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This is a postprint of an article published in

Schaub, B., Liu, J., Höppler, S., Schleich, I., Huehn, J., Olek, S., Wieczorek, G., Illi, S., von Mutius, E.
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1

2 **Maternal farm exposure modulates neonatal immune mechanisms through regulatory T**

3 **cells**

4

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19 This work was supported by Stiftung OMNIBUS, Friedrich-Baur Institut, Münchner

20 Universitätsgesellschaft (all BS) and by DFG SFB650 (J.H.).

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22 Total word count: 3283, Word count abstract: 250

23 **Abstract:**

24

25 **Background:** Cross-sectional studies suggest that maternal exposure to farming decreases the
26 risk of allergic diseases in their offspring. The potential underlying immunological mechanisms
27 are not understood.
28 **Objective:** To assess whether maternal farm exposure activates regulatory T-cells (Tregs) in
29 cord blood exerting Th2-suppressive effects following microbial stimulation.
30 **Methods:** Eighty-four pregnant mothers were recruited before delivery. Detailed questionnaires
31 (60 non-farming/22 farming mothers, 2 exclusions) assessed the farming exposures. Cord blood
32 was stimulated (microbial stimulus peptidoglycan (Ppg), mitogen phytohemagglutinin (PHA),
33 house dust mite-extracts (Derp1), combinations). Tregs ($CD4^{+}CD25^{high}$, intracellular FOXP3-
34 expression, FOXP3, LAG3 mRNA-expression, functional studies, DNA-methylation of the
35 *FOXP3*-locus), proliferation and Th2/Th1/Th17-cytokines were examined.
36 **Results:** Cord blood Tregs ($CD4^{+}CD25^{high}$ T-cells, unstimulated, PHA-stimulated) were
37 increased with maternal farming exposures associated with higher FOXP3 (Derp1+Ppg-
38 stimulation) and trendwise higher LAG3-expression (Ppg). Furthermore, Treg function was
39 more efficient with farming exposure (effector cell suppression, $p=0.004$). In parallel, Th2
40 cytokines (IL-5) were decreased, associated with decreased lymphoproliferation and increased
41 IL-6 (Ppg and/or Derp1+Ppg-stimulation, respectively, $p<0.05$). Maternal exposure to
42 increasing numbers of farm animals and stables were identified to exert distinct effects on
43 Tregs and/or Th1/Th2 cells. Additionally, FOXP3-demethylation in offspring of mothers with
44 farm milk exposure was increased ($p=0.02$).
45 **Conclusions:** Pregnancy farm exposure increases the number and function of cord blood
46 regulatory T-cells associated with lower Th2-cytokine secretion and lymphocyte proliferation
47 upon innate exposure. One fascinating speculation is that maternal farm exposure may reflect a

48 “natural model of immunotherapy” potentially including a selection of innate stimuli in
49 addition to allergen, shaping a child´s immune system at an early stage.
50

51 **Key messages:**

52 - Pregnancy farm exposure increases T regulatory cell number and function
53 - Maternal farm exposure may reflect a “natural model of immunomodulation” shaping a
54 child´s immune system in early life
55

56 **Capsule summary:**

57 Pregnancy farm exposure increases cord blood regulatory T-cells associated with lower Th2-
58 cytokine secretion upon innate microbial exposure. Maternal farming exposure may be
59 represent “a natural model of childhood immune modulation” relevant for protection of allergic
60 diseases.
61
62

63 **Keywords:** Cord blood; cytokines; farming, innate; interleukin; methylation; microbial; T
64 regulatory cells; Toll-like receptor.
65

66 **Abbreviations:** CBMC, cord blood mononuclear cells; D, Dermatophagoides pteronyssinus 1;
67 FOXP3, Forkhead/winged-helix family transcriptional repressor p3;
68 F, farming mothers; GITR, Glucocorticoid-induced tumor necrosis factor receptor;
69 IQR, interquartile range; LAG3, Lymphocyte activation gene 3; LP, Lymphocyte proliferation;
70 NF, non-farming mothers, OR, Odd´s Ratio; Ppg, Peptidoglycan; PHA, phytohemagglutinin;
71 SD, standard deviation; SI, stimulation index; TLR, Toll-like receptor; Tregs, regulatory T-
72 cells.

73 **Introduction**

74 One promising natural model of "allergy-protection" has been demonstrated by farm exposure.
75 Children growing up on a farm were shown to have a decreased risk of developing allergic
76 diseases in later child- and adulthood [1]. Several cross-sectional studies have reproduced the
77 findings that exposure to stables, barns and consumption of farm milk protects children against
78 the development of atopic diseases (e.g. ALEX, PARSIFAL study) [2]. Furthermore, the
79 exposure to an increasing number of animal species was associated with innate Toll-like
80 receptor (TLR) gene expression [3]. In this context, maternal farm exposure in pregnancy seems
81 to be critical for the effects in their offspring [3]. Clearly, the influence of farming on early
82 immune development seems to play an important role, promoting the idea to examine infant
83 immune responses at the earliest possible time, namely in cord blood.
84
85 Regarding immune modulation in the offspring, one concept of "allergy-protection" is that farm
86 exposure reflects microbial exposure inducing innate immune mechanism contributing to a less
87 allergic "anti-Th2"-immune phenotype. This process likely involves upregulation of different
88 innate receptors (e.g. TLR) [4]. For example, upregulation of TLR2-expression has been shown
89 in farm children at school age [4], and TLR2/TLR4-upregulation was strongly determined by
90 maternal exposure to stables during pregnancy [3]. These human data were supported by murine
91 studies, demonstrating decreased allergic responses following TLR2/TLR4 stimulation [5].
92 However, the specific immunological mechanisms are not well determined.
93
94 Regulatory T-cells (Tregs), which play an important role in the balance and maturation of T-
95 cell polarization, are one promising candidate contributing to this effect early in life. Neonatal
96 Tregs with a naive phenotype have been described to exert a potential immunoregulatory role in
97 intrauterine life [6, 7]. To date, Tregs are best characterized by FOXP3-expression, whose
98 transcription is fundamental for both differentiation and function [8], complemented by additional

99 markers such as CD25, GITR, LAG3 or low CD127 expression [9-11]. A default regulation or
100 reduced number of $CD4^+CD25^+$Tregs may contribute to the development or progression of
101 allergy [12-14]. Indeed, successful treatment of allergy by allergen-specific immunotherapy may
102 depend on induction of Tregs[6, 15].
103
104 The aim of this study was to investigate the immunological mechanisms contributing to this
105 natural model of "allergy- protection", namely pregnancy farm exposure. Specifically, we
106 aimed to examine which exposure may influence neonatal immune development. Next, we
107 assessed whether offspring of mothers exposed to farming during pregnancy have
108 quantitatively and qualitatively different Tregs as compared to offspring of non-farming
109 mothers. We further investigated whether this change would result in different Th1/Th2-
110 effector cell responses and lymphocyte proliferation. These experiments were performed using
111 different *in vitro*-stimulations such as microbial TLR-ligand and allergen exposure.

112

113 **Methods**

114

115 **Study population**

116 We recruited pregnant mothers in an obstetric clinic in rural southern Germany in the frame of
117 a birth-cohort study (PAULCHEN). The population is representative of the subjects involved in
118 previous farm studies [1, 3]. Study enrolment through trained midwives occurred from July 2005
119 to September 2007 in the last trimester of pregnancy. Inclusion criteria comprised healthy
120 neonates and mothers with uncomplicated pregnancy. Exclusion criteria included preterm
121 deliveries, perinatal infections, maternal use of antibiotics in the last trimester and chronic
122 diseases. From 84 mothers enrolled in the study, 82 cord blood samples (97%) were included in
123 the study. Two subjects were excluded due to perinatal infections. Mothers completed a
124 detailed questionnaire regarding rural lifestyle including detailed farming exposures [3]. Maternal
125 farm exposure was defined as the mother living and regularly working on a farm in the last 1-5
126 years and during pregnancy. Non-farming mothers lived in the same rural area but not on a
127 farm. Specific exposure to stable/ barn, animal species and milk intake were documented
128 during pregnancy. Potential covariates including delivery mode, gender, birth characteristics,
129 siblings, education, maternal atopy, smoking and miscarriage were determined by
130 questionnaire. Informed consent was obtained from the mothers for participation in the study,
131 including cord blood collection. Approval was obtained from the local human research
132 committee of the Bavarian Ethical Board, LMU Munich, Germany.
133 For details of methods see Repository.
134

135

136 **Lymphocyte proliferation, cytokine secretion**

137 Cord blood mononuclear cells (CBMC), freshly isolated within 24 hours, were stimulated with
138 peptidoglycan (Ppg 10µg/ml), phytohemagglutinin (PHA 5µg/ml) and Dermatophagoides
139 pteronyssinus1 (Derp1 30µg/ml) or a combination of Derp1 and Ppg (Derp1+Ppg) for 3 days
140 and compared to unstimulated cells [16, 17]. Dose-response and time curves were assessed in
141 optimization experiments. Significant changes through endotoxin were excluded by functional
142 assays (Repository), apoptosis was not-significantly different in farm/non-farm exposed
143 neonates in preliminary experiments. After incubation with ^{3}H-Thymidine for 8 hours, cells
144 were analyzed for lymphocyte proliferation, assessed by counts per minute (cpm), quantified by
145 stimulation index (SI), representing the ratio of mean cpm of stimulated/unstimulated
146 replicates. Cytokine concentrations were measured in supernatants using the Human Cytokine-
147 Multiplex-Assay-Kit according to the manufacturer's instructions (Bio-Rad, Munich, Germany)
148 by LUMINEX-technology.
149

150 **Flow cytometry, functional analysis of Tregs**

151 Cells were analyzed using 3-color flow cytometry (FACScan, Becton-Dickinson, Germany).
152 For surface staining, 2µl anti-human CD4-FITC, CD25-RPE-Cy5, 1µl IgG1-FITC (Dako
153 Cytomation, Denmark), 0.5µl IgG2a RPE-Cy5 (BD Biosciences, Pharmingen, Germany) was
154 added. For intracellular FOXP3 staining, 8µl anti-human CD4-FITC, 4µl anti-human CD25-
155 RPE-Cy5 antibodies ($1x10^{6}$/100ml) were used, cell permeabilization performed, and FOXP3-
156 PE/corresponding isotype-control antibodies added. Data were analyzed with CellQuest
157 software (Becton Dickinson, Germany), postacquisition analysis was performed with WinMDI
158 2.8 software (Becton Dickinson, Mountain View, CA, USA)(Fig.E1). Regarding functional
159 Treg studies, $CD3^{-}$ cells were isolated (CD3 isolation-kit, Miltenyi Biotec, Germany) and
160 irradiated. $CD4^{+}CD25^{-}$/$CD4^{+}CD25^{+}$T-cells were isolated (two-step-procedure), using depletion
161 of non-$CD4^{+}$cells, followed by positive selection of $CD4^{+}CD25^{+}$T-cells (Miltenyi Biotec,

162 Germany). $CD4^+CD25^-$T-cells (2×10^4/well), labeled with 5µM CFSE (Invitrogen, Germany),
163 were incubated for 3 days with irradiated $CD3^-$ cells in coculture with/without $CD4^+CD25^+$T-
164 cells before/after stimulation with 0.8µg/ml PHA. Division and proliferation of $CD4^+CD25^-$T-
165 cells were assessed by flow cytometry and ^{3}H-thymidin incorporation, respectively; control
166 experiments showed no significant division/proliferation of CD4+CD25+ cells; cytokine
167 concentrations were measured in supernatants using the Human Cytokine-Multiplex-Assay-Kit
168 (Bio-Rad, Germany).
169

170 **Real-time quantitative RT-PCR**

171 Total RNA, isolated with TRI-Reagent, was processed with reverse transcriptase (Invitrogen,
172 Germany). mRNA-specific oligonucleotide primer pairs were designed (Vector NTI-
173 Advance10). Direct detection of the PCR-product was monitored by measuring the increase in
174 fluorescence caused by binding of SYBR-Green to dsDNA. The determined threshold cycle
175 (CT) was set relative to the amplification plot of 18SrRNA. CT describes the number of PCR-
176 cycles required for the fluorescence signal to exceed the detection threshold, which was set to
177 the log-linear range of the amplification curve. The difference in CT-values relative to 18S was
178 used to calculate the fold difference. The formula 2^{-ddct} was applied. Data compare the relative
179 increases compared to unstimulated samples [18].
180

181 **DNA bisulfite conversion and FOXP3-methylation specific real-time-PCR**

182 Genomic DNA was isolated using the Dneasy blood-kit (Qiagen, Hilden, Germany). Bisulfite
183 treatment of genomic DNA was performed [19]. Real-time-PCR for measurement of FOXP3
184 Treg-specific demethylated region (TSDR) [20] was performed in 20µl using Roche
185 LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany) containing
186 15pmol of methylation or non-methylation-specific forward and reverse primers for TSDR,
187 5pmol hydrolysis probe, 200ng lambda-DNA (New England Biolabs, Frankfurt, Germany) and

188 30ng bisulfite-treated genomic DNA template or respective amount of plasmid standard. Each

189 sample was analyzed in triplicate using a Light-Cycler 480 System.

190

191 **Statistical analysis**

192 Data were generally not normally distributed and not all data could be transformed to

193 normality. Non-detectable cytokine concentrations were assigned to 0.01pg/ml for inclusion

194 into the analysis. Non-parametric tests (Mann-Whitney, Wilcoxon) were used to compare the

195 median of gene expression, proliferation and cytokine concentrations; parametric tests were

196 used for normally-distributed data (surface expression). Data were reported as mean±SEM or

197 median±IQR depending on the distribution. Treg suppression was assessed by comparing

198 multiple/two groups with ANOVA and t-test. Spearman's correlation was used to assess

199 associations between gene-expression and cytokine secretion. Chi2-tests were used to evaluate

200 categorical predictor variables. Linear and logistic regression analyses were calculated, and

201 included potential confounders such as maternal smoking, education. Odds Ratios (OR) were

202 reported depending on regression analysis. Statistical significance was defined by $p<0.05$. Data

203 analysis was performed with SAS 9.1, and SigmaStat 3.5 software.

204 **Results**

205 **Population characteristics**

206 Table 1 shows the characteristics of 82 included subjects (22 neonates of farming, and 60 of
207 non-farming mothers). Farming mothers smoked less and showed a trend to lower education
208 compared to non-farming mothers (p=0.04/0.06). Both factors were tested for potential
209 confounding, but revealed to be no confounders. Maternal atopy was lower in farming mothers,
210 however non-significantly.

211

212 **Regulatory T-cells were quantitatively and qualitatively increased in cord blood of**
213 **farming mothers**

214 We assessed regulatory T-cells by measuring the surface expression of $CD4^{+}CD25^{high}$ T-cells
215 (% of CBMC), in parallel expressing FOXP3 and low CD127, and examining Treg-markers
216 including FOXP3, GITR, LAG3 on mRNA-level. Treg-markers were increased following PHA
217 and Ppg-stimulation ($p<0.05$, data not shown). Comparing cord blood of farming versus non-
218 farming mothers, $CD4^{+}CD25^{high}$ T-cells, FOXP3 and trendwise LAG3 mRNA, but not GITR
219 were increased in offspring of farming mothers before or after PHA-, Derp1+Ppg- or Ppg-
220 stimulation, respectively (Fig. 1, Repository). Tregs are known to be increased in cord blood of
221 non-atopic compared to atopic mothers [17.]. We detected higher Tregs in CBMC (unstimulated
222 and PHA-stimulated) of non-atopic farming compared to non-farming mothers. We next tested
223 whether Tregs of farming children were also functionally more efficient by assessing their
224 suppressive capacity. Only non-atopic donors were chosen. Indeed, cord blood of farming
225 children showed more efficient suppression of effector T cell division (p=0.004, Fig.2, and
226 Repository).

227

228 **Different farm exposures showed heterogenous effects on immune regulation**

229 In order to disentangle the specific stimuli in the farming environment, we examined the effect
230 of exposures to stable, barn, number of animal species and maternal milk intake on immune
231 regulation (Table 2). For analysis of distinct exposures all mothers with exposure were assessed
232 and included some "non-farming mothers". These did not work or live on farms ("non-farming
233 mothers") but visited their neighbors on farms and thus had some exposure to stable, barn,
234 animals or drank farm milk during pregnancy. While farming had an effect on several Treg
235 markers including function, distinct exposures (different sample sizes) had different effects.
236 Exposure to the stable showed an increase in $CD4^{+}CD25^{high}$ (Ppg-stimulated) and lower IL-5
237 (Derp1+Ppg-stimulated). Increased $CD4^{+}CD25^{high}$ (Ppg-stimulated) expression was correlated
238 with lower IL-5 (r=0.61), however not significant (p=0.15, data not shown). Exposure to barn
239 showed non-significant effects on Th1 cytokines. A higher number of animal species increased
240 Treg markers (FOXP3 Derp1+Ppg, GITR PHA compared to unstimulated expression), resulted
241 in higher Th1 (IFN-g Derp1 and Ppg) and a trend to lower Th2-expression (IL-5 Derp1+Ppg).
242 Comparing three groups of non-farming non-exposed, non-farming exposed to milk or animals
243 and farming mothers, primarily IFN-g (Ppg and Derp1-stimulated) was highest in the non-
244 farming exposed group (p=0.04, Repos. Table 2).
245 To further investigate whether the different exposure had an effect on epigenetic regulation of
246 FOXP3-expression, we examined the relevance of exposure on the demethylation of an
247 evolutionarily conserved element within the *FOXP3*-locus (TSDR, Treg-specific demethylated
248 region) in a subset of 59 subjects (Table 3). The selected group was similar to the whole group
249 excluding a selection bias. This locus had previously been shown by us to be selectively
250 demethylated only in FOXP3 stably expressing natural Tregs, but not in transiently FOXP3
251 expressing, recently activated effector T-cells [20, 21]. Consistently, the amount of demethylated
252 TSDR was higher with any single exposure compared to no exposure, significantly only for
253 maternal intake of farm milk during pregnancy (p=0.02). When analyzing subjects with any
254 exposure compared to no exposure, a similar trend was observed (p=0.09).

255

256

257 **Lower Th2 cytokines and proliferation after Derp1+Ppg stimulation in offspring of**

258 **farming mothers**

259 Next, we aimed to investigate whether farm exposure resulted in lower lymphocyte

260 proliferation and decreased Th2 (pro-allergic) cytokines. Indeed, proliferation of offspring from

261 farming mothers was lower as compared to non-farming mothers (Table 4, p=0.04). Regarding

262 overall T-cell cytokines, there was no significant difference in both groups (Table 4, Repository

263 Table 1). However, the pro-allergic Th2-cytokines IL-5 and IL-13 were significantly or

264 trendwise decreased in offspring of farming compared to non-farming mothers (Derp1+Ppg-

265 stimulation). IL-6 was higher in offspring of farming mothers (Derp1+Ppg stimulation, p=0.01;

266 Derp1-Repository p=0.05). Of note, subset analysis revealed that IL-6 and IL-5 were expressed

267 by CD4/CD8 T- and to low percentage by NK cells, IL-13 primarily by CD4/CD8 T cells

268 besides CD3- cells (Repository).

269 When correlating Treg-markers with Th1/Th2-cytokines, in offspring of farming mothers cord

270 blood FOXP3-expression was positively correlated with higher IFN-γ levels following Ppg or

271 Ppg+Derp1 stimulation (r=0.51, p=0.047; r=0.81, p=0.01), and negatively correlated with IL-13

272 or IL-5 expression following Ppg or Ppg+Derp1 stimulation, respectively (r=-0.72, p=0.02; r=-

273 0.52, p=0.18). In non-farming CBMC, no significant correlations were observed apart from

274 FOXP3/IL-13 expression being negatively correlated following Ppg stimulation (r=-0.47,

275 p=0.002).

276

277 **Correlation between IL-17 and IFN-γ**

278 A T-cell population closely linked to Tregs, namely Th17 cells, can also influence Th1/Th2

279 responses [22]. IL-17 secretion was undetectable in unstimulated and PHA-stimulated samples

280 (Table 4). However, following innate and allergen stimulation, IL-17 secretion was weakly

281 increased, but not significantly different between the two groups. The correlation of IL-17 and
282 IL-13 secretion (Th2) was positive following Ppg stimulation (r=0.75/0.79), independent of
283 farming (Table 5). However, the correlation between IL-17 and IFN-γ (Th1) was significantly
284 positive (r>0.6), but only among offsprings of farming mothers following PHA/Ppg
285 stimulation.
286
287
288

289 **Discussion**

290 This study suggests a mechanism by which the protective effect of farming exposure in
291 pregnancy may influence neonatal immune development and could subsequently reduce the
292 development of allergic diseases. Regulatory T-cells, comprehensively assessed in number,
293 gene expression, epigenetic regulation and function were mainly higher and more efficient in
294 offspring of farming compared to non-farming mothers following specific stimulation. This was
295 associated with decreased Th2 cytokines and lower proliferation in offspring of farming
296 mothers. Exposure to several animal species had an effect on Treg markers and IFN-g
297 secretion, while exposure to stable and milk intake showed heterogenous effects with an
298 incomplete overlap suggesting diverse additive effects. In addition, Th17 cells were regulated
299 independently of farming status, but were positively correlated with Th1 lineages primarily in
300 offspring of farming mothers.

301

302 Several farming studies have reproduced the findings that farming exposure is protective
303 against the development of atopic disease [1, 2]. Two factors seem to be particularly important for
304 this effect. First, early prenatal influences on immune modulation have the strongest effect on
305 reducing later atopic development, and second, microbial exposure has been identified as a
306 crucial "entity of allergy-protective exposure" [1, 2]. These observations suggest that influences of
307 the environment modulate this very sensitive period of fetal immune development in such a
308 strong way [3] that it results in long-term immune effects, either through direct or indirect impact
309 on the maternal immune milieu, maternal-fetal immune interactions or quite likely a
310 combination of both. The early long-lasting immune effect is supported by the increase of
311 innate receptors including TLR2 and CD14 at school age in particular when exposure to stable
312 was present in pregnancy [3]. While it is remarkable that effects in utero are capable of inducing a
313 long-lasting immune modulation, the underlying mechanism is not understood.

314

315 Our study potentially adds a novel immunoregulatory mechanism by which maternal exposure
316 to this “natural model of allergy-protection” can modulate Tregs already before birth indirectly
317 reflecting early immune development. The data suggest that Tregs assessed in number and gene
318 expression may be also functionally more efficient in early immune modulation in offspring of
319 farming mothers. In addition, data on FOXP3-demethylation - primarily for milk exposure -
320 point also into this direction. While the trendwise difference in this study requires confirmation,
321 this novel “epigenetic candidate” suggests early epigenetic immune regulation through specific
322 stimulation during pregnancy.
323 Importantly, the Treg effects in this study were not only associated with more efficient
324 functional suppression in the context of farm exposure but was also associated with effector T-
325 cell modulation such as a decrease of the Th2 cytokine IL-5. This shift to an anti-Th2 “allergy-
326 protective” immune pattern was present at birth, but requires specific stimulatory conditions.
327 We chose the stimulatory conditions to mimick a part of natural farm exposure using either
328 innate stimulation (Ppg) or a combination with allergen, which may in fact reflect *in vivo*
329 exposures of the farming environment more closely than one single experimental stimulus,
330 which does not occur in real life. Indeed, stimulation with Ppg in combination with allergen
331 showed the strongest effects. Of note, LPS content in our reagents was low, and did not
332 influence the findings.
333 Murine neonatal data support the hypothesis that appropriate exposure in early life is required
334 to prevent airway allergic inflammation. Wang et al. have demonstrated that prevention of
335 airway allergy may be best achieved by specific exposure (LPS and allergen) of the airway
336 mucosa early in life to environmental antigens potentially reflecting early mucosal tolerance [23].
337 Consequently, one may speculate that subsequent development of allergic diseases results from
338 an “early immune deficiency to respond adequately to specific stimuli” due to a lack of
339 exposure, in our case microbial farm exposure.

340 Regarding the type of microbial exposure, the exposure to an increasing number of animal
341 species may potentially indicate an incremental dose-response effect contributing to more
342 pronounced immune stimulation early in life. Although we could not disentangle one specific
343 exposure as indicative of Treg induction, distinct exposures may reflect exposure to a variety of
344 microbes reflecting non-specific stimulation. Intriguingly, some effects were not restricted to
345 farming mothers. Offspring of non-farming mothers, which did not live and work on farms but
346 were occasionally exposed to farm animals or farm milk showed e.g. higher IFN-g
347 concentrations than any other group. While overall effects are still strongest in the offspring of
348 farming mothers, these data –though in small numbers- suggest a potential immunomodulatory
349 effect even present for non-continuous exposure.

350

351 While Th2 responses were decreased in offspring of farm-exposed mothers, one may expect
352 either a parallel decrease in Th1 through general suppression or an additional increase in Th1
353 cytokines reflecting selective regulation. Only animal exposure showed an increased Th1-
354 response but not "overall farming". This may be explained by the requirement of a certain level
355 of stimulation, no direct effect on Th1-lineage or more complex regulatory features than
356 Th2/Th1 pathways in "overall farming". Supporting the latter, promising candidates are Th17
357 cells, which we previously demonstrated to be immature at birth compared to adult immune
358 responses [24]. Thus, no significant differences between farmers and non-farmers children may be
359 due to low IL-17 secretion at this early stage of maturation. However, the positive correlation
360 primarily with Th1-cells in the context of farming may account for a role of Th17-cells in
361 balancing the immune system during maturation, in close association with Th1-lineages. In
362 parallel with our study, Th17/Th1 correlations were also described in Crohn´s disease [25]. While
363 Th17 cells are shown to be involved in atopic dermatitis [26], they are a promising candidate in
364 the regulation of atopic diseases. However, these hypotheses have to be confirmed in larger
365 studies.

366

367 Finally, one fascinating speculation is that farm exposure may reflect a "natural model of
368 immunotherapy" potentially including a selection of innate stimuli in addition to allergen,
369 shaping a child's immune system at an early stage. In the conventional immunotherapy
370 strategies a protective effect may be mediated through regulatory T-cells [6, 27, 28]. This
371 hypothesis clearly requires further investigation, but would offer some consideration for future
372 preventive strategies.

373

374 In summary, this study proposes a novel mechanism, by which maternal farm exposure
375 modulates offspring immune responses. Regulatory T-cells in cord blood were increased in
376 number and function in this unique model of "*in-vivo* allergy-protection" and resulted in
377 suppression of pro-allergic immune responses such as Th2-cytokines associated with lower
378 lymphoproliferation. This regulatory T-cell population keeping healthy immune responses in
379 balance and thus preventing disease development may have the potential to contribute to the
380 "long-lasting allergy-protective effect" of Tregs in older children and adults [29] [13, 30, 31] [23, 32].
381 However, only a future follow-up of these children until the age of 6 years can determine
382 whether this "neonatal immunological starting condition" is able to shape the immune
383 regulation in such a way to truly protect against the development of atopic disease.

384

385 **Acknowledgments**

386 We thank all families for study participation, and the midwifes for the participation in the
387 study. We thank the lab facilities of the Children's Hospital including Prof. Belohradskys
388 laboratory for their ongoing support.

389

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475

476 **Table 1**: Characteristics of the study population

Parameters	Non-farming CBMC N=60	Farming CBMC N=22	P*
Maternal age (years): Median (± IQR)	31.0 (27.0/34.5)	30.5 (24.0/34.0)	0.33#
Maternal smoking (n, %)			
No	**29 (48.3)**	**18 (81.8)**	
Yes, during pregnancy	**6 (10.0)**	**0 (0)**	
Yes, until pregnancy	**8 (13.3)**	**2 (9.1)**	
Yes, only before pregnancy	**17 (28.3)**	**2 (9.1)**	**0.04**
Maternal education: n (%)			
Elementary school	15 (25.0)	8 (36.4)	
High school	22 (36.7)	12 (54.5)	
Grammar school	12 (20.0)	0 (0.0)	
University	11 (18.3)	2 (9.1)	0.06
Maternal atopic diseases: (n, %)			
Total	19 (31.7)	5 (22.7)	0.43
Asthma	3 (5.0)	2 (9.1)	0.61
Hay fever	14 (23.3)	2 (9.1)	0.21
Atopic eczema	4 (6.7)	1 (4.5)	1.0
Maternal serum total IgE (IU/ml): Median (± IQR)	34.3 (12.9/121.9)	28.3 (9.7/62.0)	0.40#
Vaginal delivery: n (%)	52 (86.7)	17 (77.3)	0.32
Neonatal gender: male (n, %)	32 (53.3)	10 (45.4)	0.53
Gestational age (Weeks): Median (± IQR)	40.0 (39.7/40.4)	40.1 (39.0/40.9)	0.77#
Birth weight (g): Median (± IQR)	3555 (3305/3865)	3575 (3220/3820	0.99#
Birth length (cm): Median (± IQR)	52 (50/53)	52 (50/53)	0.96#
Siblings: n (%)			
0	30 (50)	10 (45.4)	
1	19 (31.7)	4 (18.2)	
2	5 (8.3)	5 (22.7)	
3	4 (6.7)	3 (13.6)	
4	2 (3.3)	0 (0)	0.24

477 *p: Comparison between farming and non-farming group (# wilcoxon rank sum test;
478 otherwise Chi2-test). IQR: interquartile range.

Table 2: Association of maternal exposure in pregnancy and immunological parameters in cord blood of their offspring. Bivariate analysis for each exposure separately (Median, p-values).

Variable		**Stable N=33/82 (Yes)** Median, p-values		**Barn N=20*/82 (Yes)** Median, p-values		**Farm milk N= 20*/82 (Yes)** Median, p-values		**Number of animal species (0-1, ≥2) N=40/82 (≥2)** Median, p-values	
Treg markers									
$CD4^{+}CD25^{high}$ U (%)	No Yes	1.2 1.6	0.08	1.3 2.0	0.53	1.2 1.6	0.09	1.2 1.7	0.48
$CD4^{+}CD25^{high}$ PHA (%)	No Yes	1.7 2.0	0.10	1.9 1.9	0.83	1.9 2.0	0.37	1.9 1.9	0.92
$CD4^{+}CD25^{high}$ Ppg (%)	No Yes	0.5 1.5	**0.01**	1.0 1.4	0.37	1.1 1.4	0.65	1.0 1.3	0.48
FOXP3 Derp1+Ppg (fd)	No Yes	1.4 2.5	0.17	1.6 4.3	0.09	1.4 2.7	0.06	1.4 2.9	**0.04**
GITR PHA (fd)	No Yes	4.0 7.7	0.37	4.0 8.3	0.13	4.0 7.3	0.65	4.0 11.7	**0.03**
LAG3 Ppg (fd)	No Yes	2.7 3.3	0.21	2.4 3.7	0.11	2.6 3.7	0.11	2.3 3.1	0.38
Cytokines									
IL-10 U	No Yes	0.01 0.08	0.07	0.01 0.08	0.26	0.01 0.10	0.09	0.01 0.09	**0.05**
IFN-g U	No Yes	0.08 0.01	0.60	0.08 0.01	0.26	0.45 0.01	0.10	0.01 0.77	0.33
IFN-g Derp1	No Yes	47.7 81.1	0.10	48.6 88.2	0.07	47.7 74.4	0.10	46.4 97.8	**0.002**
IFN-g Ppg	No Yes	47.0 69.0	0.21	50.2 95.1	0.06	47.8 74.4	0.10	46.3 97.8	**0.02**
IL-13 Derp1+Ppg	No Yes	52.8 27.9	0.12	51.8 25.2	0.07	43.2 28.5	0.55	42.2 34.8	0.57
IL-5 Derp1+Ppg	No Yes	49.2 31.9	**0.04**	48.8 30.6	0.10	40.1 38.4	0.13	48.9 28.6	**0.05**

Stable, barn, farm milk =exposure to the stable, barn, farm milk during pregnancy. U= unstimulated, PHA =mitogen, Ppg= microbial stimuli peptidoglycan, Derp1= allergen house dust mite. Cytokines are shown as pg/ml, gene expression of FOXP3, GITR and LAG3 as fold difference (fd). * n=20 are not the identical subjects for barn and farm milk. P-values were determined with Wilcoxon test.

487

488 **Table 3:** FOXP3 Demethylation (%) in cord blood in relation to different farming

489 exposures. Bivariate analysis for each exposure separately (min, quartiles, Median,

490 max, p-values).

	N	Min	1.Quart	Median	3.Quart	Max	P (Wilcoxon)
Farm child							
No	39	0.6	1.2	1.6	2.1	3.3	0.38
Yes	20	0.8	1.5	1.8	2.3	3.0	
Preg stable							
No	34	0.6	1.3	1.6	2.0	3.1	0.25
Yes	25	0.8	1.4	1.8	2.3	3.3	
Preg barn							
No	43	0.6	1.2	1.6	2.1	3.3	0.21
Yes	16	0.9	1.6	1.8	2.4	3.0	
Preg fmilk							
No	40	0.6	1.3	**1.6**	1.9	3.1	**0.02**
Yes	19	0.8	1.6	**2.0**	2.4	3.3	
Number of animal species							
0-1	43	0.6	1.3	1.6	2.1	3.1	0.41
≥2	16	0.9	1.5	1.8	2.3	3.3	
Any exposure							
No	32	0.6	1.2	1.6	1.9	3.1	0.09
Yes	27	0.8	1.4	1.8	2.3	3.3	

491 Pregstable, Pregbarn, Pregfmilk =exposure to the stable, barn or farm milk during

492 pregnancy.

493

494 **Table 4:** Lymphocyte proliferation (LP) and cytokine concentrations in CBMC of
495 offspring of non-farming and farming mothers after various stimulations.
496

Para-meter	Stimuli	Non-farming CBMC (Median±IQR) N=59	Farming CBMC (Median±IQR) N=21	P *
LP	U	3800 (2866/5973)	3562 (2201/6268)	0.47
	PHA	61573 (43624/94685)	64894 (44357/91877)	0.96
	Ppg	**7591 (4759/10659)**	**4423 (2899/8080)**	**0.04**
	Derp1+Ppg	**7899 (6090/11424.)**	**3830 (2491/7298)**	**0.04**
IFN-γ	U	0.1 (0.01/1.8)	0.01 (0.01/0.8)	0.27
	PHA	58 (17.7/100)	76 (29/202)	0.31
	Ppg	50 (31/119)	74 (33/128)	0.36
	Derp1+Ppg	107 (54/155)	120 (52/157)	0.71
IL-13	U	0.33 (0.01/0.8)	0.2 (0.2/0.6)	0.97
	PHA	93 (4/734)	31 (7/2013)	0.99
	Ppg	37 (18/59	22 (13/67)	0.70
	Derp1+Ppg	52 (28/64)	25 (16/41)	0.10
IL-5	U	0.01 (0.01/0.02)	0.01 (0.01/0.1)	0.61
	PHA	18 (3/36)	8 (3/36)	0.85
	Ppg	39 (19/74)	25 (15/50)	0.17
	Derp1+Ppg	**49 (32/106)**	**23 (16/39)**	**0.02**
IL-10	U	0.01 (0.01/0.1)	0.1 (0.01/0.1)	0.09
	PHA	79 (25/130)	60 (30/151)	0.89
	Ppg	1053 (772/1582)	1030 (802/1490)	0.58
	Derp1+Ppg	1978 (1251/2516)	1627 (1115/2469)	0.50
IL-6	U	3.6 (1.3/14.0)	4.8 (1.9/26.9)	0.60
	PHA	26028 (17620/35433)	34000 (21876/41998)	0.25
	Ppg	29893 (25205/43876)	34000 (26453/51814)	0.61
	Derp1+Ppg	**34100 (22575/40967)**	**64536 (34000/111784)**	**0.01**
IL-17	U	0.01 (0.01/0.4)	0.01 (0.01/0.5)	0.97
	PHA	0.01 (0.01/0.2)	0.1 (0.01/0.7)	0.25
	Ppg	3.3 (1.2/8.8)	1.5 (1.1/8.8)	0.35
	Derp1+Ppg	5.0 (1.4/7.9)	2.7 (1.7/9.8)	0.39

497 P: Comparison between non-farming and farming CBMC (wilcoxon rank sum test).
498 LP in cpm (Median ± IQR), Cytokines in pg/ml. IQR: interquartile range, U
499 (unstimulated), Ppg (Peptidoglycan), PHA (phytohemagglutinin), Derp1+Ppg
500 (Dermatophagoides pteronyssinus+ Peptidoglycan).

501
502 **Table 5:** Correlation between IL-17 and Th1/Th2, IL-10 secretion
503

Cytokines (pg/ml)	Stimuli	Non-farming CBMC (CBMC-NF) N=59		Farming CBMC (CBMC-F) N=18	
		r	p	r	p
IL-17/IL-13	**PHA**	0.10	0.46	**0.55**	**0.03**
	Ppg	**0.75**	**<.0001**	**0.79**	**0.0001**
IL-17/IFN-γ	**PHA**	0.13	0.34	**0.83**	**0.0001**
	Ppg	0.26	0.05	**0.61**	**0.01**
IL-17/IL-10	**PHA**	0.24	0.08	0.41	0.11
	Ppg	-0.09	0.51	0.23	0.37

504 Analysis using Spearman´s correlation coefficient r. PHA (phytohemagglutinin), Ppg
505 (Peptidoglycan). $P<0.05$ is significant.

506 **Figure legends:**

507

508 **Figure 1A-D:** The percentage of $CD4^{+}$CD25high T-cells (A) and expression of Treg-

509 associated genes (B-D) in CBMC of offspring of non-farming (NF) and farming mothers (F).

510 T-test was used (mean ± SEM) for surface, Mann-Whitney rank-sum test was used for gene

511 expression data (Median ± IQR and outliers). The mRNA-expression was presented as fold

512 difference of stimulated/unstimulated samples, relative to the housekeeping gene 18S. NF

513 (non-farming), F (farming), U: unstimulated; PHA: phytohemagglutinin, Ppg

514 (Peptidoglycan), Derp1+Ppg (Dermatophagoides pteronyssinus+ Peptidoglycan) (n=44

515 CBMC NF; n=19 CBMC F).

516

517 **Figure 2:** The suppressive capacity of $CD4^{+}CD25^{+}$ T reg cells on $CD4^{+}CD25^{-}$ T effector

518 cells in CBMC of non-farming (NF) and farming mothers (F). $CD4^{+}CD25^{-}$ T effector cells

519 (20000 cells/well) labeled with CFSE, cultured with irradiated $CD3^{-}$ cells (40000 cells/well)

520 and stimulated with PHA (0.8ug/ml), with/without $CD4^{+}CD25^{+}$ T reg cells

521 (20000/40000/60000 cells/well). After 72h culture, percentage of new divided $CD4^{+}CD25^{-}$ T

522 effector cells was measured with FACScan, proliferation assessed with 3H Thymidin

523 incorporation (n=7 NF/7 F). Comparison between groups was performed using ANOVA and

524 t-test (see methods).

525