamics of HIV replication in irradiated SupT1 cells.

Aim 2: Performing additional experiments in humanized mice testing lower virus inputs. In our previous animal study all infections were performed with a very high virus input dose (100,000 TCID50) [1]. Considering the interesting tendency to have a significantly lower viral replication at Week 1, it is possible that the combination of a very aggressive viral strain such as HIV-1 LAI and a relatively high viral input hides the potential efficacy of the treatment, resulting in virus suppression only in one animal. In the follow up studies, we plan to repeat the experiments with lower virus input doses (e.g., 50,000 TCID5025,000/8,000/2,000 TCID50) to

better show virus replication dynamics in order to study the condition that is associated with the desired outcome (majority of mice showing virus suppression and CD4+T cell survival).

Aim 3: Testing the treatment with an R5 virus strain. Along with testing lower virus doses, we also aim to test our therapy with an R5 virus strain using a CCR5-expressing SupT1 cell line. This is of great importance considering that the majority of HIV seropositive individuals carry an R5 virus strain of HIV.

Aim 4: Assessing the potential vaccination effect of SupT1 cell infusion therapy. In the previous study we did not perform specific tests to analyze the possible attenuation of the virus as a consequence of the treatment, which would be a vital aspect that needs to be investigated. Accordingly, in the next studies we will isolate the virus grown in mice receiving SupT1 cell infusion, which will be isolated from samples taken at different time points (1 week, 2 weeks, 3 weeks, 4 weeks post infection) and analyze virus attenuation in terms of acquired sensitivity to neutralization and reduced capacity to induce syncytia with respect to the original virus.

Aim 5: Performing a more in-depth analysis of treatment toxicity. In the previous study we based our conclusions with regard to treatment toxicity by monitoring the mice for signs of illness along with monitoring SupT1 cell blood count by FACS analysis. In this follow up study we propose to perform a more accurate examination of treatment toxicity, performing acute, subchronic, and chronic toxicity tests.

EXPERIMENT PROTOCOL

Aim 1 (in vitro culture experiments are repeated in triplicates)

Cells

Hu-PBMC
Hu-PBMC are isolated from buffy coats using a Ficoll density gradient and cultured in RPMI-1640 Glutamax™ medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and in 5% CO2.

SupT1 cell line
SupT1 cells [5] are cultured in RPMI-1640 Glutamax™ medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and in 5% CO2.

SupT1-CCR5 Cl.30 cell line
SupT1-CCR5 Cl.30 cells [6] are cultured in RPMI-1640 Glutamax™ medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and in 5% CO2.

1)In vitro assessment of viral replication efficiency in irradiated SupT1 cells

This test will have the purpose of assessing life span and viral production of irradiated SupT1 and SupT1-CCR5 Cl.30 cells after infection with the X4 LAI and R5 BaL virus strains, respectively. Briefly, 10 million SupT1 cells are X-irradiated with 30 Gy, infected with either 7,500 TCID50 HIV-1 LAI (SupT1 cells) or 9,000 TCID50 HIV-1 BaL (SupT1-CCR5 Cl.30 cells), and cultured in 5mL of RPMI-1640 Glutamax™ medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and in 5% CO2. Every 2 days, a sample is collected to measure viral load and the cells are counted. The culture is stopped once there are no more viable cells in the culture. HIV viral load is measured by qPCR and expressed as HIV RNA copy number per mL (detection threshold: 10^2 HIV-1 RNA copies per mL).

Control
As controls, cultures with unirradiated SupT1 and SupT1-CCR5 Cl.30 cells (5 million cells each) are used to compare the results in terms of viral production.

The results of this test will provide specific data regarding life span and viral production of the irradiated cells. These data will be important to determine the subsequent phase of the study.

2)In vitro assessment of SupT1 cells as a decoy target for HIV

To further investigate whether SupT1 cells can act as a decoy target for HIV, co-cultures with SupT1/PBMC cells are used. After 72 hours of PHA activation (5 micrograms/mL PHA), 10 million

PBMC are washed with medium and mixed with 10 million 30 Gy-irradiated SupT1 cells, and the cell mixture is suspended in 5 mL of complete medium. The cell mixture is then infected with either 7,500 TCID50 HIV-1 LAI (SupT1 cells) or 9,000 TCID50 HIV-1 BaL (SupT1-CCR5 Cl.30 cells) and cultured in 5 mL of complete medium. The cells are maintained in culture for up to 4 weeks, and each week 10 million of 30 Gy-irradiated SupT1 cells are added to the co-culture.
Every 3 days a sample is taken for FACS analysis and viral load quantification. Blood cell leukocytes are isolated from the cell fraction on a Ficoll density gradient. Flow cytometric analysis of blood leukocytes is performed using a monoclonal antibody cocktail (Table 2) after incubation with human FcR blocking reagents.

Table 2. Flow cytometry reagents.

Target	Label	Clone	Origin	Dilution
hCD3	E450	UCHT1	eBioscience	1:50
hCD4	PE	MEM-	ImmunoTools	1:25
hCD8	FITC	MEM-31	ImmunoTools	1:12.5

Incubations are performed in 96-well plates in the dark at 4 °C, and the following populations are successively gated: human T cells (hCD3+), and human T cell subsets based on CD4 vs. CD8 expression (CD4+CD8−, CD4−CD8+, and CD4+CD8+). Accordingly, SupT1 CD4+CD8+ T cells are distinguished from PBMC CD4+CD8−/CD4−CD8+ T cells by FACS analysis. Data acquisitions are performed with an LSR-II Fortessa flow cytometer interfaced with FACS-Diva software. Data are analyzed using FlowJo 9.8 software, and graphs are plotted with GraphPad Prism-5 software. Once all culture samples are collected and stored, HIV viral load is measured by qPCR in a single run. Viral load is expressed as HIV RNA copy number per mL of blood (detection threshold: 102 HIV-1 RNA copies per mL). Samples taken at different time points (3, 15, 30 days post infection) from the co-cultures will also be stored and used for virus isolation and subsequent test of virus attenuation.

Control experiments (control co-cultures are with irradiated SupT1 cells as well)

Positive control
Infected PBMC culture with either HIV-1 LAI or BaL.

Negative controls
Uninfected SupT1/PBMC co-culture
Uninfected SupT1 CCR5 Cl.30/PBMC co-culture
Uninfected PBMC culture

2)Analysis of virus attenuation (potential vaccination effect as a consequence of viral replication in SupT1 cells)

Analysis of neutralization sensitivity (HIV-1 BaL)

It has been previously reported that prolonged viral replication of primary HIV-1 isolates in T cell line cultures results in a virus that shows an increased sensitivity to neutralization by rsCD4 (recombinant soluble CD4) [3,4]. Accordingly, the purpose of this test is to assess whether this phenomenon is also observed with HIV-1 BaL after replicating in PBMC/SupT1-CCR5 Cl.30 co-cultures.
This experiment will be preformed only with the R5 HIV-1 BaL virus because the X4 LAI virus is already a strain that acquired sensitivity to neutralization due to prolonged culturing in T cell line cultures. Briefly, 10 million PBMC will be cultured in 5mL of RPMI-1640 Glutamax™ medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and in 5% CO2. After 72 hours of PHA stimulation, cells are resuspended in 96-well plates, each with 200,000 cells in 200 microliters of medium. 300 TCID50 of HIV-1 BaL virus are mixed with different concentrations of rsCD4 (500, 300, 100, 30, 10, 3, 1 micrograms/mL) for 60 min at 37°C and then added to the cells. Cells are then monitored for viral production (HIV RNA/mL) and visually inspected for presence of syncytia. The rsCD4 concentration at which the virus is successfully neutralized (no virus production and no presence of syncytia) is considered the baseline neutralization value (control) for the primary BaL HIV-1 isolate. Experiment is then repeated with HIV-1 BaL isolated from the PBMC/SupT1-CCR5 Cl.30 co-cultures at different time points (3, 15, 30 days post infection) and the data compared with the control experiment, to assess whether virus sensitivity to neutralization gradually increased at later time points with respect to the original BaL virus.

Analysis of virus capacity to induce syncytia in infected cells (HIV-1 LAI)

It has been previously reported that upon prolonged replication in the SupT1 cell line, the X4 HIV-1 virus LAI evolves toward a less cytopathic virus with a reduced capacity for syncytium formation [2]. Accordingly, the purpose of this test is to assess whether this phenomenon is also observed in viral isolates grown in SupT1/PBMC co-cultures. This test will be performed only with the HIV-1 LAI virus because syncytium formation is a typical characteristic of X4 virus strains. Briefly, 10 million PBMC will be cultured in 5mL of RPMI-1640 Glutamax™ medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and in 5% CO2. After 72 hours of PHA stimulation, cells are resuspended in 96-well plates, each with 200,000 cells in 200 microliters of medium. 200 TCID50 of HIV-1 BaL virus are then added to the cells. Cells are then monitored for viral production (HIV RNA/mL) and visually inspected to assess syncytium formation. A fresh stock of LAI virus not previously grown in SupT1 cells is used as a control, and results are compared with that obtained with LAI virus isolated at different time points (3, 15, 30 days post infection) from SupT1/PBMC co-cultures.

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Aims 2,3,4,5

Mice
The mice used in the study are unmanipulated male and female adult NSG mice. The animals are bred and kept in SOPF conditions in individually ventilated cages (up to seven mice per cage) of an ABSL3 facility. Sterile food and water are provided ad libitum. All animal experiments must receive approval from the local Animal Ethical Committee.

Cells
Hu-PBMC are isolated from a buffy coat using a Ficoll density gradient and kept frozen in liquid nitrogen in the presence of DMSO (10% final concentration) until further use. Cells are freshly thawed before inoculation into animals. For infection experiments with the X4-tropic HIV-1 strain LAI, the SupT1 cell line is used. For infection experiments with the R5-tropic HIV-1 strain BaL, the SupT1-CCR5 Cl.30 cell line is used. SupT1 cells are cultured in RPMI-1640 Glutamax™ medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and in 5% CO2. SupT1 cells are injected into animals after 30 Gy irradiation delivered by an X-ray generator (X-Strahl). Please note that all PBMC used for engrafting the mice belonging to the same experiment must be isolated from a single donor, in order to avoid donor variability as a source of different susceptibility to infection between treatment and control groups. Accordingly, a single PBMC donor must be used for experiments with the X4 LAI virus, and a single PBMC donor must be used for experiments with the R5 HIV-1 BaL virus.

First round of in vivo infection experiments with HIV-1 LAI (25,000 TCID50)

In vivo HIV infection
Infection is performed intraperitoneally. One syringe is used per animal to reduce the risk of an injection accident.

Treatment and control groups

For the first round of infection experiments, 18 mice are divided into three groups (n=6 mice per group): a treatment group A, a positive control group B, and a negative control group C. The animals are ear-tagged with unique numbers. One week before onset of the study, the selected mice are transferred to an ABSL3 isolator space (six cages, with three mice per cage). At the onset of the study, the six animals in treatment group A receive 20×10^6 Hu-PBMC intraperitoneally (T0), 40×10^6 30 Gy-irradiated SupT1 cells intraperitoneally at T + 2 hours, and 25,000 TCID50 of HIV-1 LAI virus intraperitoneally at T + 4 hours [14]. Mice in group A also receive three supplementary weekly intraperitoneal inoculations with 40×10^6 30 Gy-irradiated SupT1 cells each. The positive control group B does not receive SupT1 cells. The negative control group C does not receive SupT1 cells and is kept free of HIV infection. To avoid cross-contamination between the different groups, only animals belonging to the same group are placed together in cages. The three groups and injection schemes are summarized in Table 1.

Table 1. Injection schemes.

Group	n	T0	T + 2 hours	T + 4 hours	T + 1 week	T + 2 weeks	T + 3 weeks
A	6	20×10^6 PBMC	40×10^6 SupT1	25,000 TCID50 HIV LAI	40×10^6 SupT1	40×10^6 SupT1	40×10^6 SupT1
B (pos-CT)	6	20×10^6 PBMC	-	25,000 TCID50 HIV LAI	-	-	-
C (neg-CT)	6	20×10^6 PBMC	-	-	-	-	-

pos-CT: positive control; neg-CT: negative control. The animals are maintained in the study for a total of 4 weeks and monitored regularly for signs of illness, for instance as a consequence of graft-versus-host disease progression.

In the event that the results are not in line with our expectations with the possible cause being a too much high virus input, experiments with HIV-1 LAI will be repeated with a lower virus input (8,000 TCID50).

Second round of in vivo infection experiments with HIV-1 BaL (50,000 TCID50)

After performing the first round of infection experiments with HIV-1 LAI, the same protocol is repreated with HIV-1 BaL (50,000 TCID50) and the SupT1-CCR5 Cl.30 cell line. Another 18 mice are used. In the event that the resutls are not in line with our expectations with the possible cause being a too much high virus input, experiments with HIV-1 BaL will be repeated with a lower virus input (25,000 TCID).

Final round of infection experiments to consolidate the results

Once the optimal viral input dose to better show treatment efficacy is determined by the previous experiments, to consolidate the results 18 mice are used to repeat the experiments with HIV-1 LAI, and another 18 mice are used to repeat the experiments with HIV-1 BaL.

Blood sampling of the animals

Blood samples (~50–100 µL) are harvested from the animals in EDTA-coated microtubes (Microvette® CB300 K2E) at the following time points: T + 1 week, T + 2 weeks, T + 3 weeks, and T + 4 weeks. In the case of treatment group A, blood sampling is performed before SupT1 cell inoculation. The blood samples are separated by centrifugation (10 min, 1000 × g) into cell fractions and plasma fractions. The plasma fractions are kept frozen (−20°C) in microtubes until the end of the study. Blood cell leukocytes are isolated from the cell fraction on a Ficoll density gradient. Flow cytometric analysis of blood leukocytes is performed using a monoclonal antibody cocktail (Table 2) after incubation with human and murine FcR blocking reagents.

Table 2. Flow cytometry reagents.

Target	Label	Clone	Origin	Dilution
hCD45	PerCP	H130	BioLegend	1:50
mCD45	APC E780	30-F11	eBioscience	1:200
hCD3	E450	UCHT1	eBioscience	1:50
hCD4	PE	MEM-	ImmunoTools	1:25
hCD8	FITC	MEM-31	ImmunoTools	1:12.5

Incubations are performed in 96-well plates in the dark at 4 °C, and the following populations are successively gated: mouse leukocytes (hCD45−mCD45+), human leukocytes (hCD45+mCD45−), human T cells (hCD3+), and human T cell subsets based on CD4 vs. CD8 expression (CD4+CD8−, CD4−CD8+, and CD4+CD8+). Accordingly, SupT1 CD4+CD8+ T cells are distinguished from PBMC CD4+CD8− and CD4−CD8+ T cells by FACS analysis. All data acquisitions are performed with an LSR-II Fortessa flow cytometer interfaced with FACS-Diva software. Data are analyzed using FlowJo 9.8 software, and graphs are plotted with GraphPad Prism-5 software. Once all plasma samples are collected and stored, HIV viral load is measured by qPCR in a single run. Viral load is expressed as HIV RNA copy number per mL of blood (detection threshold: 10^2 HIV-1 RNA copies per mL). Blood samples will be also used for toxicological tests and virus isolation.

Analysis of virus attenuation (potential vaccination effect of SupT1 cell infusion)

Analysis of neutralization sensitivity (HIV-1 BAL)

It has been previously reported that prolonged viral replication of primary HIV-1 isolates in T cell line cultures results in a virus that shows an increased sensitivity to neutralization by rsCD4 (recombinant soluble CD4) [4]. Accordingly, the purpose of this test is to assess whether this phenomenon is also observed in viral isolates grown in mice that received SupT1 cell infusion treatment. This experiment

will be preformed only with the R5 HIV-1 BaL virus because the X4 LAI virus is already a strain that acquired sensitivity to neutralization due to prolonged culturing in T cell line cultures. Briefly, 10 million PBMC will be cultured in 5mL of RPMI-1640 Glutamax™ medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and in 5% CO2. After 72 hours of PHA stimulation, cells are resuspended in 96-well plates, each with 200,000 cells in 200 microliters of medium. 300 TCID50 of HIV-1 BaL virus are mixed with different concentrations of rsCD4 (500, 300, 100, 30, 10, 3, 1 micrograms/mL) for 60 min at 37°C and then added to the cells. Cells are then monitored for P24 production and visually inspected for presence of syncytia. The rsCD4 concentration at which the virus is successfully neutralized (no p24 production and no presence of syncytia) is considered the baseline neutralization value for the primary isolate. The same experiment will be then repeated with HIV-1 BaL virus isolated from mice that received SupT1 cell infusion treatment in order to assess whether a lower concentration of rsCD4 is required for neutralizing the virus. Specifically, virus isolated from different time points (1 week, 2 weeks, 3 weeks, and 4 weeks post infection) will be tested to assess whether virus sensitivity to neutralization gradually increased at later time points. BaL virus not previously grown in SupT1 cells is used as a control. In vitro culture experiments are repeated in triplicate.

Analysis of virus capacity to induce syncytia in infected cells (HIV-1 LAI)

It has been previously reported that upon prolonged replication in the SupT1 cell line, the X4 HIV-1 virus LAI evolves toward a less cytopathic virus with a reduced capacity for syncytium formation [2]. Accordingly, the purpose of this test is to assess whether this phenomenon is also observed in viral isolates grown in mice that received SupT1 cell infusion treatment. This test will be performed only with the HIV-1 LAI virus because syncytium formation is a typical characteristic of X4 virus strains. Briefly, 10 million PBMC will be cultured in 5mL of RPMI-1640 Glutamax™ medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and in 5% CO2. After 72 hours of PHA stimulation, cells are resuspended in 96-well plates, each with 200,000 cells in 200 microliters of medium. 150 TCID50 of HIV-1 LAI virus are then added to the cells. Cells are then monitored for P24 production and visually inspected to assess syncytium formation. LAI virus not previously grown in SupT1 cells is used as a control.

Toxicological study

The experiment protocol to test acute, subchronic, and chronic toxicity of SupT1 cell infusion therapy is still being finalized.

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2. Das, A.T.; Land, A.; Braakman, I.; Klaver, B.; Berkhout, B. HIV-1 evolves into a nonsyncytium-inducing virus upon prolonged culture in vitro. Virology 1999, 263, 55–69.
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with alterations in the responses of their envelope glycoproteins to soluble CD4. AIDS Res. Hum. Retroviruses 1993, 9, 529–539.
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TOKEN UTILITY

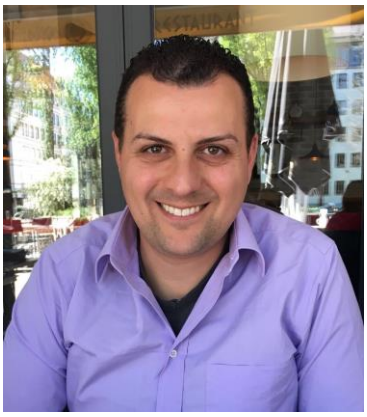
The “You’re not alone” application will have a mining software, and a mobile app that will feature visual representation of the clinical data on the fly as well as the mentioned social functions. The tokens will give early access for downloading the application before the official release. They will also give access to a series of privileged features in the app ecosystem such as a voting system for proposing new parameters (e.g., HLA patient subtype) for the database, or access to moderation roles in the social communities. Miners will also receive Tokens as a reward for providing computational power for processing the data.

Token supply will be limited to an initial 20,000,000$ cap. If the cap is reached during the pre-ICO, the ICO may not be necessary.

TEAM

Jonathan Fior

Owner and Chief Scientific Officer. Jonathan Fior is a research scientist specialized in the field of virology, immunology, cancer and regeneration*****. He founded Innovative Bioresearch and conceived SupT1 cell infusion therapy, a novel cell-based therapy for HIV that uses SupT1 cells as a decoy target for HIV-1 to prevent CD4+ T cell depletion as well as to render the virus less cytopathic. Jonathan Fior is also a successful stock trader and thus he has a great financial competence as well.



Alessandro Gatti

Chief Legal Officer. Alessandro Gatti is a legal expert and he ensures that everything we do is in accordance with the law.

***Jonathan Fior publications**

Fior J. An Initial In Vitro Investigation into the Potential Therapeutic Use of SupT1 Cells to Prevent AIDS in HIV-Seropositive Individuals. PLoS ONE. 2012;7(5):e37511. https://www.ncbi.nlm.nih.gov/pmc/articles/pmid/22701517

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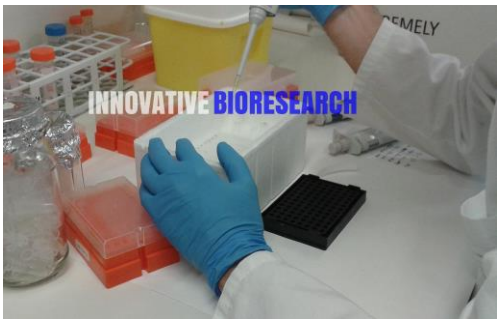

To maximize results and reducing costs, we perform our highly specialized research by contracting with state-of-the-art CROs and Institutions. Some CROs we hired in the past are listed below

AXENIS

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SOFTWARE DEVELOPMENT

Along with performing our AIDS cure research, the other important aim of this project is developing the "You're not alone" application. For developing the application, a professional software development company will be hired for the job. Some potential candidates we selected are listed below

Patacaum

WillowTree, Inc.®

Dogtownmedia