

**Short Term Scientific Mission COST BM0704**

**Emerging EMF Technologies and Health Risk Management**

Title of the mission:  
**“Immunological investigation related to early life exposure to EMF”**

Reference code: **COST-STSM-BM0704-05275**

**Beneficiary:**

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**IPB - Institut Polytechnique de Bordeaux - France**

**Host:**

**Dr. Claudio PIOLI**

**Immunology laboratory, Section Biotec-Med.**

**ENEA - Italian Agency for New Technologies Energy and Environment -  
Italia**

**Period: from 18/10/2009 to 11/11/2009**

**Place: Rome (IT)**

## Purpose of STSM

The objective of this STSM was to extend complementary immunological techniques adopted in experimental studies related to EMF<sup>1</sup>. This one-month visit was a great opportunity to share useful information and acquire specific techniques developed by the host team, and also to exchange scientific strategies for the study of the effects of *in utero* exposure of immature organisms to radiofrequency fields (RF). The study performed in the host laboratory will contribute to answer the current concerns related to the rapid increase in WLAN<sup>2</sup> use and its potential ability to alter the maturation of the immune system of newborn organisms.

## Introduction

Daily public exposure to Wi-Fi signals increases rapidly. Although the exposure level is extremely low, several investigations on its potential adverse effects are ongoing. One of the critical biological systems that could be a target as well as a potential source of markers is the immune system. As a relevant model of complexity and dynamism of this system, cellular immune responses were investigated in animals and humans, mostly with exposure to GSM signals. Lymphocyte sub-populations and their mitogenic responses have been assessed, showing a lack of alteration of lymphocyte phenotype and functionality and suggesting that low-level pulsed microwaves could not affect the integrity of the immune system in rodents (Chagnaud and Veyret, 1999, Gatta *et al*, 2003). Interestingly, some authors evoked the possibility that the immune system might adapt to RF radiation as it does with other stressing agents (Gatta *et al*, 2003). No significant changes were observed on the humoral immune response, particularly B-cell peripheral compartment, antibody production and for bone marrow efficiency (Nasta *et al*, 2006, Prisco *et al*, 2008). Recent works also showed that 2450 MHz exposure had no influence on several immune and degenerative parameters or on pre-natal development (Poullietier de Gannes *et al*, 2009).

Dr Pioli's team (host team) has a particular interest in investigating the RF health effects and already contributed to investigations of immune responses related to RF exposure. More specifically, the recent ELEYAR<sup>3</sup> project aims at studying the effects of *in utero* and early exposure to Wi-Fi signal on the immune system of mice. This project is a collaboration between the host institution ENEA<sup>4</sup> and my own laboratory (Laboratoire IMS, Institut Polytechnique de Bordeaux). At IMS, I set up immunological experiments on mice exposed *in utero* and during five weeks post-natal (total exposure of 7 weeks). This work was part of the ERYA<sup>5</sup> project and used an original exposure system for free-running animals.

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<sup>1</sup> EMF: Electric and Magnetic field

<sup>2</sup> WLAN: Wireless Local Area Network

<sup>3</sup> ELEYAR: Early Life Exposure of Young Animals to Radiofrequency fields

<sup>4</sup> Italian Agency for New Technologies and Energy and Environment fields

<sup>5</sup> ERYA: Effects of Radiofrequency fields on Young Animals

## Main objectives

The goal of this work was to take benefit of the host team contribution to the ELEYAR project, initiated by the Bioelectromagnetics team in Bordeaux, to compare and acquire knowledge that could be useful for my own work on the ERYA project. In the work performed at ENEA, we investigated the biological effects of *in utero* exposure to Wi-Fi signals (2.45 GHz) on the immune system of 6-month-old mice taking into account the following parameters:

- Health condition and weight
- Thymus total cell number, subpopulations frequencies
- Proliferative response of thymocytes
- Spleen total cell number and CD4/CD8 ratio
- Mitogen activated spleen cell responses
- Total IgG and IgM production in sera and in activated cells
- Cytokines production (IL-2 and IFN $\gamma$ <sup>6</sup>)

## Technical approach (Fig.1):

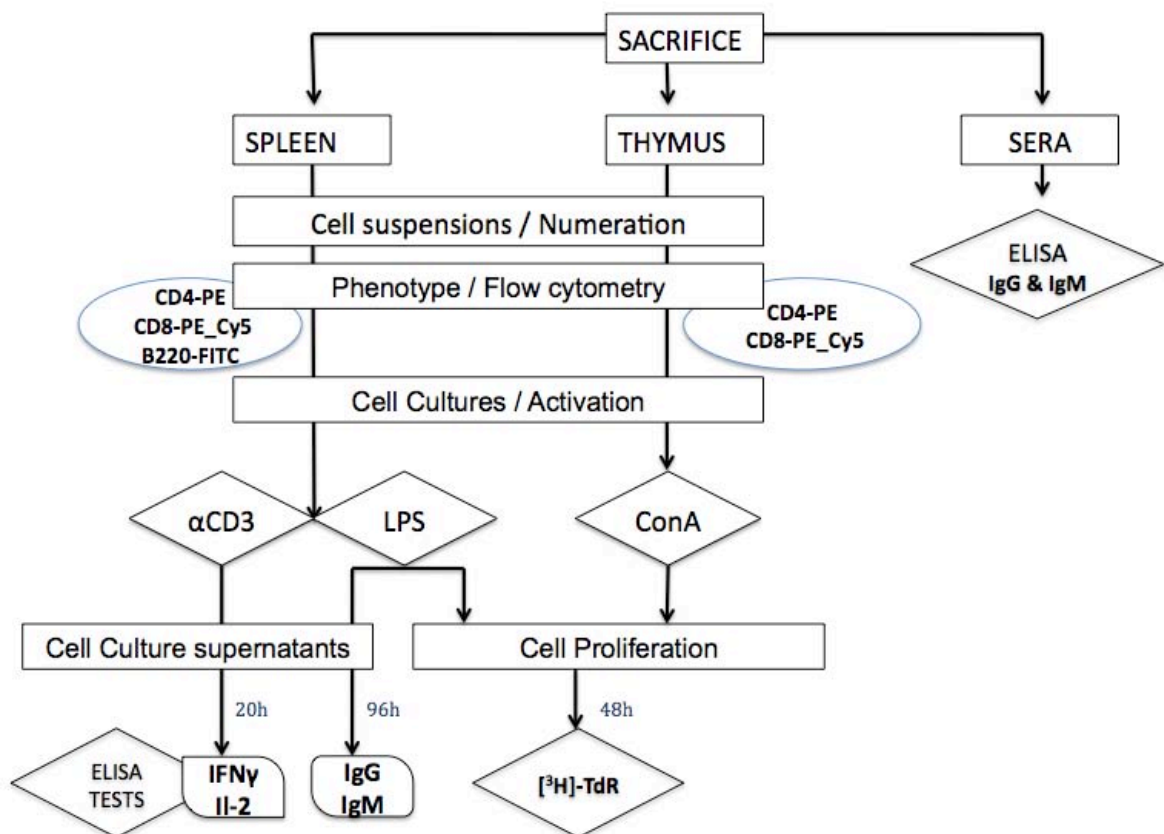


Fig.1: Experiments realised on two series of exposed and sham-exposed C57BL6 mice to Wi-Fi signal (25 males and 24 females, 6 months-old).

<sup>6</sup> Interleukin-2 and Gamma Interferon Cytokines

## 1- Exposure system:

The exposure system consisted of two nonstandard TEM (Transverse Electromagnetic) cells (12x12x120 cm) (Fig.2). The dimension was set to ensure a pure TEM mode propagation. The structure also provided air flow during animal exposure and a water-cooling system to avoid temperature elevation.

The TEM cells permitted simultaneous exposure of multiple animals to the same conditions (e.g. two groups of four mice) and experimental blind procedure performances.

The source of Wi-Fi signal was a commercial access point (AP) communicating with two PCs.

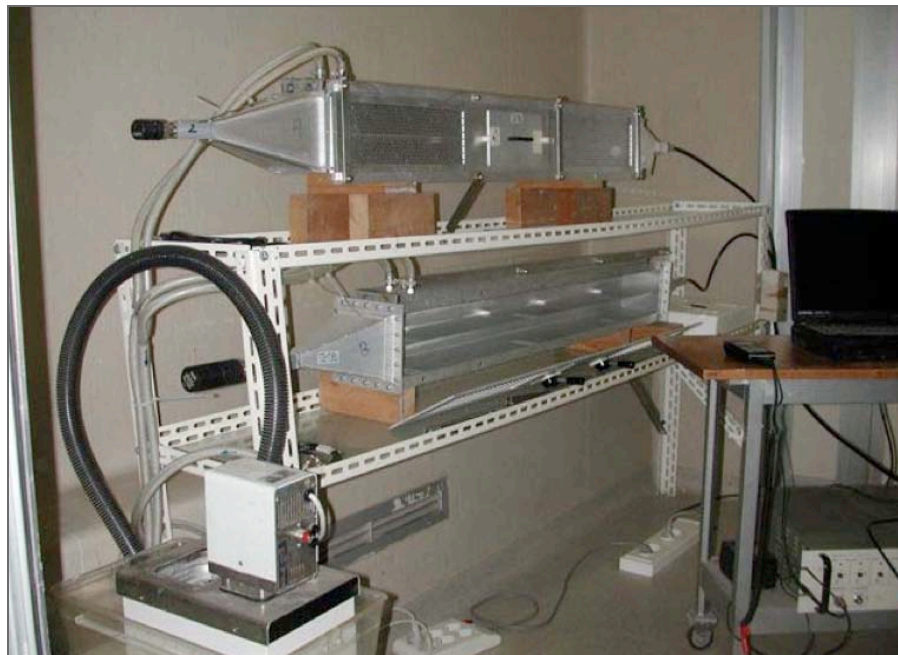


Fig.2 TEM cells system exposure (Animal facilities of ENEA laboratory)

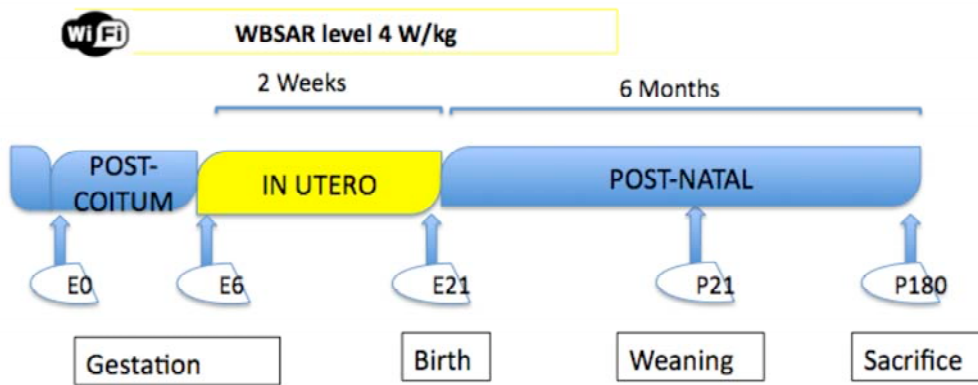
## 2/ Animals exposure

Female mice were exposed in the TEM cell system to a SAR<sup>7</sup> level of 4 W/kg (ICNIRP<sup>8</sup> critical level), 2 hrs/day, starting on 5 days post *coitum* until the day before delivery (corresponding to 14 consecutive days) (Fig.3). Animals were kept constrained within transparent Perspex tubes during exposure. Sham-exposed mice underwent the same procedures as exposed mice except for the presence of the Wi-Fi signal and cage controls were kept in animal facilities. Parallel dosimetry study provided adequate SAR to mice in the radiating structure. The ethical committee of ENEA approved these experimental procedures.

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<sup>7</sup> SAR: Specific Absorption Rate

<sup>8</sup> ICNIRP: International Commission on Non-Ionizing Radiation Protection



E: Embryonic / P: Post-natal

WBSAR: Whole Body Specific Absorption Rate

Fig.3: Animal exposure set-up (Exp. Code WiFi0903)

### 3/ Cell culture

#### ***Organ removal and cell suspensions:***

Six months after the last exposure, animals were sacrificed and lymphatic organs collected. Cell suspensions from thymus and spleen were obtained by gently pressing organs on sterile grids placed on Petri dishes. Cell aggregates were dissociated by pipetting the cell suspension. After washing, splenocytes and thymocytes were suspended in complete medium [RPMI 1640 medium, 10% Foetal Calf Serum, 2 mM glutamine, penicillin (100 U/ ml), and streptomycin (100 µg/ml)]. The number of nucleic cells as well as the distribution of cell sub-populations was determined by flow cytometry. We previously stained these cells with fluorochrome-linked antibodies: anti-CD4-PE, anti-CD8-PE-Cy5 and anti-B220-FITC to assess the frequency of lymphocyte sub-populations in thymus and spleen. These antibodies specifically recognize T Helper cells, T cytotoxic cells and B cells, respectively. Each fluorescence signal detected was analyzed and cells repartition was investigated using a FACScalibur flow cytometer (Becton Dickinson).

#### ***Cell cultures and activation:***

To induce cell proliferation, thymocytes were challenged for 48 hours with the mitogen Concanavalin A (ConA, 5 µg/ml). To induce T- and B-cell proliferation, spleen cells were stimulated with anti-CD3 antibodies (1 µg/ml) and Lipopolysaccharide (LPS, 1 µg/ml), respectively. Cells ( $2 \times 10^6$ /ml splenocytes and  $5 \times 10^6$ /ml thymocytes) were cultured in flat-bottomed 96-well plates with complete medium at 37°C, 5% CO<sub>2</sub> in air and humidified atmosphere. After 48 h, cultures were pulsed for 4 h (at 37°C, 5% CO<sub>2</sub> in air) by adding 1 µCi [<sup>3</sup>H]-thymidine to the culture medium. Cell proliferation was evaluated by tritiated thymidine uptake using a Matrix 96 Direct beta counter (Packard Instruments Co.).

## ELISA tests

**Cytokine titration:** Interleukin-2 (IL-2) and gamma Interferon (IFN $\gamma$ ) were titrated in culture supernatants of spleen cells challenged with anti-CD3 using the sandwich ELISA method. High-protein-adsorption 96-well plates were coated overnight (20 h) at 4°C with purified anti-IL-2 (1  $\mu$ g/ml) or anti-IFN $\gamma$  (1  $\mu$ g/ml). A saturation step consisting in coating plates with normal serum was done to avoid aspecific reactions. After washing, serially diluted cytokine standards (recombinant IL-2 and IFN $\gamma$ ) or culture supernatants were added to the wells and incubated overnight at 4°C. After washing, plates were incubated for 1 h at 37°C with either biotin-conjugated anti-IL-2 (2  $\mu$ g/ml) or anti-IFN $\gamma$  (1  $\mu$ g/ml). After washing, samples were incubated with peroxidase-conjugated streptavidin at 37°C for 1 h. After washing, ABTS substrate [2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)] was added and absorbance was read at 405 nm.

**Antibodies:** Total IgM and IgG antibodies were detected by titration in sera of mice and in splenocytes culture supernatants. The technique was the sandwich ELISA method, using antibody fragments directed against specific Ig G and IgM isotypes (AffiniPure F (ab) 2 Fragment goat anti-mouse IgG and IgM (H&L); 5  $\mu$ g/ml). Sera and culture supernatants were serially diluted for the titration and detection step was carried on with peroxidase-conjugated (AffinePure F (ab) 2) with goat anti-mouse IgG or anti-mouse IgM fragments. The absorbance of the product of reaction with ABTS substrate [2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)] was determined at 405 nm.

## Other Experiment:

### *Cell proliferation assay CFSE staining set-up*

An additional proliferation test was performed using CD4<sup>+</sup> cells purified by immunomagnetic cell sorting from PARP1 K.O. (Knock-Out for PARP1 gene) and WT (wild type) C57/B6 mice. After CFSE-labelling, these cells were co-cultured with irradiated (12 Gy; X-rays) antigen presenting cells. The fluorescence signal was collected using a FACScalibur cytometer (Becton Dickinson).

## Results & Discussion

We examined the biological effects of *in vivo* exposure to a 2.45 GHz Wi-Fi signal on 6-month C56BL7 mice exposed *in utero*.

Previous data obtained by the Italian team during the gestational period in pregnant mice and during 6 months after birth for pups showed no difference in body weight of mothers and pups with respect to sham-exposed mice and pups. The number of pups per litter of exposed mice was to the same as in the sham group.

Interestingly, the animal weight at the critical point of weaning was similar in all groups confirming the lack of effect of *in utero* exposure on the growth of young animals. Thus the percentage of pregnant mice was found similar in all group exposed, non-exposed, and cage controls.

During my stay at ENEA, we sacrificed the 6-month-old mice and implemented the protocol described above. Preliminary analysis of the data showed a slight difference in cell number in the thymus of the animals but not in the spleen. This difference was related to the sex ratio but no differences related to Wi-Fi exposure were observed.

The frequencies of differentiating cell subpopulations (identified by CD4/CD8 expression for the thymus and B220 for the spleen) were studied among the different groups and the statistical analysis is in progress. The mitogen-induced thymocyte and splenocyte proliferation analysis is also ongoing.

The *in vitro* cytokine production by splenocytes (IFN-gamma and Interleukin-2) of exposed mice was found similar to that of the sham-exposed mice. This result suggests that Wi-Fi exposure does not alter the ability of lymphocytes to produce cytokine and that production is not enhanced by exposure to this signal.

The humoral immune response did not seem to be altered by *in utero* exposure since IgM and IgG serum levels were not significantly different among exposed, sham-exposed, and control mice. Moreover, B cells from mice splenocytes challenged *in vitro* with LPS produced similar amounts of IgM and IgG in culture supernatant. These observations suggest that Wi-Fi exposure does not influence isotypes production *in vivo* and *in vitro*.

Taken together these preliminary results show no effect of *in vivo* exposure to Wi-Fi during the gestational period. Data will be completed (statistical analysis) and submitted for publication. These data will improve our knowledge on potential long-term consequences of *in utero* exposure, following investigations in mice aged 5 weeks and 6 months.

Moreover, this work provided useful comparison between the two projects (IMS and ENEA). For instance, exposure setups for mice and dosimetric data are available to compare between 5-week-old (early stage of sexual maturation) and 6-month-old mice (considered as sexually adult) after *in utero* exposure. My training at ENEA was an opportunity to use another exposure system with the same type of RF signal as in Bordeaux but with real-time SAR evaluation and under constrain conditions.

At IMS the spleen was used as a target organ; I had also the opportunity at ENEA to explore the thymus. This relevant lymphoid organ is involved in T cell differentiation and this approach will be useful for further studies related to cellular immune responses.

The functionality of lymphocytes, specially through their ability to produce cytokines, was tested in supernatants of cell cultures and will be compared to the intracellular production of cytokines that we plan to do as part of the ERYA project. I performed a number of technical protocols related to cell activation conditions (conA, anti-CD3, LPS; time, concentration) and cell proliferation assay. Among these techniques is the CFSE assay, a method that we use for NK cells cytotoxicity test and the use of radioactive method of [<sup>3</sup>H]-thymidine incorporation, while in Bordeaux, we currently use an enzymatic assay (MTT test) to assess cell proliferation.

Finally, I had also the opportunity to learn new methods particularly how to use CD4+ cells purified by immuno-magnetic cell sorting from PARP1 knockout and wild type C57/B6 mice. This was a very interesting training in applied immunology related to T auxiliary cells and immuno-modulation.

## **Outlook**

Seven-week exposures that began *in utero* are underway on spleen cells of C57BL6 mice at IMS. Immunological tests are programmed at the end of the year 2009. The experience gained during the STSM will benefit to these experiments as well as in future collaborative projects.

## **Acknowledgement:**

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