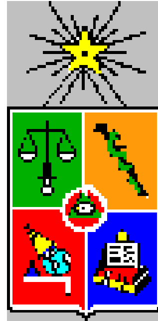

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PHASE II STUDY OF IMMUNIZATION WITH DENDRITIC CELLS AND LOW DOSE OF INTERLEUKIN-2, IN PATIENTS WITH ADVANCED MELANOMA.

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Cancer continues to be one of the leading causes of death, especially among inhabitants of highly developed countries. Modern surgical and transplant techniques, new and more effective drugs, and improved radiation methods have yielded promising results in treating some of these conditions. However, some malignant cells often survive these therapies and spread throughout the body, necessitating the development of new treatments as an alternative or complement to current ones. One such approach would be manipulating the immune system to combat tumors, known as cancer immunotherapy. The use of modified immune system cells for oncological treatment, such as dendritic cells (DCs), is well-documented by international studies that have tested and are exploring various clinical protocols.

Over the past five years, accumulated evidence has demonstrated that modified dendritic cell vaccines produce significant clinical and immunological responses in diseases such as malignant melanoma, non-Hodgkin's lymphoma, and other neoplasms in stages of the disease where no practical alternative therapy exists.

On the other hand, experimental therapies based on Interleukin-2 (IL-2) have been used with relative success in some neoplasms since 1985. However, problems related to the toxicity of IL-2 are still under investigation. Although recombinant human IL-2 like PROLEUKIN has proven to be one of the most effective strategies in treating advanced renal carcinoma and melanoma, therapy based on high-dose intravenous IL-2 is not suitable for all patients. Our direct experience, based on clinical studies conducted at the Karolinska Institute in Sweden and international experience with PROLEUKIN, indicates that low doses (2.4x106 IU/m2) administered subcutaneously maintain the same beneficial effects for patients while almost wholly eliminating adverse effects. Therefore, we have decided to present to the respective committee a second protocol, leveraging the experience gained and the recent funding obtained from FONDEF, to develop an optimized protocol that alternates dendritic cell vaccines with the use of low doses of IL-2 as a complement to strengthen the immune system.

For some time now, we have established a multidisciplinary group of professionals at the Faculty of Medicine of the University of Chile, involving the participation of preclinical and clinical researchers. We have recently completed a phase I clinical protocol for treating malignant melanoma using DCs loaded with tumor extracts. This study, the first of its kind in Chile and probably in Latin America, serves as the basis for the presentation of this project.

The results can be summarized as follows: No adverse effects were observed from the subcutaneous application of dendritic cells, except for one patient who showed mild fever and arthralgia. Immunological effects were observed in vitro in 60% of the treated patients through an increase in specific T lymphocyte precursors against tumor cells in the patient's blood measured by ELISPOT, in addition to delayed-type hypersensitivity reactions (DTH) in 50% of the treated patients when tumor extract was injected subcutaneously after vaccinations. Finally, partial regression or mixed response was observed in two patients. One showed regression of lung metastases, and the other showed regression of cutaneous metastases. In most cases, disease stability was observed, although it cannot be concluded that a significant relationship exists between vaccination and the survival of treated patients. These results motivate further exploration of the methodology, primarily intending to provide therapeutic alternatives to patients with no other options.

Theoretical Formulation of the Project

Melanoma is a malignant tumor of neuroectodermal origin whose incidence has progressively increased in the last century. The only way to cure a malignant melanoma is early detection and appropriate surgical treatment, as once it reaches an advanced state, it is highly resistant to conventional treatments such as surgery, radiotherapy, and chemotherapy (1-3). Globally, melanoma is the fastest-growing and most deadly type of skin cancer in Europe and the United States among various cutaneous cancer types. This situation is also observed in Chile; only in Santiago, between 1992 and 1996, 318 malignant melanoma cases were detected, representing 10.6% of all types of skin cancer (1). While it is true that malignant melanoma is almost entirely curable (>95%) when detected early, the survival probability in patients with metastatic melanoma decreases significantly (<15%). Although surgery, chemotherapy, and radiotherapy are the primary conventional methods for treating this disease, implementing new therapies is essential, especially in patients with stage III and IV melanoma, where chemotherapy and radiotherapy are ineffective.

Cancer remains one of the leading causes of death, especially among residents of highly developed countries. Modern surgical techniques and transplants, new and more effective drugs, and improved irradiation methods have allowed promising results in treating some diseases. A clear example of this progress is reflected in the treatment of childhood leukemia with a new generation of anti-cancer drugs (2). Often, some malignant cells survive these therapies and spread throughout the body, necessitating the development of new treatments as alternatives or complements to current ones. One such approach is manipulating the immune system to combat tumors, known as cancer immunotherapy. Since the 1980s, when cytotoxic T lymphocytes (CTL) with antitumor activity were isolated from peripheral blood, lymph nodes, and tumor tissue of melanoma patients, there has been growing interest in using the immune system in cancer treatment. Cytokines such as Interleukin-2 (IL-2) and Interferon gamma (IFN-γ) are commonly used as alternative therapies (4,5) in some neoplasms, and adoptive therapies, using in vitro stimulated CTL, have been tested in some clinical protocols with varying responses (6).

Over the last thirty years, significant progress has been made in understanding the functioning of the immune system, especially regarding antigen recognition (3,7). On the other hand, the significant advances in molecular biology techniques that facilitate gene cloning and the production of recombinant proteins have allowed for identifying tumor-associated antigens (AAT) and their subsequent characterization.

Cytotoxic T Lymphocytes and Antitumor Immune Response

Historically, pioneering experiments demonstrated that the growth of syngeneic tumors in mice could be prevented through prior immunization with the same tumor (8,9). Guided by these studies in animal models, it has been observed that human T lymphocytes have also shown the ability to lyse autologous tumors in vitro (10,11) specifically. Furthermore, T cells are capable of proliferating in response to stimulation with autologous tumor cells, secreting cytokines such as IL-2, interferon-gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-alpha (TNF-α) (12). Antitumor T cells can be expanded in large quantities in vitro and adoptively transferred to treat significant tumor burdens in mice and humans (6). These observations have enabled the identification of tumor antigens recognized by human autologous T cells using molecular cloning techniques (13,14). These results provide undeniable evidence of a T cell-mediated response against autologous tumors.

Cytotoxic T lymphocytes (CTLs, CD8+) can recognize tumors through 8-10 amino acid peptide fragments derived from cytoplasmic or nuclear proteins associated with major histocompatibility complex (MHC) class I (15). Melanoma-specific CTLs, either adoptively transferred or activated in vivo by melanoma-associated antigen epitopes, have therapeutic potential, capable of inducing the regression of tumors and their micrometastases (16,17).

Melanoma-Associated Antigens

Over the past five years, several melanoma-associated antigens (MAAs) recognized by CTLs have been identified, and their peptide epitopes are characterized using genetic (13,14) and biochemical methods (18).

The cloning of genes encoding human MAAs has allowed their characterization and classification into different types. The first category consists of so-called tumor-specific antigens, among which the MAGE gene family stands out (19-21). These antigens are derived from proteins whose genes are actively transcribed in various types of tumors but not in normal adult tissue cells and are related to the primary stages of embryonic development (19-21). Another tumor-specific antigen in melanoma consists of those originating from mutated genes. A few examples of such antigens have been described in melanoma, with one of them being derived from cyclin-dependent kinase 4 (CDK4) (22). The primary group of antigens, whose CTL response has been most frequently

described in melanoma patients, comprises differentiation antigens, also known as tissue-associated antigens. This category includes melanosomal antigens MART-1/Melan A, gp100, and Tyrosinase (23-28). Genes encoding these antigens are expressed not only in melanomas but also in normal melanocytes and the pigmented epithelium of the retina, making them autoantigens (23-28). CTLs specific for these antigens can be isolated from lymph nodes, tumor-infiltrating lymphocytes (TILs), or peripheral blood lymphocytes (PBLs) of melanoma patients (26-28). This T-cell recognition has been interpreted as a breakdown of immunological tolerance against self-antigens, perhaps due to the overexpression of these proteins in tumor cells.

Dendritic Cells: Key Professional Antigen-Presenting Cells
A T cell-mediated response induction requires antigen presentation in a specialized cellular context provided by antigen-presenting cells (APCs) (14). These specialized cells trigger a primary immune response and include macrophages, B cells, and dendritic cells (DCs). DCs play a fundamental role in generating an antitumor T cell-mediated response, as they can capture tumor antigens and present them in association with MHC molecules (15–17). Additionally, they express many co-stimulatory molecules, which provide crucial signals ensuring the effectiveness of the T cell-mediated response (18). DCs, also called professional APCs, are strategically located at antigen concentration sites, efficiently internalizing, processing, and presenting soluble antigens in the context of MHC class I and II, being the most efficient in inducing a primary T cell response (19–21). DCs were first visualized in the skin as Langerhans cells in 1868, but their characterization began only 25 years ago. DCs are also potent stimulators of B cells and mediate the isotype switching of immunoglobulins.
The main obstacles to the characterization and utilization of DCs (low relative frequency and the lack of specific cellular markers) have recently been overcome. Indeed, relatively high quantities of DCs can be obtained in vitro from peripheral blood monocytes stimulated with GM-CSF and interleukin-4 (IL-4) (29). The cells generated in this way have the same characteristics as immature primary DCs isolated in vivo. They have low expression of MHC and other co-stimulatory molecules like CD86 and CD40 but are highly well-equipped to capture antigens (29) through mechanisms that include endocytosis, pinocytosis, and membrane fusion (30).

Interleukin-2
Interleukin-2 (IL-2) is a 15 kDa glycoprotein primarily synthesized and secreted by helper T lymphocytes (Th) as a result of activation mediated by mitogens or the interaction between the TCR/CD3 complex and the MHC complex on the surface of antigen-presenting cells (31-35). The response of T lymphocytes to activation is the induction of IL-2 production and the expression of its receptor (IL-2R) on the cell membrane, leading to the clonal expansion of T lymphocytes specific to the antigen. IL-2 can act as an autocrine factor, stimulating the expansion of the producing cells themselves, or as a paracrine factor, stimulating the activation of other immune system cells, such as CD8+ T lymphocytes, B cells (36,37), and natural killer (NK) cells (38-46). Furthermore, so-called lymphocyte-activated killer cells (LAK) (47,48), used in adoptive therapies, are believed to derive from NK cells and CD8+ lymphocytes activated by IL-2 in vitro. Other cellular subtypes influenced by IL-2 include neutrophils (49,50), monocytes (51), and gamma-delta T lymphocytes (52), which have shown increased activation, enhanced effector functions, and improved cell survival. IL-2 was first biologically characterized in JURKAT cells, a human leukemia cell line. In its natural state, IL-2 is differentially glycosylated, and these changes in glycosylation levels explain the variations in its molecular weight, ranging from 15 to 18 kDa (53). The IL-2 gene was later cloned and expressed in eukaryotic cells (53) and E. coli (54). The recombinant analog of IL-2 is known as PROLEUKIN® (aldesleukin).

Given the pivotal role of IL-2's interaction with IL-2R in mediating the immune response, it is evident that research and manipulation of this system for therapeutic or diagnostic purposes are crucial. IL-2 has shown promising effects as an anti-cancer drug due to its ability to activate and proliferate LAK cells and tumor-infiltrating lymphocytes (TILs) (55-58). IL-2-mediated effects observed in renal carcinoma and melanoma since 1985 have earned recent approval by the United States Food and Drug Administration (FDA) for treating these neoplasms (59-63).

However, issues related to IL-2 toxicity are still under investigation (64). Although PROLEUKIN IL-2 has proven to be one of the most effective strategies in treating advanced renal carcinoma and melanoma, high-dose intravenous IL-2 therapy is unsuitable for all patients. Its use is restricted to adult patients in good overall health. The basic biology of IL-2-based strategies for treating infectious diseases, transplants, and AIDS has been reviewed recently (65,66).

Since 1985, PROLEUKIN IL-2 has been administered to thousands of patients. As experience with PROLEUKIN

IL-2 has grown over the years, treating physicians have become familiar with most of the side effects that have arisen and have learned to manage them appropriately. In most patients, these effects occur during treatment and are controlled within seventy-two hours after treatment completion. Therefore, it is recommended that intravenous IL-2 administration be carried out with the patient hospitalized.

Most of the side effects of IL-2 use described in the literature are based on experiences with high doses (18 million units per square meter of body surface area) and intravenous administration, as reviewed in Table 1. Our direct experience, based on clinical studies conducted at the Karolinska Institute in Sweden (67), as well as international experience (68-70), indicates that low doses (2.4 million units per square meter of body surface area) and subcutaneous administration maintain the same beneficial effects for patients while nearly eliminating adverse effects (67-70).

Significance of these Studies

Several preclinical studies have documented the ability of certain genetically modified tumors to induce protective immunity against a challenge of native tumors (71-73). Based on this data and shared antigens recognized by CTLs, several clinical studies have been developed using vaccines with modified allogeneic tumor lines expressing cytokines such as IL-2 or co-stimulatory molecules like B7-1 (74). There is ample evidence and substantial preclinical and clinical data indicating the importance of T cell-mediated responses in immune-dependent tumor regression (6,9-12). These results have stimulated research, leading to the development of vaccines against defined antigens.

Several vectors containing defined antigens, such as MAGE1, MAGE3, gp100, and Tyrosinase, have been produced for clinical studies. Due to the lack of knowledge about the best immunization methods and the absence of appropriate animal models, various immunization protocols have been clinically tested.

Recently, therapies based on immunization with peptides derived from the gp100 antigen modified to increase their HLA affinity (75) or dendritic cell vaccines pulsed with peptides (76) or melanoma extracts (77) have been shown to induce specific CTL responses in most treated melanoma patients, along with objective regressions of their tumor masses (76-79). This type of therapy has also been used in other types of neoplasms. In the case of Follicular Lymphoma, the positive effects of chemotherapy

have been combined with subsequent immunotherapy based on dendritic cells pulsed with anti-idiotypic antigens. The results in treating minimal residual disease have been more than satisfactory, with 8 out of 11 treated patients eliminating residual malignant cells after vaccination (80).

Vaccination of Melanoma Patients Using DCs Loaded with Allogeneic Melanoma Extracts

In Chile, we have completed the first phase I clinical trial for treating advanced malignant melanoma using dendritic cell vaccines loaded with allogeneic tumor extracts. Since chemotherapy and radiotherapy only offer palliative effects in advanced melanoma, resulting in an inferior prognosis for these patients, we intended to develop a protocol that would adapt to the reality of our country, allowing for a combination of high technology and low costs.

In summary, the results obtained in the Phase I clinical trial, which were published in (*Escobar et al. Clin. Exp. Immunol. 2005. doi:10.1111/j.1365-2249.2005.02948.x*), can be summarized as follows:

1- No adverse effects to the subcutaneous application of dendritic cells were observed, except for one patient who showed mild fever and arthralgia, probably not associated with the vaccine, as shown in Table 2- Immunological effects were observed in vitro in 60% of the treated patients, through an increase in precursor T lymphocytes specific to tumor cells in the patient's blood, as measured by ELISPOT.

3- Delayed-type hypersensitivity reactions (DTH) were observed in 50% of the treated patients when tumor extract was injected subcutaneously after vaccinations.

4- Partial regression or mixed response were observed in 2 patients. One showed regression of lung metastases, and the other showed regression of cutaneous metastases (Table 3). In most cases, disease stability was observed, although it cannot be concluded that a significant relationship exists between vaccination and the survival of treated patients.

The idea of this Phase I/II clinical trial will be an adaptation of the protocol by Nestle and colleagues. After obtaining approval from the human research ethics committee, we recruited 80 patients (Table 2) who were duly informed about the scope of these protocols and the realistic prospects of success. They then underwent a comprehensive health check to assess their overall health. The selected patients underwent leukapheresis at the blood bank of the Clinical Hospital of the University of Chile. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Hypaque gradient in the laboratory of Dr. Flavio Salazar at the Institute of Biomedical Sciences of the University of Chile. Some of the cells were cryopreserved in liquid nitrogen for future use. According to the protocol, approximately 1.8x108 PBMCs were placed in 6-well culture plates and incubated for two hours at 37°C and 7% CO2. The suspended cells, peripheral blood lymphocytes (PBLs), were removed and frozen for later use. The monocytes adhering to plastic were incubated for seven days in serum-free culture medium (AIM-V therapeutic, GIBCO, BRL) in the presence of 500 U/ml of IL-4 and 800 U/ml of GM-CSF, as described in detail in the Materials and Methods section. On the sixth day, the DCs were pulsed with tumor extracts from allogeneic melanoma cell lines, previously checked for sterility, and incubated for 6 hours at 37°C and 7% CO2, followed by maturation with TNF-alpha for 12 to 18 hours. Mature DCs were characterized by flow cytometry. Approximately 5 to 15 x 106 pulsed DCs were subcutaneously injected. This routine was repeated weekly for at least four times and a maximum of 6 times. After the fourth dose, the patient's general condition, possible clinical regressions, adverse side effects, and the effects of the treatment on the immune system were evaluated. This evaluation was done through in vivo delayed-type hypersensitivity (DTH) assays using melanoma extracts and their respective controls. Planned in vitro assays included the detection of anti-melanoma CTL precursors by ELISPOT (77), cytokine production, proliferation assays, and quantifying the CD4/CD8 ratio by flow cytometry.

Several obstacles must be overcome to make this type of treatment effective in patients. First, immunization is hindered in patients due to the state of immunosuppression that many experience due to tumor burden, chemotherapy (81,82), or other as-yet-undetermined factors. The heterogeneity of antigenic expression in tumors must also be considered, so vaccination with tumor extracts guarantees immunization with multiple antigens. Another difficulty to be considered is the ability of tumors to evade immune system recognition (83). These escape strategies range from inhibiting antigen presentation to producing immunosuppressive substances such as TGF-beta and IL-10 (84). The relative importance of these potential mechanisms in reducing the potential of vaccine-based immunotherapy has yet to be established. Nevertheless, it is crucial to investigate these obstacles and develop appropriate clinical protocols that harness the full potential of cellular vaccine-based immunotherapy, which has been convincingly demonstrated in murine models. Therefore, we have decided to present a second study to the respective committee, building on the experience gained and the recently obtained funding from FONDEF, to develop an optimized protocol that alternates dendritic cell vaccines with the use of low doses of IL-

2 as a complement to strengthen the immune system.

This optimization is based on studies by Steve Rosenberg of the NIH, which showed improved responses by combining dendritic cell vaccines loaded with modified antigenic peptides in the presence of IL-2 (75). Additionally, as previously described, the use of high-dose IL-2 has side effects that, while manageable, are severe and temporarily affect patients' quality of life. Therefore, based on our own experience (67) and that of others (68-70), we have decided to use low doses of subcutaneously administered IL-2, which do not produce marked adverse effects (67,70). Few studies worldwide have been conducted in this area, one recent in renal carcinoma (70). Overall, most studies demonstrate that IL-2 enhances the ability of dendritic cells to induce an immune response (68-70).

Finally, the received funding will allow us to conduct these studies in GMP (good manufacturing procedure) laboratories with high biological safety, complying with all international standards for clinical studies.

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TABLE 1: Side effects reported in clinical trials with PROLEUKIN IL-2, involving a total of 525 patients with either renal carcinoma or metastatic melanoma

System	GRADE 3	GRADE 4	ALL EVENTS
General			
Chills	70 (13%)	0 (0%)	271 (52%)
Fever	29 (6%)	5 (1%)	154 (29%)
Malaise Infection	43 (8%)	0 (0%)	143 (27%)
	29 (6.0%)	7 (1%)	66 (13%)
Cardiovascular System			
Hypotension	205 (39%)	15 (3%)	375 (71%)
Cardiovascular Disorders	36 (7)	7 (1%)	59 (11%)
Digestive System			
Diarrhea	113 (22%)	10 (2%)	350 (67%)
Vomiting	111 (21%)	7 (1%)	261 (50%)
Nausea	25 (5%)	0 (0%)	186 (35%)
Nausea and Vomiting	25 (5%)	2 (0%)	101 (19%)
Hematological			
Thrombocytopenia	59 (11%)	5 (1%)	192 (37%)
Anemia	15 (3%)	1 (0%)	150 (29%)
Metabolic and Nutritional			
Bilirubinemia	30 (6%)	13 (2%)	210 (40%)
Creatinine Increase	14 (3%)	5 (1%)	174 (33%)
SGOT Increase	17 (3%)	3 (1%)	122 (23%)
Central Nervous System			
Confusion	57 (11%)	5 (1%)	177 (34%)
Somnolence	19 (4%)	2 (0%)	115 (22%)
Respiratory System			
Dyspnea	52 (10%)	5 (1%)	224 (43%)
Respiratory Disorders	15 (3%)	14 (3%)	59 (11%)
Urogenital System			
Oliguria	137 (26%)	33 (6%)	333 (63%)
Anuria	13 (1%)	25 (5%)	36 (7%)

Grade 3 and 4 Adverse Effects in > or equal to 3% of treated patients (n=525). Grade 4 Adverse Effects are those that compromise the lives of patients.

The rate of drug-related death in 255 patients with metastatic renal carcinoma who received PROLEUKIN IL-2 was 4% (11/255). The rate of drug-related death in 270 patients with metastatic melanoma who received PROLEUKIN IL-2 was 2% (6/270).

TABLE 2: General Characteristics of Patients Before the Initiation of Phase I/II Clinical Protocol

Patient	Age	Gender	Time of Evolution Before Starting Protocol	Clinical Stage	Primary	Metastasis	Previous Treatment
CT001	34	F	36 months	IV	Thorax	In transit and gland	Surgery anterior mammary
CT002	35	M	ND	IV		Osseous (Vertebral)	
CT003	34	M	12 months	IV	Back	Lung	Surgery
CT004	69	M	60 months	IV	Back	Multiple	Surgery, IL2
CT005	57	M	36 months	IV	EII	Lung	Surgery
CT006	30	M	36 months	IV	Thorax	Subcutaneous inguinal left	Surgery
CT007	53	F	6 months	III	EII	Inguinal left lymph nodes	Surgery
CT008	37	F	36 months	IV	EII	Pulmonary	Surgery
CT009	24	F	8 months	III	Back	Axillary right lymph nodes	Surgery
CT010	39	M	60 months	IV	Malar	Local dissemination	Enucleation
CT011	68	F	30 months	IV	Back	Liver	Surgery
CT012	57	F	14 months	IV	Plant left	In transit	Surgery, IL2
CT013	62	M	36 months	IV	Scalp	Lung	Surgery

Note: ND stands for "Not Determined."

TABLE 3: Adverse Reactions and Clinical and Immunological Responses of Patients in Previous Phase I Clinical Protocol

Patient	No. DC/Dose	Adverse Reactions	Elispot	DTH	Vaccination	Objective Regressions	Post-Therapy Disease
CT001	3-5 x 10^6	Negative	+	+	2nd cycle	Regression of pulmonary nodules	Progression of pulmonary nodules
CT002	3-5 x 10^6	Negative	Nd	Nd	-	-	Deceased
CT003	3-5 x 10^6	Negative	+	+	3rd cycle	-	Deceased
CT004	3-5 x 10^6	Negative	-	-	1st cycle	-	Stable disease
CT005	3-5 x 10^6	Fever, arthralgias AAN and RF (-)	-	-	1st cycle	Regression of cutaneous metastasis	Progression of cutaneous metastasis
CT006	3-5 x 10^6	Negative	+	+	2nd cycle	-	Progression
CT007	3-5 x 10^6	Negative	+	-	1st cycle	-	Stable disease
CT008	10 x 10^6	Negative	+	-	Withdrawal (1st dose)	-	Deceased
CT009	10 x 10^6	Negative	+	+	1st cycle	-	Stable disease
CT010	10 x 10^6	Negative	Nd	-	1st cycle	-	Progression
CT011	10 x 10^6	Negative	Nd	+	1st cycle	-	Stable disease
CT012	10 x 10^6	Negative	Nd	-	2nd cycle	-	Progression
CT013	10 x 10^6	Negative	Nd	-	1st cycle	-	Progression

Note: Nd = not determined.

Treatment of Malignant Melanoma with Dendritic Cells Pulsed with Tumor Extracts. Clinical Study Design

PHASE I/II TRIAL IN STAGE IV MALIGNANT MELANOMA
This protocol is ethically justified as advanced melanoma patients, once their tumor invasion has extended beyond the lymphatic lymph node barrier, are not amenable to satisfactory treatment with traditional methods such as surgery, radiotherapy, or conventional chemotherapy. Disseminated tumors are refractory to all standard treatments. 100% of patients who develop distant metastases will die due to their disease, either from complications or cachexia. Therefore, immunotherapy based on immunological stimulation with immunocompetent dendritic cells, combined with immunological reinforcement using IL-2, may, according to evidence from ongoing clinical protocols, lead to prolonged survival with improved quality of life and, in some cases, complete tumor regression.

GENERAL AIM:
To study the clinical and immunological response of patients treated with vaccines based on autologous dendritic cells loaded with tumor antigens derived from autologous or allogeneic melanoma extracts, supplemented with the intermittent use of low doses of recombinant human interleukin 2 (rhIL2) PROLEUKIN® (aldesleukin).

MAIN SPECIFIC AIMS:

- SAFETY: Safety in administering dendritic cell preparations; local and systemic toxicity estimation. Determination of adverse reactions such as fever, nausea, allergy, neurological and cardiovascular symptoms. Local toxicity at the administration site.
- IMMUNE RESPONSE MEASUREMENT: Observe the immune response based on in vivo and in vitro parameters:
    - In vivo response: Measure the Delayed-Type Hypersensitivity response (DTH). It involves a cross-test comparing in vivo tissue response between dendritic cells sensitized with tumor extracts and their respective control dendritic cells without loading.
    - In vitro response: ELISPOT assays, measuring IFN-γ production in the peripheral blood of treated patients. Compare specific immune responses after each therapy cycle by measuring IFN-γ production by tumor-specific CTLs. Radioactive chromium releases cytotoxic assays to measure CTL and NK-mediated antitumor response. After each therapy cycle, ELISA assays for quantifying cytokines (IFN-γ, IL-10) in patient serum.
- CLINICAL RESPONSE MEASUREMENT: To observe the objective response rate. The percentage of patients with complete response (CR) and patients with partial response (PR) will be reported.

SECONDARY SPECIFIC AIMS:
To evaluate:

- Overall Survival.
- Disease-Free Survival.
- Quality of Life (assessed using EORTC global QLQ-C30 and EORTC disease-specific QLQ).
- Performance status response: The percentage of patients with a 20% or more significant increase in activity measured by the Karnofsky index will be reported.

MATERIAL AND METHODS
Dendritic Cell Generation: Patient leukocytes will be obtained through leukapheresis at the Blood Bank of the Clinical Hospital of the UNIVERSITY OF CHILE, following the standard protocols used in that unit. Peripheral blood mononuclear cells (PBMCs) will be isolated from peripheral blood using a Ficoll-Hypaque gradient. PBMCs will be cultured for 2 hours at 37ºC and 5% CO2 in a suitable serum-free clinical research medium, AIM-V Clinical Research® (Life Technologies, USA). Non-adherent cells will be discarded, and adherent cells (monocytes) will be cultured in AIM-V supplemented with 500 U/ml of recombinant IL-4 (R&D Systems, INC., MN, USA) and 800 U/ml of GM-CSF (R&D Systems, INC., MN, USA). On the sixth day, monocytes differentiated into dendritic cells will be pulsed with melanoma extracts and then matured with TNF-α (R&D Systems, INC., MN, USA). Flow cytometry will confirm mature dendritic cells' $CD14^{-}$, $CD36^{low}$, and $CD83^{high}$ phenotype characteristics. Mature DCs will be washed twice and resuspended in 300 µl of sterile saline. All cell manipulation procedures for vaccine production will be performed under GMP conditions, as indicated.
Melanoma Extracts: The melanoma cell lines used in this study were established by Dr. Flavio Salazar at The Institute of Biomedical Sciences, Universidad de Chile. These cell lines have been previously checked to ensure they are free from viruses or other contaminants. Cells are maintained under strict sterile conditions in the Immunology Laboratory of the Biomedical Sciences Institute at the University of Chile. When possible, tumor

tissue obtained from the patient will be directly treated to obtain autologous tumor antigens. Dendritic cells will be loaded with lysates from three melanoma lines, $1x10^6$ cell/per line. The lysate will be obtained by three freeze-thaw cycles, irradiated with a dose of 60 Gy, and sonicated for one 30-second cycle. The cell extract will be resuspended in 600 µl of sterile saline. Dendritic cells, approximately $6x10^6$ per 6-well plate, will be pulsed with 100 µl of this extract per culture well for 6 hours at 37ºC and 5% CO2.

Interleukin 2: PROLEUKIN (recombinant human Interleukin-2) will be administered in a dose of 2.4×10^6 IU/m^2 (millions of international units per square meter of body surface), subcutaneously, reconstituted according to the manufacturer's instructions, for two days, after each vaccination for three consecutive days. The administration will be carried out by trained personnel in the appropriate facilities of the Clinical Hospital of the UNIVERSITY OF CHILE.

Vaccination: The preparation of autologous dendritic cells loaded with tumor extract (approximately $10\text{-}20 \times 10^6$ cells/dose) will be administered subcutaneously to selected patients in a volume of 300 µl of sterile saline solution, in a plastic container, labeled with the patient identification number, date, number of dendritic cells inoculated and dose.
Patients will receive their first dose seven days after undergoing the leukapheresis procedure, which will be considered day 0, their second dose on day 10, followed by IL2 at the indicated doses on days 12, 13, and 14. Subsequently, their third dose will be injected on day 30, followed by IL2 on days 32, 33, 34, and its fourth dose on day 50, followed by IL2 on days 52, 53, and 54 (see scheme).

IL2 IL2 IL2

12 13 14 32 33 34 52 53 54

-7 **0** **10** 30 50

Leukapheresis 1st DCV 2nd DCV 3th DCV 4th DCV

DCV= Dendritic Cell Vaccination

Preclinical Trials:
CELL LINES AND MEDIA: The melanoma cell lines Mel-1, Mel-2 and Mel-3 were established at the Institute of Biomedical Sciences, Faculty of Medicine, University of Chile and screened to ensure viruses' absence. The T2 cell line, a TAP-deficient cell line derived from a T-cell/ B-cell leukemia hybrid and C1R-A2, a cell line deficient in MHC class I expression and transfected with HLA-A2, will be maintained in RPMI 1640 medium (GIBCO BRL.) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO BRL.), 1% L-glutamine (GIBCO BRL.), and 1% Penicillin-Streptomycin (GIBCO BRL.).

ISOLATION OF MONONUCLEAR CELLS FROM PERIPHERAL BLOOD: Leukocyte concentrates from patients (leukapheresis) will be mixed in equal parts with sterile PBS. Each sample will be separated into 50 ml conical tubes containing 10 ml of Limphoprep® (NYCOMED PHARMA, Oslo, Norway.) and then centrifuged for 20 minutes at 1200 rpm without brake at 18°C using a refrigerated IEC MP4R centrifuge (MA, USA). The mononuclear cell layer will then be transferred with a pipette to conical tubes and washed three times in 20 ml of PBS for 5 minutes at 1200 rpm, resuspended in a final volume of 20 ml for quantification and viability determination by Trypan Blue exclusion. 1.8×10^8 PBMCs per 6 well plate will generate DCs; the rest will be cryopreserved for future use.

ELISPOT ASSAY FOR IFNγ RELEASE BY ANTIGEN-SPECIFIC CTLs: In brief, 96-well plates (Multiscreen; Millipore, MAIP N45) will be sensitized with 2 μg/ml of primary anti-IFNγ monoclonal antibody (acMo) (Mabtech 1-DIK) overnight at 4°C, washed, and blocked with 1% PBS/BSA for 2 hours at 37°C. Effector and target cells will be added to the wells for 4 hours at 40°C, followed by washing before incubation for 20 hours at 4°C with 75 μl of secondary antibody (0.75 μg/ml biotin-conjugated anti-IFNγ acMo; Mabtech, 7-B6-biotin). Streptavidin-ALP will be added for 1 hour at room temperature in the dark. The wells will then be washed and incubated for 5 minutes with NBT/BCIP for complex visualization. Spots will be counted using a microscope. The response will be considered positive if there are >10 spot-forming cells (SFC) per $2x10^5$ respective controls.
TYPING OF CD8, CD4, AND NK CELL POPULATIONS IN PATIENT BLOOD: To detect cellular markers in PBMCs from melanoma patients, the cells to be analyzed will be washed three times with ice-cold PBS and then incubated with 2 μg/ml of either FITC- or PE-conjugated anti-CD3, anti-CD4, anti-CD8, and anti-CD56 monoclonal antibodies (Pharmigen). The tests will be analyzed using the FAScan instrument (Beckton Dickinson, CA, USA) at the Disciplinary Program of Immunology at the ICBM, University of Chile.
ELISA ASSAY FOR THE DETERMINATION OF IL-10 AND IFNγ IN PATIENT SERUM: In brief, plates sensitized with primary anti-IFN-γ monoclonal antibody (acMo) (OptEIA, Pharmigen) and primary anti-IL-10 monoclonal antibody (acMo) will be blocked with 1% PBS/BSA for 1 hour at room temperature. Then, 100 μl of each patient's serum will be added to each well and incubated for 2 hours at room temperature. The wells will then be washed and incubated for one hour at room temperature with 100 μl of secondary antibody (0.75 μg/ml biotin-conjugated anti-IFN-γ acMo Avidin-HRP (Pharmigen)). Substrate (TMB and hydrogen peroxide) will be added for 30 minutes at room temperature in the dark. The wells will then be washed, and the STOP solution will be added. Absorbance at 450 nm will be read using an ELISA reader (BIORAD, model 3550) at the Disciplinary Program of Immunology at the ICBM, University of Chile.

Dose and Dosing Rationale: Selected patients will be treated with four cycles of therapy over 60 days. In each dose, 10 to 20 x 10^6 DCs loaded with extracts from $3x10^6$ allogeneic melanoma cells will be injected. The extracts will be prepared from irradiated melanoma cells lysed through continuous freezing, thawing, and sonication. This dose is estimated to be sufficient to induce an antitumor immune response, as preclinical and clinical trials have demonstrated clinical and Laboratory immune responses, supporting the method's safety. To support the patient's immune system stimulation, interleukin-2 (IL-2) will be administered subcutaneously at 2.4 x 10^6 IU/m2 two days after the second vaccine dose for three consecutive days. This regimen will be repeated after each vaccination. Currently, standard protocols administer between 18 x 10^6 and 9 x 10^6 IU/m^2 of IL-2 per dose (recommended by distributor CHIRON BV Holland). The low doses used in this study are based on the project director's experience, supported by studies published in international journals and recent literature (67-70). The intention is to avoid IL-2-mediated side effects while preserving its beneficial effects.

Treatment:

a) Treatment Regimen: For administering each dendritic cell dose, the treating investigator will observe and treat patients on an outpatient basis in an adequately equipped outpatient clinic. The 300 μl dose in a sterile saline solution will be injected subcutaneously, preferably near the primary tumor or near unaffected lymph nodes.
b) Immediately after treatment, the patient must be monitored. Vital signs should be taken 15 minutes before and after each treatment, and the patient should be observed in the clinic for at least one hour. After the observation period, the patient will be sent home.
c) Interleukin-2 will be administered subcutaneously, on an outpatient basis, in a properly equipped outpatient clinic for three consecutive days. Vital signs will be monitored, and the patient will be observed in the clinic for 30 minutes.
d) Patients will be followed to detect and evaluate potential toxic reactions to treatment. The next dose can be repeated the following week if: - There was no grade IV toxicity with the previous treatment, - There was no obvious disease progression - The patient does not meet the exclusion criteria for the protocol.

Inclusion Criteria:
• Patients with histologically confirmed melanoma of the skin: Breslow, Clark, histological type. Location of the primary, clinical, or pathological regional status.
• Complete staging demonstrating the presence of distant metastases, either visceral or in soft tissues or bone tissue: Brain, Lung, Abdomen, and Pelvis CT and bone scintigraphy.
• Objectively measurable disease by clinical or radiological means
• Karnofsky's Performance Status is more significant than 70%
• Life expectancy greater than three months
• Patients over 18 years old
• Informed consent from the patient to participate in the protocol

Exclusion Criteria:
Patients meeting any of the following criteria will be removed from the study:

• Age greater than or equal to 65 years
• Ongoing active infections, including viral immunodeficiency
• Previous chemotherapy within less than two months
• Concomitant malignant tumors (e.g., Chronic Lymphocytic Leukemia, etc.)
• Uncontrolled concomitant diseases (Hypertension, unstable Diabetes mellitus, renal diseases requiring dialysis)
• Situations or conditions requiring urgent surgical intervention, such as intestinal obstruction due to metastasis
• Pregnancy or lactation
• Concurrent participation in other therapeutic research protocols
• Any condition that compromises the objectives of this study.

Removal Criteria from the Study:
Patients will be removed from this study if any of the following conditions occur:

• By the patient's decision to discontinue the study.
• Intolerable adverse reactions, according to WHO Criteria Grading Toxicities
• Intercurrent illness that may compromise the patient's life or interfere with the treatment study evaluation
• Requirements for concomitant medication that may interfere with study results.
• Failure to complete the study entirely
• Failure to follow up on the patient.

Sample Size

Recruitment will be conducted to terminate the study after a specific number of patients have been enrolled if a minimum number of responses have not been observed. The lowest significant response rate will be 20% (alpha = 0.10; beta 0.10). If at least the minimum number of objective responses are observed to complete recruitment, 30 evaluable patients will be enrolled. For example, if there is no response in any of the first 12 treated patients or only 1 out of the first twenty, the study should be terminated. The confidence interval for the response rate is >20%.

Evaluable Patient: An evaluable patient meets the inclusion criteria, does not meet any of the removal criteria, receives all four vaccination cycles, and has been clinically and radiologically followed until the end of this trial.

Study Conduct

• Signing of informed consent
• Complete medical history
• Complete physical examination
• Karnofsky Performance Status measurement
• Estimation of survival quality using the EORTC scale
• If applicable, photographs of the tumor and metastases
• Hematological studies, including albumin and total proteins.
• Electrocardiogram
• CD3, CD4, CD8 cell counts, and total lymphocyte count
• Blood sample collection for in vitro immunological studies.
• Staging radiological studies: Computed tomography of the brain, lungs, abdomen, and pelvis, and bone scintigraphy
• Estimation of metastasis size through physical and radiological examination
• Delayed hypersensitivity test (DTH)

Enrollment
After the procedures mentioned above, each eligible patient will be enrolled. Each patient will be given an identification number. Data will be maintained in a computerized file with restricted access for the research team for each patient.

Patient Monitoring
Patients will be clinically monitored once a week through the following parameters:
- Weight
- Karnofsky Index
- Vital signs
- EORTC Quality of Life Index
- Comprehensive physical examination
- Monitoring for toxicity symptoms according to WHO Criteria Grading Toxicities and reporting adverse effects

Additionally, they will be evaluated through ELISPOT and ELISA assays, with blood samples taken before each vaccination, to assess the response of specific CTLs to DC stimulation and the presence of cytokines in the patient's serum.

Measurement at the End of Treatment
- Clinical evaluation of the patient
- Estimation of clinical or radiological tumor response: determining tumor volume
- Comprehensive physical examination
- Karnofsky Index
- Photography
- Quality of life and pain assessments
- Delayed hypersensitivity test (DTH): for in vivo response measurement
- Hematological and chemical tests: Assays for measuring in vitro immune response.

Long-Term Follow-Up
Once the patient has completed the full four-cycle treatment, they must be followed up every four weeks for one year. Every two months during the second year. The date of death must be determined in applicable cases. During each visit:
- Directed physical examination
- Karnofsky Index
- Estimation of clinical and radiological tumor size
- Photography
- EORTC Quality of Life Assessment
- Reporting of adverse effects

Clinical Response Assessment

TUMOR RESPONSE CRITERIA
CR: Complete disappearance of the tumor
PR: Tumor regression > 50%
MR: Tumor regression > 25% - <50%
SD: Decrease or growth < 25%
PD: Increase in tumor size during treatment

Statistical Analysis
The primary objective of this study is to estimate the objective response and toxicity associated with treatment using dendritic cells loaded when administered to patients with disseminated melanoma. A stratified recruitment design will be used in this study. In the first stage, 12 patients will be enrolled and treated with loaded dendritic cells. If none of the patients responds to the treatment, enrollment for this study will be terminated. If at least one patient responds to the treatment, the treatment will continue. If, after the enrollment of patient number 20, at least two patients respond to the treatment, the study should continue until a minimum of 30 evaluable patients with 3 out of 4 completed cycles. Assuming that the study will be completed with thirty patients as planned, the 90% confidence interval for the estimated response rate will be 12%, assuming a 20% response rate. If sufficient evidence indicates that the valid response rate is greater than 20%, it would be given if the lower limit of the confidence interval is more significant than 0.20 (alpha = 0.05, 1-tailed -test).

Primary Efficacy Analysis
Because this uncontrolled study enrolls a small number of patients, formal hypothesis tests are not planned. However, comparing different subgroups may generate hypotheses that can be studied in subsequent clinical trials.

Infrastructure and Equipment Provided by the Sponsor Academic Units for the Project

This project will primarily utilize the Tumor Immunology Laboratory's infrastructure and the Immunology Program facilities at the Institute of Biomedical Sciences, Faculty of Medicine, University of Chile. This Laboratory is equipped for the culture and expansion of cell lines in a Good Manufacturing Practice (GMP) compliant culture room. This system ensures that our vaccines are produced and continuously monitored according to international standards, verifying sterility and quality control (World Health Organization TRS 908, Annex 4). Additionally, there are suitable conditions for molecular and biochemical studies. The Laboratory is equipped for working with low-activity radioisotopes such as 3H and 51Cr. Furthermore, the group of researchers involved has the necessary experience, as reflected in their publications.

1. Tumor Immunology Laboratory: Analytical and precision balances, spectrophotometer, refrigerators, -20°C and -70°C freezers, immunodot and immunoelectrotransfer multi-analysis system, gradient former, Eppendorf centrifuge, refrigerated centrifuge, magnetic stirrers, vortex, thermostatic bath with agitation, power supplies, PCR thermocycler, microscope, digital camera, equipment for immunohistochemistry and immunofluorescence, chemical hood. Cell Culture Room: GMP-compliant culture room, temperature-regulated filtered air, laminar flow hoods, regulated CO2 incubators, inverted microscope, thermostatic bath, refrigerator, and liquid nitrogen tanks. The project includes a wide range of human cell lines, cytokines such as IL-2, IL-4, IL-10, GM-CSF, TNF, IFN-γ, IL-12, and IL-15, hybridomas, monoclonal antibodies specific for MHC class I and different haplotypes (HLA-A2, HLA-B7, among others), gene expression plasmids for receptors (MC1R, HER2/neu), human and murine IL-2, IL-4, and IL-10, as well as primers for T cell receptor repertoire detection with PCR and synthetic peptides of various melanoma-associated antigens.
2. High-Efficiency and Conventional Chromatography Laboratory: FPLC/HPLC system and columns, cold chamber, conventional columns, peristaltic pump, UV flow detector, fraction collectors, recorder.
3. Laboratory for Biological Probes Radiolabeled with High and Low-Energy Isotopes: Negative flow safety hood and volatile radioisotope absorption system with activated carbon, orbital shaker, visible light microscope, and safety cabinet for storing and decaying radioactive waste.
4. Flow Cytometry Room: Flow cytometer (FACSort), analysis computers.
5. Darkroom for Radioautographic Processing will be available, including a Polaroid camera, developing reagents, and radioautography cassettes.
6. Washing, Sterilization, Distilled Water, and Ice Room: Material drying oven, floor autoclave, flake ice maker, water distillers, water deionizer, ultra-pure water production system.
7. A Central Laboratory containing pH meters, a spectrophotometer, an ELISA reader, and a 10-head gamma counter will be available.
8. Immunology Library includes ten journal subscriptions, a database for bibliographic reference search, and over 100 immunology and related books.
9. Others: Word and graphics processing computer systems, fax, direct phone line, email, and internet access.
10. Central Services: In addition to the infrastructure within the Immunology Program, the Faculty of Medicine has other general-use equipment that may be relevant to this project, such as an ultra-centrifuge, scintillation counter, confocal microscope, peptide, and oligonucleotide synthesis and sequencing, among others. There is also a central animal facility designed for maintaining pure strains of mice, infected and immunized mice, and rabbits.
11. Clinical Hospital of the University of Chile: The infrastructure of the blood bank at the Clinical Hospital of the University of Chile is available, featuring equipment for leukapheresis sample collection and the Cryopreservation Laboratory equipped with a modern automated biological sample freezing system.
12. Associated Companies in the Project: Oncobiomed Ltda., Oncomed Ltda., and Tecnofarma S.A. have provided their clinics for the selection and treatment of some patients and their administrative teams for technological transfer, respectively.

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ONCOMBIOMED Ltda. (85 million Chilean pesos) Furthermore, Tecnofarma S.A. (35 million Chilean pesos) has committed to providing funds through personnel financing and cytokine grants such as IL-2.