Identification of Mechanisms Correlating with Susceptibility for Avian Influenza

Reporting

Project Information

IMECS

Grant agreement ID: 201169

Project website

Project closed

Start date
1 April 2008

End date
31 December 2012

Funded under
Specific Programme "Cooperation": Health

Total cost
€ 3 668 819,60

EU contribution
€ 2 797 287,00

Coordinated by
RIJKSINSTITUUT VOOR VOLKSGEZONDHEID EN MILIEU
Netherlands

Final Report Summary - IMECS (Identification of Mechanisms Correlating with Susceptibility for Avian Influenza)

Executive Summary:

1. EXECUTIVE SUMMARY

The immune response against influenza is influenced by many variables, many of which have been unknown. A main distinction is made between immunity against the yearly, so-called seasonal influenza

and non-seasonal influenza, such as pandemic influenza strains or influenza strains of avian origin. Whereas the immune response against seasonal influenza renders a basic form of protection to almost all individuals, this is often absent for avian or pandemic strains. The partners in this study, called “Identification of Mechanisms Correlating with Susceptibility for Avian Influenza”, investigated what parts of the immune response against avian influenza strains were protective. The project was based on the assumption that people are generally protected against a specific influenza strain, after having survived an infection. Therefore, immune responses in several groups of individuals were characterized against avian and non-human influenza. In the course of the project from 2008 - 2012, pandemic broke out. Therefore, the scope was shifted to also include responses against pandemic (H1N1pdm) influenza.
We published extensive data on the humoral immune response against H1N1 pandemic influenza. The findings were reported in a series of papers by the group of prof. Julkunen (THL, Finland), showing that elderly individuals had protective humoral responses against H1N1pdm due to previous exposure to Spanish influenza and its immediate viral descendants, young adults (20-48 years) responded better to vaccination than older adults (49-64 years), and small but crucial changes in the influenza proteins induced a major reduction in antibody recognition of the virus. In Vietnam, extensive studies were done in individuals having occupational exposure to avian influenza. Significant progress was made in identifying H5N1 subclinical infection in a collaboration between the group of Dr. Vu Tan Trao (NIHE, VN) and prof. Lanzavacchia (IRB, CH). We revealed that seasonal influenza vaccination might induce neutralizing antibodies against other virus strains such as highly pathogenic avian and pandemic influenza (Corti et al, J. Clin. Invest, 2010)
Identification of cellular immune responses against highly pathogenic H5N1 influenza has been facilitated for flow cytometry applying patented technology (Methods for diagnosis of immune responses against viruses, WO2011/049448). Furthermore, we discovered that elderly individuals have an impaired dendritic cell function, leading to a lower Tcell immune response against influenza (Liu et al, Vaccine 2012).
Extensive data were generated to develop a completely synthetic broadly protecting influenza vaccine in a collaboration of Prof. Ossendorp (Leiden University, NL) and RIVM (NL). Expanding on earlier work, ISS and FAC (Rome, I) showed that chloroquine treatment may broaden and improve T cell responses after vaccination (in part published as Garulli, J Biomed Biotech 2011).
Overall, 19 scientific publications, 33 scientific presentations and one patent family have been generated so far. The findings are expected to have a major impact on influenza research and vaccine development.

Project Context and Objectives:
2. PROJECT CONTEXT AND OBJECTIVES

The project context
The IMECS initiative was introduced in 2008, since the H5N1 avian influenza vaccine trials, results of which had been published in international scientific journals had shown limited success in inducing a protective immune response as compared to the standard human influenza vaccine. This, despite large investments and multiple vaccine formulations tested. These results had made clear that the mechanisms of immunity to avian influenza were essentially different from those for human/seasonal influenza. The IMECS initiative aimed to elucidate these mechanisms and aimed to provide essential knowledge for

development of breakthrough pandemic vaccines as a result. The initiative comprised a research programme for the development of avian influenza-specific correlates of protection, the screening of vaccine candidates in vitro, the understanding of the origin of subclinical avian influenza (AI) infection in humans and the clinical screening of vaccine candidates in healthy adults and in different target groups.

The DG-Research project IMECS started in April 2008 to study the immune mechanisms that mediate protection against avian influenza. At that time, the likely origin for a pandemic influenza outbreak was avian influenza, including the H5N1 avian influenza strain as the most eminent exponent. Since the onset of the project, studies had been initiated to study the major aspects of influenza-specific immunity, including humoral, cellular and innate responses in the general population as well as in different focus groups. In addition, these responses had been investigated as part of vaccination trials, to determine their relevance for vaccine-induced protection.

In 2009, H1N1pdm pandemic influenza emerged that was not of avian but of swine origin. The outbreak of H1N1pdm pandemic influenza evoked a major reaction of health authorities, including the Commission, EMA and ECDC, but also influenced the IMECS project. Since the project was specifically put in place to monitor and investigate immune responses in humans to new (potentially) pandemic viruses, it was evident that IMECS was to study these responses against H1N1pdm. Inevitably, some of the studies in IMECS included the response to H1N1pdm, e.g. a study on the immune response to natural infection automatically focused on H1N1pdm after the pandemic started in 2009. We believed that IMECS was uniquely positioned to investigate immune responses to H1N1pdm, as it was specifically designed to study immune responses to pandemic influenza. Since the IMECS project started to generate an extensive clinical repository just before the pandemic outbreak, these cell and serum samples were of value as a control for immune responses against H1N1pdm. Consequently, the project was amended to study immune responses against H1N1pdm pandemic influenza, aiming to make the project more valuable as it enabled identification of protective mechanisms against pandemic influenza, which had been the main goal of the IMECS project. The focus of IMECS however remained on studying avian influenza virus infections in humans.

The project objectives
The overall project objectives as formulated in the application, before the amendment were:

To address the overall project objectives three research objectives were formulated:

• Determine the basis of the homologous and non-homologous mechanisms of protection
The objective is to determine critical mechanisms of protection from infection with avian or pandemic influenza in humans, by classifying the convalescent immune response. Since the immune response to a natural infection is often considered as the ‘golden standard’ the humoral and cellular immune responses of previously infected individuals will be analyzed directly ex vivo. An example is the recent identification of protective B-cells in PBMC of individuals who were infected with H5N1 in Vietnam. Based on homologous avian influenza specific responses in infected individuals, cross-specific responses to other avian influenza

viruses will be qualified. Within the group of H5N1 infected individuals (clade 1 infected from Vietnam; clade 2 infected from Turkey) cross clade specific responses will be determined. Heterologous avian influenza specific responses will be identified in H7N3 infected individuals from Italy. In addition, cross-specific responses to avian influenza will be qualified in individuals who had not been infected with avian influenza.
Special emphasis will be given to identifying sub-clinical infections with H5N1 in Vietnam. Based on an extensive local research programme in 9,000 people, these individuals will be identified applying internationally accepted technologies that were published by the IMECS partner ISS.
The humoral response will be qualified using standard serological techniques and advanced B-cell cloning techniques will be applied to identify homologous or heterologous protective antibody responses. Alternatively, if specific B cell clones cannot be generated, immune responses will be typed based on serology alone.
The cellular response will be qualified based on validated techniques resulting from the FLUSECURE project, including the determination of absolute and relative levels of cytokine productions in PBMC cultures (IFNγ/IL-10). In addition, multiplex cytokine assays will be used to characterize and compare inflammatory responses and Th1 and Th2 cytokine levels in PBMC culture supernatants. CTL cytotoxicity will be determined based on granzyme B detection assays.

• Determine the mechanisms of protection in specific target groups, including infants & the elderly
The objective is to determine how the influenza-specific immune response relates to the protective responses in the most vulnerable groups, such as infants, the elderly and immunocompromised hosts. To this end, several approaches will be followed applying to both the avian influenza-specific response and the human influenza-specific response:

Avian and H1N1 pandemic influenza-specific response:
In work package 2, the humoral immune responses against pandemic and avian influenza will be determined.
In work package 3, the cellular immune responses against seasonal, pandemic or H5N1 influenza will be typed, in specific target groups.
In work package 5, the innate response of H5N1-pulsed DCs in stimulating T-cells will be investigated in vitro.
In work package 6, the vaccination response in elderly people (n=50) will be investigated in H1N1pdm and H5N1 vaccination trials. In addition, a trial in children and immunocompromised hosts is planned.

Human influenza-specific response:
In work package 4, the humoral and cellular immune responses of infants will be investigated after their first influenza infection, applying techniques as developed in work package 2 and 3.
In work package 4, the humoral and cellular immune responses of elderly individuals will be investigated, applying techniques as developed in work package 2 and 3.
In work package 5, the innate response of human influenza-pulsed DCs (from infants, elderly and healthy adults) in stimulating T-cells will be investigated in vitro.

• Determine the avian and H1N1pdm influenza-specific response after vaccination in clinical trial studies

The objective is to qualify the protection that is induced by vaccination with avian and H1N1pdm influenza vaccines. To this end, an in vitro model will be designed, and human clinical trials will be executed.
In the human clinical trials, the safety, long term persistence of humoral and cell-mediated immunity in response to H5N1 vaccination will be studied until 2 years after vaccination.
The in vitro model will be designed to qualify the efficacy of DCs to stimulate T-cells after pulsing with influenza vaccine antigens. The outcomes of the model will be directly correlated to protection in vivo by the vaccine antigen in an animal model. In addition, the optimal adjuvant formulations will be screened and the optimal cross-presentation-inducing technology will be designed.

Project Results:
3. THE MAIN SCIENCE AND TECHNOLOGY RESULTS

3.1 Work package 2: "Mechanisms of humoral protection to avian and pandemic H1N1pdm influenza (H1N1pdm, H5N1, H7N3), ex vivo"
Introduction This work package focuses on the humoral response of clinically and sub-clinically infected individuals. The immune response to a natural infection is often considered as the ‘golden standard’ for protection. Research conducted in this work package will make use of this paradigm by investigating the homologous B-cell response of individuals who were infected with avian influenza or pandemic influenza. In addition, cross-specific immune responses will be characterized. The research data resulting from this work package may describe the homologous and heterologous protective antibody response to non-human influenza. The outcome may indicate which responses are homologous, heterologous or cross-protective, specifically focusing on the antibody response to the NA antigen.
A summary of progress and results For determining the humoral responses, several new technologies were established, including:
- A novel microneutralization method for analysing anti-influenza A virus antibodies as developed by THL (Lehtoranta, Villberg et al. 2009).
- A panel of ten human and avian H5 pseudoviruses spanning clades 0, 1, 2.1 2.2 and 2.3 was established by IRB for the assessment of virus neutralizing monoclonal antibodies and sera breadth of reactivity.
- A new ELISA using baculovirus-expressed proteins for H5 proteins of clade 1 and clade 2.1. The ELISA was adapted to be used as an inhibition ELISA for indicating whether individuals had been infected with H5N1. Several human clinical studies were executed including: • Clinical studies in Vietnam, including H5N1 avian influenza infected individuals as well as individuals potentially infected with H5N1 and a negative control group; • in Italy, H7N3 avian influenza infected individuals were included • in Finland, H5N1 vaccinated individuals were included. From all individuals blood samples were taken. • in Finland, H1N1pdm vaccinated individuals were included. From all individuals blood samples were taken. Humoral Responses Against Pandemic H1N1 Influenza THL collected serial serum samples from health care professionals who were vaccinated with pandemic monocomponent influenza vaccine (Pandemrix) followed by vaccination with seasonal trivalent influenza vaccine 1 year later. Humoral responses against the California/7/2009 H1N1pdm vaccine virus as well as against multiple circulating wild type viruses isolated in epidemic seasons 2009/2010 and 2010/2011 were analysed. The data indicated that the vaccines induced very good humoral immune responses, but there was some variation in immune responses against different viruses (Strengell, Ikonen et al. 2011). There was also more than 60%

reduction in geometric mean antibody levels one year after Pandemrix vaccination (Strengell, Ikonen et al. 2013). Based on the HA sequences of influenza A(H1N1)2009 viruses, we selected 13 different strains for antigenic characterization. The analysis included the vaccine virus, A/California/07/2009 and multiple California-like isolates from 2009-2010 and 2010-2011 epidemic seasons. These viruses had two to five amino acid changes in their HA1 molecule. The mutation(s) were located in antigenic sites Sa, Ca1, Ca2 and Cb region. Analysis of the antibody levels by hemagglutination inhibition test (HI) indicated that vaccinated individuals and people who had experienced a natural influenza A(H1N1)2009 virus infection showed good immune responses against the vaccine virus and most of the wild-type viruses. However, one to two amino acid changes in the antigenic site Sa dramatically affected the ability of antibodies to recognize these viruses. (Strengell, Ikonen et al. 2011) (Strengell et al., 2013). In contrast, the tested viruses were indistinguishable in regard to antibody recognition by the sera from elderly individuals who had been exposed to the Spanish influenza or its descendant viruses during the early 20(th) century. Cross-Specific Humoral Responses Against Neuraminidase Of Avian H5N1 Influenza RIVM applied a technology to determine neuraminidase inhibiting responses as described previously with several modifications. The NI assay was specifically modified to determine cross-specific NI responses against the neuraminidase of highly pathogenic A/Vietnam/1194/2004 (H5N1) avian influenza. We found that cross-specific NI responses against H5N1 were significantly induced after seasonal influenza vaccination, and that seasonal NI responses might contribute to protection against seasonal influenza infection (Zolyomi et al, manuscript in preparation). Humoral Responses After Infection With Avian Influenza. In Vietnam, individuals who were at risk for contacting H5N1 were asked to participate in a longitudinal study. In addition, vegetarian monks who were not in contact with poultry were asked to participate as a negative control group. Serum specimens were collected at three different time points. In total 202, 191 and 168 people participated, respectively. As a positive control, sera from H5N1 contacts in Vietnam were collected and were analysed for H5N1-specific responses. Ms. Mai Ha Thi Phuong (M.Sc.) from NIHE, Hanoi determined NI responses at RIVM, against seasonal H1N1, seasonal H3N2 and highly pathogenic avian H5N1. Significant differences in NI responses against N1 NA of A/Vietnam/2004 (clade 1) were found between individuals who had contacted infected poultry and negative controls. The analyses remains to be completed. At NIHE, HI has been used to evaluate the changes of humoral responses of H5N1 contacts at two time points to 5 locally isolated H5N1 viruses H5N1 clade 1 (03-04):A/ck/VN/16/03, A/ck/VN/31/04, H5N1 clade 2.3 (07-10): A/MD/VN/67/07 and A/MD/VN/4/10. Antibody respondes to any of the 4 tested H5 antigens among contacts by HI at two time points was 11.70% (18/154) and 10.40 % (14/135) respectively. The numbers of responders depended on the tested viruses. The positive responders were highest (4/154 and 11/135) in response to A/Ck/VN/6/03, then lowered to A/Ck/VN/31/04 (10/154 and 7/135), A/MD/VN/67/07 (18/154 and 14/135) and A/MD/VN/4/10 (5/154 and 6/135). Anti-H5 antibodies in poultry raisers, poultry sellers and slaughterers, and avian flu patients and their family and professional contacts were 6/84 and 4/78, 2/19 and 1/19, 3/4 for two time points, 4/9 and 2/8, 2/35 and 3/35 by HI and 3.66 (19/519), 6.15 (4/65) and 7.08 (8/113) respectively. - The highest titers of Abs to H5 (1/80) tested by HI to were found in two people: one young man (born in 1993) in a poultry raiser family and one H5N1 patients who had been treated for 80 days in 2005. Both had highest titers of Abs to the H5N1 strain, isolated locally in 2003 – A/Ck/VN/6/03. The follow up of the changes of responses to H5 antigens showed that the Abs could be found long after the first encounters to the viruses (from 1 year to 8 years) in some of the responders. - Anti-H5 antibodies were tested by HI using 4 H5N1 strains isolated from 2003 to 2010 in Thai Binh province showed that the responses to A/Ck/VN/6/03 were highest in titers of antibodies to H5 and were staying detected for the longest time (up 8 years). The boosting could be

specific and cross reactive, but after 2005 would be mainly cross reactive because this strain has been replaced by the clade 2 at the study sites. Human viruses had been received from RIVM. They will be cultured for determining HI and MN titers on the three sets of samples together with avian flu viruses for specific and cross protection on the H5N1 naturally infected population in Vietnam . At the same time, Western Blot also will be performed at NBI. Five samples will be sent to IRB for B epitope selections. To determine unique responses against H5N1, IRB analysed the sera from Vietnamese individuals who were at risk for having been exposed to avian influenza, applying a newly developed ELISA expressing: H5 HA from the A/Anhui/1/5 isolate (clade 2.3) A/Vietnam/2004 (clade 1) and A/Indonesia/2005. (clade 2.1). As a positive control for group 1 responses H9 of HK99 and for group 2 responses H7 of NE03 was used, respectively. In addition, H5 pseudoviruses of the VN04, IN05 and AN05 were produced and used to assess the neutralization by immune sera. Furthermore, an inhibition ELISA was developed and serum samples were identified from individuals containing antibodies able to compete with a stem-specific mAb (FE43) for binding to Anhui H5 HA. Applying 2 globular head specific (A/Indonesia/2005 specific) monoclonal antibodies (FV29 and FS9) in a competition test on AN05 HA with reactive sera for the presence of polyclonal competing antibodies, 11 out of 191 sera were able to compete with FS9 and FV29 for binding to H5 AN05 HA. Two of these sera were from individuals who survived an infection with H5N1. Furthermore, 9 out of 11 sera also showed a detectable virus neutralizing titer against H5 AN05 pseudovirus. These individuals -none of the negative controls scored positive - might have been indeed exposed to a sub-clinical infection with H5 viruses. The concomitant presence of heterosubtypic antibodies (FE43 competition) could be explained (similarly to what recently observed with H1N1pdm) by the boosting of crossreactive antibodies as a result of the exposure to a novel antigenically distant virus. Finding Humoral Cross-Reactive Responses Against H5N1 Individuals with a strong reactivity to both Group 1 and 2 were identified based on the analyses as described above. PBMC of these individuals may valuable as a source of memory B cells to isolate Group 1 and 2 reacting antibodies. For future perspectives, PBMC from selected donors will be used to interrogate their memory B cell repertoire for the isolation of H5 specific antibodies as a POC of H5N1 exposure and for the isolation of broadly neutralizing antibodies. The target of neutralizing antibodies that protect against influenza virus infection is the viral protein HA. Genetic and antigenic variation in HA has been used to classify influenza viruses into subtypes (H1–H16). The neutralizing antibody response to influenza virus is thought to be specific for a few antigenically related isolates within a given subtype. However, while heterosubtypic antibodies capable of neutralizing multiple influenza virus subtypes have been recently isolated from phage display libraries, it is not known whether such antibodies are produced in the course of an immune response to influenza virus infection or vaccine. We report that, following vaccination with seasonal influenza vaccine containing H1 and H3 influenza virus subtypes, some individuals produce antibodies that cross-react with H5 HA. By immortalizing IgG-expressing B cells from 4 individuals, we isolated 20 heterosubtypic mAbs that bound and neutralized viruses belonging to several HA subtypes (H1, H2, H5, H6, and H9), including the pandemic A/California/07/09 H1N1 isolate. The mAbs used different VH genes and carried a high frequency of somatic mutations. With the exception of a mAb that bound to the HA globular head, all heterosubtypic mAbs bound to acid-sensitive epitopes in the HA stem region. Four mAbs were evaluated in vivo and protected mice from challenge with influenza viruses representative of different subtypes. These findings reveal that seasonal influenza vaccination can induce polyclonal heterosubtypic neutralizing antibodies that cross-react with the swine-origin pandemic H1N1 influenza virus and with the highly pathogenic H5N1 virus (Corti, Suguitan et al. 2010). Deviations and corrective actions Further analyses of PBMC and humoral responses will be done, based on available sera and cells. Individuals

taking part in the studies in Vietnam will be asked to participate supply a last sample in Spring 2013.
Deliverables (brief description and month of delivery)
D2.1 Obtained approval of medical ethical commissions from Vietnam (6 M), Italy, and Finland, finished (others: 3 M)
D2.2 Serological data on Vietnamese (12 M), Italian, and Finnish donors/patients, finished. (others 6 M)
D2.3 Panel of H5 and/or H7 neutralizing monoclonal antibodies, finished (30 M)
D2.4 Serological data on half of the Vietnamese cohort (36 M; N = min 100)
D2.5 Data on neutralization of non homologous viruses (57 M)
D2.6 Epitope mapping of crossprotective antibodies (48 M)
D2.7 Serological data on the complete Vietnamese cohort (57 M N = min 200, max 400)
M2.1 Submission of clinical study protocol to independent ethical commission from Finland, Italy, Vietnam, finished (1 M) M2.2 Recruitment of 10 high responder vaccinated volunteers from Finland, finished (3 M) M2.3 Establishment of assays for identification of antibodies specific for the homologous virus (HI, microneutralization, ELISA using baculovirus-expressed proteins, finished. (6 M) M2.4 Successful recruitment of five H5N1 patients from Turkey, 10 Vietnamese exposed individuals with high H5 neutralizing titres, H7N3 immune donors from Italy, finished (9 M) M2.5 Establishment of assays for identification of neutralizing activity on heterologous viruses, finished. (12 M) M2.6 Establishment of methods for epitope mapping (36 M)

3.2 WorkPackage 3: "Mechanisms of cellular protection to pandemic H1N1pdm or avian influenza (H5N1, H7N3), ex vivo"
Introduction This work package focuses on the T cell response against pandemic of avian influenza. The importance of cytotoxic CD8+ T cells (CTLs) for recovery from pulmonary influenza infection has been well recognized. Humans experimentally infected with influenza were shown to rely on CTL for recovery, even in the absence of strain-specific antibodies. In contrast to the antibody response, the CTL response is specific for viral epitopes that are remarkably conserved between different influenza subtypes. In addition, the CD8 T-cell epitopes are less susceptible to antigenic drift than the B-cell epitopes . This work package focuses on four tasks: • Determining and identifying homologous T cell responses. • Determining and identifying cross-specific T cell responses. • Determining and qualifying cross-protective T cell responses. • Determining the cross protective immune induction by conventional inactivated vaccines. A summary of progress and results For determining cellular immune responses, new technologies were established and existing technologies were adapted for determining responses against avian or pandemic influenza, including: - A novel technique to determine T cell responses against whole influenza virus, using inactivated influenza virus (Jonges, Liu et al. 2010) - The identification of a panel of influenza-specific peptides for HLA-A201 and HLA-A24, containing previously unknown influenza sequences (FAC). In addition, a panel of T cell epitopes was determined by mass spectrometry. - The usage of mass spectrometry to identify T cell epitopes. - The application of Luminex and Elispot assays Cellular Responses Against Seasonal Influenza In Adults During the 2006/2007 influenza season, vaccinated volunteers were monitored for ILI and blood samples were drawn (before, 3 weeks after vaccination, and at the end of the influenza season). The samples were isolated as part of the Flusecure project, which was the predecessor of IMECS and funded by DG Sanco. A subset of samples (N=23) were selected for cellular immune responses based on high (N=12) or low (N=11) antibody titers in HI and NI analysis and infection status. PBMCs of the selected donors were stimulated with homologous H1N1 and H3N2 influenza strains of that season and cellular immune responses were measured by multiplex cytokine

analyses (LUMINEX) for IL-2, IL-4, IL-5, IL-10 and IFN gamma, and intracellular cytokine flowcytometry for IFN gamma and CD107a. We detected IL-2, IL-10 and IFN gamma after in vitro stimulation of the PBMCs. IL-2 correlated before and after vaccination with HI and NI responses, while Il-10 only correlated with responses after vaccination. If we looked into more detail, we observed a significant higher IL-2 response in individuals having a high humoral (HI) response versus those with a low humoral response. For IFN gamma, IL-10 and CD107a similar trends were observed. However these responses were also observed before vaccination. Therefore, we conclude that these responses were independent of vaccination and reflected a general immune status of the subjects (Manuscript in preparation). Cellular Responses Against Avian H5N1 Influenza (D3.2) PBMC from 52 individuals were taken who were exposed to H5N1 in Vietnam. NIHE has insisted that these samples were either analysed by their own personnel at RIVM or at the NIHE site applying the technology as described above. Getting NIHE personnel at RIVM was unsuccessful, thus analyses were to be done at NIHE. However, due to tightened restrictions on transport of influenza viruses and related materials across the Vietnamese border the samples have not yet been analysed. Cellular Immune Response Against Pandemic H1N1 In Elderly (D3.3) RIVM determined the cross-reactive HI antibody titers and cellular responses against pandemic influenza from 13 young healthy adults (20 – 40 years) and 19 healthy elderly (≥ 65 years), that were isolated before the outbreak of pandemic H1N1 influenza. We determined cytokine production and the cytotoxic response after stimulation with seasonal or pandemic influenza strains. In general, elderly individuals showed higher CD4+ and CD8+ T cell responses against both seasonal and pandemic influenza viruses. Also elderly individuals had significantly more cytotoxic T cells cross-reacting with pH1N1 compared to younger adults. Together, these data demonstrate that elderly individuals had increased numbers of cross-reactive memory T cells against both seasonal and pandemic influenza, and had a stronger cross-reactive cytotoxic T cell response against the pH1N1 virus compared to younger adults (Liu et al, manuscript in preparation). Identification Of T Cell Epitopes Of Seasonal And Pandemic Influenza (D3.2 and D3.3) Applying mass-spectrometry, RIVM did an extensive study to identify T cell epitopes for seasonal and avian influenza. HLA class A2 and B7 restricted epitopes were found for seasonal H1N1, seasonal H3N2 and pandemic H1N1. For HLA-A2 corresponding epitopes were found for all these three virus strains / subtypes having one or more amino acid changes. Cellular Responses Against Avian H7N3 Influenza (D3.4) Cellular immune responses using human cells. To determine the cross-protective immune induction by conventional inactivated vaccines in the WP3, CTL clones from PBMC of H7N3 infected individuals were supposed to be generated and used in the study. The poor viability and failure to generate Flu-specific reactive CTL clones from a small number of H7N3 subclinically infected individuals have obliged ISS and FAC to approach two alternative strategies to the original one described in the project to accomplish the goals and objectives of this study. The alternative strategy performed by FAC concerned a comparative analysis of memory CD8+ T specificities, revealed with peptides from PBMC of Flu-positive healthy donors, with the respective recall responses measured after stimulation with inactivated Flu virus-loaded DC. By this means, we were able to determine whether and to what extent whole virus-based candidate vaccines would be able to recall naturally acquired immunity against influenza. In particular, by using a panel of 28 peptides derived from the H7N3 virus (7 peptides from PB1 protein, 4 peptides from PB2 protein, 4 peptides from M protein, 5 peptides from NP protein, 8 peptides from PA protein), we found significant responses, in terms of IFN-g production, in PBMC of 6 out of 10 HLA-A2 positive healthy individuals. All of them were recognizing the M52 epitope (GILGFVFTL) and some of them were also reacting with peptides specific to the polymerase complex. So far, we cloned specific lines and obtained three specific CD8+ T cell clones against M52, M53 and PB1-244. By using

these clones, we set the system to determine the cross-presentation of inactivated influenza virus by dendritic cells. In the second part of the study of CD8+ immune response to flu epitopes, we started to study healthy individuals response with different class I molecules to define conserved epitopes for different alleles, so we analyzed more HLA haplotypes using another panel of 201 H7N3 derived peptides matching HLA A01, A03, A11, B07, B35 and B44. These peptides were derived from the H7N3 proteins: HA, NA, M, PB1,PB2, NP, PA, NS1. At the moment we are testing these peptides in PBMC of Italian healthy individuals in Elispot assay (data are in progress), in order to obtain specific CD8+ T cells clones. Until now, we tested 7 healthy individuals and found specific response in term of IFN-? production against the peptides of NP, NS1, NP and HA (on A3 haplotype). Cellular immune responses against avian H7N3 applying a transgenic mouse model. The alternative strategy performed by ISS concerned the use of Tg HLA-A2 mice and a panel of H7N3-specific peptides, available from FAC, to determine the CD8+ T cell responses inducible upon immunization of mice with inactivated H7N3 avian virus, and subsequently to evaluate the extent of cross-reactive CTL responses detectable between the avian strain and the PR8 virus upon viral infection. So far, the results showed that by this means, we could effectively determine the cross-reactivity between PR8-based inactivated whole virus and heterologous Flu viral strains, such as the H7N3 of avian origin. In particular, priming of Tg HLA-A2 mice with the inactivated H7N3 virus was eliciting CD8+ T cell responses specific to M52 epitope (GILGFVFTL) (HLA-A2-restricted) and to the NP366 epitope (Db-restricted). When boosting with PR8 virus, the frequencies of both the M52-and the NP366-specific CD8+ T cells were increasing. However, we could not detect any recall of NP-specific CD8+ T cells restricted to HLA-A2 molecules. Moreover, the frequencies of polymerases-specific CD8+ T cells in mice boosted with PR8 were slightly lower than those measured in unprimed mice, indicating that the T cell cross-reactivity in HLA-A2 mice is essentially related to the M52 epitope. We also determined the frequency of HLA-A2 restricted FLU-specific CD8+ T cells in HLA-A2 mice with pre-existing immunity to PR8 virus. A relevant recall response to inactivated H7N3 virus was obtained only for the M52-(GILGFVFTL) and the NP366-specific CD8+ T cells. Overall, these data suggest that cross-reactive T cell responses to avian viruses could be generated during immunization with whole PR8 virus-based vaccines but they would be restricted to just the conserved immunodominant epitopes (Garulli et al, manuscript in preparation).

Deliverables (brief description and month of delivery) D3.1 Obtained approval of medical ethical commissions from Vietnam, Italy (6M). This Deliverable was submitted with the 1st and 2nd project report D3.2 Homologous CTL responses for AI or pandemic influenza phenotyped and an indication for the correlates of protection determined (48 M) D3.3 Heterologous CTL responses for AI influenza or pandemic influenza phenotyped, and an indication of the level of cross-protection determined (57 M). D3.4 The level of CTL mediated protection by inactivated influenza vaccines determined (57 M). M3.1 Submission of clinical study protocol to independent ethical commission from Vietnam, Italy M3.2 Homologous CTL responses for AI phenotyped in AI infected individuals. Delivery date: 24M M3.3 Heterologous CTL responses for AI phenotyped. Delivery date: 48M This Milestone was submitted with the 3rd project report M3.4 Determination of level of CTL mediated protection by inactivated influenza Delivery date: 48M

3.3 Work Package 4: "Mechanisms of protection in specific target groups" Introduction The gold standard to evaluate influenza vaccine efficacy is antibody titers by the hemagglutination inhibition assay, but the standard is limited as a sole measure in older adults. Based on the technologies that are exploited within the IMECS programme, mechanisms and regulation of the humoral response, the cellular response and the interaction of DC’s and lymphocytes in vitro was determined in specific target groups including the elderly. Application of the technologies included the identification of the homologous and heterologous

humoral protective immune responses as well as cellular responses. In addition, the in vitro DC – lymphocyte model that was designed in WP5, was exploited in this WP4 to monitor the efficacy of DC to stimulate T cells using cells of specific target groups. Work package 4 identified of the mechanisms of immunity against (avian) influenza in different target groups. Differences in these mechanisms from the response in healthy adults were clarified. In addition, the mechanisms of the response after vaccination of the target groups was identified. Objectives To determine the immune response to infection with human (H1 and H3 viruses) and avian influenza virus in specific target groups, using the immunological mechanisms as determined in WP 2, 3 and 5. The focus groups included infants, elderly people, immunocompromised people, or pregnant women. A summary of progress and results Observational studies were executed in children and elderly individuals, who are primarily vulnerable to influenza infection. Analysis of immune responses included humoral, cellular and innate immunity. Altogether, several techniques were developed, including: • Characterization of pandemic virus strains. THL analysed and published the characterization of ca 140 2009 pandemic H1N1pdm viruses (Ikonen, Haanpaa et al. 2010). The data revealed that the viruses experienced a clear evolution and moderate level of amino acid changes appeared in different wild type virus strains. • THL developed and applied the use of a very sensitive quantitative RT-PCR assay for the detection of pandemic virus genetic material. (Ronkko, Ikonen et al. 2011). The assay can be used in clinical vaccination trials to analyse the protective efficacy of 2009 pandemic influenza A virus vaccines. • RIVM developed technology to determine CD4 and CD8 T cell responses using inactivated influenza virus (Jonges, Liu et al. 2010). • Characterization of human dendritic cell responses to infection with 2009 pandemic and certain seasonal influenza (H1N1 and H3N2) was carried out and published (Osterlund, Pirhonen et al. 2010). The data revealed that the pandemic virus replicated well in human DCs, but induced a poor antiviral interferon response. T cells of very young children were still not fully developed and therefore weaker than those of older children (5-9). Analysis of T cell responses showed that elderly individuals in general have better T cell immunity than adults (20-40 years), implicating that these mechanisms of immunity increase in strength with age and may partly compensate for reduced humoral immune responses in the elderly. Several human clinical studies were executed including: Avian Influenza-Specific Responses In The Elderly (D4.4) In a vaccination cohort that included approximately 80 health care professionals (as described for WP2) we observed that individuals older than 45-50 years of age showed a weaker humoral immune response after Pandemrix vaccination as compared to younger age groups (25-45 year olds; (Strengell, Ikonen et al. 2012; Strengell, Ikonen et al. 2013)). Another larger Pandemrix vaccination cohort (n=199) showed a significant age-related humoral immune responses with the age group of 18-29 year-olds having the highest vaccine induced antibody levels. These individuals showed 5-fold higher antibody levels as compared to the elderly (<60 year-olds). Overall age groups showed a directly linear decrease in geometric mean antibody titers with age (Strengell et al., unpublished). Since May 2009, the pandemic influenza A(H1N1) virus has been spreading throughout the world. Epidemiological data indicate that the elderly are underrepresented among the ill individuals. Approximately 1,000 serum specimens collected in Finland in 2004 and 2005 from individuals born between 1909 and 2005, were analysed by haemagglutination-inhibition test for the presence of antibodies against the 2009 pandemic influenza A(H1N1) and recently circulating seasonal influenza A viruses. Ninety-six per cent of individuals born between 1909 and 1919 had antibodies against the 2009 pandemic influenza virus, while in age groups born between 1920 and 1944, the prevalence varied from 77% to 14%. Most individuals born after 1944 lacked antibodies to the pandemic virus. In sequence comparisons the haemagglutinin (HA) gene of the 2009 pandemic influenza A(H1N1) virus was closely related to that of the Spanish influenza and 1976 swine influenza viruses. Based on the three-dimensional

structure of the HA molecule, the antigenic epitopes of the pandemic virus HA are more closely related to those of the Spanish influenza HA than to those of recent seasonal influenza A(H1N1) viruses. Among the elderly, cross-reactive antibodies against the 2009 pandemic influenza virus, which likely originate from infections caused by the Spanish influenza virus and its immediate descendants, may provide protective immunity against the present pandemic virus. Cross-Specific Responses Against Pandemic Influenza In The Elderly RIVM determined the cross-reactive HI antibody titers and cellular responses against pandemic influenza from 13 young healthy adults (20 – 40 years) and 19 healthy elderly (≥ 65 years), that were taken before the outbreak of pandemic H1N1 influenza. We determined cytokine production and the cytotoxic response after stimulation with seasonal or pandemic influenza strains. In general, elderly individuals showed higher CD4+ and CD8+ T cell responses against both seasonal and pandemic influenza viruses. Also elderly individuals had significantly more cytotoxic T cells cross-reacting with pH1N1 compared to younger adults. Together, these data demonstrate that elderly individuals had increased numbers of cross-reactive memory T cells against both seasonal and pandemic influenza, and had a stronger cross-reactive cytotoxic T cell response against the pH1N1 virus compared to younger adults (Liu et al, manuscript in preparation). Influenza-Specific Responses In Children (D4.3 D4.5) A clinical trial was performed from 2008 until 2010 (3 seasons) in a cohort of children who had been hospitalized for influenza infection (cases) or for minor surgery (controls). Serum and PBMCs were collected from all age groups. In the age group 0-4 years old, 13 children with influenza infection were included and 20 without influenza infection. In the age group 5-9 years of age, 6 influenza infected children and 17 controls were included. In addition, PBMCs were obtained from 27 healthy adults. The samples of infected individuals were taken the 2 years before and during the pandemic H1N1 outbreak. All negative control samples were taken before the fall of 2009/2010. In 2 out of 30 control children, we observed a titer for pandemic H1N1, while in the infected individuals 4 out of 6 individuals had antibodies against pandemic H1N1. For seasonal H3N2 we observed in most subjects titers to the recent H3N2 strain (A/Brisbane/10/2007) and in a subset of the individuals to the older strain (A/Wisconsin/67/2005). In addition, we did not observe age-related differences in the levels of antibodies, but differences could be explained by exposure due to date of birth to the specific influenza subtypes. Next, we looked into the cellular immune response against pandemic H1N1 and the seasonal H3N2 (A/Brisbane/10/2007). We developed an assay combining IFN gamma ELISPOT followed by LUMINEX to analyse cytokine responses after 18 hours of stimulation with live virus. In addition, Granzyme B responses were also analyzed by a similar ELISPOT. We observed no significant differences for the influenza strains used in the assays or for the infection status of the children. We did observe significant higher immune responses in the older children as indicated by IFN gamma, IL2 and IL5 responses. In contrast, the IL-10 induction showed a trend to be higher in the young children. These results indicate an effect of a higher immune response in general in older children and does not indicate an influenza specific respons. A manuscript of these results is in preparation. Antibody Responses Against Pandemic Influenza (D4.6): Influenza A(H1N1)pdm09 virus has been circulating in human population for three epidemic seasons. During this time, monovalent pandemic and trivalent seasonal influenza vaccination against this virus have been offered to Finnish healthcare professionals. It was, however, unclear how well vaccine-induced antibodies recognize different strains of influenza A(H1N1)pdm09 circulating in the population and whether the booster vaccination with seasonal influenza vaccine would broaden the antibody cross-reactivity. Influenza vaccine-induced humoral immunity against several isolates of influenza A(H1N1)pdm09 virus was analyzed in healthcare professionals. Age-dependent responses were also analyzed. Influenza viruses were selected to represent viruses that circulated in Finland during two consecutive influenza epidemic

seasons 2009-2010 and 2010-2011. Serum samples from vaccinated volunteers, age 20-64 years, were collected before and after vaccination with AS03-adjuvanted pandemic and non-adjuvanted trivalent seasonal influenza vaccine that was given 1 year later. Single dose of pandemic vaccine induced a good albeit variable antibody response. On day 21 after vaccination, depending on the virus strain, 14-75% of vaccinated had reached antibody titers (≥1:40) considered seroprotective. The booster vaccination 1 year later with a seasonal vaccine elevated the seroprotection rate to 57-98%. After primary immunization, younger individuals (20-48 years) had significantly higher antibody titers against all tested viruses than older persons (49-64 years) but this difference disappeared after the seasonal booster vaccination. In conclusion, even a few amino acid changes in influenza A HA may compromise the vaccine-induced antibody recognition. Older adults (49 years and older) may benefit more from repeated influenza vaccinations. Deviations and corrective actions Adjuvanted H5N1 vaccine was not given to children, since it was considered unethical to give such novel vaccines to children, as these vaccines had not been used in children before and there were serious adverse events reported for the adjuvanted avian influenza vaccine in children (pandemrix). Deliverables (brief description and month of delivery) D4.1 Obtained approval of medical ethical commission of Finland and the Netherlands or studies in children (6 M). D4.2 Obtained approval of medical ethical commission of Finland and the Netherlands for studies in elderly (9 M). D4.3. Analysis-report of humoral and cell-mediated immune responses in children suffering from an infection with a seasonal influenza A virus strain (48 M, report) D4.4. Analysis of the activation of humoral and cell-mediated immune responses against seasonal and pandemic influenza A virus infection in the elderly. (48 M, report). D4.5. Analysis-report of heterologous immunity against relevant heterologous influenza A virus types in children who had undergone a primary influenza A virus infection (57 M, report)). D4.6. Analysis of humoral immune responses against heterologous viruses (H1, H3, H5) with special emphasis on the ability of antibodies of the elderly to neutralize avian influenza viruses. (57 M, report). M4.1 Submission of clinical study protocol to independent ethical commission of Finland and the Netherlands for studies in children M4.2 Submission of clinical study protocol to independent ethical commission of Finland and the Netherlands for studies in elderly M4.3 Study on influenza infection in children executed M4.4 Nursing home study in elderly executed M4.5 DC functional assays performed 3.4

Work Package 5: "Mechanisms of innate protection to AI, development of an in vitro model" Introduction The immune response to influenza infection and vaccination is established through close contact between the innate and the specific immune system. These contacts between major exponents of the innate response, e.g. dendritic cells, or alveolar macrophages/monocytes, and T cells may steer the immune response significantly. The contacts are therefore essential steps in the development of protective immunity after vaccination, and may play a role in steering an adequate immune response after natural infection. In WP5, in vivo and in vitro systems were developed based on contacts between innate immune cells and specific immune cells, mimicking an essential stage in the immune response to influenza. This in vitro system was be subjected to diverse conditions representing essential immunologic responses in vivo, e.g. infection of cells with live influenza virus or pulsing of the in vitro system with influenza vaccine antigens, with or without adjuvants. Our aim is to steer and control the immune response to adequately raise CD8 T cell immunity against well-defined influenza antigens. The Work Package consists of: • Design of in vivo and in vitro systems on a murine and a human background. The development of the in vitro systems resulted in a model that was efficient in using DCs pulsed with flu antigens to induce CD8 T cell responses in vitro. • Application of the in vitro system to determine the protective response for adjuvanted

influenza vaccines in a mouse model In this workpackage, the dendritic cell (DC) as a major exponent of the innate immune system is targeted, first to determine T cell specific responses and second to induce T cell responses in a highly sophisticated endeavour to develop a synthetic influenza vaccine. As part of this vaccine development program, the vaccine was designed to be broadly active against multiple influenza strains and to be specific for individuals having different HLA haplotypes. Furthermore, the vaccine was optimized for inducing DC maturation. Initial vaccination experiments in mice showed significant T cell responses induced by the vaccine. • Application of the in vitro and the in vivo systems to determine cross-presentation of influenza immunogens to CD8+ T lymphocytes and improvements by drugs. The research conducted in this work package is expected to determine the ability of chloroquine and other candidate drugs on cross-presentation of whole inactivated influenza virus by human and murine DC ex vivo to elicit virus-specific CD8+ T cell responses against conserved internal proteins. In addition it evaluates the efficacy of chloroquine and other potential candidate drugs to elicit primary CD8+ T cell responses in mice when administrated with whole inactivated flu viruses. In a second task within WP5, DCs were employed to determine Tc responses in vivo, showing the use of the drug chloroquine to enhance T cell immunity against influenza. Objectives To develop an in vitro model for the human immune system that mimics the response to vaccination in vivo, and allows the pre-clinical assessment of a candidate vaccine. A summary of progress and results Design Of In Vitro / In Vitro Systems And Usage Thereof To Develop A Synthetic Influenza Vaccine. In vivo assessment of the efficacy of peptide-based vaccines against Influenza A was carried out in C57BL/6 mice. The mice were immunized with long peptides encoding the murine Flu CTL and T helper epitopes combined with the TLR2 ligand PAM3CysSK4 s.c. Our results showed that the combination with this TLR2ligand results in significant CD4 and CD8 T cell responses. This optimal vaccination protocol, which induced functionally active CD8 T cells that were able to kill Flu antigen loaded target cells in vivo, was used for in vivo virus challenge experiments. Sublethal HKx31 virus doses were optimized in a collaborative effort between LUMC and RIVM. This dose 10e5 PFU was used for challenging mice which were vaccinated with synthetic peptide/TLR2 ligand vaccines. Three different peptide-based vaccine formulations were selected for in vivo viral protection analysis. First, a vaccine formulation based on two long peptides carrying the CTL and T helper epitopes from the NP and HA viral proteins respectively, as described before, which could optimally prime CTL and T helper cells in the C57BL/6 mouse strain. Secondly, a peptide-based vaccine was developed which included two conserved B cell epitopes which were directly coupled to the HA T helper sequence. These B cell epitope containing peptides were able to induce strong specific antibody responses in C57BL/6 mice after prime-boost vaccination sc. This peptide vaccine was also combined with first T cell based peptide vaccine. Thirdly, an overlapping long peptide vaccine was designed based on conserved areas in the M1, NP and PB1 viral proteins and the presence of known (previously described) cytotoxic and T helper epitopes in mice and mostly in man. This cocktail of 25 long peptides will be mixed and adjuvanted with Incomplete Freunds Adjuvant. If effective this vaccine formulation from conserved areas of the viral genome would be of great advantage to raise stable immunity against Flu in outbred populations. LUMC in collaboration with RIVM performed a proof of concept study involving C57BL/6 mice, which were vaccinated with the three different peptide-based vaccines and challenged with the HKx31Flu virus. Mice were analyzed for body weight, virus titers, lung histology and T cell immune responses. One week after challenge maximal weight loss was observed in all vaccinated groups after which recovery took place. No significant differences in weight loss and recovery were observed. However, in the vaccinated groups in which the conserved B cell epitopes were included significant decrease in virus titers in the lung (more than 10 fold lower titers) was detectable. Also the vaccine formulation based on overlapping long peptides of conserved areas in the

M1, NP and PB1 viral proteins showed a similar decrease in virus titers. The anti-virus T cell responses related to the vaccine formulations correlated with the observed decrease in lung viral titers, although a causal relationship needs to be proven. In a new project, these promising peptide-based vaccines are now combined and investigated in the BALB/c mouse strain. Analysis of these mice revealed similar results as found in the C57BL/6 mice showing partial reduction of lung viral titers but no significant differences in body weight reduction. Future evaluations of these promising vaccines will be performed in the ferret model outside this project. Cross-Presentation Of Influenza Immunogens To CD8+ T Lymphocytes And Improvement Of Dendritic Cells (DC) Crosspresentation Studies in human cells ex vivo Setting up of efficient antigen-presentation assay addressed to identify novel molecules or drugs improving cross-presentation. CTL clones from PBMC of H7N3 infected individuals were supposed to be generated and used also in this study. Although our attemps to get them failed, we cloned specific lines and obtained different T cells clones from PBMC of Flu- and HLA-A201-positive healthy individuals exposed to seasonal flu. • Development of different tools for analysis of cellular immunity, including FACS, ELISPOT and Luminex.In particular, we have obtained and characterized three influenza virus specific CD8+ T cell clones: two clones specific for M gene (M52 and M53 peptides) and one clone specific for PB2 gene (PB1-244 peptide). These CTL clones obtained will be used to set up an efficient antigen-presentation assay to test novel drugs and molecules improving cross-presentation, according to the objectives of this WP. In particular, we have performed the first crosspresentation experiment using citofluorimetric analysis detection of intracellular IFN-□ production of CD8+ specific M52 T cell clone and i-DCs, pulsed with inactivated influenza virus or peptide in the presence or absence of inhibitor of endosomal proteases (chloroquine). Preliminary results showed a low crosspresentation of inactivated virus, while a relevant crosspresentation of M52 peptide was shown in the presence of chloroquine. The crosspresentation experiment with the other M59 and PB244 CD8+ specific clones, using also Dc pulsed with inactivated virus and others inhibitors of endosome movement, including nocodazole (which induces microtubule depolymerization),wortmannin (which inhibits early endosomes), Baf-A1 (which blocks early endosomes from fusing with late endosomes, and U18666A (which arrests late endosome movement), did not show critical differences. At the moment, we are studying the response in healthy individuals with different class I molecules to define conserved epitopes for different alleles. This study is required to verify if the improvement of response to HLA-A2 restricted epitopes, using DC cross-presented inactivated H7N3 in the presence of chloroquine, is observed for other epitopes binding different alleles too. We'll try to obtain, in healthy individuals, another specific CD8+ T cells clones using HLA A01, A03, A11, B07, B35 and B44 binding peptide, in order to perform crosspresentation experiment using chloroquine. Studies in mice. We set up a system in vitro to assess the effect of chloroquine treatment on the capacity of splenic DCs to process and present inactivated Flu virus by using proliferative and IFN-γ ELISPOT assays. In particular, the recombinant Flu virus bearing both the CD8+ T cell epitope (OVA257-264) and the CD4+ T cell epitope (OVA323-339) of ovalbumin, named WSN-OVAI/II, was generated and used for an vitro proliferation assay of OVA-specific transgenic OT-I and OT-II cells, upon heat-inactivation. Our results show that chloroquine was improving cross-presentation of OVA257-264 epitope to naïve OT-I cells at the late time (day 4) by reducing antigen degradation and thus prolonging antigen processing and presentation to MHC class I molecules. A similar increase in antigen presentation was also revealed by using the ELISPOT assay as read-out. However, the presence of low concentration of chloroquine throughout the in vitro assay was reducing the proliferation of naïve OVA-specific transgenic OT-II cells. Following the in vitro studies, we then determined the effect of chloroquine during a single immunization of mice with inactivated X31 virus. Similarly to the OVA257-264 epitope exposed on the mature HA

described above, our results show that a short course chloroquine treatment was improving the primary Flu-specific CD8+ T cell response specific to the internal NP366-374 epitope, as determined by either an ex-vivo ELISPOT assay or a CTL assay upon Flu-specific restimulation of lymphocytes in vitro. Importantly, our recent data show that chloroquine treatment was also improving the primary Flu-specific CD4+ T cell responses in vivo, as determined by proliferation assays performed with inactivated Flu virus in vitro. Moreover, the normal serum antibody levels measured in chloroquine treated mice provide further evidence that CD4+ T cells were functional in sustaining efficient adaptive immune responses. Thus, the results suggest that a short-course treatment of mice with chloroquine during a single immunization with inactivated influenza virus was improving the virus-specific T cell responses. To corroborate further our data, we compared the extent of the antigen-specific CD8+ T cell recall responses of both chloroquine-treated and untreated immunized mice upon intranasal challenge with the antigenically distinct A/PR8 virus. As expected, the spleens and lymph nodes draining the respiratory tract showed higher numbers of CD8+ T effectors in mice immunized in the presence of chloroquine than in those immunized without treatment. We finally examined whether the chloroquine treatment was also improving the recovery of mice from challenge with 2MLD50 of the heterologous PR8 virus. We found that in three out of four experiments, two out of seven (28.5%) of the vaccinated mice in the presence of chloroquine lost weight until day 8 and then started to recover and survived the lethal challenge, whereas all mice vaccinated in the absence of choloroquine died by day 8. Moreover, lower lung viral titers were consistently measured in vaccinated mice in the presence of chloroquine at day 6 p.i. as compared to the unprimed or the untreated-vaccinated mice. Overall, concurrent immunization of mice with a single doses of HI-X31 virus and chloroquine treatment was supporting higher CD8+ T cell expansion of primary effectors, that were rapidly recruited and able to reduce viral load following pulmonary challenge with PR8 virus. This deliverable concerning the evaluation of chloroquine in improving cross-priming in mice has been finalized. Further studies are needed to evaluate the efficacy of other drugs. Deliverables (brief description and month of delivery) D5.1 Obtained approval for study in humans from medical ethical commission in Italy (mo 6). D5.2 Optimal in vitro DC maturation and CD8 T cell activation protocol (mo 18) D5.3 Isolation and expansion of flu antigen-specific CD8 T cell clones by stimulation of PBMC of immune subjects (mo 24) D5.4 Vaccination protocol in HLA transgenic mice (mo 30) D5.5 Optimal adjuvanted vaccination protocol for CD8 priming and virus protection in mice (mo 36) D5.6 Evaluation of chloroquine and other drugs in improving cross-priming in mice (mo 42) D5.7 Setting-up of efficient antigen-presentation assay addressed to identify novel molecules or drugs improving cross-presentation (mo 48) M5.1 Presentation of optimal TLR ligands for CD8 T cell activation in vitro. Delivery date: 24 M M5.2 Presentation of optimal vaccine formulation for CD8 T cell activation and virus protection in vivo. Delivery date:48M M5.3 Presentation of epitope repertoire generated during direct presentation or Delivery date: 48 M M5.4 Presentation of novel molecules or drugs improving cross-presentation to CD8 T cells. Delivery date: 48 M
3.5

Work Package 6: "Avian Influenza vaccine trials, in humans" Introduction Based on the findings in work package 2, 3, 4 and 5, the immune responses after vaccination was investigated in humans and qualified in terms of protection to homologous challenge and heterologous challenge. This was extended to different target groups. In addition, the vaccine industry was invited to participate. Objectives - To determine homologous and heterologous protection after H5N1 and H1N1pdm09 vaccination of human beings, and the contribution of the humoral and cellular immune response thereto. - To carry out an initial avian (H5N1), pandemic and seasonal influenza A virus vaccination trials with voluntary healthy adults. The

inclusion of pandemic (Pandemrix) and seasonal vaccines was included along with the pandemic and thus the goals are equally important for avian and human seasonal vaccines and immune responses. - To obtain information on the safe and long term persistence of humoral and cell-mediated immunity in response to H5N1 avian, pandemic and seasonal influenza A virus vaccine - To carry out a clinical avian influenza A virus vaccination trial on a specific population group, the elderly A summary of progress and results Within WP6 several essential clinical trials were executed using avian, pandemic and seasonal influenza vaccines, both in adults and elderly individuals. The preliminary analysis of the data added to the understanding of immunity induced by avian influenza vaccines and for future vaccine development. Specifically, it was found that H5N1 vaccination using a standard adjuvanted (AS03; GSK vaccine) vaccine induced an immune response that was specific for the homologous strain and closely related strains, but that was relatively low cross-specific with other H5N1 clades. In addition, it was found that the responses declined considerably over a 6 month period. After the initial vaccination the same vaccination cohort received a nonadjuvanted whole inactivated H5N1 vaccine (see below) and the cohort has now been followed for nearly five years. A vaccination trial with avian H5N1 vaccine in human and animal healthcare professionals was successfully conducted. The first part of the trial included ca. 70 individuals who received two doses of GSK Indonesia/5/2005 H5N1 adjuvanted vaccine. Serum samples were collected at day 0, 21, 42 and 6, 12 and 27-29 months after vaccination. Humoral immune responses were analysed by HI test against five different clade H5N1 vaccine virus strains. In the second part of the trial mostly the same individuals (n=55) as well as a group previously H5N1 virus-unvaccinated individuals (n=15) received Baxter Vietnam/1203/2004 H5N1 nonadjuvanted whole virus vaccine. Serial serum and cellular samples were taken at days 0, 21 and 42 days and 6 and 12 months after the vaccination. Serum specimens are ready to be analysed for humoral immune responses against multiple H5N1 vaccine virus strains. Presently the list of different clade H5N1 vaccine viruses has grown to nine members and thus entensive work is expected. The analyses are to be carried out especially by the neutralization test, since HI test is often poorly suited for different clade avian viruses. Neutralization tests for analyzing neutralizing antibody responses and cross-protective responses against different clade H5N1 vaccine viruses are to be started in September 2013 and the analyses are expected to be completed by spring 2014. The methodology has now been set-up in the laboratory and adopted for the analysis against H1N1pdm09 viruses. This enables us to carry out a very extensive analysis of H5N1 vaccine induced cross-reactive responses with the developed microneutralization test. The methodology and reagents for analysing cell-mediated immunity is still under development at RIVM and THL. Due to the appearance of the 2009 influenza pandemic a lot of research efforts were directed to this novel pathogen and immunity induced by natural infection and vaccination. THL successfully analysed and published the characterization of ca 140 2009 pandemic H1N1pdm viruses (Ikonen, Haanpaa et al. 2010). The data revealed that the viruses underwent a clear evolution and moderate level of amino acid changes appeared in different wild type virus strains. Some of the changes appeared at key antigenic epitopes of the virus HA and NA proteins affecting the ability of serum specimens collected from H1N1pdm09 virus vaccinated individuals or those undergone a natural H1N1pdm09 virus infection to recognize drifted viruses (Strengell, Ikonen et al. 2011; Strengell, Ikonen et al. 2013); Strengell et al., manuscript in preparation). These vaccination cohorts also included elderly people aged 60 to 75 years of age. Interestingly, the elderly showed almost five-fold lower geometric mean antibody titers after Pandemrix vaccination as compared to the youngest group (18-29 year-olds) (Strengell et al., manuscript in preparation). In addition, the protective efficiency of Pandemrix and seasonal vaccines against H1N1pdm09 virus infection was carried out. The data showed very good protection of Pandemrix vaccination even against the next season H1N1pdm09 infection (with vaccine

efficiency, VE of ca. 80%) and practically 100% protection in individuals that had been vaccinated both with Pandemrix (previous season) and the seasonal vaccine (Syrjänen et al., submitted). Deviations and corrective actions Initial analysis of the H5N1 vaccination trials was done with the HI assay. The results could not be interpreted completely and unambiguously. Therefore, a micro neutralization (MN) assay was adapted to analyze the data. The final D6.5 report depend on the analysis of the MN data. Deliverables (brief description and month of delivery) D6.1 Obtained approval for study in healthy adult volunteers (4 mo) finished This Deliverable was submitted with the 1st and 2nd project report D6.2 Initial analysis of humoral and cell-mediated immune response against H5N1 vaccine (6 mo, report) D6.3 Obtained approval for vaccine study in elderly (12 mo) finished D6.4 Successful completion of a clinical H5N1 vaccination trial in healthy voluntary adults has been successfully completed (48 mo). D6.5 Analysis of the H5N1-specific neutralization activity and persistence of humoral immunity in response to H5N1 vaccination is expected to be fully completed by spring 2014. () D6.6 Successful completion of a clinical H1N1pdm09 vaccination trial in the elderly was successfully completed (48 mo, report under preparation) D6.7 Specificity and persistence of cell-mediated immunity against H5N1 antigens in response to vaccination is still under technological development due to initially observed weak cell-mediated influenza-specific immune responses. The data is expected to be completed by fall 2014 (72 mo) D6.8 Specificity and persistence H1N1-specific humoral immune responses in adults and the elderly were successfully analysed and data is being prepared for publication. Influenza-specific cell-mediated immune responses were initially observed to be weak or missing and thus it is unclear whether relevant data is expected. M6.1 Submission of clinical study protocol to independent ethical commission for study in healthy adult volunteers Delivery date: 18 M (finished) M6.2 Submission of clinical study protocol to independent ethical commission for vaccine study in elderly Delivery date: 18 M (finished) M6.4 Characterization and persistence of vaccine-induced immunity in adults Delivery date: 30 M (finished)

3.6 The Ethical Issue Report

Explanation and justification of the research design
The aim of IMECS was to identify the mechanisms of homologous and non-homologous protection from avian influenza that will the enable the development of an effective vaccination strategy to protect the people of the European Union in response to a pandemic influenza outbreak". Since the immune response for avian influenza (AI) in humans is unique, studies were performed including humans and investigating human clinical material ex vivo. A thorough analysis was done whether a clinical study was necessary and ethically justified based on the most recent data, leading to the cancellation of the study if a clinical study in humans was found not justifiable. In such case, the EC was informed of this cancellation as well as stating the reasons for the decision. Ethical considerations were based on the application of regulations and international codes as stated below (vide infra). In two cases, this decision process led to the cancellation of clinical studies (vide infra).
For pre-clinical studies necessitating transgenic animals, it was determined whether the study was needed in the light of the most recent scientific data. Such pre-clinical study was carefully designed taking into account the relevant international regulations and including the principles of 3Rs, i.e. 'replacement, reduction and refinement' (vide infra).

The research projects
Human specimens were used for studies directly ex vivo, for a concise number of research projects:

 The quantification of the extent of subclinical infections with avian influenza in people who were professionally exposed to the virus.
 The homologous immune response specific for avian influenza.
 The non-homologous immune response specific for avian influenza.
 The influenza-specific responses in certain human target groups, including children and the elderly.
 Using human white blood cells, an in vitro system was designed to identify basic mechanisms at a cellular and sub-cellular level to determine the AI vaccine efficacy. In addition, the mechanisms that lead to exaggerated immune response and fatal infection with avian influenza H5N1 was investigated.
 The immune responses were determined in humans who were participating in clinical trials for the evaluation of AI vaccines to qualify these.

In addition to the research programme in humans, a limited number of animal experiments were be performed in mice that were transgenic for human HLA molecules.

Adherence to national regulations and international codes of conduct for clinical studies
In the design and execution of clinical studies, the consortium fulfilled the legal requirements of each state, the relevant EU legislation and international legislation, including the Helsinki declaration in its latest version.

As part of the IMECS project management, the submission of an application for a clinical study was monitored and generally taken up as a milestone in the project. The studies were submitted to the national authority research ethics committee and the studies were only started after approval had been received from the research ethics committee. The actual permission of the ethical committee was a deliverable of the IMECS project and subsequently submitted to the EC as a deliverable of the project, enabling a careful guidance and monitoring on the ethical compliance by the IMECS management and EC. The various deliverables with respect to the clinical studies were as indicated below:

Deliverable Approval Document (Organization), country of execution Ethical permission (purpose)
2.1 Institutional Review Board NIHE, Hanoi,Vietnam Blood collection from healthy adults for ex vivo analysis, from individuals working with poultry
2.1 Ethics Committee of ISS, Rome, Italy Blood collection from healthy adults for ex vivo analysis
2.1 - Helsinki-UUsima Health District

- the National Agency for Medicines, Finland - Study vaccine induced cellular and humoral responses in health professionals
- Permission to use H5N1 vaccine for laboratory personnel for health protection purposes
3.1 Institutional Review Board NIHE, Hanoi,Vietnam Blood collection from healthy adults for ex vivo analysis, from individuals working with poultry
3.1 Ethics Committee of ISS, Rome, Italy Blood collection from healthy adults for ex vivo analysis
4.1 - the South-Western Finland Health District Ethical Review Committee, Finland
- Helsinki-Uusimaa Health District, Finland - studies of respiratory infections in children

- seroepidemiological analysis of sera from children
4.1 Medical Ethical Committee, University Medical Center Utrecht, the Netherlands Ethical permission for study of T cell responses in children after seasonal influenza infection

4.2 - Helsinki-UUsima Health District, Finland

- Helsinki-Uusimaa Health District, Finland
- Pirkanmaa HealthD istrict

- Helsinki-Uusimaa Health District, Finland - Study vaccine induced cellular and humoral responses in health professionals
- seroepidemiological analysis of sera from children

- clinical influenza vaccination study

- permission to study all vaccine preventable diseases
4.2 Medical Ethical Committee, University Medical Center Utrecht, the Netherlands
- Elderly people, natural infection with seasonal influenza A, 2 consecutive years, the Netherlands
- Blood collection, Nasopharyngeal specimens
5.1 Ethics Committee of ISS, Rome, Italy Blood collection from healthy adults for ex vivo analysis
6.1 - Helsinki-UUsima Health District, Finland
- the National Agency for Medicines, Finland - Study vaccine induced cellular and humoral responses in health professionals
- Permission to use H5N1 vaccine for laboratory personnel for health protection purposes
6.3 Pirkanmaa HealthD istrict, Finland clinical influenza vaccination study

Specifics of the studies involving human participants
People participating in the studies were reimbursed for the costs that were made by them. In the Netherlands participating subjects had no benefits from participating in the trials.The people taking part in the studies were given a unique study code number. The code was used throughout the study. In the reports from the study, it was assured that the participants were unidentifiable. This design verified tracking of HLA data only to the unique study code number. Subjects were only recruited if they wished to be informed about coincidental findings.

Ethical guidance
Prof. Dr. van Delden, professor of medical ethics at the Medical School of Utrecht University, advised the IMECS project how to fulfil the ethical requirements and reviewed the design of the IMECS study with respect to ethical issues.

Control of ethical procedures by IMECS management
All submissions and approvals by the ethical committee were included in the project design, as milestone and deliverable reports and were sent by the IMECS partners to the IMECS management. Through this project design, the submission as well as the ethical approval were monitored by the IMECS management

thoughout the project. All approvals by the Ethical Committees were submitted to the EC as a deliverable of the project.

Informed consent
The participants in the clinical studies were asked for informed consent. In WP2 and WP3, new participants were recruited to determine immune responses against avian influenza in specimens. Specimens of individuals taking part in the studies were given a code which prevented tracking the samples back to the respective individuals. In WP4, studies were executed in specific target groups for influenza, including elderly and children. These studies were done specifically in these age groups, since the immune response of elderly and children was found to be different from adults in general, leaving these individuals highly susceptible to infection and complications thereof. Regarding the informed consent procedure, parents of children younger than 18 years of age who were not able to give informed consent were approached to do so. During the procedure, parents were present.
Furthermore, in WP4 elderly people were recruited for clinical studies. These elderly individuals were taking part voluntarily and were able to give informed consent.

Studies in children and elderly
- In case of clinical trials in children, the procedures and the amount of blood that was collected was minimized and justified by the relevant EU regulations. The relevant approvals were provided to the EC before the start of the studies, as deliverables of the IMECS project. In case of studies in elderly, only individuals were recruited who were able to give informed consent.

Research in co-operation with developing countries and less developed countries
For determining the immunological mechanisms of protection, IMECS cooperated with the Vietnamese National Institute of Hygiene and Epidemiology (partner 2). The inclusion of a partner from a disease endemic country was essential for successfully completing the IMECS project.
As indicated in Annex 1 of the IMECS project, specific measures were taken to guarantee an effective transfer of knowledge and technology to Vietnam. Furthermore, measures were taken to make sure that the local community would benefit from the project. The measures included:
- a twinning structure in which the Vietnamese partner received direct technical advice, expertise, materials, and assistance from the partners in Europe. During the complete program four visits were made by RIVM to NIHE to assist in the design and execution of the studies. As a result of these visits and exchanges by students (below), flow cytometry equipment was taken into practice by NIHE which had not been used before for analysis of immunity against avian influenza.
- an exchange program was executed for partners from the NIHE in Vietnam to visit laboratories in Europe to perform assays and implement these later in Vietnam. One MSc and one PhD student from Vietnam visited the European partners and stayed for several months to take up technology and implement this at NIHE.
- The health care community in Hanoi and the area of Thai Binh in general was underpinned by the IMECS program. This area was specifically struck by H5N1 since 2004. Apart from technologies to investigate immune responses in those people who were infected with H5N1, the local healthcare program in the province of Thai Binh was supported by organizing the ‘First workshop on influenza immune responses province’ which was held from 10-11 September 2009 in Thai Binh, Vietnam. This workshop convened all parties that having responsibilities in the protecting the community from avian influenza. The goal of the

program was to determine which measures were needed to protect the community. Representatives included scientists from the Vietnamese partners in IMECS as well as from affiliated institutes including Thai Binh Hospital Center for Preventive Medicine which provides secondary care to the community, the Hospital for Tropical Diseases which is providing secondary and tertiary care and the Provincial veterinary station, which is responsible for local veterinary services as well as the Netherlands Vaccine Institute (NL).
- the IMECS general meeting was held in Hanoi in June 2011 updating the findings of the first workshop.

As such the IMECS project complied with the relevant international regulations including:
- Declaration of Helsinki, paragraph 19: “Medical research is only justified if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the results of the research.”
- International Ethical Guidelines for Biomedical Research Involving Human Subjects (Council for International Organizations of Medical Sciences, CIOMS) guideline 10: as the research was responsive to the health needs of the community and knowledge generated was made available to the population
- Universal declaration on Bioethics and Human rights (UNESCO) Article 15 – Sharing of benefits: as benefits were shared in the form of support for health services, access to scientific and technological knowledge and capacity building facilities for research purposes.
- No product was made as a result of the study.

Animal studies

Experimental design
- To minimize the number of animals needed, our experimental approach was based on optimization of the potential vaccines and cross presentation conditions first in vitro. Only the optimal conditions were tested in vivo. All animal experiments were approved by official institutional animal experimental committees which judge the proposed experiments on experimental and ethical aspects. Vaccination and ex vivo read out experiments were judged to have virtually no impact on the animal suffering. Protection experiments in which pre-vaccinated mice were challenged with live influenza virus lead to lethality in control groups. The human endpoint in these experiments was determined before start of the experiments. Animals were closely watched during the experiments. Animals that showed signs of suffering according to the human endpoint conditions were sacrificed in a controlled procedure. In conclusion, clinical symptoms were monitored and, animals were sacrificed before non-allowed suffering was observed.

Specifics of the studies involving transgenic animals
- Transgenic animals expressing human HLA molecules were included in pre-clinical studies. The vaccines that were designed in the IMECS project were specifically targeted at these human molecules which are not present in mice. The immune responses determined in these transgenic mice for specific influenza epitopes were found representative of the immune response to these epitopes in humans. As there was no other opportunity to study these responses, the studies were executed in transgenic mice, after having received approval of the relevant Animal Experimental Committee. Therefore, preclinical studies in mice necessitated the inclusion of transgenic mice expressing human HLA molecules.
- The number of animals that were included in the studies was kept to a minimum based on power calculations and as approved by the Animal Experimental Committee.

- The work was carried out in accordance with the International Guiding Principals for Biomedical Research Involving Animals, as developed by the Council Organisations of Medical Sciences and the Guide for the Care and Use of Laboratory Animals.

Potential Impact:

4. THE POTENTIAL IMPACT

Socio-economic impact

Pandemic influenza remains a global threat, and may have a severe impact on world health and on the global economy. The disease is characterised by the fact that immunity is virtually absent in large parts of the population and thus may disperse easily around the globe. Epidemiologically important is that its origin is often in Asia. Recent outbreaks of avian influenza were in southern China – most recently indicated by the outbreak of H7N9 – and close to the Chinese – Vietnamese border.
IMECS addressed these issues by
1) identifying characteristics of protection against avian and/or pandemic influenza. 2) developing technologies to determine immune responses. 3) developing tools to monitor viral characteristics of pathogenicity. 4) developing vaccine leads and 5) enhancing collaboration between Asian and European research institutes.

1) Characteristics of protection against avian and/or pandemic influenza.

Characteristics of protection may be associated with humoral, cellular or innate immune cells. It was found that a potentially protective humoral response against pandemic H1N1pdm influenza was present mostly in elderly individuals who had encountered a similar H1N1 strain originating from 1918. Based on pre-existing humoral immunity, the elderly had sero-protective immune responses against the virus inducing the H1N1pdm outbreak of 2009. Although the elderly had high pre-existing antibody levels against the pandemic virus, they showed a weak response after vaccination with the pandemic vaccine. This weak response after vaccination in the elderly is similar how they usually respond after seasonal influenza vaccination. Consequently, elderly may be protected against pandemic influenza if they have humoral memory responses from similar infections in early life, but if they are not protected, standard vaccination procedures may induce insufficient immune responses.
Furthermore, it was shown in a recent paper by one of the IMECS partners that some young individuals were protected against pandemic influenza after seasonal vaccination, but elderly were not (Luytjes, Enouf et al. 2012). On top of that, seasonal vaccination not only protected against pandemic H1N1pdm influenza,

but also protected young individuals against avian H5N1 influenza. Specifically, findings by Corti et al revealed that seasonal influenza vaccination could induce polyclonal heterosubtypic neutralizing antibodies that cross-react with the swine-origin pandemic H1N1 influenza virus and with the highly pathogenic H5N1 virus (Corti, Suguitan et al. 2010). Altogether, these data indicate that in the absence of a pandemic vaccine, younger individuals may benefit from cross-protective responses induced by seasonal vaccination. Elderly individuals might benefit from humoral immunity induced by similar infections in early life. Surprisingly, if elderly are vaccinated after a pandemic outbreak they will need more potent vaccines than younger individuals. Societal implications of these data are that an emergency vaccination after a pandemic outbreak using seasonal vaccine may be beneficial for some young adults. Furthermore, potent pandemic vaccinations need to be developed specifically for the elderly.
The innate immune response was addressed in a study by Liu et al, showing that innate immune cells of elderly individuals had a reduced capability to induce T cell immunity (Liu, Nahar et al. 2012). Strategies to overcome this impairment were suggested, such as the usage of specific cytokines.
The socio-economic impact of these data may imply that in the absence of a pandemic vaccine, an emergency vaccination with seasonal vaccine may be beneficial for younger individuals. More importantly, development of a vaccine addressing broadly protective epitopes contained in the seasonal influenza antigens is a promising goal for the new generation vaccine. The induction of a viable immune response in the elderly however may need the usage of specific adjuvants that are able to enhance the efficacy of innate immune cells. Consequently, vaccine development for elderly may need the usage of adjuvants or other approaches enhancing the vaccine efficacy.

2) Development of technologies to determine immune responses.
The existing assays to determine influenza vaccine efficacy are based on HI and virus neutralizing (VN) assays. These existing assays are not sufficient as correlates of protection. Specifically for the elderly better assays are needed. In addition, HI responses against avian H5N1 are often only marginally induced. Therefore, assays are needed that better address humoral protection. In addition, assays are needed that specifically address other arms of the immune response, such as cellular immunity and innate immunity. IMECS introduced several new technologies targeting the various arms of the immune response. Furthermore, IMECS introduced complex in vitro assays mimicking the interaction between cells of various arms of the immune response. On top of that, pre-clinical models were designed to test protective efficacy of newly designed vaccine-leads in the intact organism. Altogether these technologies may allow to better determine the protective state of a specific individual against influenza. Furthermore, the assays have commercial value in the development of new vaccines, specifically where patent protection was sought. Technologies include:

Humoral immunity

• A novel microneutralization method for analysing anti-influenza A virus antibodies as developed by THL (F) (Lehtoranta, Villberg et al. 2009).

• A panel of ten human and avian H5 pseudoviruses spanning clades 0, 1, 2.1 2.2 and 2.3 of avian influenza H5N1 was established by IRB (CH) for the assessment of virus neutralizing monoclonal antibodies and sera breadth of reactivity.

• A new ELISA using baculovirus-expressed proteins for H5 proteins of clade 1 and clade 2.1. The ELISA was adapted to be used as an inhibition ELISA for indicating whether individuals had been infected with H5N1.

Cellular immunity
• A novel technique to determine CD4+ and CD8+ T cell responses against complete influenza virus (Jonges, Liu et al. 2010). The assay allows to be executed under standard BSL-2 conditions, also for determining T cell responses against pandemic or highly pathogenic influenza viruses. A patent application was filed with priority date 21.10.2009 (PCT/NL2010/050699), which received a favorable search report by the European Patent Office.
• A panel of influenza-specific CD8+peptides for HLA-A201 and HLA-A24, containing previously unknown influenza sequences was identified (FAC and RIVM).
• Influenza-specific T cell lines were cloned from PBMC of Flu- and HLA-A201-positive healthy individuals exposed to seasonal flu.

Innate immunity
• Characterization of human dendritic cell responses to infection with 2009 pandemic and certain seasonal influenza (H1N1 and H3N2) was carried out and published (Osterlund, Pirhonen et al. 2010).
• An assay was developed for determining the efficacy of human dendritic cells to activate a CD8+ T cell response against influenza. The assay allows to investigate the effect of various cytokines on the innate immune response against influenza.
• In vivo assessment of the efficacy of peptide-based vaccines against Influenza A was carried out in C57BL/6 mice. The mice were immunized with long peptides encoding the murine Flu CTL and T helper epitopes combined with the TLR2 ligand PAM3CysSK4 s.c. Our results showed that the combination with this TLR2ligand results in significant CD4 and CD8 T cell responses.

3) Developing tools to monitor viral characteristics of pathogenicity.
THL developed technology to quantify influenza virus in human samples. The highly sensitive quantitative RT-PCR assay for the detection of pandemic virus genetic material (Ronkko, Ikonen et al. 2011) can be used in clinical vaccination trials to analyse the protective efficacy of 2009 pandemic influenza A virus vaccines.

4) Developing vaccine leads
LUMC in collaboration with RIVM performed a proof of concept study involving C57BL/6 mice, which were vaccinated with peptide-based vaccines and challenged with the HKx31Flu virus. It was found that peptides for specific B cell epitopes were partially protective as well as peptides specific for T cell epitopes. An overall conceptual vaccine may follow from these investigations based on specific epitopes targeting humoral and cellular immune responses. It is expected that such an epitope based vaccine will be advantageous above the standard vaccines in terms of stability and easiness to produce. Furthermore, the vaccine may have antigenic properties that have not been present to date in current vaccines. This new technology is expected to have commercial potential.

5) Enhancing collaboration between Asian and European research institutes.The Asian – European collaboration was enhanced leading to the option for a sustained interaction between the various partners and the option to proceed with vaccine development and lead identification between the partners. Specifically, the collaboration between Vietnam and Europe was intensified. Partners in Hanoi included the National Institute of Hygiene and Epidemiology (NIHE), which has a main task in protecting the Vietnamese population from infectious diseases,as well as the third party institutes: the Institute of Biotechnology (IBT) from Hanoi, which is involved in basic biotechnology research; National Institute of Veterinary Research (NIVR) from Hanoi, which has a main task in protecting the Vietnamese livestock from infectious diseases and the Thai Binh Health Center, which is located a two-hours drive from Hanoi in Thai Binh and which has a main task in providing primary care to the population of Thai Binh area. Several visits were made to the partners in Hanoi by the IMECS partners to strengthen the cooperation. The visits accumulated in the 'First workshop on influenza immune responses in Thai Binh province' which was held from 10-11 September 2009 in Thai Binh, Vietnam. This workshop convened all parties necessary to investigate the immune responses against avian influenza in the region. Representatives included scientists from the Vietnamese partners in IMECS as well as from affiliated institutes including Thai Binh Hospital Center for Preventive Medicine which provides secondary care, the Hospital for Tropical Diseases which is providing secondary and tertiary care and the Provincial veterinary station, which is responsible for local veterinary services all from Vietnam as well as the Netherlands Vaccine Institute (NL). The meeting exhibited the launch of the IMECS project in Thai Binh province. In addition, the 3rd IMECS general meeting was held in Hanoi allowing an intensive scientific interaction between the Vietnamese and European partners. Furthermore, one MSc and one PhD student from Vietnam visited the European partners and stayed for several months to take up technology and implement this at NIHE.
European laboratories collaborated with the National Institute of Hygiene (NIHE) in Hanoi. Specifically, Vietnamese MSc and PhD students were trained in the Netherlands to use and apply flow cytometry for investigating immune responses in humans. This technology was applied at NIHE after a technology transfer programm involving training of the students at RIVM and subsequent application of the technology on a FACS-Calibur machine at NIHE.

The Main dissemination activities

The technology of the IMECS project was disseminated through various channels, amounting to 60 activities altogether. In total 19 scientific papers were made, in journals chosen and targeted at the audience that is of interest for the message conveyed in the manuscript. Several other publications are submitted or planned. Journals included those specifically targeted at influenza, vaccinology, immunology, virology and medicine. Several publications were included in top-ranking journals within their field, such as journal of clinical investigation and Plos Pathogens. Several papers were shown to have a significant impact. A case in point is the publication from IRB by Corti et al (2010) in J.Clin.Invest. which was cited 135 times indicating a high uptake of the findings in the scientific community (Google Scholar, July 2013). Examples from other IMECS research groups are from THL by Ikonen et al (2010) in Eurosurveillance, which analysed the protective disposition of elderly individuals in Finland against pandemic influenza and which was cited 131 times. Other examples include Liu et al (2011) from RIVM which discussed the

background of impaired immunity in the elderly and which was cited 17 times. An overview of publications is listed below.
In total 33 presentations were given to scientific and general audiences and expected to have reached more than 8,500 researchers. In addition, 11 conferences and workshops were organized which were partly or completely related to IMECS.

2009
- Franceschini, D., et al 2009. PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. J. Clin. Invest 119:551.
- Lehtoranta L, et al (2009) A novel, colorimetric neutralization assay for measuring antibodies to influenza viruses. J Virol Methods. 2009 Aug;159(2):271-6.

2010
- Corti, D., et al (2010). Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J. Clin. Invest 120:1663.
- Ikonen, N., et al (2010). High frequency of cross-reacting antibodies against 2009 pandemic influenza A(H1N1) virus among the elderly in Finland. Euro. Surveill 15.
- Jonges, M., et al (2010). Influenza virus inactivation for studies of antigenicity and phenotypic neuraminidase inhibitor resistance profiling. J. Clin. Microbiol. 48:928.
- Sallusto, F., et al. (2010). From vaccines to memory and back. Immunity, 33(4): 451-463.
- Ikonen, N. Et al (2010). Genetic diversity of the 2009 pandemic influenza A(H1N1) viruses in Finland. PLoS ONE, 5, e13329.
- Soethout EC et al (2010) Phacilitate, Barcelona Vaccine Forum 2010: focus on correlates of protection against influenza. Hum Vaccin. 2010 Dec;6(12):964-5

2011
- Liu WM, et al (2011) Aging and impaired immunity to influenza viruses: implications for vaccine development. Hum Vaccin. 2011 Jan-Feb;7 Suppl:94-8.
- Strengel M, et al (2011) Minor Changes in the Hemagglutinin of Influenza A(H1N1)2009 Virus Alter Its Antigenic Properties. PLoS One. 2011;6(10):e25848. Epub 2011 Oct 11.
- Esa Rönkkö, et al (2011) Validation and diagnostic application of NS and HA gene-specific real-time reverse transcription-PCR assays for detection of 2009 pandemic influenza A (H1N1) viruses in clinical specimens. J Clin Microbiol. 2011 May;49(5):2009-11.
- Bruno Garulli et al (2011) Immunogenicity of a Recombinant Influenza Virus Bearing Both the CD4+ and CD8+ T Cell Epitopes of Ovalbumin. Journal of Biomedicine and BiotechnologyVolume 2011 (2011), Article ID 497364,
- Strengell M., Ikonen N., Ziegler T., Kantele A., Anttila V-J. and Julkunen I. (2011) Antibody responses against influenza A(H1N1) pdm09 virus after sequential vaccination with pandemic and seasonal influenza vaccines in Finnish health care professionals. PLoS One. 2011;6(10):e25848.

2012
- Liu W.M. et al Impaired production of TNF-α by dendritic cells of older adults leads to a lower CD8+ T cell response against influenza. Vaccine. 2012 Feb 21;30(9):1659-66.
- Luytjes W, Enouf V, Schipper M, Gijzen K, Liu WM, van der Lubben M, Meijer A, van der Werf S,

Soethout EC. HI responses induced by seasonal influenza vaccination are associated with clinical protection and with seroprotection against non-homologous strains. Vaccine. 2012 Jul 27;30(35):5262-9.
- The ambiguity in immunology. Barnaba V, Paroli M, Piconese S. Front Immunol. 2012;3:18.
- Mari Strengell, Niina Ikonen, Thedi Ziegler, Anu Kanteleb, Veli-Jukka Anttilab, Ilkka Julkunen, (2012) Antibody responses against influenza A(H1N1)pdm09 virus after sequential vaccination with pandemic and seasonal influenza vaccines in Finnish health care professionals. Influenza Other Respi Viruses. 2012 Aug 23.
- Polyfunctional type-1, -2, and -17 CD8(+) T cell responses to apoptotic self-antigens correlate with the chronic evolution of hepatitis C virus infection. Franceschini D, Del Porto P, Piconese S, Trella E, Accapezzato D, Paroli M, Morrone S, Piccolella E, Spada E, Mele A, Sidney J, Sette A, Barnaba V. PLoS Pathog. 2012 Jun;8(6):e1002759.

2013
- Garulli B, Di Mario G, Sciaraffia E, Accapezzato D, Barnaba V, Castrucci MR. Enhancement of T cell-mediated immune responses to whole inactivated influenza virus by chloroquine treatment in vivo. Vaccine 2013, in press.

List of Websites:

The Project website

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Last update: 7 August 2014

Permalink: https://cordis.europa.eu/project/id/201169/reporting