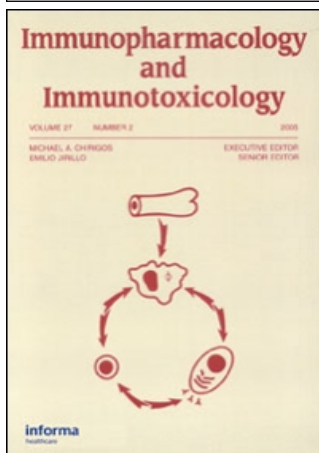


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***Mycobacterium vaccae* Immunization to OVA Sensitized Pregnant BALB/c Mice Suppressed Placental and Postnatal IL-5 and Inducing IFN- γ Secretion**

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Although the development of atopy in the newborn is determined by a multitude of factors, an intense Th1 stimulus early in life could be protective by facilitating a switch away from Th2. Aimed to determine the effect of single *Mycobacterium vaccae* (*M. vaccae*) immunization to OVA-sensitized pregnant mice on IL-5 and IFN- γ secretion from placental lymphocytes and splenocytes of offspring.

Pregnant BALB/c mice were divided into 4 groups, OVA-sensitized + *M. vaccae* immunized, OVA-sensitized, *M. vaccae* immunized and controls. Sensitization with OVA was initiated before mating, and aerosol OVA challenge were performed during pregnancy. *M. vaccae* immunization was performed on the 12th day of pregnancy. IL-5 and IFN- γ levels of placental lymphocytes were analyzed on the 18th day of pregnancy and splenocytes of offspring on the 2nd and 28th days during postnatal period. A single administration of *M. vaccae* to OVA-sensitized pregnant mice down-regulated IL-5 secretion and induced IFN- γ secretion from placental lymphocytes. On the other hand, after *M. vaccae* immunization downregulation of IL-5 levels and upregulation of IFN- γ secretion persisted in offspring when determined on 2nd and 28th days of life. Vaccination with *M. vaccae* to OVA-sensitized pregnant BALB/c mice prevented Th2 immune responses by enhancing secretion of IFN- γ and lowering IL-5 levels during pregnancy and the effect persisted during the postnatal period in offspring.

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Keywords Hygiene hypothesis, Interferon gamma, Murine model of asthma, *Mycobacterium vaccae*, Ovalbumin, Pregnancy.

INTRODUCTION

The prevalence of asthma and atopic disease has increased in recent decades, particularly in industrialized countries.⁽¹⁾ The main reason for this increase has been ascribed to environmental factors, including the reduction of early-life exposure to microorganisms hence termed as “hygiene hypothesis.”⁽²⁾

Allergic asthma is associated with an increase in secretion of Th2 cytokines such as IL-4, IL-5 and IL-13, leading to the induction of asthma through the production of allergen-specific IgE hence inducing pulmonary eosinophilic inflammation and increase mucus production in the airways.⁽³⁾

The rationale behind the “hygiene hypothesis” is that children infected with microorganisms early in life may not develop asthma or atopic diseases which is supported by a significant clinical and experimental data on the inverse link between mycobacterial antigens and atopy in which exposure to mycobacteria had prominent Th2 limiting effects. These studies demonstrated the efficacy of mycobacterial species in ameliorating the severity of atopy, downregulating IgE response and inhibiting asthmatic histopathologic changes in the lung.^(4–7)

Several studies have suggested that, *in utero* environment plays an important role in the onset of sensitization to environmental allergens. Pregnancy is known to be characterized by Th2 predominance which is an essential prerequisite for a successful outcome as the Th2 environment prevents fetal-allograft rejection.⁽⁸⁾ As a consequence, neonatal immunity is Th2 skewed and allergen specific T cell responses are common already at birth.⁽⁹⁾ Although the development of atopy in the newborn is determined by a multitude of factors, an intense Th1 stimulus early in life could be protective by facilitating a switch away from Th2.

Mycobacterium vaccae, a non-pathogenic, fast-growing saprophytic mycobacterium has been shown to have a potential role in immunoregulation when delivered by subcutaneous injection by promoting Treg cells and upon transfer can protect against recipient mice from airway inflammation.⁽¹⁰⁾ Recently, Hunt et al. reported local and systemic immune responses after administration of *M. vaccae* by an increase of IL-10 and IFN- γ simultaneously downregulating IL-5 and in turn reducing the severity of allergic inflammation.⁽¹¹⁾

In this study, our aim is to determine the impact of *Mycobacterium vaccae* (*M. vaccae*) immunization in OVA-sensitized and challenged BALB/c mice during pregnancy on cytokine production both from placental lymphocytes and splenocytes of offsprings.

METHOD

Mice

Five- to 6-week-old BALB/c mice were obtained from The Scientific and Technical Research Council of Turkey (TUBITAK) and housed according to the National Institutes of Health Guidelines. Experimental protocols were ethically approved and complied with the Animal Research Committee of Marmara University (Istanbul, Turkey).

Study Design

Pregnant mice were divided into 4 groups; OVA-sensitized and *M. vaccae*-immunized group (OVA + *M. vaccae*, n = 5), OVA-sensitized group (OVA, n = 5), *M. vaccae* immunized group (*M. vaccae*, n = 5) and non-sensitized, non-immunized control group (n = 5). OVA-sensitization of female BALB/c mice was initiated before mating, and OVA challenges were performed during pregnancy in OVA + *M. vaccae* and OVA groups. Immunization with *M. vaccae* was carried out on the 12th day of pregnancy in the OVA + *M. vaccae* and *M. vaccae* groups. Placental lymphocytes were obtained on the 18th day of pregnancy, while isolation of splenocytes of offspring was performed on the 2nd and 28th days post-natally. Placental lymphocytes and splenocytes were cultured with OVA, *M. vaccae* and PHA and cytokine determinations were performed by ELISA. Study design is shown in Figure 1.

Ovalbumin Sensitization and Challenge

Female BALB/c mice in OVA + *M. vaccae* group and OVA group were sensitized by intraperitoneal injection of 10 µg ovalbumin (OVA, Grade V, Sigma, St. Louis MO, USA) on days -28, -14 and -7 prior to mating (day 1). During the gestational period, pregnant mice were challenged (20 min) with aerosolized OVA (1% in saline) for every second day using ultrasonic nebulization (AeroSonic ultrasonic nebulizer; DeVilbiss, Somersed, PA). Non-sensitized control animals received only the saline.

M. vaccae Preparation and Immunization

Mycobacterium vaccae (ATCC 29678 strain) was supplied by North Carolina State University and was cultured in Lowenstein-Jensen medium (REMEL, Lenexa, KS). *Mycobacterium vaccae* was cultured and the vials were autoclaved at 120°C for 25 minutes. Aliquots of 1×10^7 Colony Forming Units (CFU)/ml in medium for subcutaneous administration were prepared and frozen at -20°C until needed. Mice in group OVA + *M. vaccae* and *M. vaccae* were immunized subcutaneously (s.c.) on the abdominal area with 1×10^7 CFU of heat-killed *M. vaccae* in 100 µl of PBS on the 12th day of pregnancy.

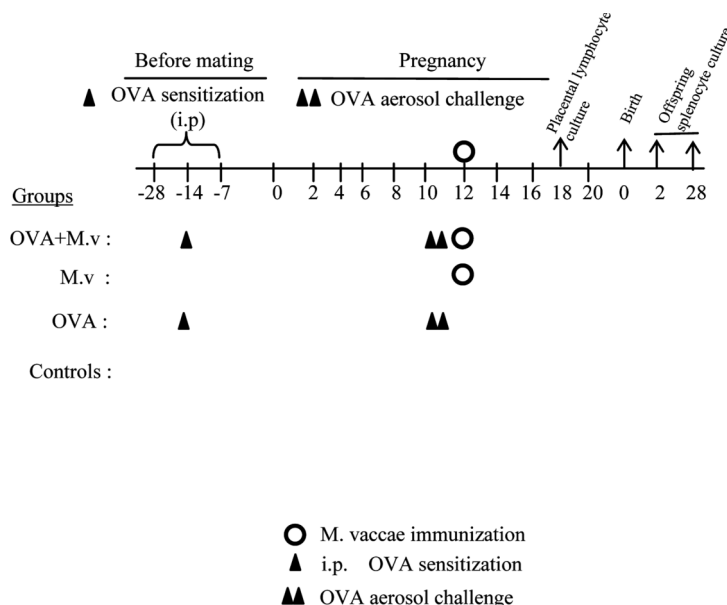


Figure 1: Study Design: ○*M. vaccae* immunization, ▲i.p. OVA sensitization, ▲▲OVA aerosol challenge.

In vitro Culture of Placental Lymphocytes and Splenocytes

Placental lymphocytes were obtained from pregnant mice on day 18.⁽⁹⁾ Both decidua and fetal placenta digested with collagenase D (1 mg/ml, type 1, Sigma, St. Louis, MO, USA) and Dnase (25 µg/ml, Sigma, St. Louis, MO, USA). A total of 10 deciduas and placentas were placed in 10 ml of RPMI 1640 (Sigma, St. Louis, MO, USA) medium, and digestion was carried out on the vertical rotating mixer at 37°C for 45 min. Single cell suspensions were obtained by low speed centrifugation (40 × *g* for 1 min). Red blood cells were removed by hypotonic lysis. The cells were washed three times with RPMI 1640/10%FCS, and put into 24 well tissue culture plates at 5 × 10⁶/ml in RPMI 1640 / 10% FCS.

Spleens of offspring were obtained on the 2nd and 28th days post-natally, and single-cell suspensions were prepared by passing the cells through cell strainer. Splenocytes were isolated with ficoll gradient centrifugation. After three washes in RPMI 1640, 5 × 10⁶ splenocytes were placed in complete medium composed of RPMI 1640 supplement with 10% fetal calf serum, L-glutamine and 1% of penicillin/streptomycin (all from Sigma, St. Louis, MO, USA). Cultures were carried on 24-well flat bottom plate with 1 × 10⁶ cells/ml per well.

Cells were stimulated with OVA (40µg/ml), *M. vaccae* (2 × 10⁷ CFU/ml) and PHA (1µg/ml, Sigma, St. Louis, MO, USA). Supernatants were obtained 24 hours later for IFN-γ, and 48 hours later for IL-5 cytokine analysis. Cytokine

levels of culture supernatants were determined with commercial ELISA kit (Endogen, Rockford, USA).

Statistical Analyses

For statistical analyses, SPSS package (release 10.1 SPSS, Chicago, IL, USA) program was used. Kruskal-Wallis test was used for comparison of all groups. When differences were statistically significant, Mann-Whitney U-test was used for between-group comparisons. A $p < 0.05$ was considered statistically significant.

RESULTS

Cytokine Production of Placental Lymphocytes

Placental lymphocytes of mice in the OVA-sensitized *M. vaccae* immunized (*M. vaccae* + OVA) and the *M. vaccae* immunized (*M. vaccae*) groups demonstrated significantly lower IL-5 levels on stimulation with OVA ($p < 0.05$, respectively) in culture supernatants when compared with non-sensitized, non-immunized controls (Fig. 2A). Moreover when compared with the OVA-sensitized group placental lymphocytes of mice in the *M. vaccae* immunized group demonstrated significantly higher IFN- γ levels in the culture supernatants on stimulation with *M. vaccae* ($p < 0.05$) (Fig. 2B). In addition, the placental lymphocytes of mice in the *M. vaccae* group produced significantly higher OVA-stimulated IFN- γ than the OVA-sensitized *M. vaccae* immunized group ($p < 0.05$) (Fig. 2C).

The assessment of IL-5 / IFN- γ ratio revealed that *M. vaccae* immunization of both OVA-sensitized and non-sensitized pregnant mice had decreased placental IL-5 / IFN- γ ratio when compared to non-immunized OVA-sensitized and control groups (Fig. 3).

Cytokine Production of Newborn Mice on the 2nd Day of Life

Comparison of IL-5 and IFN- γ production from splenocytes of the newborn mice on the 2nd day of life revealed statistically significant differences. Newborns of the OVA-sensitized *M. vaccae*-immunized group demonstrated significantly lower IL-5 levels on stimulation with *M. vaccae* on culture supernatants of splenocytes when compared to newborns of healthy controls ($p < 0.05$) (Fig. 4A). Meanwhile, IFN- γ secretion in offspring of the *M. vaccae* immunized group was significantly higher than controls on stimulation with *M. vaccae*, PHA and in unstimulated culture supernatants of splenocytes ($p < 0.05$, respectively) (Fig. 4B, C, D). Moreover, *M. vaccae* stimulated IFN- γ production from newborn splenocytes of the OVA-sensitized *M. vaccae* immunized mothers was significantly higher than controls.

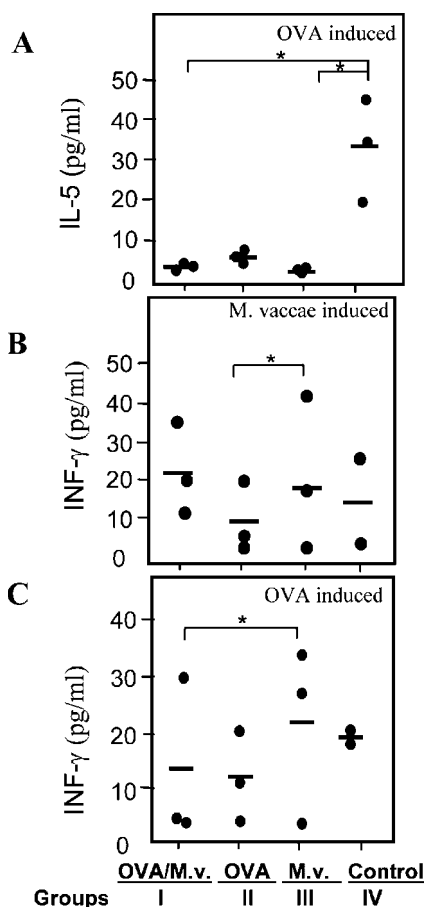


Figure 2: Comparison of placental cytokine levels. Placental lymphocytes of pregnant BALB/c mice were obtained on the 18th day of pregnancy cultured with OVA, *M. vaccae* and PHA and IL-5 and IFN- γ levels were determined in culture supernatants and in unstimulated (u.s.) supernatants. A. IL-5 levels *OVA+ *M. vaccae* group vs controls $p < 0.05$; **M. vaccae* group vs controls $p < 0.05$. B. IFN- γ levels *OVA group vs *M. vaccae* group $p < 0.05$. C. IFN- γ levels *OVA + *M. vaccae* vs *M. vaccae* group $p < 0.05$.

The ratio of IL-5 / IFN- γ was found to be lower in offspring of *M. vaccae*-immunized OVA-sensitized and non-sensitized mothers on the 2nd day of life when compared to OVA-sensitized groups non-immunized and controls (Fig. 3).

Cytokine Production of offspring on the 28th Day

Cytokine levels of splenocytes of 4-week-old offspring were determined in order to explore the effect of *M. vaccae* immunization of mothers during pregnancy. In offspring of *Mycobacterium vaccae* immunized healthy pregnant (*M. vaccae*) group, a significant decrease in levels of IL-5 levels were detected when compared to controls on the 4th-week of life ($p < 0.05$) (Fig. 5A). Also

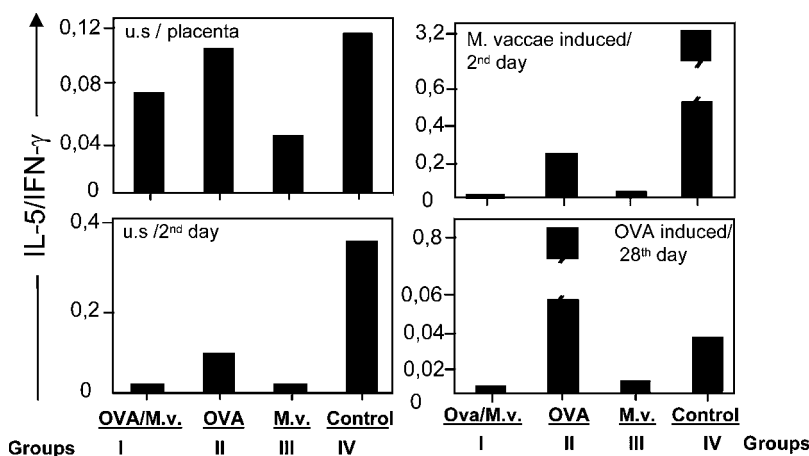


Figure 3: IL-5/IFN- γ ratio of placental lymphocytes of mothers and splenocytes of offsprings. IL-5 / IFN γ ratio was lower in the *M. vaccae* immunized and *M. vaccae* immunized OVA-sensitized groups when compared to the OVA-sensitized and non-sensitized, non-immunized controls both in placental lymphocytes of mothers and splenocytes of offsprings on the 2nd and 28th days of life.

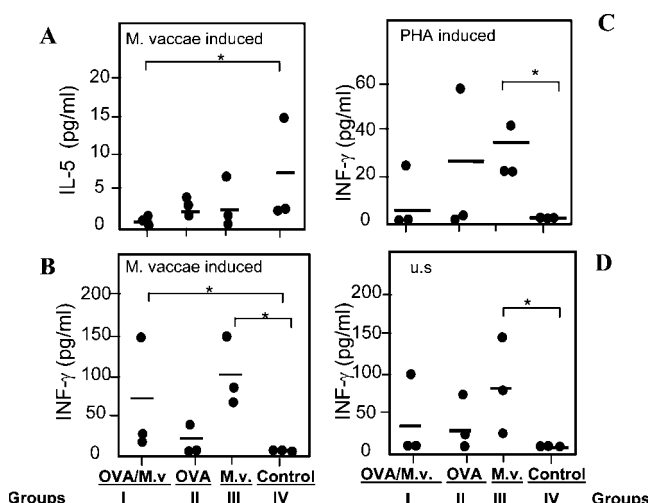


Figure 4: Comparison of cytokine production from splenocytes of offsprings on the 2nd day of life. Splenocytes were obtained on the second day of life from newborns of the 4 groups of pregnant mice. Splenocytes were cultured with *M. vaccae*, OVA and PHA and cytokine levels were determined in cultured and unstimulated supernatants. A. *OVA+ *M. vaccae* group vs. controls $p < 0.05$. B. **M. vaccae* group vs controls $p < 0.05$. C. *OVA + *M. vaccae* group vs controls $p < 0.05$. **M. vaccae* group vs controls $p < 0.05$. D. *M. vaccae* group vs controls $p < 0.05$.

offspring of *M. vaccae* immunized-OVA sensitized (OVA + *M. vaccae*) group demonstrated an enhanced PHA-stimulated IFN- γ levels compared to those of healthy controls ($p < 0.05$) (Fig. 5B). The ratio of IL-5/IFN- γ of offspring whose

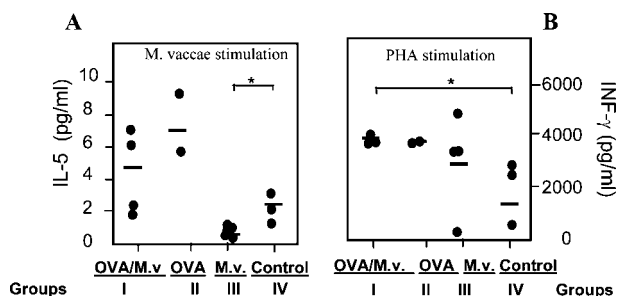


Figure 5: Comparison of IL-5 and IFN- γ production from splenocytes of offsprings on the 28th day of life. Splenocytes were obtained on the second day of life from newborns of the 4 groups of pregnant mice. Splenocytes were cultured with *M. vaccae*, OVA and PHA and cytokine levels were determined in cultured and unstimulated supernatants. A. **M. vaccae* group vs controls $p < 0.05$. B. *OVA+ *M. vaccae* group vs controls $p < 0.05$.

mothers had been immunized by *M. vaccae*, were lower when compared to the non-immunized group (Fig. 3).

DISCUSSION

The findings in this study demonstrated that a single dose of heat-killed *M. vaccae* immunization administered to OVA-sensitized Balb/c mice during pregnancy was able to alter the cytokine secretion from splenocytes of offspring away from Th2 profile by up-regulating IFN- γ and downregulating IL-5 production.

The initial event responsible for the development of allergic diseases is the generation of allergen-specific CD4⁺ T helper cells. Under the influence of interleukin 4 (IL-4), naive T cells activated by antigen-presenting cells (APC) differentiate into Th2 cells. Th2 cells produce IL-4, IL-5 and IL-13, which are responsible from many stigmata of the disease, including high IgE production, eosinophil and mast cell activation.^(3,12,13) Intrauterine Th2 cells and cytokines predominance has been shown to be an important factor determining the survival of the fetus in maternal uterus for preventing fetal allograft rejection.⁽¹⁴⁾

It is well known that expression of the allergic phenotype depends on the genetic predisposition and environmental factors. Environmental factors may influence the differentiation of allergen-specific T cells into Th-2 cells by acting both before and after birth. Several studies showed that in utero, environment play important role as an initial step towards Th2 development of allergic phenotype.⁽¹⁵⁾ Moreover, maternal inheritance is considered as an important factor on the development of asthma in the offspring.⁽¹⁶⁾ In human, an intense Th1 stimulus, such as Bacillus Calmette-Guérin (BCG) vaccination right after birth has been shown to reduce the prevalence of atopy compared to non-vaccinated newborns.^(17,18) Previously, our group demonstrated that

immunization of newborn mice with a fast growing saprophytic mycobacteria, *M. vaccae*, prevented allergic and asthmatic manifestations more efficiently than BCG in murine models of asthma.^(4,6)

Therefore, in the current study, we immunized mice during pregnancy by a Th1 inducer in order to dampen the Th2 dominance of offspring early in life. For this purpose, we chose to use *M. vaccae*, a saprophytic mycobacteria, ubiquitously present in the environment. In animal models of asthma, mycobacterial antigens have been shown to directly suppress allergic response. Wang and Rook were the first to demonstrate that heat-killed *M. vaccae* treatment inhibited not only serum IgE, but also ovalbumin-induced IL-5 production by spleen cells.⁽¹⁹⁾ Zuany et al. indicated that the immunological mechanisms behind the inhibitory effects of *M. vaccae* might be much more complex than is predicted by Th1-Th2 paradigm. They showed that the subcutaneous treatment with *M. vaccae* was able to reduce symptoms of allergic pulmonary inflammation through the induction of immunotherapy mechanisms such as the development of IL-10 secreting CD4⁺CD45RB^{low} Tregs.⁽²⁰⁾ Furthermore, our findings are in part consistent with a recent study on atopic dermatitis children, which have demonstrated that administration of a single dose of *M. vaccae* stimulates a significant increase in Th1-type activity which lasts between 1 and 3 months.⁽²¹⁾

Tukenmez and coworkers have shown that preimmunization with *M. bovis* and *M. vaccae* on the first day of life in Ova-sensitized mice resulted in the suppression of IgE production in response to aerosolized allergen.⁽⁴⁾ With the hypothesis that interventions before the sensitization period would be most beneficial in terms of preventing those changes, we have demonstrated before that treatment of newborn mice with heat-killed *M. vaccae* significantly reduced the histopathologic changes in murine model of asthma.⁽⁶⁾

In the current study we observed an up-regulation of IFN- γ along with down-regulation of IL-5 production as early as the 2nd day of life, which still persisted on day 28 in the offspring of both *M. vaccae*-immunized OVA-sensitized and non-sensitized mothers. In accordance with these findings ratio of IL-5 / IFN- γ in these *M. vaccae*-immunized groups was found to be lower, on the same days of life in offspring. These results suggest the potential long-term effect of *M. vaccae* immunization which was first demonstrated by Zuany-Amorim.⁽¹⁰⁾ In that study, heat-killed *M. vaccae* induced long-term protection in a murine model of allergic pulmonary inflammation, which was shown to be antigen specific.

Our findings supports the idea that *M. Vaccae* immunization during pregnancy might be able to halt Th2-type immune response in mothers and subsequently might have transferred its protective effect to newborns by enhancing the IFN- γ secretion from placenta and newborns splenocytes that persisted up to the 4th week of life.

In conclusion, we demonstrated that one dose of *M. vaccae* immunization during pregnancy was able to modulate the immune response away from Th2 in offspring as early as the 2nd day of life with the persistence until the 28th day of life, suggesting that *M. vaccae* immunization may have a long-term prophylactic effect, which warrants further investigation.

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