

Effects of Feeding Bt Maize to Sows during Gestation and Lactation on Maternal and Offspring Immunity and Fate of Transgenic Material

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Abstract

Background: We aimed to determine the effect of feeding transgenic maize to sows during gestation and lactation on maternal and offspring immunity and to assess the fate of transgenic material.

Methodology/Principal Findings: On the day of insemination, sows were assigned to one of two treatments (n = 12/treatment); 1) non-Bt control maize diet or 2) Bt-MON810 maize diet, which were fed for ~143 days throughout gestation and lactation. Immune function was assessed by leukocyte phenotyping, haematology and Cry1Ab-specific antibody presence in blood on days 0, 28 and 110 of gestation and at the end of lactation. Peripheral-blood mononuclear cell cytokine production was investigated on days 28 and 110 of gestation. Haematological analysis was performed on offspring at birth (n = 12/treatment). Presence of the *cry1Ab* transgene was assessed in sows' blood and faeces on day 110 of gestation and in blood and tissues of offspring at birth. Cry1Ab protein presence was assessed in sows' blood during gestation and lactation and in tissues of offspring at birth. Blood monocyte count and percentage were higher ($P < 0.05$), while granulocyte percentage was lower ($P < 0.05$) in Bt maize-fed sows on day 110 of gestation. Leukocyte count and granulocyte count and percentage were lower ($P < 0.05$), while lymphocyte percentage was higher ($P < 0.05$) in offspring of Bt maize-fed sows. Bt maize-fed sows had a lower percentage of monocytes on day 28 of lactation and of CD4⁺CD8⁺ lymphocytes on day 110 of gestation, day 28 of lactation and overall ($P < 0.05$). Cytokine production was similar between treatments. Transgenic material or Cry1Ab-specific antibodies were not detected in sows or offspring.

Conclusions/Significance: Treatment differences observed following feeding of Bt maize to sows did not indicate inflammation or allergy and are unlikely to be of major importance. These results provide additional data for Bt maize safety assessment.

Citation: Buzoianu SG, Walsh MC, Rea MC, O'Donovan O, Gelencsér E, et al. (2012) Effects of Feeding Bt Maize to Sows during Gestation and Lactation on Maternal and Offspring Immunity and Fate of Transgenic Material. PLoS ONE 7(10): e47851. doi:10.1371/journal.pone.0047851

Editor: Alejandra Bravo, Universidad Nacional Autonoma de Mexico, Instituto de Biotecnologia, Mexico

Received: July 4, 2012; **Accepted:** September 21, 2012; **Published:** October 16, 2012

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Funding: The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007–2013) under grant agreement number 211820 and the Teagasc Walsh Fellowship Programme. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The continuous increase in the area cultivated with genetically modified (GM) crops [1] and consequently their ubiquitous presence makes it increasingly difficult for consumers to avoid GM products. However, the dispute regarding the safety of GM crops is far from resolved [2] and consumer opinions vary over time and from country to country [3,4].

Bt MON810 maize is resistant to attack by *Lepidopterae* pests, as it expresses the Cry1Ab protein [5]. This protein is produced in all plant tissues as a result of introduction of the bacterial *cry1Ab* transgene into the maize genome [6]. While the bacterial Cry1Ab protein has been extensively used as an organic insecticide [7], its expression in transgenic maize could potentially alter its structure which may render it allergenic or otherwise harmful upon

ingestion [8]. Fears are expressed by consumers regarding the safety of transgenic compounds following long-term consumption [9,10]. As pregnancy-related hormonal changes may result in immunosuppression [11,12], the immune system of pregnant females may respond differently to dietary antigens. Maize is a major component of animal feed and the safety of feeding GM maize to breeding livestock is also of paramount importance.

Having been marketed in the US since 1996 and grown in market penetration since then [1] with no evidence to suggest harmful effects, Bt MON810 maize has a relatively long history of safe use [13]. Furthermore, numerous controlled studies have investigated the effects of dietary Bt maize in different animal species [14]. However, while several studies have investigated effects over multiple generations in rodents and ruminants [15],

multi-generational studies in pigs are notably lacking from the literature. It is well known that the digestive physiology of pigs is very similar to that of humans [16–18]. Therefore, studies in pigs may provide some insight into the expected effects of trans-generational Bt maize consumption in humans, although the limitations of any animal model must always be taken into account.

The aim of the present study was to investigate the effects of feeding Bt MON810 maize to nulliparous sows during pregnancy and lactation on maternal and offspring immune function and to assess the presence of transgenic material in the blood of sows as well as in the blood and tissues of offspring at birth.

Methods

Ethical approval

The pig study complied with European Union Council Directives 91/630/EEC (outlines minimum standards for the protection of pigs) and 98/58/EC (concerns the protection of animals kept for farming purposes) and was approved by, and a license obtained from, the Irish Department of Health and Children (license number B100/4147). Ethical approval was obtained from Teagasc and Waterford Institute of Technology ethics committees.

Maize and diets

Seeds derived from GM Bt MON810 and non-GM parent line control maize (PR34N44 and PR34N43, respectively; Pioneer Hi-Bred, Sevilla, Spain) were grown simultaneously side by side in 2007 in Valtierra, Navarra, Spain by independent tillage farmers. The Bt and non-Bt control maize were purchased by the authors from the tillage farmers for use in this animal study.

Diets were manufactured as previously described by Walsh et al. [19]. All diets were formulated to meet or exceed the National Research Council requirements for pigs of given weights [20]. The Bt and non-Bt control maize, as well as the whole diets, were sampled in accordance with international guidelines [21] and tested for chemical, carbohydrate and amino acid composition as well as for presence of pesticide contaminants, the *cry1Ab* transgene, and mycotoxins, as previously described by Walsh et al. [19].

Animals and experimental design

Twenty four sows (Large White × Landrace) were purchased from Hermitage AI (Kilkenny, Ireland) as weanling pigs (~28 days old) and raised to ~165 kg on diets free of GM ingredients. On the day of insemination, sows were blocked by body weight and insemination date and randomly assigned to one of two dietary treatments: 1) non-Bt control parent line maize diet (Pioneer PR34N43) or 2) Bt maize diet (Pioneer PR34N44 event MON810). Sows were fed experimental diets from insemination throughout gestation and lactation until weaning at ~28 days post-farrowing (~143 days in total). Diets used in this animal study are presented in Table 1.

Synchronization of oestrus was achieved by administering 20 mg of altrenogest (Regumate™, Intervet/Schering-Plough Animal Health, Bray, Ireland) per gilt in the feed for 18 days. A sexually mature boar was housed adjacent to the gilts to stimulate oestrus. Nine days prior to predicted oestrus, the gilts were flush fed (4 kg/day) gilt developer diet (6.0 g/Kg lysine and 13.67 MJ/Kg DE). Sows were inseminated with pooled semen from five Hylean MaxGro™ boars (Hermitage AI) as soon as oestrus was detected and again 24 h later. Following insemination, sows were penned in individual gestation stalls (2.4 m × 0.6 m; O'Donovan

Engineering, Coachford, Ireland) until day 110 of gestation. Environmental temperature was maintained between 20 and 22°C and sows were provided with *ad libitum* access to water from one drinker per pen (Arato, Köln, Germany). Supplementary feed was not offered to suckling piglets.

A vaccine to prevent *E. coli* neonatal enterotoxigenosis in piglets (Porcoli Diluvac Forte™; Intervet/Schering-Plough Animal Health) was administered to all sows on days 74 and 99 of gestation. Zerofen™ (4% powder, Chanelle Animal Health Limited, Liverpool, UK) was administered to sows in feed (0.125 g/kg body weight) on day 110 of gestation for routine deworming.

From day 110 of gestation until weaning, sows were housed in three farrowing rooms with 10 farrowing crates (O'Donovan Engineering) per room. Control and treatment sows were allotted to farrowing rooms in equal numbers to minimise environmental influences. However, to minimise potential cross-contamination, control and treatment sows were penned together on either side of a central dividing passageway. Room temperature was maintained at 20°C and increased to 24°C for 48 h around farrowing. Sows had access to feed from a Daltec feeder (Daltec A/S, Egtved, Denmark) and were fed 2 kg/day immediately post-farrowing. Feed allowance was then increased by 500 g/day until day 7 post-farrowing when sows were offered *ad libitum* access to feed. Unlimited access to water was provided throughout lactation via a single nipple drinker per pen. Induction of farrowing was achieved by administering 2 mL of Enzaprost™ (5 mg/ml; CEVA Animal Health Ltd, Chesham Bucks, UK) on day 114 of gestation.

At farrowing, the fourth piglet born alive from each sow was euthanized before suckling occurred and samples were obtained, as outlined below.

At all times throughout the study sows were observed closely at least twice daily and any showing signs of ill health were treated as appropriate and all veterinary treatments recorded. Data from these animals was examined on a case by case basis and if necessary, removed from the data set.

Blood, faeces and tissue sampling

Blood samples were taken from the external jugular vein of sows at insemination (day 0), at days 28 and 110 of gestation and on day 28 of lactation (n = 12/treatment). Blood samples were also collected from offspring at birth. Whole blood from sows and offspring was sampled in K₂EDTA evacuated tubes (Vacuette, Greiner Bio One Ltd, Gloucestershire, UK) and stored at room temperature for whole blood haematological analysis (within 6 hours of sampling). Haematological analysis was performed using a Beckman Coulter Ac T Diff analyser (Beckman Coulter Ltd., High Wycombe, UK) for determination of white blood cell counts (WBC#) and counts and percentage of lymphocytes (LY# and LY%), monocytes (MONO# and MONO%) and granulocytes (GRAN# and GRAN%). Calibration of the analyser was performed in accordance with the manufacturer's instructions and accuracy was determined by testing control samples of known values.

Blood was also collected from sows in evacuated tubes containing a silica coagulation activator (Vacuette) and allowed to coagulate for 2 hours at room temperature. Serum was then collected following centrifugation at room temperature, at 1500 ×g for 10 minutes. The serum was analysed for presence of the Cry1Ab protein and its specific antibodies, as outlined below.

White blood cells for gene detection were isolated from sow and offspring blood collected in K₂EDTA evacuated collection tubes. To prevent DNA degradation, the tubes were stored on ice following sampling and were centrifuged within 2 hours at 4°C, at

Table 1. Composition of diets fed to sows during gestation and lactation (fresh weight basis, %).

Ingredient	Gestation		Lactation	
	Non-Bt control	Bt	Non-Bt control	Bt
Maize	86.55	86.55	74.42	74.42
Soybean meal (non-GM)	10.33	10.33	19.30	19.30
Soybean oil	-	-	3.02	3.02
L-Lysine HCl	0.16	0.16	0.25	0.25
DL-Methionine	-	-	0.10	0.10
L-Threonine	-	-	0.06	0.06
Vitamin and mineral premix ¹	0.10	0.10	0.10	0.10
Salt	0.40	0.40	0.40	0.40
Di-calcium phosphate	1.36	1.36	1.29	1.29
Limestone flour	1.10	1.10	1.06	1.06
Analysed chemical composition				
Dry matter	88.40	87.80	89.50	88.60
Crude protein	11.80	11.00	15.60	15.00
Fat	3.00	3.20	5.90	6.00
Crude fibre	1.70	1.60	1.60	1.90
Ash	4.10	4.30	4.60	4.30
Lysine	0.64	0.68	1.01	0.96
Ca ²	7.60	7.60	7.50	7.50
p ²	6.10	6.10	6.20	6.20
Digestible energy, MJ of DE/kg ²	13.80	13.80	14.50	14.50

¹Premix provided per kg of complete diet: Cu, 15 mg; Fe, 70 mg; Mn, 62 mg; Zn, 80 mg, I, 0.6 mg; Se, 0.2 mg; retinyl acetate, 3.44 mg; cholecalciferol, 25 µg; DL-alpha-tocopheryl acetate, 100 mg; vitamin K, 2 mg; vitamin B₁₂, 15 µg; riboflavin, 5 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 500 mg; vitamin B₁, 2 mg; and vitamin B₆, 3 mg.

²Calculated values.

doi:10.1371/journal.pone.0047851.t001

1500×g for 10 minutes. The separated plasma from offspring blood was also collected for Cry1Ab protein detection. White blood cell and plasma samples were stored at -20°C until analysed.

Blood samples were also collected from sows in sodium heparin tubes (BD Vacutainer Systems, Franklin Lakes, NJ, USA) for isolation of peripheral blood mononuclear cells (PBMC), as outlined below.

Prior to all blood sampling the skin at the sampling site was thoroughly swabbed with 70% ethanol to prevent contamination of blood samples with feed dust.

Colostrum samples for detection of Cry1Ab-specific antibodies (as outlined below) were collected from sows immediately prior to parturition in 30 ml sterile containers (Sterilin Limited, Newport, UK) and stored at -20°C until analysis. Sow teats were sanitised with 70% ethanol prior to sampling to prevent environmental contamination.

On day 110 of gestation, faecal samples were collected from sows and stored at -20°C prior to gene detection (n=12/treatment).

The heart, kidney, spleen, liver, *semitendinosus* muscle, brain and navel tissue were collected from offspring at birth for Cry1Ab protein and transgene detection and stored at -20°C until analysed. Every effort was made to prevent environmental and cross-contamination; Scalpel blades and gloves were changed and all instruments were sanitised using 70% ethanol after each pig was sampled. Furthermore, each organ was placed on a single-use aluminium foil sheet to avoid cross-contamination and the outer

surface was removed and samples were taken from an inner portion. In addition, to avoid contamination with feed dust, sampling of all tissues was performed in a room separate to the farrowing house.

Immune cell populations and cytokine production

Sow PBMC were isolated and stimulated using a combination of phorbol 12-myristate 13-acetate and ionomycin on days 28 and 110 of gestation, as described by Walsh et al. [22]. Prior to and following stimulation, the cell culture supernatant was removed and stored at -80°C for analysis of cytokines (IL-4, IL-6, IL-8 and TNF-α). Cytokine concentrations were determined using multiplex porcine-specific ELISA kits (Meso Scale Discovery, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions.

Staining of PBMC with fluorescent antibodies for flow cytometric analysis was also performed as described by Walsh et al. [22,23] for determination of the percentages of white blood cells, B lymphocytes, monocytes and CD3⁺, CD4⁺, CD8⁺ and CD4⁺CD8⁺ T lymphocytes. Proportions of monocytes and B and T lymphocytes were calculated from the total number of white blood cells. Proportions of CD4⁺, CD8⁺ and CD4⁺CD8⁺ T lymphocytes were calculated from the number of CD3⁺ T lymphocytes. These cells were analysed in sows at day 0, 28 and 110 of gestation and at day 28 of lactation using a BD FACSCanto flow cytometer (BD Biosciences, Franklin Lake, NJ, USA) and FACSDiva software (BD Biosciences).

Table 2. Effects of feeding Bt MON 810 maize to nulliparous sows during gestation and lactation on haematological parameters of sows¹.

	Transformation ²	Treatments				P – value
		Non-Bt control		Bt		
		Mean	95% CI ³	Mean	95% CI ³	
WBC⁴, ×10³/μL	–0.5					
Day 28 of gestation		20.4	18.3–23.0	21.3	19.0–24.0	0.61
Day 110 of gestation		16.2	14.7–18.0	16.4	14.9–18.3	0.90
Day 28 of lactation		18.0	16.2–20.1	17.8	16.0–19.8	0.83
Overall		18.0	16.9–19.3	18.3	17.1–19.6	0.82
Lymphocytes, %	1.75					
Day 28 of gestation		54.3	50.7–57.7	53.5	49.9–57.0	0.76
Day 110 of gestation		46.0	41.9–49.9	49.5	45.7–53.2	0.21
Day 28 of lactation		38.8	34.1–43.1	39.6	34.9–43.9	0.81
Overall		46.7	43.9–49.4	47.8	45.1–50.4	0.55
Monocytes, %	–0.5					
Day 28 of gestation		5.7	4.4–7.8	5.3	4.1–7.1	0.68
Day 110 of gestation		3.9	3.1–5.0	5.7	4.4–7.8	0.04
Day 28 of lactation		5.2	4.0–6.9	5.8	4.4–7.9	0.57
Overall		4.8	4.2–5.7	5.6	4.8–6.6	0.19
Granulocytes, %	none					
Day 28 of gestation		40.8	35.6–46.0	40.3	35.1–45.5	0.89
Day 110 of gestation		50.7	45.4–55.8	41.0	35.3–45.8	0.01
Day 28 of lactation		57.7	52.2–63.1	54.3	48.9–59.7	0.39
Overall		49.7	46.3–53.0	45.1	41.7–48.4	0.06
Lymphocytes, ×10³/μL	0.25					
Day 28 of gestation		10.9	10.0–11.9	11.6	10.6–12.7	0.34
Day 110 of gestation		7.3	6.6–8.0	8.2	7.4–9.0	0.09
Day 28 of lactation		7.0	6.3–7.8	7.0	6.3–7.7	0.93
Overall		8.3	7.8–8.8	8.8	8.3–9.3	0.14
Monocytes, ×10³/μL	–0.25					
Day 28 of gestation		1.2	0.9–1.6	1.2	0.9–1.6	0.89
Day 110 of gestation		0.6	0.5–0.8	1.0	0.8–1.4	0.03
Day 28 of lactation		0.9	0.7–1.2	1.0	0.8–1.4	0.38
Overall		0.9	0.7–1.0	1.1	0.9–1.3	0.09
Granulocytes, ×10³/μL	0.5					
Day 28 of gestation		8.6	7.0–10.4	8.8	7.2–10.6	0.88
Day 110 of gestation		8.3	6.7–10.0	6.5	5.1–8.0	0.11
Day 28 of lactation		10.4	8.6–12.3	9.7	8.0–11.5	0.57
Overall		9.1	7.9–10.3	8.3	7.2–9.5	0.33

¹Sows were fed either a non-Bt control or a Bt maize-based diet (n = 12/treatment).

²Lambda used for Box Cox transformation of data to ensure normal distribution. Means and 95% confidence limits for data which were normalised using the Box Cox transformation were back transformed and are presented on the original scale.

³95% CI – 95% confidence interval (the interval that contains the true population mean in 95% of cases).

⁴WBC – white blood cells.

doi:10.1371/journal.pone.0047851.t002

Cry1Ab protein and transgene detection in faeces, blood and tissues

The presence of the Cry1Ab protein in the serum of sows at day 28 and 110 of gestation and day 28 of lactation and in the plasma and tissues of offspring euthanized at birth was assessed using the QuantiPlate kit for Cry1Ab/Cry1Ac detection (Envirologix, Maines, USA) in accordance with the manufacturer's instructions.

Cry1Ab protein was extracted from serum of sows and from organs (heart, kidney, spleen, liver, muscle, liver, navel cord and brain) of pigs euthanized at birth as previously described by Walsh et al. [24].

Extraction of DNA from sow and offspring white blood cells and offspring tissues (kidney, liver and muscle) was also conducted as previously outlined by Walsh et al. [23]. The presence of two

cry1Ab transgene fragments (149 and 211 bp), the *sw* (porcine growth hormone) gene (108 bp), the *shrunk 2* gene (*sh2*; a maize-specific ADP glucose pyrophosphorylase gene; 213 bp) and the *rubisco* gene (maize-specific ribulose biphosphate carboxylase; 173 bp) was assessed by PCR as previously described by Walsh et al. [23,24].

Cry1Ab-specific antibody response

The presence of Cry1Ab protein-specific immunoglobulins (IgA and IgG) was investigated in the serum at day 28 and 110 of gestation and day 28 of lactation and colostrum of sows and in the plasma of the offspring sacrificed at birth, as described by Walsh et al. [23].

Statistical analysis

Statistical analysis was performed using SAS 9.2 [25]. To ensure normality, haematology data (except for sow GRAN% and offspring WBC# and LY# which were initially normally distributed) were transformed using the Box Cox transformation [26] $x = (y^\lambda - 1)/\lambda$ where y is the initial variable and λ is a constant (values of λ are presented in Tables 2 and 3). Data which were initially normally distributed or which were normalised using the Box Cox transformation were analysed using PROC MIXED, with treatment and day as fixed effects and block as a random effect. Day 0 values were used as a covariate in the model and day was included in the model as a repeated variable. Due to unequal spacing between sampling days and as indicated by the model fit criteria a spatial power covariance structure was fitted to the data [27]. The *slice* option was used to test for simple effects at each time point. The denominator degrees of freedom were computed using the Satterthwaite approximation. For analysis of offspring haematology, body weight was used as a covariate in the statistical model. To assess model suitability, data were examined using the influence diagnostics provided within PROC MIXED in SAS and by investigation of normality of scaled residuals using the Shapiro-Wilk test within the UNIVARIATE procedure in SAS. Least squares means were computed and P values were adjusted for multiple comparisons using the Tukey-Kramer adjustment. Means and 95% confidence limits for data which were normalised using the Box Cox transformation were back-transformed and are presented on the original scale. Data which were non-normal (cytokine production) were analysed using the non-parametric Kolmogorov-Smirnov test within PROC NPAR1WAY [28]. The *exact* option was used for non-parametric tests as recommended by Mundry and Fischer [29]. Non-parametric data is presented as medians and the 5th and 95th percentiles which were computed using PROC UNIVARIATE. Significance is reported for $P \leq 0.05$ and tendencies towards significance are reported for $0.05 < P \leq 0.10$. For all response criteria, the individual pig was the experimental unit.

Results

Health of animals

One sow from the control treatment was observed to have a lack of appetite and fever at day 105 of gestation and received injectable penicillin between days 105 and 107 of gestation. Following treatment, the sow made a complete recovery. As all haematological and immunological parameters investigated for this sow had similar values to other sows from the same treatment, data from this sow were not removed from the data set.

Maize and diets

The Bt maize and its non-Bt control counterpart had similar proximate and amino acid composition [19]. Likewise, amino acid content and proximate composition were similar for the non-Bt control and Bt diets used in the present study (Table 1).

Effects of feeding Bt and non-Bt control maize to sows on sow and offspring haematology

Results of the haematological analysis are presented in Tables 2 and 3 for sows and offspring at birth, respectively. For sow haematology, no treatment \times time interaction was observed ($P > 0.05$; data not shown). WBC# was not different between treatments at any time point or overall ($P > 0.05$). A time effect was observed for WBC#, with values decreasing up to day 100 of gestation and increasing thereafter ($P < 0.05$; data not shown). Likewise, no treatment differences were observed for LY% overall or at individual time points. LY% decreased throughout the study ($P < 0.05$; data not shown). On day 110 of gestation MONO% was higher in sows on the Bt treatment compared to sows on the control treatment ($P < 0.05$). However, MONO% was not different between treatments at any other time point or overall ($P > 0.05$) and no time effect was observed ($P > 0.05$; data not shown). GRAN% was lower on day 110 of gestation ($P < 0.05$) and, as a result, a tendency towards lower overall GRAN% ($P = 0.06$) was also observed for sows fed Bt maize diets compared to sows fed non-Bt control maize diets. GRAN% increased throughout the study ($P < 0.05$; data not shown). LY# was not significantly different between treatments overall or at any time point ($P > 0.05$). However, a tendency towards higher LY# on day 110 of gestation was observed in sows fed Bt maize diets compared to sows fed non-Bt control maize diets ($P = 0.09$). LY# decreased throughout the study ($P < 0.05$; data not shown). MONO# was higher on day 110 of gestation ($P < 0.05$) and this led to a tendency towards a higher overall MONO# in sows on the Bt treatment compared to the control treatment ($P = 0.09$). A time effect was also observed for MONO#, with values decreasing up to day 110 of gestation and increasing thereafter ($P < 0.05$; data not shown). No treatment effects were observed for GRAN# ($P > 0.05$); however, values decreased up to day 110 of gestation and thereafter increased up to day 28 of lactation ($P < 0.05$; data not shown).

At birth, WBC# was lower ($P \leq 0.05$) and LY% was higher ($P \leq 0.05$) for offspring of Bt maize-fed sows. GRAN% and GRAN# were lower for offspring of sows fed Bt maize ($P < 0.05$). MONO# tended to be lower for offspring of sows fed Bt maize ($P = 0.07$). No treatment effects were observed for MONO% and LY# in offspring at birth ($P > 0.05$).

Effects of feeding Bt and non-Bt control maize to sows on immune cell populations from isolated peripheral blood mononuclear cells

The results from flow cytometric analysis of immune cell populations present in peripheral blood are presented in Table 4. No treatment effects were observed for the percentage of white blood cells. However, a treatment \times time interaction ($P < 0.05$; data not shown) and a time effect ($P < 0.05$; data not shown) were observed, with white blood cells increasing up to day 110 of gestation and decreasing thereafter. Similar values were observed for both treatments at all time points and overall for the percentage of peripheral blood B lymphocytes ($P > 0.05$). B lymphocyte percentage increased up to day 110 of gestation and decreased thereafter ($P < 0.05$; data not shown). A treatment \times time interaction ($P < 0.05$; data not shown) was observed for monocyte percentage and values increased over time ($P < 0.05$).

Table 3. Effects of feeding Bt MON810 maize to nulliparous sows during gestation and lactation on offspring haematological parameters at birth¹.

	Transformation ²	Treatments				P - value
		Non-Bt control		Bt		
		Mean	95% CI ³	Mean	95% CI ³	
WBC ⁴ , ×10 ³ /μL	none	5.0	3.9–6.1	3.4	2.4–4.5	0.05
Lymphocytes, %	1.5	60.6	47.9–72.2	76.6	65.4–87.0	0.05
Monocytes, %	0.75	15.7	11.7–19.9	13.4	9.6–17.5	0.33
Granulocytes, %	–0.25	11.6	6.0–25.3	4.7	2.8–8.8	0.01
Lymphocytes, ×10 ³ /μL	none	2.9	2.2–3.7	2.4	1.7–3.2	0.34
Monocytes, ×10 ³ /μL	–0.25	0.7	0.5–0.9	0.4	0.3–0.6	0.07
Granulocytes, ×10 ³ /μL	–0.5	0.4	0.2–1.0	0.2	0.1–0.3	0.01

¹Sows were fed either a non-Bt control or a Bt maize-based diet and blood samples were collected at birth from the 4th live born piglet from each litter (n = 12/ treatment).

²Value of lambda used for Box Cox transformation of data to ensure normal distribution. Means and 95% confidence limits for data which were normalised using the Box Cox transformation were back transformed and are presented on the original scale.

³95% CI –95% confidence interval (the interval that contains the true population mean in 95% of cases).

⁴WBC – white blood cells.

doi:10.1371/journal.pone.0047851.t003

On day 28 of lactation the Bt maize-fed sows had lower peripheral blood monocyte percentages than non-Bt control maize-fed sows ($P < 0.05$). No treatment effects were observed for the percentages of CD3⁺ T lymphocytes and CD4⁺ T lymphocytes ($P > 0.05$); however, both increased up to day 110 of gestation and then decreased up to day 28 of lactation ($P < 0.05$; data not shown). CD8⁺ T lymphocyte percentages in peripheral blood were not affected by feeding Bt maize ($P > 0.05$); however, values decreased throughout the study ($P < 0.05$; data not shown). A decrease in CD4⁺CD8⁺ T lymphocyte counts was observed for sows on the Bt treatment compared to sows on the control treatment on day 110 of gestation, day 28 of lactation and overall ($P < 0.05$). Likewise, the percentage of CD4⁺CD8⁺ T lymphocytes in peripheral blood increased over time ($P < 0.05$; data not shown).

Effects of feeding Bt and non-Bt control maize on cytokine production from isolated PBMC

Cytokine production data from resting and stimulated PBMC are presented in Table 5. No treatment effect was observed for IL-4 and IL-6 production by resting PBMC on day 28 or 110 of gestation ($P > 0.05$). However, production of both IL-4 and IL-6 by resting cells increased over time ($P < 0.05$; data not shown). A tendency towards lower production of IL-8 by resting PBMC was observed on day 28 and 110 of gestation in sows fed Bt maize ($P = 0.10$). No time effect was observed for IL-8 ($P > 0.05$; data not shown). TNF- α production by resting PBMC was not affected on day 28 of gestation ($P > 0.05$); however, a tendency towards lower TNF- α production was observed in Bt maize-fed sows on day 110 of gestation ($P = 0.10$). The production of TNF- α by resting PBMC increased over time ($P < 0.05$; data not shown).

The production of IL-4, IL-6, IL-8 or TNF- α by stimulated PBMC was not different between treatments at any time point ($P > 0.05$). However, values for all cytokines increased over time ($P < 0.05$; data not shown). The production of all cytokines increased with mitogen stimulation ($P < 0.05$; data not shown).

Cry1Ab protein and transgene detection in blood, faeces and tissues

The Cry1Ab protein was not detected in the serum of sows at day 28 and 110 of gestation or at the end of lactation (data not shown) or in the plasma, heart, kidney, spleen, muscle or brain of offspring from sows fed either non-Bt control or Bt maize throughout gestation (data not shown).

The frequency of detection of both transgenic and maize and porcine endogenous gene fragments is presented in Tables 6 and 7 for sows and offspring at birth, respectively. The 108 bp gene fragment of the pig-specific *sw* gene was detected in white blood cells of all sows from both treatments on day 110 of gestation. The 173 bp fragment of the maize-specific multi-copy *rubisco* gene was detected in the white blood cell samples of 2 of 12 sows fed the non-Bt control maize diet and in 1 of 12 sows fed the Bt maize diet. In faeces, the same gene fragment was detected in 7 and 5 of 12 sows on the non-Bt control and Bt treatments, respectively. The 213 bp fragment of the maize-specific single-copy *sh2* gene was not detected in white blood cells or faeces of sows from either treatment. The single-copy 149 or 211 bp fragments of the *cry1Ab* transgene were not detected in the blood or faeces of sows from either treatment group.

In the offspring at birth, the pig-specific *sw* gene fragment was detected in all white blood cell samples from the control treatment and in 11 of 12 white blood cells samples from the Bt treatment. The *sw* gene fragment was also detected in all kidney, liver and muscle samples from both treatment groups. The maize-specific multi-copy *rubisco* gene fragment was present in the white blood cells of two animals from each treatment group and in the muscle of one animal from each treatment group but was not detected in any kidney or liver samples analysed. The maize-specific single-copy *sh2* gene fragment was not detected in any of the white blood cell, kidney, liver or muscle samples from either the control or Bt treatment group. Neither the 149 bp nor the 211 bp transgene fragments were detected in white blood cells, kidney, liver or muscle of offspring at birth from either non-Bt control or Bt maize-fed sows.

Table 4. Effects of feeding Bt MON 810 maize to nulliparous sows during gestation and lactation on peripheral blood immune cell populations^{1,2}.

	Treatments		SEM ³	P – value
	Non-Bt control	Bt		
WBC⁴				
d 28 of gestation	42.1	40.5	3.88	0.84
d 110 of gestation	57.1	50.4	3.04	0.28
d 28 of lactation	46.0	52.3	3.07	0.31
Overall	48.4	47.7	3.79	0.90
B lymphocytes				
d 28 of gestation	2.8	2.0	0.47	0.42
d 110 of gestation	4.4	4.0	0.35	0.53
d 28 of lactation	3.4	2.9	0.36	0.54
Overall	3.5	3.0	0.36	0.29
Monocytes				
d 28 of gestation	9.1	6.8	1.25	0.37
d 110 of gestation	13.5	15.8	0.95	0.25
d 28 of lactation	19.1	13.5	0.95	0.01
Overall	13.9	12.1	0.94	0.17
CD3⁺ T lymphocytes				
d 28 of gestation	51.8	52.0	2.44	0.97
d 110 of gestation	60.6	63.1	1.86	0.52
d 28 of lactation	58.1	60.5	1.83	0.53
Overall	56.8	58.5	1.97	0.57
CD4⁺ T lymphocytes				
d 28 of gestation	7.3	7.4	1.89	0.99
d 110 of gestation	21.9	22.5	1.33	0.82
d 28 of lactation	22.3	19.3	1.35	0.28
Overall	17.2	16.4	1.44	0.70
CD8⁺ T lymphocytes				
d 28 of gestation	38.4	40.0	1.91	0.68
d 110 of gestation	36.1	40.7	1.36	0.11
d 28 of lactation	22.2	24.9	1.38	0.33
Overall	32.2	35.2	1.59	0.20
CD4⁺CD8⁺ T lymphocytes				
d 28 of gestation	12.9	11.5	1.45	0.56
d 110 of gestation	19.5	15.0	1.15	0.01
d 28 of lactation	19.3	15.4	1.15	0.03
Overall	17.2	14.0	1.17	0.02

¹Sows were fed either a non-Bt control or a Bt maize-based diet (n = 12/ treatment).

²Values are given as percentages of the total peripheral blood mononuclear cell population for all parameters, except the CD4⁺ CD8⁺ and CD4⁺CD8⁺ lymphocytes which are given as percentages of the CD3⁺ lymphocytes.

³SEM – standard error of the mean.

⁴WBC – white blood cells.

doi:10.1371/journal.pone.0047851.t004

Cry1Ab-specific antibody response

Neither Cry1Ab-specific IgA nor IgG were detected in serum from sows at any sampling point, in colostrum taken from sows immediately prior to parturition or in plasma from offspring from either treatment group (data not shown).

Discussion

Most studies that have previously investigated the safety of GM feed ingredients with respect to animal health have generally involved only one generation of animals. For this reason multi-generational studies are needed to fully establish the safety of feeding GM feed ingredients over multiple generations. To our knowledge, this is the first study to investigate immune function and to assess the presence of transgenic products in blood and tissues of Bt maize-fed pregnant sows and their offspring. Although there are no data to suggest that the Cry1Ab protein is toxic to mammals (most likely because they lack specific receptors in the intestinal tract [5]), nonetheless consumers are concerned about potential toxicity [2]. However, Cry1Ab occurs in relatively low quantities in Bt maize (e.g. 0.6 µg/g in this study). Furthermore, incorporation of Bt maize in complete animal feedstuffs results in dilution. For these reasons, subtle effects may not be evident unless multiple health indicators are examined following high-level long-term exposure [15,30]. Therefore, in the present study, Bt maize was included at high levels in pig diets (74–87%), and the duration of the study was extended beyond the 90-day standard recommended by the European Food Safety Authority [31]. In addition, a wide array of health indicators was assessed in sows and their offspring to determine the long-term and trans-generational safety of feeding Bt maize.

Food allergies are characterised by intestinal inflammation and are associated with diarrhoea and malabsorption [32,33]. However, no such symptoms were observed in pregnant sows fed Bt maize [34]. Prolonged allergen exposure increases the magnitude of an inflammatory response and compromises the integrity of the intestinal epithelium, thereby allowing the allergen to enter the circulatory system which may lead to development of a systemic immune response [32,35,36]. Previous studies have demonstrated that the quantity of Cry1Ab protein that survives intestinal digestion is very low and have also confirmed that the Cry1Ab protein is absent from the blood and organs of livestock fed Bt maize for extended periods [23,24,37–40]. However, even at low doses, the Cry1Ab protein has the potential to be allergenic, as, in general, only minute quantities of allergens are required for an allergic response in sensitized individuals [36].

In the present study, an increase in the proportion of circulating T lymphocytes and, more specifically, an increase in CD4⁺ T or B lymphocytes, which would be indicative of allergy [36] were not observed at any time point. The decrease in monocytes and in immature CD4⁺CD8⁺ T lymphocytes from PBMC observed at day 28 of lactation in response to feeding Bt maize to sows is unlikely to denote an inflammatory/allergic response. This is because only an increase of these cell populations associated with an increase in B and CD4⁺ T lymphocytes and accompanied by production of Cry1Ab-specific antibodies would indicate such a response [33,36]. An increase in the cytokines involved in allergic/inflammatory responses, such as IL-4, IL-6, IL-8 and TNF-α [41,42], was also not observed at any time point during the study. Furthermore, Cry1Ab-specific IgG and IgA were not detected in response to Bt maize feeding, making it unlikely that treatment differences observed would indicate an allergic or inflammatory response. The absence of treatment differences for cytokine production by stimulated PBMC also indicates that Bt maize-fed animals are likely to respond in a similar fashion in an *in-vivo* challenge situation. These results are in agreement with our previous findings in pigs fed Bt maize for 31 [23] or 110 days [24], where no differences in IL-4, IL-6, IL-8 and TNF-α production were found between treatments in either resting or stimulated PBMC. In contrast, differences in cytokine production and

Table 5. Effects of feeding Bt MON810 maize to nulliparous sows during gestation on cytokine production from resting and stimulated peripheral blood mononuclear cells (pg/mL)¹.

	Non-Bt control	5–95 th percentiles ²	Bt	5–95 th percentiles ²	P - value ³
<i>Resting</i>					
Day 28 of gestation					
IL-4	0.5	0–8.1	0.1	0–5.2	0.77
IL-6	1.2	0–14.1	0.7	0–3.2	0.52
IL-8	1666.8	66.6–10795.5	1150.3	278.7–1506.4	0.10
TNF- α	10.5	0.4–71.7	8.5	3.0–36.2	0.54
Day 110 of gestation					
IL-4	108.0	0–1049.4	72.8	0–121.9	0.24
IL-6	19.9	0–1184.6	22.8	0–45.5	0.85
IL-8	3691.7	0–21749.2	1233.0	0–4127.1	0.10
TNF- α	222.1	0–25555.2	105.1	16.2–319.2	0.10
<i>Stimulated</i>					
Day 28 of gestation					
IL-4	12.8	0–127.2	16.9	1.3–45.1	0.87
IL-6	38.5	9.3–203.3	34.3	14.3–198.7	0.99
IL-8	6527.7	1742.0–13819.8	6801.2	1703.5–18474.8	0.87
TNF- α	9331.6	3056.7–28898.4	11725.9	3519.3–23946.0	0.87
Day 110 of gestation					
IL-4	447.0	0–1227.4	457.9	300.4–656.5	0.87
IL-6	400.3	0–753.7	475.9	260.1–672.5	0.87
IL-8	11757.8	0–36328.5	11882.7	8010.8–15270.9	0.54
TNF- α	18765.3	133.2–130654.7	18185.9	12167.8–22784.5	0.54

¹Sows were fed either a non-Bt control or a Bt maize-based diet (n = 12/treatment).

²The 5th percentile is larger than 5% of the values and the 95th percentile is larger than 95% of the values.

³Computed on untransformed data using the Kolmogorov-Smirnov non-parametric test within PROC NPARIWAY in SAS.

doi:10.1371/journal.pone.0047851.t005

immune cell populations were observed in mice fed Bt maize for 30 or 90 days compared to controls [43]. Although the biological significance of those findings was questioned by the authors, the responses observed were believed to be as result of a higher quantity of the maize allergen zein due to *cry1Ab* insertion [43].

While this may be true, environment, growing location and developmental stage have all been shown to have a greater influence on maize allergens and metabolites than the genetic modification itself [44–46].

Table 6. Detection of endogenous maize and porcine gene fragments and transgene fragments in blood and faeces of sows fed non-Bt control or Bt maize-based diets during gestation and lactation¹.

Gene (fragment length)	Treatments			
	White blood cells		Faeces	
	Non-Bt control	Bt	Non-Bt control	Bt
Endogenous				
<i>sw</i> – pig-specific (108 bp)	12	12	NA ²	NA ²
<i>rubisco</i> – multicopy maize-specific (173 bp)	2	1	7	5
<i>sh2</i> – single copy maize-specific (213 bp)	0	0	0	0
Transgenic (single copy)				
<i>cry1Ab</i> (149 bp)	0	0	0	0
<i>cry1Ab</i> (211 bp)	0	0	0	0

¹Number of samples out of 12 that tested positive for the gene fragment of interest. One sample was tested per sow (n = 12 sows/treatment).

²Not assessed.

doi:10.1371/journal.pone.0047851.t006

Table 7. Detection of endogenous maize and porcine gene fragments and transgene fragments in blood and organs of offspring from sows fed non-Bt control or Bt maize-based diets during gestation and lactation^{1,2}.

Gene (fragment length)	Treatments							
	Non-Bt control	Bt	Non-Bt control	Bt	Non-Bt control	Bt	Non-Bt control	Bt
	White blood cells		Kidney		Liver		Muscle	
Endogenous								
<i>sw</i> – pig specific (108 bp)	12	11	12	12	12	12	12	12
<i>rubisco</i> – multicopy maize specific (173 bp)	2	2	0	0	0	0	1	1
<i>sh2</i> – single copy maize specific (213 bp)	0	0	0	0	0	0	0	0
Transgenic (single copy)								
<i>cry1Ab</i> (149 bp)	0	0	0	0	0	0	0	0
<i>cry1Ab</i> (211 bp)	0	0	0	0	0	0	0	0

¹Samples were collected at birth from the 4th live born piglet from each litter.

²Number of samples out of 12 that tested positive for the gene fragment of interest. One sample was tested per piglet (n = 12 piglets/treatment).
doi:10.1371/journal.pone.0047851.t007

An inflammatory response is also characterised by an increased white blood cell count and increased granulocytes and monocytes (which are both white blood cell types), representing the innate immune response. This is then followed by an increase in lymphocytes during the development of the adaptive immune response [36]. On the contrary, in the present study, the proportion of monocytes within the PBMC decreased on day 28 of lactation in response to feeding Bt maize, although this was not reflected in the findings for monocytes within whole blood. In fact, an increase in monocytes was observed during haematological analysis of Bt maize-fed sows during gestation. This could be explained by the higher body weight observed in these sows in the second half of gestation [34], as monocyte recruitment and increased cytokine production are known to be associated with an increase in body weight [47]. However, no differences in fat deposition or cytokine production were observed between treatments. Furthermore, monocyte counts remained within the normal range for pigs and this difference did not persist up to day 28 of lactation. While an increase in granulocyte percentage could be indicative of inflammation, in the present study we observed only a decrease in granulocyte percentage in whole blood for Bt maize-fed sows on day 110 of gestation, which was mirrored in the blood of offspring at birth. A lower abundance of the potentially pathogenic *Proteobacteria* observed in faeces of Bt maize-fed sows (Buzoianu et al., unpublished) may be the cause of the lower granulocytes and tendencies towards lower IL-8 and TNF- α observed in these sows, as *Proteobacteria* have been positively correlated with increased blood granulocytes and cytokine production [48,49]. Illness was not the reason for the treatment differences in immune cell populations observed in sows, as all animals were in good health, except for one sow in which immune parameters were unaffected (as outlined above). Likewise, the variability between animals on the same treatment was small, as evidenced by the confidence intervals. While no differences were observed for total white blood cell counts in sows fed Bt maize, a lower WBC# was observed in their offspring at birth. Only an increase in WBC# is associated with an allergic or inflammatory response. However, a higher LY% was observed at birth in the blood of offspring of Bt maize-fed sows and lymphocytes are a subset of white blood cells, but this was not mirrored in sows. The absence of the Cry1Ab protein in sow or foetal blood indicates that there was no contact between the Cry1Ab and the foetal immune

system which was also evidenced by the absence of Cry1Ab-specific antibodies in blood of either sows or their offspring. Furthermore, an increase in spleen weight which is known to accompany a systemic immune response [50] was not observed in these piglets at birth [34]. An additional study using littermates of piglets from the present study found no increase in spleen weight later in life (~143 days of age) and an absence of health abnormalities (Buzoianu et al, unpublished). Therefore, while treatment differences were observed in offspring at birth for certain haematological parameters in this study, they are not believed to indicate an allergic/inflammatory response to Bt maize ingestion by the sows. Furthermore, the values in sows generally remained within the normal range reported for pigs [50]. However, in offspring, lymphocyte and monocyte percentages were higher and granulocyte count and percentage were lower than the normal ranges [50,51]. Stress is known to have a major influence on haematological parameters [50] and as these samples were taken immediately after birth, a time of great stress for the piglet, this may help to explain the deviations from the normal ranges.

Differences in immune response have previously been observed in sheep following Bt maize consumption for three years [52] and in mice after 30 and 90 days of Bt maize consumption [43]. Similar to results from our study, biological significance of these findings was also questioned by the authors and the inconsistency in results between studies is likely to be due to the use of different animal models. However, while neither of the above studies investigated Cry1Ab-specific antibody production, our results, as well as those of Adel-Patient et al. [53] for mice, indicate that no antibodies to the Cry1Ab protein are produced as a result of oral exposure to Bt MON810 maize.

Although present in the diet, both transgenic and native single-copy gene fragments (*cry1Ab* and *sh2*) were degraded along the gastrointestinal tract (GIT), as they were not detected in the faeces of sows or in the organs of the offspring. However, the multicopy *rubisco* maize gene was present in sow faeces and was detected at low frequency in the blood and in the muscle and blood of offspring, but did not reach the organs. Extensive DNA degradation was expected, as high DNase activity is found along the porcine GIT [18,54]. In agreement with our previous results [23,24] and those of others [37,40,52,55], the present study confirms that DNA from single-copy genes is degraded along the

GIT of livestock and does not reach the organs. There is no clear explanation why Mazza et al. [56] detected plant genes (both endogenous and transgenic) in the organs of 35 kg pigs, but discrepancies may be related to age/weight of the pigs, maturity of the intestine, dietary inclusion rate and time since the last meal. While DNA transfer to tissues and across the placenta has been confirmed in the present study, the frequency of detection appears to be more related to the number of copies of the gene of interest than to its endogenous or transgenic nature. Our results confirm that there are no differences in degradability and uptake of transgenic DNA compared to that of native plant DNA. In addition, incorporation and stable expression of transgenic DNA into the host genome and detrimental effects on the host as a consequence have not been demonstrated.

The fact that responses to Bt maize consumption are inconsistent across different species highlights inter-species variability and calls into question the relevance of these findings to humans. Therefore, the biological relevance of findings in the animal model should first be addressed before attempting to extrapolate results to humans. Furthermore, as biological processes are complex and interrelated in nature, investigating a wide range of parameters simultaneously allows for a more definitive assessment of biological relevance. Ruminants and rodents are frequently used as models for humans; however, they are not without shortcomings [57,58]. As porcine intestinal physiology and microbiota as well as aspects of immune function are similar to that of humans [16,17,57], pigs are increasingly used as models for human immune response and even as candidates for xenotransplantation [32,59,60]. Therefore, our results in pigs, which demonstrate a lack of biologically significant effects on immune function as a result of feeding Bt maize could potentially be considered of more relevance to humans than findings from studies which use less appropriate animal models.

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Acknowledgments

The authors would like to acknowledge Tomas Ryan, Charlotte Amdi, Kathleen Morton, Julie Ledoux, Julia Chaumier, Lidia Teixeira, John Walsh and Pat Twomey for technical assistance.

Author Contributions

Conceived and designed the experiments: PGL RPR. Performed the experiments: SGB MCW PGL GEG MCR. Analyzed the data: SGB. Contributed reagents/materials/analysis tools: SGB MCW GEG PGL MCR EG GU ES AN OO. Wrote the paper: SGB. Revised the manuscript: SGB MCW GEG PGL. Read and approved the final manuscript: SGB MCW MCR OO EG GU ES AN RPR GEG PGL.

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