**PROJECT FINAL REPORT**

**Grant Agreement number:** 252508

**Project acronym:** (SIMULATA)

**Project title:** System for Immunological Modelling as an Ultimate tool to Link Adjuvant

 function To Adaptive immune responses

**Funding Scheme:** FP7-PEOPLE-2009-IEF

**Period covered: from** 02/06/2010 **to** 01/06/2012

**Name of the scientific representative of the project's co-ordinator[[1]](#footnote-1), Title and Organisation:**

Robbert van der Most

Director, translational Science, Vaccine Research; GSK Vaccines

**Tel:** 02/6569875

**Fax:** 02/6564070

**E-mail:** robbert.x.van-der-most@gsk.com

**Project website address:**

## Final publishable summary report

This section must be of suitable quality to enable direct publication by the Commission and should preferably not exceed 40 pages. This report should address a wide audience, including the general public.

The publishable summary has to include **5 distinct parts** described below:

* An executive summary (not exceeding 1 page).
1. A summary description of project context and objectives (not exceeding 4 pages).
* A description of the main S&T results/foregrounds (not exceeding 25 pages),

1. The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results (not exceeding 10 pages).

1. The address of the project public website, if applicable as well as relevant contact details.

Furthermore, project logo, diagrams or photographs illustrating and promoting the work of the project (including videos, etc…), as well as the list of all beneficiaries with the corresponding contact names can be submitted without any restriction.

**EXECUTIVE SUMMARY**

The immune system uses specific molecular receptors to decide with what kind of pathogen it is dealing. For instance, this recognition system can distinguish whether the invading pathogen is a virus, a fungus or a bacterium. Much progress has been made in understanding how exactly this system recognizes the different pathogens and which molecular structures from the pathogens are important. The discovery of such immune-stimulatory molecules from pathogens has opened the possibility to design vaccines that use this property of the immune system to help generate immune responses to vaccine antigens. One important compound among this group of immune-stimulatory molecules is monophosphoryl lipid (MPL), which is a de-toxified derivative of the bacterial molecule lipo-polysaccharide (LPS). MPL is currently used in several vaccines. When molecules such as MPL are used in vaccines to enhance immune responses, they are referred to as ‘vaccine adjuvants’. It is important to better understand how these adjuvants work: what is the mechanism by which they enhance immune responses, e.g., production of antibodies or generation of T cells. So far, much of the work in this field has been carried out in experimental animals. However, the immune systems from experimental animals might be different from the human immune system, so it may not always be easy to interpret such results. On the other hand, the analysis of vaccine adjuvants in humans is not so straightforward either, as it is constrained by ethical considerations, genetic diversity and costs of human clinical trials. Therefore, we aimed to build an *in vitro* human immune system, in which we can study the behavior of vaccine adjuvants and antigenic proteins in a test tube. In order to mimic the actual vaccine injection site, we designed a three-dimensional (3D) *in vitro* in vitro vaccination system. The design of this model, called SIMULATA, is loosely inspired by work published by G Randolph and co-workers (Randolph *et al.*, Science 282:480, 1998). The *in vitro* model has three different components: (1) a collagen layer, (2) a thin layer of endothelial cells on top of the collagen (mimicking the wall of a blood vessel) and (3) peripheral blood mononuclear cells (PBMC). The basic assumption is that an important cell type in the PBMC population, monocytes, will migrate through the endothelial cell layer into the collagen. , It is expected that these monocytes will migrate through the endothelial cell layer. In the process, monocytes are hypothesized to pick up antigenic proteins and to be exposed to any adjuvants that are present in the system. This process imitates that migration of monocytes from blood vessels into infected tissues or tissue in which the vaccine has been injected and then back into the blood vessel or into the lymphatics. Our results show that indeed monocytes migrate into the collagen and then back to the supernatant (where they came from). In the process, we observed that these monocytes differentiated into so-called dendritic cells (DC). DCs are antigen-presenting cells: they pick-up proteins (antigens) from pathogens or vaccines and process these into smaller peptides. These peptides are presented by DCs to other immune cells, such as T cells. We have repeated this process in our in vitro system by harvesting the monocytes, after they migrated back to the supernatant, and incubating them with T cells. This process indeed resulted in the activation and proliferation of the T cells. Next, various antigens and/or adjuvants were added and the impact of adjuvants on monocyte migration, DC activation and T cell responses was measured. We found that several immune-stimulatory molecules increased the migration of monocytes from the PBMC in the supernatant into the collagen. These migrating cells then acquired dendritic cell properties, indicating that they underwent at least partial differentiation in the process of migrating, encountering adjuvant and picking up antigen. Using this system, we measured T cell responses in healthy donor PBMC to different viral and bacterial proteins, including several different subsets of CD4 T cells, some of which are difficult to detect with conventional methods (e.g., so-called Th17 cells). Based on these observations, we conclude that our 3D *in vitro* system has sufficient promise to warrant further investigation.

**SUMMARY DESCRIPTION OF PROJECT CONTEXT AND OBJECTIVES**

The context in which antigens are presented to the immune system controls the immunological outcome of antigenic challenge, ie, the precise nature of the immune response that follows exposure to antigens. The innate immune system uses a specific set of receptors that recognize molecular patterns from pathogens, known as pattern recognition receptors, to decode the nature of the antigen (*e.g.*, viral, bacterial, fungal) and to translate this into an appropriate adaptive immune response. Different microbial compounds are recognized by Toll-like receptors (TLR) but there are also compounds that can activate the immune system in a TLR-independent manner. The use of immune-stimulatory molecules that engage pattern recognition receptors as vaccine adjuvants opens the door towards the rational design of vaccines that induce defined and optimized immune responses. Thus, an important objective for adjuvant research, both from the efficacy and safety perspectives, is to better understand the links between the recognition of adjuvants by the innate immune system and the nature of the subsequent adaptive response. In other words: how does the innate immune system use the signals through pattern recognition receptors to generate a specific type of immune response? So far, much of the work in this field has been carried out in animal models such as mice, but these data are not readily extrapolated to human responses. On the other hand, the analysis of vaccine adjuvants in humans is constrained by ethical considerations, genetic diversity and costs of human clinical trials. Therefore, the *in vitro* re-creation of a human immune response would be ideal to begin bridging the gap between pre-clinical animal models and human clinical trials. One emerging concept of adjuvant-stimulated immune activation is that blood monocytes are recruited to the injection site and differentiate into either macrophages or dendritic cells (DCs) after which the latter migrate to the draining lymph nodes to activate adaptive immunity.

The **overall objective** of our proposal was to construct a 3D *in vitro* model to recapitulate this sequence of events and address fundamental questions related to vaccine adjuvants and adaptive immune response outcomes. The basic assumptions of our model derive from the work published by Randolph *et al* (Science 282:480, 1998). In short, PBMC or CD14+ blood monocytes are cultured on top of a collagen matrix that is covered by a monolayer of human umbilical cord endothelial cells (HUVEC). Monocytes will then migrate through the HUVEC monolayer into the collagen matrix. This migration through the HUVEC layer triggers differentiation into either macrophages or DCs. These DCs will then migrate back from the collagen layer towards the supernatant (‘reverse transmigration’) and will have a different activation status. DCs can be exposed to immune-stimulants and antigens in the system. To generate adaptive immune responses, antigen-loaded and immuno-stimulant exposed DC can be used to stimulate T cells. Based on the work from Randolph *et al* (Science 282:480, 1998) and with an emphasis on automation, a somewhat simplified system, referred to as MIMIC™, was further developed in a commercial vaccine-testing setting by the Florida-based company Vaxdesign (Higbee *et al*, Altern Lab Anim 37:19, 2009; Ma *et al*, Immunology 130:374, 2010). Moreover, Gaucher *et al* (J Exp Med 205:3119, 2008) used an in vitro immunology system to show, using microarray RNA expression analysis, that clear overlaps existed between early immune responses induced by the YFV-17D vaccine in the in vitro model versus in vivo. However, these authors used a highly simplified in vitro system which was more reminiscent of in vitro stimulation of PBMC. A major advantage of the in vitro immunization systems, such as the ones described by Randolph and coworkers (Science 282:480, 1998), Higbee *et al* (Altern Lab Anim 2009) and Gaucher *et al* (J Exp Med 205:3119, 2008) was that different vaccine formulations can be tested using PBMC from a single donor. Figure 1 schematically explains the MIMIC™ model, as described Higbee *et al* (Altern Lab Anim 37:19, 2009). Briefly, in the MIMIC model, peripheral blood mononuclear cells (PBMCs) are isolated by leucopheresis from consenting adult healthy donors. To set up the culture system, a collagen matrix is created in a flat bottom 96 well plate. Then, a HUVEC monolayer is grown on top of the collagen matrix and PBMCs (open and red circles) are added for 2h to the culture to allow monocyte (red circles) to migrate. After the 2 hours, cultures are washed to remove non-migrated lymphocytes and monocytes and incubated for 2-5 days after which transmigrated DCs are harvested. Addition of antigen and/or adjuvants into the system results in activation of the transmigrating inflammatory DCs which can then either prime an autologous naïve CD4 T cell (blue circles) response or stimulate a pre-existing memory response.

The **first objective** of the current project was to set up an in vitro immunization system and explore the parameters that control its functionality. Amongst others, we studied the behaviour of the HUVEC monolayer and its imact on the ensuing immune responses. Next, we studied the behavior of monocytes and PBMC when cultured on the HUVEC/collagen layer and the impact of immune stimuli/adjuvants on monocyte migration was analyzed. Different adjuvants and vaccine formulations were tested, focusing on Alum, AS01, AS02, AS03 (Garcon & Van Mechelen, Exp Review Vaccines 10:471, 2011) and zymosan. Subsequently, we studied activation profiles of migrating monocytes as well as production of inflammatory cytokines. Finally, the capacity of DCs to take up antigens was analyzed. Using fluorescently labeled antigen, it was found that cells carrying the fluorescent label could be detected in the supernatant, suggesting that antigen had been taken up. Thus, in Objective 1, we established and optimized the basic parameters of the system. The **second objective** was to evaluate the impact of vaccine adjuvants on T cell quality and magnitude of primary CD4 T cell responses to a candidate HIV vaccine in the *in vitro* model. The question addressed here is whether reverse-transmigrated monocyte-derived cells (ie, antigen-carrying and activated DCs) were capable of triggering primary CD4 T cell responses. In addition, the quality of these responses was studied as a function of adjuvant. However, we elected to not assess the immune responses of healthy donors to HIV candidate vaccine proteins (because this test could then be mistaken for an HIV-positivity test). Instead, we used the *Mycobacterium* *tuberculosis* candidate vaccine antigen formulated with different adjuvants. The vaccine consists of a fusion protein named M72 containing part of the Mtb32a and Mtb39a proteins from M. *tuberculosis* (Leroux-Roels *et al*, Vaccine 2012, Epub ahead of print) and formulated with Alum, AS01, AS02, AS03 or zymosan. Cell cultures were established and the vaccine was administered to the system using the optimized procedures described above. Monocyte-derived cells were harvested and cultured with autologous PBMC. Responses were measured after 4 weeks of culture by stimulating cells with a set of overlapping peptides spanning the vaccine antigen, followed by intracellular cytokine staining (ICS). We analyzed production of IFN-, TNF- and IL-17 in combination with CD40L by multiparameter flow cytometry. This setup provides the unique opportunity to compare different adjuvant formulations in the same donor, since multiple cultures will be set up using the same PBMC batch and established how adjuvant impacts on the cytokine profiles and magnitude of the primary response. Finally, the **third objective** was to assess the impact of adjuvanted priming on recall responses to drifted influenza virus vaccines. For reagent availability reasons, we decided to first focus experiments on the question whether adjuvant could affect the cytokine profile and the magnitude of the secondary response to antigens from Varicella Zoster Virus (VZV), Hepatitis B virus (HBV), S. *aureaus* and S. *pneumoniae* bacteria. The gE protein from VZV (Dendouga *et al*, Vaccine 30:3126, 2012; Leroux-Roels *et al*, J Inf Disease 2012, Epub ahead of print) and the HBs protein from HBV (Vandepapeliere *et al*, Vaccine 26:1375, 2008) were used in the *in vitro* system. To study the behavior of anti-bacterial T cells, antigens from both S. *aureaus* and *S.* *pneumonia*were tested in the system as well. For reasons of confidentiality, these proteins will be referred to as *S.* *aureus* and *S.* *pneumoniae* proteins 1 and 2. We analyzed the immune responses to these candidate vaccine antigens formulated with different adjuvants in individuals likely to have pre-existing CD4 T cell immunity to VZV, HBV, *S.* *aureus* and *S.* *pneumoniae*. To confirm pre-existing immunity, healthy donors were first screened for a detectable recall responses to VZV gE, HBV surface antigen (HBs) and the *S. aureus* and *S. pneumoniae* proteins, using a long term *in vitro* PBMC restimulation culture with overlapping peptides spanning the antigens. The best responders were selected and used in the in vitro system. The main findings from this work were (1) that CD4 T cell responses can be detected and that adjuvants can affect these responses, and (2) that the system revealed Th17-type responses with *S. aureus* proteins. The identification of S. aureus specific Th17 responses is consistent with a recent report (Zielinski *et al*, Nature 484:514, 2012). Interestingly, inclusion of IL-2 in the culture medium prevented expansion/induction of Th17 responses, consistent with the plasticity of the Th17 phenotype (Zielinski *et al*, Nature 484:514, 2012). Induction of such Th17 responses was not an artifact of the system, since this was not observed with other antigens.



**Fig 1: Schematic of the 3D *in vitro* MIMIC system from Vaxdesign. Left panel indicates the different step of the immune response that are modelled in the system, ie, the innate response (“PTE”) and the adaptive response (“LTE”). Right panel indicates the actual immune response to illustrate how the model reflects on ‘real’ immune responses.**

**DESCRIPTION OF THE MAIN S&T RESULTS/FOREGROUNDS**

**OBJECTIVE 1**

**1/ Construction of the in vitro SIMULATA system (set-up)**

The first challenge of this project was to set up an in vitro immunization system when only a few research papers on the topic were available (Randolph *et al*, Science 282:480, 1998; Qu *et al*, J Imm 182:3650, 2009; Higbee *et al*, Altern Lab Anim 37:19, 2009; Ma *et al*, Immunology 130:374, 2010). To get the system operational, we had to optimize multiple system parameters such astissue culture plates, endothelial cells (HUVEC), timing of the different steps and quantitation of antigens and adjuvants.

Optimization yielded a protocol in which 96 well plates were used and collagen was added. The HUVEC cells were seeded on top of the collagen matrix. The original research paper describing the in vitro system published by Randolph and colleagues (Science 282:480, 1998) demonstrated that 2 days was sufficient to get DC reverse trans-migration and differentiation. Here we tested this two day incubation period and also longer incubation times of monocytes in the collagen matrix (2 and 5 days). Finally, a titration of the dose of adjuvants added to the system was done to avoid HUVEC cell mortality and get good DC viability. The optimized system will be referred to as SIMULATA.

Overall, in the project, several different vaccine adjuvants or combinations of immune stimuli were used:

* Alum: Al(OH)3 or AlPO4, used to bind antigens; also activates the innate immune system
* Zymosan: a fungal compound that is a ligand for TLR2 and dectin-1
* LPS: a bacterial compound that is a ligand for TLR4
* MPL: detoxified LPS and also a ligand for TLR4
* AS01 (Adjuvant System 1): MPL and QS21 in a liposome formulation
* AS02 (Adjuvant System 2): MPL and QS21 in an oil-in-water emulsion
* AS03 (Adjuvant System 3): an oil-in-water emulsion containing -tocopherol
* AS15 (Adjuvant System 15): MPL, QS21 in a liposome formulation and CpG oligonucleotides

More details on the Adjuvant Systems can be found in the following publications:

* Garcon & Van Mechelen, Exp Review Vaccines 10:471, 2011 (review on Adjuvant Systems)
* Morel et al, Vaccine 29:246, 2011 (AS03)

**2/ Evaluation of the impact of vaccine adjuvants on monocyte migration**

Intra-muscular injection of adjuvanted vaccines is known to recruit innate immune cells such as monocytes from the blood through the induction of chemokine expression. We therefore decided to assess the impact of various vaccine adjuvants on monocyte migration using the SIMULATA system. 10µg/ml of antigen (from HBV amongst others) formulated with or without adjuvant or the adjuvant alone (Alum, AS01, AS03, AS15 or 1% zymosan) (see Garcon & Van Mechelen, Exp Review Vaccines 10:471, 2011 and Morel et al, Vaccine 29:2461, 2011 for an overview on the adjuvant systems AS01 and AS03) were at a 1/100 dilution. After reaching HUVEC confluency, PBMC were added on top of the HUVEC monolayer. The frequency of monocytes in the PBMC was determined by FACS using CD14 staining.

After migration, non-migratory cells, i.e., those cells that remained in the supernatant, were extensively washed and kept for an additional CD14 staining in order to calculate the percentage of monocyte migration through the endothelium using the following formula:

% of monocyte migration= % CD14+ monocytes in the PBMC - % CD14+ after migration

 % CD14+ monocytes in the PBMC

The results were as follows: we showed that addition of the TLR2 ligand zymosan (Randolph et al, Science 282:480, 1998) increased the migration of monocytes thought the endothelium compared to the condition where nothing was added to the system (None conditions). In contrast, no impact of Alum, AS01 and AS03 on monocyte migration was observed.

**3/ Evaluation of the impact of vaccine adjuvants on reverse transmigrating cell differentiation and activation**

Even though the original research paper describing the *in vitro* system published by Randolph and colleagues (Science 282:480, 1998) demonstrated that 2 days was sufficient to get DC reverse trans-migration and differentiation, we decided to test longer times of incubation of monocytes . After migration through the endothelium, monocytes were left in the collagen matrix to allow the cells to reverse transmigrate. The phenotype of the reverse transmigrating cells was analysed by flow cytometry using different surface markers.

Reverse transmigrating cells collected after incubation in the SIMULATA system showed higher expression of DC activation markers, reflecting a “DC like” phenotype. Moreover, these cells seemed to mature more efficiently when the immunostimulant zymosan was included. Indeed, a proportion of cells showed reduced expression of monocyte markers and increased expression of activated DC markers.

Another way to assess the activation of antigen-presenting cells (APC) (i.e., the monocytes and DCs) following contact to adjuvant is to measure their production of pro-inflammatory cytokines. Hence, cytokine production in the medium overlying the endothelial cell layer (the supernatant) was measured by multiplex cytokine analysis (luminex) after 5 days of differentiation. No effect of AS03 or AS01 on the production of several pro-inflammatory cytokines (such as IL-6, GMCSF, TNF-, MCP-1 or IL-1) was observed. The cytokine IL-8 was induced by AS03 in the *in vitro* system. Zymosan was able to induce the production of all cytokines mentioned above by monocytic cells. Even though zymosan promoted migration of a higher proportion of monocytes, this increased migration alone probably does not account for the difference in cytokine production observed in these experiments. IFN production (at very low levels) was only found using zymosan activated APCs. No IL-18 or IL-22 was detected in any of the conditions.

The capacity of adjuvants to increase reverse transmigrating cell maturation can also be evaluated through their capacity to increase antigen uptake. Fluorescent HBV surface antigen (HBs antigen) was used to detect cells that would take up antigen. Thus, fluorescent HBs antigen was added to the SIMULATA system and after 5 days of differentiation, we quantified antigen uptake by flow cytometry. The presence of AS01 in the system led to an increase of antigen uptake compared to the condition with fluorescent Ag alone. There was no impact of zymosan on antigen uptake. Cells that had taken up higher amount of proteins displayed a more mature phenotype with higher expression of the DC activation marker HLA-DR.

The conclusion from this part of the project was that monocytes can migrate through the HUVEC monolayer into the collagen and then reverse transmigrate back into the supernatant. In this process, the monocytes acquire several properties, such as expression on DC markers,and production of cytokinesthat suggest that they differentiated into bona fide antigen-presenting cells. However, to confirm that, a functional test is essential, in which the capacity of the reverse-transmigrated cells to activate T cells is tested.

**OBJECTIVE 2**

**Evaluation of the impact of vaccine adjuvants on primary T cell quality and magnitude**

Having established that the *in vitro* systems reproduced key aspects of the innate immune response, the next step was to assess whether the reverse transmigrating cells, which were assumed to have differentiated into professional APCs, i.e. DCs, were capable of inducing CD4 T cell responses. In addition, the phenotypic characteristics (the ‘quality’) of the CD4 response were measured as a function of adjuvant.

To achieve this, we originally planned to use the GSK candidate HIV vaccine antigen (Leroux-Roels et al, Vaccine 28:7016, 2010) formulated with different adjuvants (Alum, AS01, AS02, AS03, zymosan at 2 different dilutions in collagen). However, we changed that and used the *M.* *tuberculosis* candidate vaccine antigen, the fusion protein M72 (Leroux-Roels et al, Vaccine 2012), because we concluded that our experiments in this system could not be used or be misinterpreted as a surrogate HIV test. We assumed that by using healthy donor PBMC, from donors with low probability of being exposed to TB, we would be able to assess primary T cell responses using the M72 antigen.

We set up the system with the M72 antigen and the different adjuvants and adjuvant systems in the system. After differentiation, reverse transmigrating cells were collected. These cells would comprise the differentiated DCs containing antigen and that had been exposed to the adjuvants. These cells were then co-cultured with autologous PBMC (1:20 ratio) in order to assess the capacity of the M72/adjuvant exposed DCs to stimulate a T cell response. This culturing was done for 4 weeks in the absence of IL-2 to avoid any skewing of the immune response. Cells were then re-stimulated overnight with either medium or M72 peptides, in order to identify responding T cells and intracellular cytokine expression was assessed by flow cytometry. Thus, the 4 week culturing step evaluates the capacity of the DCs harvested from the collagen/HUVEC system to stimulate T cells. The short (overnight) incubation with peptides serves to visualize the responding T cells, such that they can be measured by flow cytometry. In these experiments, M72 specific responses induced by antigen-loaded reverse transmigrating cells were compared with responses obtained from ‘simple’ PBMC stimulation with M72 protein. We observed that M72-loaded APCs harvested from the *in vitro* SIMULATA system were better at stimulating a specific primary CD4 T cell response than PBMC that were simply incubated with the M72 protein. Formulation of M72 with AS02 and zymosan further increased the capacity of the monocyte derived APCs (from SIMULATA) to prime a specific CD4 T cell response. While the magnitude of the CD4 T cell response was affected by the adjuvants, the cytokine profiles (quality) of these cells were similar.

The conclusion from this part of the project was that the reverse transmigrating monocytes (that differentiated into DCs) behaved as antigen-presenting cells as evidenced by their capacity to stimulate naïve T cells.

**OBJECTIVE 3**

**Evaluation of the impact of vaccine adjuvants on recall T cell quality and magnitude**

*A. Responses specific for VZV gE and HBV surface antigen*

We next focused on detecting secondary CD4 T cell responses, i.e., by studying responses in the *in vitro* system using antigens for which immune memory was already present. Thus, here, we addressed whether existing memory T cells could be re-activated in the *in vitro* system. For this, we used PBMC samples from healthy donors with defined memory responses against VZV antigen gE and HBV surface antigen HBs. Healthy donor PBMC samples were first screened for their gE and HBs memory responses. This was done as follows: PBMC were stimulated for 5 days or overnight with gE or HBs peptides, respectively. For the long term culture, cells were re-stimulated overnight with either medium or peptides. gE and HBs responders were selected based on their antigen-specific CD4 T cell responses as measured by flow cytometry.

To test whether the *in vitro* system was capable of activating memory CD4 T cells, the VZV gE antigen was formulated with different adjuvants (1/10 dilution for all adjuvants except for zymosan 1/100) and these were added to the system. Then, PBMC from healthy donors that were VZV memory CD4-positive in the first screening test were added to the collagen/HUVEC wells and incubated. After differentiation, reverse transmigrating cells, were collected. These cells were co-cultured with autologous PBMC during 8 days in the presence of interleukin-2 (IL-2) and IL-7. In this set-up, the harvested cells, harbouring antigen-presenting cells that picked-up antigen and that were exposed to adjuvant, present the antigen to T cells in the PBMC preparation. Responses were measured by intracellular cytokine staining after overnight stimulation of the cells to peptides spanning the gE protein followed by flow cytometry. Antigen-specific responses induced by antigen-loaded and adjuvant-exposed reverse transmigrating cells were compared with responses obtained from PBMC stimulated with peptides covering the selected antigens. Cells were re-stimulated overnight with either medium or overlapping peptide pools and intracellular cytokine expression was assessed by flow cytometry. The results show that antigen-loaded transmigrating cells were better than overlapping peptides at stimulating specific CD4 memory responses. In addition, formulation of the antigen with either AS03 or zymosan further increased the capacity of the “DC like” cells to activate a specific a CD4 T cell response. From these results, it was concluded that the SIMULATA system was capable of re-activating CD4 memory T cells.

Next, memory CD4 T cell responses specific for the HBV surface antigen HBs were assessed using the *in vitro* system. To do this, HBs antigen was formulated with different adjuvants and Adjuvant Systems and added to the system. After differentiation, reverse transmigrating cells were collected from the SIMULATA cultures and co-cultured with autologous PBMC during 8 days in the presence of IL-2 and IL-7. After this incubation, peptides spanning the HBs protein sequence were added to the PBMC cultures to allow flow cytometric detection of responding T cells. Antigen-specific responses induced by reverse transmigrating cells (i.e., the cells harvested from the in vitro system) were compared with responses obtained from PBMC stimulated with peptides covering the selected antigens. Cells were re-stimulated overnight with either medium or overlapping peptide pools and intracellular cytokines expression was assessed by flow cytometry. In contrast to the above-described results with the VZV gE antigen, antigen-loaded transmigrating cells were not better than overlapping peptides at stimulating specific CD4 memory responses in the setting of the HBs antigen. This result suggested either that recall of HBs-specific memory T cell responses does not need SIMULATA-generated antigen- loaded APCs or that the amount of HBs protein added to the system was not sufficient to get an optimal loading of the antigen on the APCs. Nevertheless, formulation of the antigen with AS03 was able to further increase the capacity of the “DC like” cells to activate a specific CD4 T cell response.

From this part of the project it was concluded that the *in vitro* system is capable of picking up antigens and presenting these to memory CD4 T cells. Whereas for one antigen, the VZV gE protein, it appeared that the *in vitro* system led to better CD4 T cell expansion as compared to long-term peptide stimulation, this was not observed for the second antigen, the HBV HBs antigen. It is unclear why these two antigens behave different. Nevertheless, it can be concluded that the SIMULATA system achieved T cell expansion for both antigens, suggesting that it can be used to assess recall responses.

*B. Responses specific for S. aureus and S. pneumoniae*

A newly described CD4 T cell subset expressing the cytokine IL-17 has been shown to play a key role in host protection against bacterial infections such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. This novel CD4 T cell lineage is now known as ‘Th17’. During life, exposure to these bacteria is common and most people are likely to harbour immunological memory specific for these pathogens. This memory could comprise Th17 cells. Indeed, in a different *in vitro* expansion system, Sallusto and coworkers recently described Th17-type CD4 memory responses specific for *S aureus* in healthy donor PBMC (Zielinski et al; Nature 2012). We were interested to evaluate how the SIMULATA system would detect such potential Th17 responses.

Healthy donors were screened for their *S. aureus* and *S. pneumoniae* memory T cell responses. To this end, PBMC were stimulated for 8 days in presence of IL-2 and IL-7 with either inactivated bacteria, the protein antigens ‘protein 1’ and ‘protein 2’ or peptide pools spanning these proteins. Cells were then re-stimulated overnight with either medium or overlapping peptides and intracellular cytokine expression was assessed by flow cytometry. Most of the healthy donors displayed *S aureus* specific Th1-type memory CD4 T cell responses (TNF-+/ IFN-+). No IL-17 expression was observed in the experiment, indicating that PBMC stimulation (note: this is not in the SIMULATA system) with inactivated bacteria, proteins or peptides does not readily reveal specific Th17 responses, despite the fact that these were shown to exist for at least *S aureus* (Zielinski et al; Nature 2012). There are several potential explanations for this. First, it is possible that the donor samples used do not contain Th17 memory cells specific for the bacterial antigens tested. Alternatively, it is possible that the presence of IL-2 during the co-culture could have blocked Th17 responses. Indeed, IL-2 has recently been shown to inhibit the production of IL-17 by memory Th17 cells (Zielinski et al; Nature 2012). The same experiment, i.e., direct PBMC stimulation with different antigens, was therefore repeated in the absence of IL-2 and IL-7 but this gave similar results.

To determine whether the SIMULATA system could reveal potential pre-existing Th17 memory responses against S. *aureus*, two bacterial proteins, protein 1 and protein 2, were formulated with different adjuvants or Adjuvant Systems (1/10 dilution for all adjuvants except for zymosan 1/100), with protein only (no adjuvants) as a control. These formulations were added to the SIMULATA system. After incubation, reverse transmigrating cells were collected and co-cultured with autologous PBMC (1:20 ratio) during 8 days in the presence or absence of IL-2. Antigen-specific responses induced by antigen-loaded reverse transmigrating cells were compared with responses obtained with PBMC stimulation with overlapping pool of peptides. After this expansion period, cells were re-stimulated overnight with pools of peptides to allow flow cytometry detection by intracellular cytokine staining. As before, stimulation of PBMC with overlapping pools of peptides resulted in a Th1 response (TNF-+/ IFN-+). Interestingly, incubation of PBMC with antigen-loaded reverse transmigrating cells from the SIMULATA system revealed antigen-specific Th17 responses, characterized by the production of IL-17 in the intracellular cytokine staining assay. Some effects of the Adjuvant Systems ASO1 and AS02 and of the TLR2 adjuvant zymosan were observed on both Th1 and Th17 responses. Interestingly, the Th17 profile was clearly different when zymosan was added to the system: under these conditions, no antigen-specific IL17+ IFN+ cells were observed.

No IL-17 production was detected when IL-2 was added to the co-culture suggesting that IL-2 might either amplify the Th1 response to the expense of Th17 responses and/or inhibit Th17 cell expansion as described by Zielinski et al; *Nature* 2012).

The SIMULATA system was also used to analyse potential pre-existing Th17 memory responses specific for *S* *pneumoniae*. By screening an expression library containing >96% of predicted pneumococcal proteins, a recent report identified two *S*. *pneumoniae* antigens recognized by Th17 cells from human PBMC (Moffitt et al, *Cell Host Microbe* 2011). We produced these proteins SP0128 and SP0148 and formulated them with various adjuvants and Adjuvant Systems (1/50 dilution for all adjuvants except for zymosan 1/100) and added these to the system. After 5 days in the system, reverse transmigrating cells, were collected and co-cultured with autologous PBMC (1:20 ratio) during 8 days in the absence of IL-2. Responses were compared with responses obtained with PBMC stimulation with protein (i.e., no SIMULATA). Cells were then re-stimulated overnight with the protein and intracellular cytokine expression was assessed by flow cytometry. While no IL-17 was detected with formulated SP0148 protein, SP0128 loaded APCs generated in the SIMULATA system were able to stimulate specific Th17 responses. As observed for *S aureus* antigens, no Th17 or Th1 specific responses were observed after classical PBMC stimulation with protein SP0128, further emphasizing the capacity of SIMULATA-derived APCs to specifically stimulate, or rescue, such Th17 responses.

From the results generated in this part of the project, it was concluded that the *in vitro* SIMULATA system has the capacity to expand pre-existing Th17 cells that are specific for *S aureus* or *S pneumoniae*. It is important that such Th17 responses were not detected in parallel cultures in which PBMC were stimulated directly with the proteins, i.e., without the DC differentiation step in the SIMULATA system. This suggest that the *in vitro* system, in which APCs are generated by monocytes that migrate through HUVEC monolayers, does capture a specific, as yet undefined, property that is important to expand Th17 cells. This is of clear interest for the further study of Th17-type immune responses.

**DISCUSSION**

We were able to set up an *in vitro* immunization system, referred to as the SIMULATA system, after optimizing multiple parameters, such as HUVEC cell handling, PBMC and timing... We measured the capacity of monocytes to migrate through the endothelial monolayer into the collagen matrix. We observed that the TLR2 agonist and immune-stimulant zymosan had a clear positive effect on monocyte migration in this system. This was not as evident with other adjuvants or with Adjuvant Systems tested.

We demonstrated that inclusion of adjuvants, starting with zymosan, in the SIMULATA sysatem induced reverse transmigrating cell differentiation and maturation. We also observed the production of pro-inflammatory cytokines in the culture system when zymosan was added to the system. IL-8 production was observed when the Adjuvant System AS03 was added to the system. We also assessed the capacity of adjuvants to affect reverse transmigrating cell maturation through their aptitude to increase antigen uptake. Additional work is needed to further characterize reverse transmigrating cells and to conclude on the impact of adjuvants on antigen uptake by these cells.

Perhaps most importantly, we evaluated the functional capacity of reverse transmigrating cells to stimulate a primary CD4 T cell response. We demonstrated that in contrast to M72 stimulated PBMC cultures, antigen-loaded APCs generated in the SIMULATA system were able to prime an antigen-specific CD4 T cell response that was further increased by the presence of adjuvants in the system.

In the next step, we assessed the capacity of SIMULATA-generated antigen-loaded APCs to stimulate CD4 memory T cell ‘recall’ responses to VZV or HBV antigens. We observed that gE loaded APC were capable of stimulating a memory responses and that both zymosan and AS03 had an adjuvant effect on these antigen-specific memory T cell responses. Here, the SIMULATA system performed better that ‘simple’ PBMC/antigen stimulation. A different observation was made with the HBV surface antigen. In this setting, SIMULATA-generated DCs were capable to expand HBs-specific memory CD4 T cells. However, in contrast to the VZV gE experiment, the SIMULATA-generated APCs were not better than simple PBMC stimulation with peptides covering the HBs. Antigen-specific T cell responses had a Th1 phenotype with no IL-17 detected by intracellular staining.

Based on our increasing understanding of the potential role of Th17 responses in host protection against bacterial infection, we aimed to extend our preliminary data on secondary CD4 T cell responses using antigens from S. *aureus* or *S. pneumoniae*. Given the recently published inhibitory role of IL-2 on memory Th17 responses (Zielinski et al; *Nature* 2012), co-culture experiments were done in the presence or absence of IL-2. Interestingly, we demonstrated that SIMULATA-derived antigen-loaded APC were able to reveal *S. aureus* and *S pneumoniae* -specific memory Th17 responses only in the absence of IL-2. The capacity to induce Th17 responses was unique to SIMULATA-derived APCs and was not observed after long term PBMC co-culture with *S. aureus* or *S. pneumoniae* proteins or with overlapping peptide pools, even in the absence of IL-2.

As a next step and to better recapitulate the intramuscular vaccination injection site, it would be of interest to evaluate the impact of incorporating of (apoptotic) antigen-carrying cells in the collagen matrix (Qu et al, J Immunol 182:3650, 2009) on phenotypic and functional properties of SIMULATA derived APCs.

## Use and dissemination of foreground

A plan for use and dissemination of foreground (including socio-economic impact and target groups for the results of the research) shall be established at the end of the project. It should, where appropriate, be an update of the initial plan in Annex I for use and dissemination of foreground and be consistent with the report on societal implications on the use and dissemination of foreground (section 4.3 – H).

The plan should consist of:

* Section A

This section should describe the dissemination measures, including any scientific publications relating to foreground. **Its content will be made available in the public domain** thus demonstrating the added-value and positive impact of the project on the European Union.

* Section B

This section should specify the exploitable foreground and provide the plans for exploitation. All these data can be public or confidential; the report must clearly mark non-publishable (confidential) parts that will be treated as such by the Commission. Information under Section B that is not marked as confidential **will be made available in the public domain** thus demonstrating the added-value and positive impact of the project on the European Union.

**Section A (public)**

This section includes two templates

* Template A1: List of all scientific (peer reviewed) publications relating to the foreground of the project.
* Template A2: List of all dissemination activities (publications, conferences, workshops, web sites/applications, press releases, flyers, articles published in the popular press, videos, media briefings, presentations, exhibitions, thesis, interviews, films, TV clips, posters).

These tables are cumulative, which means that they should always show all publications and activities from the beginning until after the end of the project. Updates are possible at any time.

|  |
| --- |
| **template A1: list of scientific (peer reviewed) publications, starting with the most important ones** |
| NO. | Title | Main author | Title of the periodical or the series | Number, date or frequency | Publisher | Place of publication | Year of publication | Relevant pages | Permanent identifiers[[2]](#footnote-2) (if available) | Is/Will open access[[3]](#footnote-3) provided to this publication? |
| 1 | **N/A** |  |  |  |  |  |  |  |  |  |
| 2 |  |  |  |   |  |  |  |   |  |  |
| 3 |  |  |  |   |  |  |  |   |  |  |
|  |   |  |  |   |  |  |  |   |  |  |

|  |
| --- |
| **template A2: list of dissemination activities** |
| NO. | Type of activities[[4]](#footnote-4) | Main leader | Title  | Date  | Place  | Type of audience[[5]](#footnote-5) | Size of audience | Countries addressed |
| 1 | **N/A** |  |  |  |  |  |  |  |
| 2 |  |  |  |  |  |  |  |  |
| 3 |  |  |  |  |  |  |  |  |
|  |   |  |  |  |  |  |  |  |

**Section B (Confidential[[6]](#footnote-6) or public: confidential information to be marked clearly)**

**Part B1**

The applications for patents, trademarks, registered designs, etc. shall be listed according to the template B1 provided hereafter.

The list should, specify at least one unique identifier e.g. European Patent application reference. For patent applications, only if applicable, contributions to standards should be specified. This table is cumulative, which means that it should always show all applications from the beginning until after the end of the project.

|  |
| --- |
| **Template B1: List of applications for patents, trademarks, registered designs, etc.** |
| Type of IP Rights[[7]](#footnote-7):  | Confidential Click on YES/NO | Foreseen embargo datedd/mm/yyyy | Application reference(s) (e.g. EP123456) | Subject or title of application | Applicant (s) (as on the application) |
| **N/A** |  |  |  |   |   |
|  |  |  |  |   |   |
|  |  |  |  |   |   |
|   |  |  |  |   |   |

**Part B2**

Please complete the table hereafter:

| **Type of Exploitable Foreground[[8]](#footnote-8)** | **Description****of exploitable foreground** | **Confidential****Click on YES/NO** | **Foreseen embargo date****dd/mm/yyyy** | **Exploitable product(s) or measure(s)** | **Sector(s) of application[[9]](#footnote-9)** | **Timetable, commercial or any other use** | **Patents or other IPR exploitation (licences)** | **Owner & Other Beneficiary(s) involved** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ***N/A*** |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |

In addition to the table, please provide a text to explain the exploitable foreground, in particular:

* Its purpose
* How the foreground might be exploited, when and by whom
* IPR exploitable measures taken or intended
* Further research necessary, if any
* Potential/expected impact (quantify where possible)

## Report on societal implications

Replies to the following questions will assist the Commission to obtain statistics and indicators on societal and socio-economic issues addressed by projects. The questions are arranged in a number of key themes. As well as producing certain statistics, the replies will also help identify those projects that have shown a real engagement with wider societal issues, and thereby identify interesting approaches to these issues and best practices. The replies for individual projects will not be made public.

|  |
| --- |
| A General Information *(completed automatically when Grant Agreement number is entered.* |
| Grant Agreement Number: |  |
| 252508 |
| Title of Project:  |  |
| SIMULATA |
| Name and Title of Coordinator: |  |
| Dr Robbert van der Most |
| B Ethics  |
| **1. Did your project undergo an Ethics Review (and/or Screening)?**1. If Yes: have you described the progress of compliance with the relevant Ethics Review/Screening Requirements in the frame of the periodic/final project reports?

Special Reminder: the progress of compliance with the Ethics Review/Screening Requirements should be described in the Period/Final Project Reports under the Section 3.2.2 *'Work Progress and Achievements'*1. \* Note that work with human biological samples was restricted to using healthy donor PBMC which were obtained under a protocol and ICF that allowed use of those samples for research purposes in an industry setting. The two study protocols under which this was done are (i) RD-HBS-001 (114183) and (ii) HDPV (BB0038/001).
 | *0Yes* 0**No\*** |
| **2. Please indicate whether your project involved any of the following issues (tick box) :** | ***YES*** |
| **Research on Humans** |
| 1. Did the project involve children?
 |  |
| 1. Did the project involve patients?
 |  |
| 1. Did the project involve persons not able to give consent?
 |  |
| 1. Did the project involve adult healthy volunteers?
 | X |
| 1. Did the project involve Human genetic material?
 |  |
| * Did the project involve Human biological samples?
 | X |
| * Did the project involve Human data collection?
 |  |
| **Research on Human embryo/foetus** |
| 1. Did the project involve Human Embryos?
 |  |
| 1. Did the project involve Human Foetal Tissue / Cells?
 |  |
| 1. Did the project involve Human Embryonic Stem Cells (hESCs)?
 |  |
| 1. Did the project on human Embryonic Stem Cells involve cells in culture?
 |  |
| 1. Did the project on human Embryonic Stem Cells involve the derivation of cells from Embryos?
 |  |
| **Privacy** |
| 1. Did the project involve processing of genetic information or personal data (eg. health, sexual lifestyle, ethnicity, political opinion, religious or philosophical conviction)?
 |  |
| 1. Did the project involve tracking the location or observation of people?
 |  |
| **Research on Animals** |
| 1. Did the project involve research on animals?
 |  |
| 1. Were those animals transgenic small laboratory animals?
 |  |
| 1. Were those animals transgenic farm animals?
 |  |
| 1. Were those animals cloned farm animals?
 |  |
| 1. Were those animals non-human primates?
 |  |
| **Research Involving Developing Countries** |
| 1. Did the project involve the use of local resources (genetic, animal, plant etc)?
 |  |
| 1. Was the project of benefit to local community (capacity building, access to healthcare, education etc)?
 |  |
| **Dual Use**  |  |
| * Research having direct military use
 | 0 Yes **0 No** |
| 1. Research having the potential for terrorist abuse
 | 0 Yes **0 No** |
| C Workforce Statistics  |
| 3. Workforce statistics for the project: Please indicate in the table below the number of people who worked on the project (on a headcount basis). |
| **Type of Position** | **Number of Women** | **Number of Men** |
| Scientific Coordinator  |   |  1 |
| Work package leaders |   |   |
| Experienced researchers (i.e. PhD holders) |  1 |   |
| PhD Students |   |   |
| Other :(**technician**) |  1 |   |
| 4. How many additional researchers (in companies and universities) were recruited specifically for this project? | 0 |
| Of which, indicate the number of men:  |  |
| D Gender Aspects  |
| **5.**  **Did you carry out specific Gender Equality Actions under the project**? | ⭘🟓 | YesNo  |
| 6. Which of the following actions did you carry out and how effective were they?  |
|  |  |  | **Not at all effective** |  |  |  | **Veryeffective** |  |
|  |  | ❑ | Design and implement an equal opportunity policy | ⭘ | ⭘ | ⭘ | ⭘ | ⭘ |
|  |  | ❑ | Set targets to achieve a gender balance in the workforce | ⭘ | ⭘ | ⭘ | ⭘ | ⭘ |
|  |  | ❑ | Organise conferences and workshops on gender | ⭘ | ⭘ | ⭘ | ⭘ | ⭘ |
|  |  | ❑ | Actions to improve work-life balance | ⭘ | ⭘ | ⭘ | ⭘ | ⭘ |
|  |  | ⭘ | Other: |  |
| 7. Was there a gender dimension associated with the research content – i.e. wherever people were the focus of the research as, for example, consumers, users, patients or in trials, was the issue of gender considered and addressed? |
|  |  | ⭘ | Yes- please specify  |
|  |  | 🟓 | **No**  |
| E Synergies with Science Education  |
| 8. Did your project involve working with students and/or school pupils (e.g. open days, participation in science festivals and events, prizes/competitions or joint projects)? |
|  |  | ⭘ | Yes- please specify  |
|  |  | 🟓 | **No** |
| 9. Did the project generate any science education material (e.g. kits, websites, explanatory booklets, DVDs)?  |
|  |  | ⭘ | Yes- please specify  |
|  |  | 🟓 | **No** |
| F Interdisciplinarity  |
| 10. Which disciplines (see list below) are involved in your project?  |
|  |  | ⭘ | Main discipline[[10]](#footnote-10): **3.1** |
|  |  | ⭘ | Associated discipline10: | ⭘  | Associated discipline10: |
| G Engaging with Civil society and policy makers |
| **11a**  **Did your project engage with societal actors beyond the research community?**  *(if 'No', go to Question 14)* | ⭘🟓 | YesNo  |
| 11b If yes, did you engage with citizens (citizens' panels / juries) or organised civil society (NGOs, patients' groups etc.)?  |
|  |  | ⭘ | No |
|  |  | ⭘ | Yes- in determining what research should be performed  |
|  |  | ⭘ | Yes - in implementing the research  |
|  |  | ⭘ | Yes, in communicating /disseminating / using the results of the project |
| 11c In doing so, did your project involve actors whose role is mainly to organise the dialogue with citizens and organised civil society (e.g. professional mediator; communication company, science museums)? | ⭘⭘ | YesNo  |
| **12.**  **Did you engage with government / public bodies or policy makers (including international organisations)** |
|  |  | 🟓 | No |
|  |  | ⭘ | Yes- in framing the research agenda |
|  |  | ⭘ | Yes - in implementing the research agenda |
|  |  | ⭘ | Yes, in communicating /disseminating / using the results of the project |
| 13a Will the project generate outputs (expertise or scientific advice) which could be used by policy makers? |
|  |  | ⭘ | Yes – as a **primary** objective (please indicate areas below- multiple answers possible) |
|  |  | 🟓 | Yes – as a **secondary** objective (please indicate areas below - multiple answer possible) |
|  |  | ⭘ | No |
| 13b If Yes, in which fields? Public Health |
| [Agriculture](http://europa.eu/pol/agr/index_en.htm) [Audiovisual and Media](http://europa.eu/pol/av/index_en.htm) [Budget](http://europa.eu/pol/financ/index_en.htm) [Competition](http://europa.eu/pol/comp/index_en.htm) [Consumers](http://europa.eu/pol/cons/index_en.htm) [Culture](http://europa.eu/pol/cult/index_en.htm) [Customs](http://europa.eu/pol/cust/index_en.htm) [Development](http://europa.eu/pol/dev/index_en.htm) [Economic and Monetary Affairs](http://europa.eu/pol/emu/index_en.htm) [Education, Training, Youth](http://europa.eu/pol/educ/index_en.htm) [Employment and Social Affairs](http://europa.eu/pol/socio/index_en.htm) |  | [Energy](http://europa.eu/pol/ener/index_en.htm) [Enlargement](http://europa.eu/pol/enlarg/index_en.htm) [Enterprise](http://europa.eu/pol/enter/index_en.htm) [Environment](http://europa.eu/pol/env/index_en.htm) [External Relations](http://europa.eu/pol/ext/index_en.htm)[External Trade](http://europa.eu/pol/comm/index_en.htm)[Fisheries and Maritime Affairs](http://europa.eu/pol/fish/index_en.htm) [Food Safety](http://europa.eu/pol/food/index_en.htm) [Foreign and Security Policy](http://europa.eu/pol/cfsp/index_en.htm) [Fraud](http://europa.eu/pol/fraud/index_en.htm)[Humanitarian aid](http://europa.eu/pol/hum/index_en.htm) |  | [Human rights](http://europa.eu/pol/rights/index_en.htm) [Information Society](http://europa.eu/pol/infso/index_en.htm)[Institutional affairs](http://europa.eu/pol/inst/index_en.htm) [Internal Market](http://europa.eu/pol/singl/index_en.htm) [Justice, freedom and security](http://europa.eu/pol/justice/index_en.htm) [Public Health](http://europa.eu/pol/health/index_en.htm) [Regional Policy](http://europa.eu/pol/reg/index_en.htm) [Research and Innovation](http://europa.eu/pol/rd/index_en.htm) Space[Taxation](http://europa.eu/pol/tax/index_en.htm) [Transport](http://europa.eu/pol/trans/index_en.htm) |  |
| 13c If Yes, at which level? |
|  |  | ⭘ | Local / regional levels |
|  |  | ⭘ | National level |
|  |  | ⭘ | European level |
|  |  | 🟓 | International level |
| H Use and dissemination  |
| 14. How many Articles were published/accepted for publication in peer-reviewed journals?  | 0 |
| To how many of these is open access[[11]](#footnote-11) provided? |  |
|  How many of these are published in open access journals? |  |
|  How many of these are published in open repositories? |  |
| To how many of these is open access not provided? |  |
|  Please check all applicable reasons for not providing open access: |  |
|  ❑ publisher's licensing agreement would not permit publishing in a repository ❑ no suitable repository available ❑ no suitable open access journal available ❑ no funds available to publish in an open access journal ❑ lack of time and resources ❑ lack of information on open access ❑ other[[12]](#footnote-12): …………… |  |
| 15. How many new patent applications (‘priority filings’) have been made? *("Technologically unique": multiple applications for the same invention in different jurisdictions should be counted as just one application of grant).* | 0 |
| 16. Indicate how many of the following Intellectual Property Rights were applied for (give number in each box).  | Trademark | 0 |
| Registered design  | 0 |
| Other |  |
| **17. How many spin-off companies were created / are planned as a direct result of the project?**  | **0** |
| ***Indicate the approximate number of additional jobs in these companies:*** |  |
| **18. Please indicate whether your project has a potential impact on employment, in comparison with the situation before your project:**  |
|  | ❑ | Increase in employment, or | ❑ | In small & medium-sized enterprises |
|  | ❑ | Safeguard employment, or  | ❑ | In large companies |
|  | ❑ | Decrease in employment,  | ❑ | None of the above / not relevant to the project |
|  | ❑ | Difficult to estimate / not possible to quantify  |  |  |
| **19. For your project partnership please estimate the employment effect resulting directly from your participation in Full Time Equivalent (*FTE = one person working fulltime for a year*) jobs:**Difficult to estimate / not possible to quantify | *Indicate figure:*■ |
| I Media and Communication to the general public  |
| 20. As part of the project, were any of the beneficiaries professionals in communication or media relations? |
|  |  | ⭘ | Yes | 🟓 | No |
| 21. As part of the project, have any beneficiaries received professional media / communication training / advice to improve communication with the general public? |
|  |  | ⭘ | Yes | 🟓 | No |
| 22 Which of the following have been used to communicate information about your project to the general public, or have resulted from your project?  |
|  | ❑ | Press Release | ❑ | Coverage in specialist press |
|  | ❑ | Media briefing | ❑ | Coverage in general (non-specialist) press  |
|  | ❑ | TV coverage / report | ❑ | Coverage in national press  |
|  | ❑ | Radio coverage / report | ❑ | Coverage in international press |
|  | ❑ | Brochures /posters / flyers  | ❑ | Website for the general public / internet |
|  | ❑ | DVD /Film /Multimedia | ❑ | Event targeting general public (festival, conference, exhibition, science café) |
| 23 In which languages are the information products for the general public produced?  |
|  | ❑ | Language of the coordinator | ■ | English |
|  | ❑ | Other language(s) |  |  |

***Question F-10*:** Classification of Scientific Disciplines according to the Frascati Manual 2002 (Proposed Standard Practice for Surveys on Research and Experimental Development, OECD 2002):

**Fields of science and technology**

1. Natural Sciences

1.1 Mathematics and computer sciences [mathematics and other allied fields: computer sciences and other allied subjects (software development only; hardware development should be classified in the engineering fields)]

1.2 Physical sciences (astronomy and space sciences, physics and other allied subjects)

1.3 Chemical sciences (chemistry, other allied subjects)

1.4 Earth and related environmental sciences (geology, geophysics, mineralogy, physical geography and other geosciences, meteorology and other atmospheric sciences including climatic research, oceanography, vulcanology, palaeoecology, other allied sciences)

1.5 Biological sciences (biology, botany, bacteriology, microbiology, zoology, entomology, genetics, biochemistry, biophysics, other allied sciences, excluding clinical and veterinary sciences)

2 Engineering and technology

2.1 Civil engineering (architecture engineering, building science and engineering, construction engineering, municipal and structural engineering and other allied subjects)

2.2 Electrical engineering, electronics [electrical engineering, electronics, communication engineering and systems, computer engineering (hardware only) and other allied subjects]

2.3. Other engineering sciences (such as chemical, aeronautical and space, mechanical, metallurgical and materials engineering, and their specialised subdivisions; forest products; applied sciences such as geodesy, industrial chemistry, etc.; the science and technology of food production; specialised technologies of interdisciplinary fields, e.g. systems analysis, metallurgy, mining, textile technology and other applied subjects)

3. Medical Sciences

3.1 Basic medicine (anatomy, cytology, physiology, genetics, pharmacy, pharmacology, toxicology, immunology and immunohaematology, clinical chemistry, clinical microbiology, pathology)

3.2 Clinical medicine (anaesthesiology, paediatrics, obstetrics and gynaecology, internal medicine, surgery, dentistry, neurology, psychiatry, radiology, therapeutics, otorhinolaryngology, ophthalmology)

3.3 Health sciences (public health services, social medicine, hygiene, nursing, epidemiology)

4. Agricultural sciences

4.1 Agriculture, forestry, fisheries and allied sciences (agronomy, animal husbandry, fisheries, forestry, horticulture, other allied subjects)

4.2 Veterinary medicine

5. Social sciences

5.1 Psychology

5.2 Economics

5.3 Educational sciences (education and training and other allied subjects)

5.4 Other social sciences [anthropology (social and cultural) and ethnology, demography, geography (human, economic and social), town and country planning, management, law, linguistics, political sciences, sociology, organisation and methods, miscellaneous social sciences and interdisciplinary , methodological and historical S1T activities relating to subjects in this group. Physical anthropology, physical geography and psychophysiology should normally be classified with the natural sciences].

6. Humanities

6.1 History (history, prehistory and history, together with auxiliary historical disciplines such as archaeology, numismatics, palaeography, genealogy, etc.)

6.2 Languages and literature (ancient and modern)

6.3 Other humanities [philosophy (including the history of science and technology) arts, history of art, art criticism, painting, sculpture, musicology, dramatic art excluding artistic "research" of any kind, religion, theology, other fields and subjects pertaining to the humanities, methodological, historical and other S1T activities relating to the subjects in this group]

1. Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement. [↑](#footnote-ref-1)
2. A permanent identifier should be a persistent link to the published version full text if open access or abstract if article is pay per view) or to the final manuscript accepted for publication (link to article in repository). [↑](#footnote-ref-2)
3. Open Access is defined as free of charge access for anyone via Internet. Please answer "yes" if the open access to the publication is already established and also if the embargo period for open access is not yet over but you intend to establish open access afterwards. [↑](#footnote-ref-3)
4. A drop down list allows choosing the dissemination activity: publications, conferences, workshops, web, press releases, flyers, articles published in the popular press, videos, media briefings, presentations, exhibitions, thesis, interviews, films, TV clips, posters, Other. [↑](#footnote-ref-4)
5. A drop down list allows choosing the type of public: Scientific Community (higher education, Research), Industry, Civil Society, Policy makers, Medias ('multiple choices' is possible. [↑](#footnote-ref-5)
6. Note to be confused with the "EU CONFIDENTIAL" classification for some security research projects. [↑](#footnote-ref-6)
7. A drop down list allows choosing the type of IP rights: Patents, Trademarks, Registered designs, Utility models, Others. [↑](#footnote-ref-7)
8. 19 A drop down list allows choosing the type of foreground: General advancement of knowledge, Commercial exploitation of R&D results, Exploitation of R&D results via standards, exploitation of results through EU policies, exploitation of results through (social) innovation. [↑](#footnote-ref-8)
9. A drop down list allows choosing the type sector (NACE nomenclature) : <http://ec.europa.eu/competition/mergers/cases/index/nace_all.html> [↑](#footnote-ref-9)
10. Insert number from list below (Frascati Manual). [↑](#footnote-ref-10)
11. Open Access is defined as free of charge access for anyone via Internet. [↑](#footnote-ref-11)
12. For instance: classification for security project. [↑](#footnote-ref-12)