

# EUREKA PROJECT E!3746- PACRIMA

## 1. General description

<b>Project</b>	E! 3746- PACRIMA	<b>Status</b>	Announced- 26-Jun-2006
<b>Title</b>	<b>Development Of Cancer Immunotherapy And Stem Cell Therapy Based On An Artificial Thymus Technology Platform</b>		
<b>Class</b>	Project	<b>Technological area</b>	Medical and Biotechnology
<b>Start date</b>	01-Apr-2006	<b>End date</b>	01-Apr-2009
<b>Duration</b>	36months	<b>Total cost</b>	3.69Meuro
<b>Partner sought</b>	No		
<b>Summary</b>	We Aim To Develop Antigen Specific T Cells Against Different Muc1 Glycoforms By Means Of Differentiating Stem Cells In An Artificial Thymus Towards Dcs And T Cells. These Dcs Will Be Transduced With Ad-Muc1 And Given To T Cells To Generate Antigen Specific T Cells That Can Be Cloned.		

## Budget and duration

Phase	Budget(Meuro)	Duration (Months)
Implementation phase	3.69	36
<b>Total</b>	<b>3.69</b>	<b>36</b>

## Member contribution

Member	Contribution	Position	Since
<b>The Netherlands</b>	<b>57.00%</b>	<b>Contact Member</b>	<b>01-Jun-2006</b>
Belgium	17.00%	Participating Member	26-Jun-2006
Japan	16.00%	Participating Member	26-Jun-2006
Singapore	10.00%	Participating Member	26-Jun-2006

## Participants

Company	Country	Type	Role
<b>Pharmacell B.V.</b>	<b>The Netherlands</b>	<b>Large company</b>	<b>Main</b>
Department Of Internal Medicine / Division Haemato-Oncology University Hospital Maastricht	The Netherlands	University	Partner
Department Of Internal Medicine / Division Haemato-Oncology University Hospital Maastricht	The Netherlands	University	Partner
Cygenics Ltd	Singapore	SME	Partner
Riken Research Center For Allergy And Immunology Riken Yokohama Institute	Japan	Research Institute	Partner
Maia-Scientific N.V.	Belgium	SME	Partner

## 2. Project outline

### Project description

Since current treatment options (surgery, chemotherapy and radiotherapy) unfortunately fail in over 50% of cancer patients, new treatment options are needed. One of these options is immunotherapy. This can be realised by several methods. One of them is making use of a donor immune system, such as in allogeneic transplantation. Another option is to stimulate the body's own immune system (active vaccination). Other possible options are antibody treatments and the adoptive transfer of immuno-competent cells. The principal idea of an immune vaccine in general is to stimulate the patient's immune system against certain threats like infectious agents or cancer. In cancer, however, it is difficult for the immune system to develop an anti-cancer response. The cancer cell by itself does not spontaneously activate the immune system in most of the cases. However in the scientific world, evidence is accumulating and showing that cancer vaccines can be realised. In clinical trials worldwide it has been demonstrated that a certain percentage of patients (10-20%) responds to a cancer vaccine with partial or complete disappearance of the disease. However further improvements have to be made. Among the various techniques used to improve cancer vaccines, the dendritic cell (DC) plays a crucial role. The DC is essential in the activation of the whole immune system in-vivo. By loading DC with tumour parts (peptides, proteins), it is possible to activate the immune system against the whole tumour. Through this activation, a subset of white blood cells called T cells are mobilised to kill the tumour cells.

The AZM is developing a cancer vaccine using the Mucine-1 protein for use in patients with breast cancer. Mucine-1 is usually present on healthy epithelial cells. However it is also present in over 80% of tumours like breast cancer, lung cancer, ovarian cancer, multiple myeloma and acute myeloid leukaemia. In cancer cells the protein Mucine-1 is differently glycosylated and therefore has a different glycopeptide structure. These glycopeptides will demonstrate new antigenic epitopes to the immune system and therefore can serve as a target for attack by antigen specific T cells in-vivo recognising these new epitopes when properly activated by DCs.

The perspectives of a cancer vaccine in breast cancer are enormous. It can be used at various - different - stages of the disease, first of all, to prevent breast cancer in women with a high risk of developing this disease because of a specific chromosomal constitution in the family. In addition, women undergoing treatment still have a high risk (30 - 70%) of developing meta-static diseases. Vaccines might be highly valuable at this point in time for prevention of meta-static diseases. Finally, a vaccine might be used to treat patients that already have meta-static disease to induce tumour responses and possibly prolong life and/or improve quality of life. In other words the perspectives of using a vaccine in breast cancer is enormous with possible high value for health care.

So far DC vaccines have been administered to patients with the aim of activating T cells in the patient. A possibility why the DC vaccines have met only limited success so far is an improper T cell activation in-vivo. CYGENICS has developed an in-vitro system called the Cytometrix(R) in which de novo T cell development from adult blood stem cells occurs, including the proper selection of T cells. Including in-vitro generated antigen specific T cells with the DC vaccines should lead to a strong and very specific anti-tumour immune response. In this proposal we will compare the T cells generated de novo from stem cells versus peripheral blood derived T cells as the repertoire of T cells might be different due to negative selection or tolerisation in-vivo.

The other approach the AZM is pursuing is an allogeneic stem cell transplantation. In this system, donor immune cells are the most active component of an anti-tumour response. Traditionally the cells most relevant are the T lymphocytes. However there is recent evidence that especially NK cells might be very relevant as well. In our research group we focus on a haploidentical (semi-HLA-identical (Histocompatibility antigen) family donor available in nearly all patients) transplantation. In mouse models, we recently observed that this treatment can cure breast cancer in about 50% of cases (manuscript submitted). In humans it is known that this might be a very good treatment for acute leukaemia. We are presently exploring the role of NK cells in various tumour types. The hypothesis is that adoptive transfer of NK cells might have an anti-tumour response in a variety of cancers.

One of the disadvantages of haploidentical transplantation is the severe immune-deficiency in these patients for a certain period of time after the treatment. Patients become susceptible to and often die from opportunistic Cytomegalo Virus (CMV) infection. The treatment of CMV-virus specific T cells might be part of the solution.

The current technologies of the partners needed in this research proposal include the generation of dendritic cells, NK cells and antigen specific T cells (AZM), the Cytometrix(R), a thymus-like environment supporting generation of T cells (CYGENICS), in-vitro systems and animal models to study T cell development (RIKEN-RCAI) and a GMP (Good Manufacturing Process) facility and manufacturing technology for generating clinical grade DCs, T cells and NK cells (PHARMACELL).

By combining these technologies, we have a central research question for both lines of research:  
Can antigen specific T cells suitable for clinical applications in cancer be obtained in the Cytometrix(R) system?

Specific aims in this project will be:

1. Optimisation of DC cultivation in the Cytometrix(R).
2. Induction tumour-specific T cells in the Cytometrix(R).
3. Scaling up of cell cultures under GMP conditions.
4. Understanding the molecular mechanism.
5. Realisation of NK cell induction and expansion in the Cytometrix.
6. Induction of CMV-specific T cells in the Cytometrix.
7. Automation of process of quality assessment by microscopic imaging.

The work packages for aims 1+2+5+6 will be carried out by the AZM. The work package for aim 3 as well as the administration for this project will be carried out by PHARMACELL. The work package for aim 4 will be carried out by the RIKEN INSTITUTE. CYGENICS will make the Cytometrix(R) system available to the partners and will teach the other partners about its use. MAIA-SCIENTIFIC will execute the work package for aim 7.

## Technological development envisaged

Current anti-cancer therapies cure only 50% of all patients and pose serious side-effects. To cure more cancer patients or to down-modulate side-effects, new therapies are needed. Immunotherapy using dendritic cells (DC) as tumour vaccine might be an important development in establishing new treatment modalities for cancer. In recent studies some clinical success (tumour responses) has been reported, but many improvements are necessary. DC can be obtained ex-vivo and infused back into the patient after being loaded with tumour antigens. Much knowledge exists in culturing DC from monocytes present in peripheral blood or mobilised stem cells of healthy volunteers or patients. These cells can be transduced with tumour specific or associated antigens. We have succeeded in transducing DC with an adenoviral construct containing the tumour antigen Mucin-1. After this DC transduction the cells express mucin-1 with a tumour specific pattern. Mucin-1 is present on a majority of tumours with a different glycosylation pattern compared to healthy epithelial cells. This tumour specific pattern is characterised by a variety of antibodies recognizing the different glycosylation form. Therefore a powerful option for inducing tumour specific immune responses is available.

### A. DENDRITIC CELL CULTURES

DCs are obtained from two different cell systems: blood monocytes and mobilised stem cells. Blood monocytes are by far the most used cells for culturing DC. This can be done using a variety of cytokines for culturing immature DC and then maturing these cells with other cytokines or bacterial products. These cells further show all characteristics of DC using immunophenotyping and have the ability to give adequate in-vitro allo-immune responses (for details see Cloosen et al. *Int Immunol* 16(11): 1561-1571, 2004).

In addition we cultured stem cell derived DC. These stem cells were obtained from G-CSF mobilised stem cells of healthy volunteers. This method gives access to large numbers of stem cells. We were able to obtain about 50% DC using this method. These DC show a similar immuno-phenotype and in vitro allo-immune stimulatory capacity including the feasibility to stimulate naive cord blood cells, a capacity of DC cells only. We will test both monocyte derived DC as well as stem cell derived DC in the Cytometrix(R) system to select the most powerful DC antigen specific T cell induction and for in-vivo use.

### B. TRANSDUCTION OF DC WITH THE TUMOUR ANTIGEN MUCIN-1 USING AN ADENOVIRUS.

Mucin-1 has been selected as a tumour antigen. Mucin-1 is present on most epithelial cells, like breast cancer, and some haematological malignancies, including Multiple Myeloma and Acute Myeloid Leukaemia. It has been well established that on tumour cells like breast cancer, the glycosylation pattern of Mucin-1 is different from normal epithelial cells, making this mucin-1 a potential tumour antigen.

In order to let DC present tumour antigens we have used viral transduction. Above other loading mechanisms it has been demonstrated in mice models that this type of loading gives better anti-tumour responses. The prime reason being a prolonged expression of the tumour antigen on the DC.

By using the adenovirus Ad5F35 the transduction reaches nearly 100% of monocyte derived DC. These DC express MUC1 for at least 48 hours, which is crucial since antigen presentation by DC should be present for at least 24 hours to give an adequate anti-tumour response in-vivo.

Similar high expression levels could be obtained more recently using stem cell derived DC. This will make it possible to compare these two different types of DC in their capability to induce antigen specific T cells for an anti-tumour response.

Both types of DCs show not only fully glycosylated MUC1 on their surface, but also shorter forms and forms that only appear on tumour cells. It is expected that there will be a relation between surface expression of under-glycosylated MUC1 and presentation of such processed and under-glycosylated peptides in the MHC

(Major Histocompatibility Complex) molecules on the DCs.

It has been demonstrated that the different glycosylation patterns of Muc1 are relevant for T cell recognition. In addition it has recently been demonstrated that it is possible to induce an immune response in Mucine-1 transgenic mice using aberrantly glycosylated peptides, while this is not possible using physiological Mucine-1 (O. Finn, DC meeting Brugge, 2004).

#### C. CYTOMETRIX SYSTEM.

Cytomatrix Devices are patented three-dimensional cell growth scaffold, ideal for expansion of stem cells and the growth of other cell lines and primary cell isolates. Through a sophisticated vapour deposition process, the carbon scaffold is uniformly coated with a biocompatible metal. Its unique architecture mimics the bone trabeculae with a high porosity of more than 80%, continuous channels and open ends, providing an ideal micro-environment for cell growth.

The matrix is available in 2 forms, the Statamatrix and the Dynamatrix, for static and dynamic cultures respectively.

The artificial thymus system utilises human skin stromal cells in co-culture with human haematopoietic progenitor cells to produce de novo T cells outside the body. A significant body of data demonstrates that these cells exhibit molecular, phenotypic, and functional traits associated with mature T cells and normal T cell development.

The system uses human skin obtained through surgical resection, cut in small pieces and allowed to grow in the Statamatrix Units. When very early progenitors are seeded in such units in the presence of a combination of cytokines, T cells have grown after 2-3 weeks. These data demonstrate that human skin stroma has the capacity to support T cell differentiation ex-vivo in the artificial thymus system.

In addition to T cell development, B cell (CD19+, IgM+), natural killer (NK) cell (CD56+, CD14<sup>+</sup>), and myeloid cell development (CD14+, CD33+) were observed. These data suggest that the artificial thymus system is capable of producing the cellular and soluble factors for both lymphoid and myeloid differentiation ex-vivo. Similar results were obtained when CD34+ and AC133+ cells were used in the artificial system established with human skin stroma.

Using Concanavalin A (5 microgram/mL) and IL-2 (10 IU/mL), a 17- to 100-fold increase in the number of T cells was observed after seven days in culture.

A spectratype profile of TCR (Thermal Coefficient of Resistance) families in T cells cultured with human skin stroma in the artificial thymus system reveals an extremely diverse population. This demonstrates that the T cell generation is consistent with what occurs in-vivo, and results in a population of cells that is diverse and appropriate for immune reconstitution.

During thymic T cell development, epithelial elements contribute to negative selection and myeloid elements contribute to positive selection. To determine how T cell neogenesis in a chimeric environment (i.e., stroma and HSCs from MHC mismatched individuals) impacts MHC restriction and positive and negative selection, mlr assays evaluating the proliferative responses of the derived T cells to: 1) the skin donor; 2) the HSC donor; and 3) a third-party donor, were performed. It was shown that the manufactured T cells were responsive to 3rd party antigen (cells), but were not responsive to cells from the skin donor or the CD34+ progenitor-cell donor. This demonstrates that the manufactured T cells function in a manner consistent with in-vivo T cells (i.e. developed in an environment that has facilitated positive and negative selection). This also indicates that the manufactured T cells are not likely to elicit responses to the self (i.e. the recipient) when administered in a clinical setting.

In conclusion, the artificial thymus system allows de novo T cell generation with a complete T cell repertoire due to normal positive and negative selection, making it possible to use the system to make the last step in the process: generation of tumour and virus antigen specific T cells.

This system does not work only in humans. Normal mouse T cell development is observed to the same extent as for human T cells, making this system also extremely useful in the study to determine the molecular mechanisms involved in T cell development.

#### D. MOLECULAR MECHANISM OF T CELL DEVELOPMENT.

T cells are produced from haematopoietic stem cells. During haematopoiesis, HSCs lose their potential for self-renewal and finally become uni-potential progenitors. Each point of losing a certain potential is called lineage commitment. The RIKEN-RCAI group has developed an assay system in which the T, B and myeloid lineages can be studied. By using this system, the basic framework of the process of lineage commitment in haematopoiesis was deduced. Recently, it was revealed that the Notch signal is essential for early T cell development. However, the precise molecular mechanism of how the Notch signal supports T cell generation, as well as the target progenitor stages of the Notch signal remain unclear.

#### E. NK CELLS IN ALLOGENEIC STEM CELL TRANSPLANTATION.

Recently, Italian research has given a strong impulse towards clinical perspectives in haploidentical transplantation. They demonstrated that NK cells could play a crucial role in haploidentical transplantation after intensive conditioning with chemo- and radiotherapy.

NK cells do have two groups of killer-immunoglobulin-like receptors (KIRs): activating receptors, which activate the NK cell after binding their ligand, and inhibitory receptors that down-regulate NK activation after binding their ligand. In case the inhibitory receptor does not see its ligand, the NK cell will be activated

('missing self' principle). This missing self principle is an interesting concept in allogeneic transplantation when there is a mismatch between donor and recipient class I antigens: missing expression of the inhibitory KIR ligand on the patient's cells can thereby trigger donor NK cell activity. Thus far, three HLA class I phenotypes have been found to be involved in this missing self concept. About 60% of patients have a HLA class I constellation which allows for finding a donor whose NK cells will miss the ligand for the inhibitory KIR on their NK cells, making them susceptible to this beneficial NK allure-activity.

We recently performed a first haploidentical transplantation in our hospital. Furthermore we are presently exploring the possible role for NK cell activity in tumours other than leukaemia. We are analysing the role of NK cells in multiple myeloma, breast cancer and ovarian cancer. The expectations and first results demonstrate that NK cells might kill these tumours. This program will explore the Biopharmaceutical culturing of NK cells with associated quality control aspects, including analysing the possible benefit and use of the Cytometrix system in a pharmaceutical/industrial application.

The major drawback thus far of haploidentical transplantation is the severe immuno-suppression, resulting in viral infections in treated patients, often leading to the death of a patient. Possibly, adding viral antigen specific T cells early after transplant might overcome this clinical problem.

#### F. AUTOMATED MICROSCOPY AND MULTIMEDIA DATA EVALUATION TOOLS

MAIA's current microscopy reader technology supports automated high speed, high resolution image capture for up to 13x microscopy modes. In addition to multiple fluorescent reading modes typically encountered in all reader technology, MAIA readers also feature two unique technologies.

(1) Weak fluorescent signals can be captured at video image capture rate by virtue of an intensified camera and proprietary auto-focus on objects with very faint signals, invisible to the human eye. This unique enabling feature facilitates real-time, high-speed exploration of the 'low abundance proteome' that represents over 90% of the cellular proteins at nascent protein concentration in the living cell.

(2) A second unique MAIA technology is brightfield (daylight) microscopy image acquisition and image analysis: cells, tissues and small animals can be observed alive and without interference. MAIA's label-free cell-based applications have replaced manual (visual) and surrogate biochemical assays to monitor live culture confluence, cell proliferation and inhibition thereof, cytotoxicity, clonality, and colony size assessment. This market-proven technology today supports full automation of cell culture and cloning by e.g. Cello, a new robot and co-development with The Automation Partnership. MAIA delivered an adapted reader technology and specific applications for e.g. therapeutic antibody (hybridoma) selection and bio-production.

A key factor to these successful developments has been the eaZYX-IMAGING software. It supports the analysis of images and video from the MIA readers and other imaging sources alike. Most important is eaZYX's unique capacity to analyse images that are noise and background intensive, by virtue of sophisticated Scale Space mathematics (Van Osta et al., Proc Royal Microscopical Soc, 37(3), 161-166, 2002). In this way, proper object identification can be made from noise intensive images from intensified cameras and from brightfield images that are distorted by the effect of a liquid meniscus. These principles were shown to be applicable to a wide range of primaries and to cell lines; both adherent and non-adherent. These technology principles will now be developed into robust, automated and dedicated image acquisition and analysis tools for high throughput T- and dendritic cell (co-) cultures that constitute autologous tumour vaccination processes.

#### DETAILED DESCRIPTION OF THE PROJECT GOALS

From our current experience with the generation of suitable DC from healthy volunteers we have the following aims:

Aim 1. Ex-vivo generation of DC from healthy volunteers and patients with solid tumours of the breast and multiple myeloma in conjunction with the Cytometrix(R) system and in comparison to the standard method.

Aim 2. Transduce DC with adenoviral vector Ad35 containing the human MUC1 tumour associated antigen in-vitro measurements of MUC1 specific T cell generation in the Cytometrix(R) system.

Aim 3. Ex-vivo generation of DC and T cells by scaling-up under GMP conditions.

Aim 4. Understanding the molecular mechanism of T cell development using in-vitro models.

Aim 5. Realisation of NK cell culture at the GMP level, and possible integration with the Cytometrix system.

Aim 6. Induction of CMV-virus specific T cells in the Cytometrix system.

Aim 7. Automation of process quality assessment by microscopic imaging.

Ad Aim 1:

The starting point of the current application is the generation of DCs from blood of healthy volunteers and comparing the culture conditions to the generation of DCs from blood obtained from patients with solid tumours of the breast and multiple myeloma. Most clinical trials have been started without analysing if DC derived from cancer patients behaves the same as DC from healthy volunteers. There are arguments in literature that DC obtained from patients with cancer, and maybe even between different forms of cancers, will behave differently and we plan to carry out a detailed analysis and comparison of Monocyte derived-DC (Mo-DC) from healthy volunteers versus the selected cancer types. We hypothesise that the Cytometrix system will have a positive effect on DC generation allowing enough cells to be used for AIM 2. Using this system, basic conditions for DC generation will be studied, like the necessity of additional cytokines or whether much lower doses can be used.

In the UNIVERSITY HOSPITAL MAASTRICHT, three members of the project are actively involved in the haematology and oncology clinic and see patients on a daily basis. They will select and recruit patients to participate in the current proposal. A condition required for these patients to be recruited is that these patients should have an active disease so tumour cells can be obtained by biopsy or from the blood, ascitis, pleural fluid or bone marrow. They are also asked to donate enough blood to obtain Mo-DC. The tumour cells will be analysed with a panel of 9 different anti-MUC1 monoclonal antibodies to check the MUC1 expression and the glycosilation status. MUC1 positive tumour cells will be frozen for later use. Patients with MUC1 positive tumours will be asked to donate blood through a regular blood donation. From the blood, monocytes will be obtained and cultured in serum-free medium with or without the Cytometrix system, in the presence or absence of IL-13 and GM-CSF to obtain immature DC for 5-6 days followed by a 40-hour maturation of DC with Ribomunyl and Interferon-gamma. Full characterisation of these DC is planned: phenotype (CD1a, CD11c, CD11b, CD40, CD80, CD83, CD86, HLA-DR, CCR7), cytokine production (IL12 versus IL-10), and their general T cell stimulation capacity measured by MLR as well as MUC1 specific T cell stimulation evaluated by tetramer staining, FACS analysis and the Cello system (MAIO-SCIENTIFIC) (further described under Aim 2).

#### Ad Aim 2

Monocytes will be isolated from buffy coats from healthy volunteers and from peripheral blood obtained from cancer patients with active disease. Cells will be cultured in the Cytometrix(R) system with IL4 or IL13 and GM-CSF to obtain immature DC. Immature DC will be transduced with Ad35-MUC1 followed by maturation of DC with Ribomunyl and Interferon-gamma. Transduced DC will then be co-cultured with either isolated T cells from donor peripheral blood or isolated stem cells induced in the Cytometrix system towards the T cell lineage. This stimulation will be repeated three times, with weekly intervals, to increase the frequency of MUC1 reactive T cells in the original population. The read-out for the expansion of specific T cells is a MUC1-tetramer staining followed by FACS analysis. In parallel we will also stimulate - for quality control - T cells against a different peptide for which we know that T cells can be usually obtained (melan-A). Functional T cell responses will be tested on EBV immortalised cells of the patient loaded with synthetic MUC1 peptide as well as on the tumour cells of the patients. Read-outs of T cell responses induced by DC through both MHC class I and class II will be carried out by ELISA (Enzyme-Linked Immuno-Sorbent Assay) cytokine release (IFN-gamma), ELISPOT (IFN-gamma; more sensitive than ELISA), FACS analysis for intracellular cytokine release (IFN-gamma) and radioactive Chromium release as a measure of killing capacity of CD8+ cytotoxic T lymphocytes.

Antigen-specific T cells will be identified and cloned by the Cello fully automated cell culture and cloning system.

For this study, we plan to use buffycoats from HLA-A2 typed volunteers as well as recruit 10 patients for each tumour: breast and multiple myeloma. The analysis will focus on 3 aspects:

- Testing the functionality of these DC against a general recall antigen as tetanus toxoid, showing the general capacity of the patients' immune response.
- The development of a MUC1 specific T cell response.
- The development of a MUC1 specific T cell response, leading to recognition and killing of the tumour cells from the patients.

#### Ad Aim 3

To be successful in developing a therapeutic approach using (stem) cell technology it is of extreme importance to scale up the laboratory scale technologies and prove that developed methods can be modified for use in a pharmaceutical manufacturing environment. The ability to scale up the required cell culture technologies for industrial use must be demonstrated. The availability and/or development of required quality assessment parameters together with associated assays are most important for pharmaceutical manufacturing. For isolation, propagation, and differentiation of stem cells to the required cell populations a robust and controllable process must be established.

All required culture systems, starting materials, testing procedures and packaging materials must be well defined and validated for use. During the course of this project these specifications will be developed. Better understanding of the requirements for systems and methods will be gained by culturing all cells to be used for the project in a GMP environment. The procedures for obtaining cells and controlling the quality of these cells must notably be well defined in order to develop a consistent culturing process. This will finally result in a reliable therapy. It should be clear that the development of a biopharmaceutical therapy requires in-depth knowledge of the behaviour of cell cultures in the proposed culture systems in order to define the quality attributes that are required to finally release a product for administration to the patient. It is well known that biological products, and especially living products, show a high degree of variation and depend on the quality of the used starting materials and culturing systems. Small-scale systems are normally not suitable for large-scale routine applications. The culturing and testing systems must be developed in such a way as to obtain a reliable and cost effective manufacturing method. PHARMACELL will use its expertise in the field of selecting, developing and using culturing systems for pharmaceutical application to design and establish the industrial technology required for this therapy.

Right from the start of this project, much attention will be given to identification, preparation and storage of

the appropriate reference standard material. This will be necessary for establishing quality control strategies to be used once the cancer immune therapy is applied in the clinic.

For this project the following GMP related procedures/technologies need to be developed:

- Isolation and qualification of (donor) cell material for isolation of required stem cells.
- Systems for propagation and differentiation of stem cells; i.e. generation of DC and T cells.
- Isolation, characterisation and propagation of the required DC/T cell population.
- Aseptic preparation of cell suspensions according to GMP regulations, ready for direct administration to patients.
- All required quality control testing for starting materials, product intermediates and final product.

Ad Aim 4

The aim of this work package is to clarify the target stage of the Notch signal, and whether the Notch signal controls cell fate choice or whether it only acts as a growth factor. Transcription factors induced or suppressed by the Notch signal that dictate T cell fate will also be studied. To this aim, a co-culture system with a mono-layered murine stromal cells transduced with a Notch ligand will be used as well as the Cytometrix system for comparison. As progenitor source, a stem cell enriched fraction of murine bone marrow cells or fetal liver cells will be used. After cultivation, the commitment status of the cultured cells will be assessed by either limiting dilution analysis or by clonal analysis. T cell cloning can be optimised using automated microscopy (from MAIA SCIENTIFIC). Furthermore, gene expression profiles will also be analysed. In the later stages of this project, human cord blood progenitors will also be tested. The information obtained by this study will be useful for manipulating de novo T cell generation in-vitro.

Ad Aim 5

It is important to assess the in-vitro susceptibility to NK allureactivity of various haematological (multiple myeloma, lymphoma) and solid tumours (ovarian carcinoma, breast carcinoma, renal carcinoma, lung carcinoma etc.). This will be studied using the Granzyme B Elispot as well as the Cr51-release assays. In the first instance, cell lines will be used as target cells. HLA-typing of the cell lines will be done by the department of histo-compatibility typing. Effector NK cells are derived from previously HLA-typed volunteers by TCD (Trace Chemical Detection) of peripheral blood by a standard immuno-magnetic method. The available donors do have a panel of NK activity based on the donor HLA type, so that all possible relevant HLA types of tumours can be tested in our laboratory. In case of susceptibility of one or more cell lines, we will test susceptibility on fresh human tumour single cell suspensions.

The number of NK cells present in peripheral blood is not so high. For the preclinical work it is necessary that NK cells can be expanded. In this proposal we aim to expand the NK cells using the Cytometrix. This system seems extremely useful as it has recently been described that only a portion of the NK cells are the real effector cells: the adherent fraction. We envisage that these cells will attach to the Cytometrix and proliferate. The other NK cells will likely also proliferate, but can easily be separated by removing the Cytometrix. These NK cells will then be used in various assays. When effectivity is established, NK cells can also be expanded under GMP conditions and used along with allogeneic stem cell transplantation.

Ad Aim 6

The experiments to be performed to generate CMV antigen specific T cells will copy the procedure as described above for tumour antigen specific T cells. DCs will be cultured in the Cytometrix system and loaded with CMV peptides, followed by 3x stimulation of T cells to obtain CMV specific T cells. T cells can be identified and cloned by the Cello fully automated cell culture and cloning system. T cells can potentially be expanded in the Cytometrix. After GMP culturing and expansion of such T cells, they can be used to treat patients with CMV infections after aSCT.

Ad Aim 7

The key objective is to build, customise and bring into operation an automated platform technology for evaluating cell culture processes involving tumour vaccination. It will be created in the form of a microscopy reader, an image analysis computer workstation and a dedicated image handling and image analysis toolkit. The microscopic image acquisition and analysis system (MIAS-2) will be functionalised for the purpose of dedicated automated image capture from initial and advanced cultures of dendritic cells, T-cells (and their co-cultures). The image analysis workstation (iBOX) will support the analysis of images taken from the project's microscopy reader and perhaps other imaging devices of the partners.

In order to create dedicated, automated tools that allow for significantly reducing hands-on time spent in the tumour vaccination process, the tasks and activities described in appendix 1 will be sequentially executed, with some overlap in order to bring the automated platform into operation for the partners.

## Markets application and exploitation

The development of a cancer vaccine is the final goal of this research project. More precisely the vehicle adeno-Mucine-1 to target dendritic cells together with antigen specific T cells. As a target model we selected Mucine-1 as a tumour antigen. The vaccine can potentially be used in a large group of cancer patients

(breast cancer, lung cancer, ovarian cancer, myeloid leukaemia, multiple myeloma, etc.). The combination with NK cell infusion may increase the efficiency of the tumour vaccine.

Though the vaccine can be used in this large proportion of patients, we will ourselves focus on two diseases, breast cancer and multiple myeloma. Breast cancer is one of the most frequent cancer malignancies. Breast cancer can be treated if it is discovered early and limited to the breast and/or a small number of lymph nodes.

However after spread of the breast cancer cells either locally or to other organs, cure is not possible anymore. The prospect of a cancer vaccine in breast cancer is enormous. It can be used at various - different - stages of the disease. First of all, to prevent breast cancer in women with a high risk of developing this disease due to a specific chromosomal constitution in the family. In addition an ideal moment to consider a vaccine is in women who undergo surgery, chemotherapy and/or radiotherapy but who still have a high risk (30 - 70% depending on this specific risk group) of developing metastatic disease. Vaccines might be highly valuable at this point in time for preventing metastatic disease, especially given that once metastases are present, cure is no longer possible. Finally a vaccine might be used to treat patients that already have metastatic disease to induce tumour responses and possibly prolong life and/or improve quality of life. In other words the prospects of using a vaccine in breast cancer is enormous with possible high value for health care.

Multiple myeloma is not as frequent as breast cancer but it is nevertheless very relevant for studying vaccine development in this disease. In fact it is one of the few diseases in which the immune system has proven to be active against the tumour. This has in clinical practice been proven after allogeneic (donor) stem cell transplantation. In these situations it has been demonstrated that multiple myeloma cancer cells can be sensitive for the immune system. Therefore this disease is a very interesting disease for studying the prospect of a cancer vaccine. In addition, at this point in time there is no cure possible for multiple myeloma, despite various active chemotherapy drugs and new treatment options like Bortezomib.

Usually patients with multiple myeloma live for several years with their disease. This is in fact an ideal clinical situation for testing the concept of a vaccine since the tumour does not grow very rapidly and therefore there is time for the immune system to be activated and demonstrate an anti tumour response. We are presently testing the presence of Mucin-1 on these multiple myeloma cancer cells and a large proportion of patients do indeed show Mucin-1 on their tumour cells.

The incidence in THE NETHERLANDS for breast cancer is about 11,000 new cases per year. For multiple myeloma this is about 700 new cases per year. For the European community it can be calculated that nearly 200,000 new women will suffer from breast cancer every year and nearly 15,000 persons from multiple myeloma. In THE NETHERLANDS about 35% of patients with breast cancer will finally succumb to their disease. In multiple myeloma the percentage of patients that will die because of the disease is nearly 100%. Survival time is a median period of about 4-5 years. From these figures it can be calculated that the value of new drugs can be extremely high. If a vaccine induces prolongation of life or improvement of quality of life, then these drugs will have high potential.

Cancer vaccines are not yet used on a commercial basis. Nevertheless cancer vaccines are developed for clinical studies. Complete vaccines will cost (rough estimation) between 15,000 and 20,000 euro per patient treatment program. The commercial prospects of such a product can be easily calculated. If it has clinically relevant effects in multiple myeloma, the value is about 15 million euro per year in Europe. If it has any effect in 50% of women with breast cancer, the commercial value is estimated to be 100 million euro per year in Europe alone.

Speculation of the potential final value is however early and mainly depends on the number of diseases and the different stages of the disease where the drug might be worthwhile.

Marketplace for NK cells in allogeneic stem cell transplantation

The prospect for allogeneic transplantation is enormous. If the concept of NK cells works in solid tumours and if the cytometrix system improves the suitable number of effective cells, then the prospects are very good.

The same holds true for the production of virus specific T cells. They are now expected to be produced for several clinical protocols. If the Cytometrix system improves the production of these cells, then this would be of great medical value.

## Project codes

**BSI**

**NACE**

### 3. Main participant

<b>Company</b>	<b>Pharmacell B.V.</b> Oxfordlaan, 70 6229 Ev Maastricht The Netherlands  Tel +31 433 88 58 44 Fax +31 433 88 58 89  www.pharmacell.nl
<b>Contact</b>	<b>Mr. Rene Lardenoije</b> C.E.O.  Tel +31 650 20 70 28 Fax  r.lardenoije@pharmacell.nl
<b>Organisation type</b>	Large company
<b>Participant role</b>	Main

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### Contribution to project

The organisation is a Biotech company developing innovative stem cell therapies and providing contract research and manufacturing services to Life Science companies, thereby using its know-how and resources for pharmaceutical product and process development. PHARMACELL was founded in 2005 by SYNTIRO PHARMA SUPPORT INTERNATIONAL BV, BIOPARTNER CENTER MAASTRICHT, UNIVENTURES, LIOF and D.I. VENTURES. The company is located in the Biopartner Centre Maastricht and operates a commercial cGMP manufacturing facility for Biotech products. Close cooperation with MAASTRICHT UNIVERSITY and the associated ACADEMIC HOSPITAL AZM gives PHARMACELL the unique capability to apply new cell therapies in the emerging field of regenerative medicine. PHARMACELL'S CONTRIBUTION TO THE PROJECT: PHARMACELL will develop state-of-the-art GMP manufacturing procedures based on the developed technology for dendritic and T cell generation/isolation. During the course of the project PHARMACELL will obtain and treat all required tissue samples and perform the cell culturing work including the generation of required reference material.

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### Expertise

The organisation operates GMP facilities for the preparation of cell cultures for application in clinical cell therapy protocols. Ample experience is available with respect to regulatory requirements in the application of live cell products for therapeutic means. Cells are obtained from donors or patients. Stem cells can be isolated from the umbilical cord of newly born babies. From these sources, precursors of DC and T cells can be obtained and differentiated in-vitro under GMP conditions. In collaboration with the AZM, suitable samples from cancer patients will be obtained. All cells are grown in-vitro using specific culture media. As a partner in this collaboration, PHARMACELL will supply patients with autologous dendritic cells and antigen specific T cells for cancer vaccines as immunotherapy. To expand the activities in this area, PHARMACELL is conducting research activities in order to develop second-generation cell therapy protocols for cancer vaccines and to explore other applications of tissue engineering in the field of cardiac repair. Rene J.G.M. Lardenoije has over 15 years of experience in the field of Biopharmaceutical product development and manufacturing. He served as head of the GMP pilot manufacturing group of both CENTOCOR and CRUCCELL in Leiden, THE NETHERLANDS. The main responsibilities were the development and manufacturing of monoclonal antibodies for applications in cancer therapies as well as viral vectors for gene therapy applications in the field of cardio vascular disorders. In 2002 he founded SYNTIRO PHARMA

SUPPORT INTERNATIONAL, a consulting company in the field of (bio) pharmaceutical manufacturing and process design. PHARMACELL works in close cooperation with the research division of the Hemato-Oncology department of the UNIVERSITY HOSPITAL OF MAASTRICHT, which is the primary referral clinic in Limburg with expertise in the field of blood cancer and solid tumours, with emphasis on breast cancer.

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## 4. Partner

<b>Company</b>	<b>Department Of Internal Medicine / Division Haemato-Oncology University Hospital Maastricht</b> P. Debyelaan, 25 6202 Az Maastricht The Netherlands
	Tel +31 433 87 70 25 Fax +31 433 87 62 81
	www.azm.nl
<b>Contact</b>	<b>Md Ph.D. Gerad Bos</b> Hematologist
	Tel +31 437 70 25 Fax
	gbos@sint.azm.nl
<b>Organisation type</b>	University
<b>Participant role</b>	Partner

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## Contribution to project

The AZM division of haematology will contribute to work packages 1 and 2. The group is 5 years old now and has built up expertise in dendritic cell culture, ex-vivo adenoviral transduction and the generation of tumour antigen specific T cells. One published article, 2 articles in the press, and 2 other articles describing the techniques and results were expected by December 2005. Within this collaboration, the team will culture DCs within the Cytometrix(R) system, optimise the culture conditions and generate antigen specific T cells from adult peripheral blood cells and also from haematopoietic stem cells delivered by CYGENICS. The other package is to establish NK cell culture and expansion conditions using the Cytometrix system.

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## Expertise

The AZM division of haemato-oncology consists of a clinical department as well as a preclinical research group that started 5 years ago. In the clinic, cancer patients are treated with emphasis placed on breast cancer as well as haematological cancers such as multiple myeloma, AML and CML. The research group has 2 lines of investigation. The first one comprises the development of dendritic cell vaccines for breast cancer and multiple myeloma, while the second line is the development of allogeneic stem cell transplantation of haplo-identical donors. Both intended therapies could profit from a new third line of research: NK cell production and use in an allogeneic transplant. Gerard M.J. Bos completed a PhD in immunology after his MD in 1984 (Maastricht, THE NETHERLANDS). This PhD (1989) was in the field of experimental allogeneic stem cell transplantation (Department Immunology, Maastricht, THE NETHERLANDS Promoter: Prof. Peter van Breda Vriesman and Prof. R. Reneman). He qualified as an immunologist shortly thereafter (SMWBO). After his training as a clinical haematologist (1995) he worked in the DANIEL DEN HOED CANCER CENTRE, Rotterdam. In 1997 he obtained a grant from the DUTCH CANCER FOUNDATION to perform pre-clinical research on immunotherapy (1998 + 1999). He worked two

years full-time in the well known laboratories of Prof. Dr. Cees Melief (Leiden, THE NETHERLANDS) and Prof. Dr. T. Boon, (Brussels, BELGIUM) on T cell directed immunotherapy. A third period of research was performed in Utrecht, THE NETHERLANDS on allogeneic transplantation (Prof. Dr. A. Hagenbeek). After this pre-clinical training, he started a clinical position as haematologist and his own research group on tumour-immunology in Maastricht. Wilfred T.V. Germeraad is an immunologist with a main interest and experience in the development of the immune system, both the normal as well as that in cancer. He received his PhD in 1994 under Prof. Dr. Yoshimoto Katsura (Kyoto, JAPAN) on T cell development and gene transfer methods followed by a post-doctoral position (Los Angeles, U.S.A.) on transgenic mouse models for leukaemia. In the post-doctoral role, he has worked on the crosstalk between the developing thymus and T cells under the guidance of Prof. Dr. Willem van Ewijk (Rotterdam, THE NETHERLANDS). Until now, this work continued in collaboration with Prof. Y. Katsura (now in Tokyo, JAPAN) and Dr. Hiroshi Kawamoto (RIKEN, Yokohama, JAPAN). Over the last 7 years he has worked on the development of tumour vaccines, including DC vaccines and monoclonal antibodies, first at CRUCCELL (Leiden, THE NETHERLANDS) before joining the AZM team in Maastricht in April 2003. The research team presently consists of a group of 10 people working on these projects. A senior immunologist, a senior haematologist, Post-Doc, 3 PhD students, 3 technicians and Gerard Bos as clinician and co-ordinator. Furthermore 2 clinicians will be involved in the clinical studies (Dr. P. Hupperets and Dr. M. van Gelder). The expertise of the team consists mainly of cellular culture techniques, standard immunology techniques (immunophenotyping, cytokine assays, ELISPOT, killing assays, etc.), molecular biology techniques (sub-cloning, expression vectors, transduction, transfection) and biochemistry (antibody production and purification, etc).

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## 4. Partner

<b>Company</b>	<b>Department Of Internal Medicine / Division Haemato-Oncology University Hospital Maastricht</b> P. Debyelaan, 25 6202 Az Maastricht The Netherlands  Tel +31 433 87 70 25 Fax +31 433 87 62 81  www.azm.nl
<b>Contact</b>	<b>Ph.D. Wilfred Germeraad</b> Immunologist  Tel +31 434 08 49 20 Fax +31 434 88 41 64  w.germeraad@immuno.unimaas.nl
<b>Organisation type</b>	University
<b>Participant role</b>	Partner

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## Contribution to project

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## 4. Partner

### Company

#### **Cygenics Ltd**

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### Contact

#### **Mr. Boon Sing, Steven Fang**

Group C.E.O. And Executive Director

Tel +65 9666 43 30  
Fax

[steven.fang@cygenics.com](mailto:steven.fang@cygenics.com)

### Organisation type

SME

### Participant role

Partner

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## Contribution to project

The organisation will contribute the following to the project: 1. Cytomatrix(TM) devices, patented three dimensional cell growth scaffold. 2. Paddle(TM) Systems for suspension cell cultures. 3. Starwheel(TM) System for adherent cell cultures. 4. Peripheral blood cells. 5. Haematopoietic stem cells. 6. Technical support as required. All materials and manpower required will be provided at cost to the project. Use of the artificial thymus system is for development purposes only. CYGENICS' participation should not be construed as allowing any of the project collaborators to use its technology for commercial purposes.

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## Expertise

The organisation is a cell therapy company focused on the development and commercialisation of adult stem cell-related products, services, applications and technologies. From its headquarters in AUSTRALIA, CYGENICS operates four subsidiaries: CORDLIFE, CELL SCIENCES, CYTOMATRIX and CYTOVATIONS. CYGENICS is listed on the Australian Stock Exchange, under the symbol CYN. \* CORDLIFE is a fee-for service in a tissue banking company. It is accredited by the American Association of Blood Banks. From its base of operations in SINGAPORE, the company has been rapidly expanding its cellular banking services across the Asian region. \* CYTOMATRIX has been successful in developing cell-based application based on its patented three-dimensional growth scaffold. The company has established strong business relationships with research institutions, hospitals and federal government bodies in the UNITED STATES. \* CELL SCIENCES manufactures the Group's proprietary products and drives the sales, marketing and distribution of biomedical products. In 2004, the company launched a patented 3-D growth scaffold and the market's first disposable cell culture spinner systems. \* CYTOVATIONS drives the development of products for the Group. In addition, it provides consulting services to the biotechnology and pharmaceutical industry in the niche areas of bioprocess development and quality assurance, and identifies suitable business partners.

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## 4. Partner

<b>Company</b>	<b>Riken Research Center For Allergy And Immunology Riken Yokohama Institute</b> 1-7-22 Suehiro-Cho, Tsurumi-Ku 230-0045 Yokohama Japan  Tel +81 45 50 39 111 Fax +81 45 50 39 113  <a href="http://www.rcai.riken.jp/indexE.html">www.rcai.riken.jp/indexE.html</a>
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<b>Organisation type</b>	Research Institute
<b>Participant role</b>	Partner

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## Contribution to project

## Expertise

The goal of the laboratory is to clarify the process of lineage restriction from multi-potent haematopoietic stem cells to unipotent progenitors, and to understand the molecular mechanisms that regulate the cell fate decisions. All hematopoietic cells, including T cells and B cells, are derived from hematopoietic stem cells. However, today it is still unclear how pluripotent stem cells become restricted to unipotent progenitors. We previously established a clonal assay system which reveals the developmental potential of individual progenitors toward T, B and myeloid cell lineages (multi-lineage progenitor assay; MLP assay). Based on the findings obtained with this method, we have clarified the lineage restriction pathways in early haematopoiesis. Our major findings are as follows; 1) The process of specifying murine hematopoietic stem cells towards T, B and erythroid lineages proceeds with accompanying myeloid cell-generating potential, 2) Pre-thymically committed T cell progenitors migrate to the thymus, 3) The earliest T cell progenitors in the thymus retain the potential to generate both NK cells as well as dendritic cells. The major aim of our present research is to elucidate molecular mechanisms that regulate cell fate decisions at each branching point we have so far identified. We now propose to answer the following questions; 1) Which molecules are involved in each lineage restriction process?, 2) Does the lineage restriction process proceed instructively or selectively? Our research also includes studies on human lympho-haematopoiesis, using cord blood cells as a source of progenitors. The information and technology that will be acquired through these studies can directly be applied to regeneration therapy and gene therapy. For example, it is now already possible to induce the differentiation of T lineage cells on mono-layered stromal cells, from cord blood progenitor cells. Importantly, such an in-vitro induction of de novo T cells would provide a novel strategy for immunotherapy of cancer and immune diseases.

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## 4. Partner

<b>Company</b>	<b>Maia-Scientific N.V.</b> Cipalstraat, 3 2440 Geel Belgium  Tel +32 14 57 06 20 Fax +32 14 57 06 21  www.maia-scientific.com
<b>Contact</b>	<b>Mr. Johan Geysen</b> President  Tel Fax  jgeysen@maia-scientific.com
<b>Organisation type</b>	SME
<b>Participant role</b>	Partner

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## Contribution to project

The organisation will develop, build for operation, and deliver to the partners, an automated microscopy platform and a dedicated toolkit for the analysis and quality control of tumour vaccination processes. The platform will capitalise on MAIA's unique image capture technology for whole cells and the image analysis technology for live, unlabeled cell cultures. Furthermore, weak signal fluorescent reading using intensified cameras will enable cost-effective identification of T-clones with potent tumour antigen specificity and optimal

proliferation.

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## Expertise

Image and video play an increasingly important role in research, development, quality assurance and production processes in the life sciences and biomedical industry. An established and growing niche herein is known as high information content or cell-based screening. A trend in this market is one to fewer instrumentation platforms in which a broadening range of biological assays are being executed, enabling wider but more flexible applicability of less instruments across the R & D value chain. MAIA orients its biology research, engineering and software development activities at building high content instrumentation, automation and application software in concert with a wide scale of matching bio-assays to explore for animal, plant and the human cytochrome at the molecular, cellular, tissue and organism level. MAIA's focus is on providing speed while maintaining flexibility, resolution and sensitivity and on supporting drastic miniaturisation of multimode and multiplexed assay, to facilitate cost effective high content screening, cytochrome research and bio-production processes.

**KEY PERSONNEL**

- \* Johan Geysen, PhD, President. Johan graduated from the UNIVERSITY OF LEUVEN with a PhD in Zoology, Developmental Biology. He became an experienced leader of target discovery, 'gene to screen', 'hit to lead', mechanisms of action and biomarker projects at Janssen Pharmaceutica from 1988-2000. Coordinated multiple drug discovery technology projects and lead several industry-academia collaborations (with VIB, The Sanger Institute, ICRF-London). Johan was visiting professor and co-chair of the Paul Janssen chair Molecular Cell Biology (University of Antwerp). Johan will supervise the MAIA contribution to this project.
- \* Kris Ver Donck, Vice-President Technology. Designed, engineered and managed all generations of high throughput research platforms and facilities for drug discovery at JOHNSON & JOHNSON, Beerse, BELGIUM. Biochemical, cell-based and small model organism-based high throughput screening campaigns for cardiovascular, inflammatory and neuro-degeneration drug discovery projects. Kris also masters all up- and downstream logistics for automation, coordination, data acquisition, data flow (LIMS) & reporting. Kris will be the contact person for technical (H/W & S/W - Hardware and Software) issues of the MAIA contribution.
- \* Marc Moeremans, Principle Scientist Biology Research. Marc will coordinate the biology activities on the project's MIA-2 instrument when operational in Geel.
- \* Bieke Govaerts, Laboratory Technician Biology & (To be appointed), Engineer, Automation & Advanced Image Analysis in executive contributions for the MAIA contribution.