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## Intestinal and Peripheral Immune Response to MON810 Maize Ingestion in Weaning and Old Mice

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This study evaluated the gut and peripheral immune response to genetically modified (GM) maize in mice in vulnerable conditions. Weaning and old mice were fed a diet containing MON810 or its parental control maize or a pellet diet containing a GM-free maize for 30 and 90 days. The immunophenotype of intestinal intraepithelial, spleen, and blood lymphocytes of control maize fed mice was similar to that of pellet fed mice. As compared to control maize, MON810 maize induced alterations in the percentage of T and B cells and of CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ T, and  $\alpha\beta$ T subpopulations of weaning and old mice fed for 30 or 90 days, respectively, at the gut and peripheral sites. An increase of serum IL-6, IL-13, IL-12p70, and MIP-1 $\beta$  after MON810 feeding was also found. These results suggest the importance of the gut and peripheral immune response to GM crop ingestion as well as the age of the consumer in the GMO safety evaluation.

**KEYWORDS:** MON810; transgenic maize; mice; intestinal immune response; lymphocytes subpopulations

### INTRODUCTION

Interest in genetically modified (GM) crops is continuously increasing due to the possibility of higher agronomic productivity and more nutritious food without the use of pesticides (1, 2). The safety issues of GM food are crucial for their acceptance into the market. Although several studies have been conducted to evaluate the safety of GM crops, there is still a debate on the risk of GM consumption and a demand for additional evidence of GM food safety (3, 4).

Many trials with animals fed different GM foods such as maize, potatoes, rice, soybeans, and tomatoes have been conducted, and parameters such as body weight, food consumption, organ weight, blood chemistry, and histopathology have been measured. The majority of these experiments did not indicate abnormalities in such parameters (5, 6). However, consumption of transgenic pea- $\alpha$ -amylase inhibitor predisposed mice to CD4<sup>+</sup> Th2-type inflammation and elicited immunoreactivity to concurrently consumed heterogeneous food antigens (7).

The transgenic MON810 maize was produced by insertion of a DNA sequence that encodes a bioactive form of *Bacillus thuringiensis* (Bt) Cry1Ab protein, which is toxic to the corn borer. Protection against corn borer damage may improve yields without the need for chemical insecticide use and reduces the risk of toxigenic fungus infection such as *Fusarium* species (8, 9). The safety of MON810 has been evaluated by previous studies that reported no toxicologically significant differences in clinical and neurobehavioral signs, ophthalmology, clinical pathology,

organ weights, and gross and microscopic pathology between transgenic and commercial maize fed animals (5, 6). The Cry1Ab protein has been also assayed for possible allergenicity. Some authors found that sensitive subjects did not react differently to GM and non-GM samples by skin prick test and IgE immunoreactivity (10). However, other authors have reported an increased anti-Bt IgG and IgE response in farm or greenhouse workers (11, 12). In addition, a recent study revealed a significant anti-Bt IgG1 response in rats fed a transgenic Bt rice spiked with purified Bt toxin and a tendency to a dose-related response for Bt-specific IgA (13).

Until now, assessment of GMO immune adverse effects was based on the potential allergenic evaluation of the pure recombinant proteins, and only a recent study has considered the potential immunotoxicological effects of whole GMO given to rats for different periods (13). In addition, no studies have considered the intestinal immune response for such a purpose. However, the intestine interacts continuously with food-derived antigens, allergens, pathogens, and other noxious agents, and the gut immune system, which is the largest lymphoid tissue of the body, is crucial for mounting a correct immune response while maintaining a quiescent status toward innocuous antigens.

In the present study we have evaluated the intestinal and peripheral immune response to long-term MON810 maize consumption, as compared to its parental control and a commercial nontransgenic maize in mice. As gut immune cells, we have considered the intraepithelial lymphocytes (IELs) that form a highly specialized lymphoid compartment and that are the first cells to encounter luminal antigens. These lymphocytes are considered to play an important role in the regulation of immune responses (14). We have used both weaning and old mice,

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because they are more susceptible to immunological insults than adult nonaged animals and their immune response may be less efficient.

## MATERIALS AND METHODS

**Test Materials.** Planted seeds derived from MON810 and its parental control maize (PR33P67 and PR33P66 varieties, respectively) were grown simultaneously in neighboring fields in Landriano, Italy (Azienda Agraria Didattico Sperimentale Angelo Menozzi, Milano, Italy). The PR33P67 and PR33P66 seeds were provided by Seeds Emporda (Girona, Spain).

The presence of the Cry1Ab gene in the transgenic maize flour and its absence in the parental control maize flour were checked by MON810 event-specific PCR reactions. DNA was extracted using a CTAB-based protocol (15), and real-time PCR was performed using primers, TaqMan probes, and PCR conditions previously described by Kuribara et al. (16). The presence of GMO in the control flour was found to be  $0.29 \pm 0.09\%$ . The results showed the expected DNA band corresponding to the Cry1Ab insertion in the MON810 and not control maize (not shown).

The presence of the protein Cry1Ab in the GM maize was checked and confirmed by ELISA kit (Agdia/Biofords, Evry Cedex, France), according to the manufacturer's instructions.

The presence of mycotoxins aflatoxins B1, B2 G1, and G2, fumonisin B1 (FB1), deoxynivalenol (DON), ochratoxin, and zeralenol was analyzed in the MON810 and control maize by HPLC (Miraglia et al., personal publication). The values were below the maximum allowable concentration, with the exception of FB1, being 1350 and 2450  $\mu\text{g}/\text{kg}$  in the transgenic and control maize, respectively (maximum allowable concentration = 2000  $\mu\text{g}/\text{kg}$ ), and DON, being 1300 and 650  $\mu\text{g}/\text{kg}$  in the transgenic and control maize, respectively (maximum allowable concentration = 750  $\mu\text{g}/\text{kg}$ ).

The micro- and macronutrients compositions of MON810 and its parental maize are reported in a previous study (17).

The purified Cry1Ab protein was provided by M. P. Carey (Department of Biochemistry, Case Western Reserve University, Cleveland, OH).

**Experimental Diets.** The diets were formulated according to the AIN-93G standard diets (18) and contained 50% MON810 or its parental control maize flour. A standard pellet diet (Mucedola, Milano, Italy), containing about 50% of a commercial nontransgenic maize, was also used. The absence of Cry1Ab in the pellet diet was confirmed by PCR assay, as described above.

**Animals.** Male Balb/c mice were used in all of the experiments. Mice at weaning (21 days of age) were obtained from Charles River Laboratories (Como, Italy), whereas old mice (18–19 months of age) were kindly provided by E. Mocchegiani (IRCA, Ancona, Italy). Mice were kept at 23 °C with a 12 h light–dark cycle. Food intake and body weight were recorded every other day. The weaning mice were fed with the different experimental diets for 30 and 90 days, whereas the old mice received the diets for 90 days. The weaning mice fed for 30 days were younger (51 days old) than the 90 day fed mice (111 days old), with different degrees of immune system maturation. Mice had free access to food and water. At the end of the experimental periods, animals were anesthetized with pentobarbital injection (10 mg), blood was drawn via cardiac puncture, and the spleen and small intestine were excised and placed in cold PBS. Animal studies were performed under conditions approved by the National Health Ministry (Department of Food, Nutrition and Public Animal Health).

**Lymphocytes Preparation.** IELs were isolated from the small intestine according to the method of Corazza et al. (19). Briefly, the intestine was washed twice with cold PBS, longitudinally opened, and cut into small size pieces after removal of Peyer patches. Intestinal pieces were washed in Hank's balanced salt solution  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free (HBSS-CMF) and stirred twice for 45 min at 37 °C in HBSS-CMF added with 10% fetal calf serum (FCS, Euroclone, Milano),  $1 \times 10^5$  units/L penicillin, 100 mg/L streptomycin, 5 mM HEPES (pH 7.4), 1 mM EDTA, and 1 mM dithiothreitol. The eluted cells were passed through 100 and 40  $\mu\text{m}$  nylon cell strainers (Becton Dickinson, BD-Falcon, Milano, Italy) and centrifuged at 650g. Lymphocytes were

isolated by discontinuous 44/67% Percoll (Percoll, GE Healthcare, Milano, Italy) gradient. Spleens were smashed with a 1 mL plastic syringe piston. The released lymphocytes were washed with PBS, separated on Ficoll gradient (Ficoll plaque-plus, GE Healthcare), and resuspended in PBS.

**Antibodies for Flow Cytometry.** Each antibody was titrated to determine the optimal concentration for maximal staining. The following antibodies were used: FITC anti-CD3 (clone 17.12), PE anti-CD19 (clone 1D3), PerCP anti-CD45 (clone 30-F11), PE anti-CD4 (clone GK1.5), PE-Cy5 anti-CD8 (clone 53–67), PE anti-TCR $\gamma\delta$ , (clone GL3), PE-Cy5 anti-TCR- $\alpha\beta$  (clone H57-597), anti-CD16/CD32 (clone 2.4G2). All antibodies were purchased from BD-Pharmingen.

**Flow Cytometry.** IELs and spleen lymphocytes ( $1 \times 10^6$  cells) were preincubated for 20 min with anti-CD16/CD32 to block Fc receptors and avoid nonspecific binding. Cells were then washed and labeled with an appropriate mixture of antibodies or isotype matched controls for 30 min, centrifuged at 650g, and resuspended in 0.5 mL of FACSFlow. Blood lymphocytes were analyzed according to the "lyse no wash" protocol from BD. Briefly, 0.1 mL of blood was incubated with an appropriate mixture of antibodies for 30 min and then incubated with erythrocyte lysing solution (155 mM  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$ , and 1 mM EDTA) on ice until complete lysis. After centrifugation at 650g, the pellet was washed and resuspended in 0.5 mL of FACSFlow. Flow cytometry analysis was performed using a FACScalibur flow cytometer (BD Biosciences). To exclude dead/dying cells and therefore nonspecific antibody-binding cells, lymphocytes were gated according to forward and side scatter. The percentage of T and B lymphocytes was calculated on leukocyte gate ( $\text{CD45}^+$ ), whereas the  $\text{CD4}^+$ ,  $\text{CD8}^+$ ,  $\alpha\beta\text{T}$ , and  $\gamma\delta\text{T}$  cell subsets were calculated on  $\text{CD3}^+$  gate. At least 10000 events were acquired and analyzed. Data were analyzed using CellQuest software (BD Biosciences).

**Proliferative Assay.** The splenic lymphocytes were centrifuged at 250g for 5 min and resuspended in RPMI-1640 medium supplemented with 10% FCS,  $1 \times 10^5$  units/L penicillin, 100 mg/L streptomycin, 4 mM glutamine, 1% nonessential amino acids, and 50 mM 2-mercaptoethanol (Sigma, Milano, Italy). Cells were cultured at  $3 \times 10^5$ /well in 96-well flat-bottom plates (Corning, Roma, Italy) at 37 °C in a humidified atmosphere with 5%  $\text{CO}_2$ . Cells were stimulated with 2.5 mg/L of concanavalin A (ConA; Sigma) for 72 h or with pure Cry1Ab (5 mg/L) for 120 h and labeled with 5 mCi/L of  $^3\text{H}$ -thymidine (6.7 Ci/mmol; NEN, Zaventem, Belgium) for the last 18 h of incubation. After harvesting, radioactivity was counted in a scintillator counter (Microbeta Trilux, Perkin-Elmer, Milano, Italy).

**Cytokine Analysis.** The levels of serum cytokines were analyzed using a mouse CBA Soluble Flex Set system (BD Biosciences) for interleukin (IL)-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFN (interferon)- $\gamma$ , TNF (tumor necrosis factor)- $\alpha$ , MCP-1 (monocyte chemoattractant protein-1), and MIP-1 $\beta$  (macrophage inflammatory protein-1 $\beta$ ) detection, according to the manufacturer's specifications. Briefly, multiplexed antibody-conjugated beads were incubated with serum samples or serial dilutions of cytokine standards for 1 h. After PE detection reagent addition, samples were incubated for an additional 1 h, washed, and analyzed by FACScalibur. Results were analyzed using the FCAP1.1 software (BD Biosciences).

**Statistical Analysis.** The significance of the differences has been tested using the parametric analysis of variance (ANOVA). The SAS statistical package (version 6.12) was used to perform statistical analyses. Differences were considered to be statistically significant when the *P* value was below 0.05.

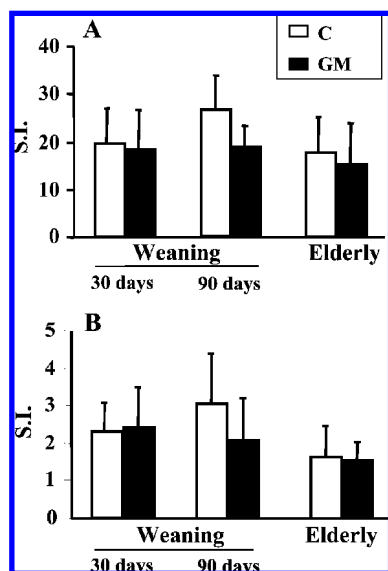
## RESULTS

**Effect of Feeding with MON810 or Its Parental Control Maize on Body Weight and Food Consumption.** There were no differences in the mean body weight between mice fed MON810 or its parental control maize for either 30 or 90 days, independent of the age of the animals. No difference was found in the food consumption of weaning and elderly mice fed the MON810 or control maize (Table 1).

**Table 1.** Body Weight and Food Intake of Weaning and Old Mice Fed MON810 (GM) or Parental Control (C) Maize for 30 or 90 Days<sup>a</sup>

treatment	body wt (g)		food intake (g/day)
	initial	final	
weaning, 30 days			
C	11.3 ± 1.35	22.7 ± 1.35	3.8 ± 0.28
GM	11.2 ± 1.59	23.3 ± 0.90	4.0 ± 0.41
weaning, 90 days			
C	10.3 ± 1.15	30.7 ± 2.60	4.0 ± 0.28
GM	10.3 ± 1.67	29.8 ± 4.22	3.9 ± 0.47
old, 90 days			
C	33.7 ± 2.14	34.8 ± 3.44	4.1 ± 0.31
GM	32.7 ± 3.00	34.4 ± 3.43	4.1 ± 0.38

<sup>a</sup>Data are the means ± SD of at least 15 animals for each group.



**Figure 1.** Proliferation of spleen lymphocytes from weaning and old mice fed MON810 (GM) or its parental control maize (C) for 30 or 90 days and stimulated in vitro with ConA (A) or Cry1Ab (B). The proliferative response was measured as <sup>3</sup>H-thymidine incorporation and is expressed as stimulation index (SI, ratio of cpm of stimulated/cpm of unstimulated lymphocytes). Data are the means ± SD of at least 10 animals for each group.

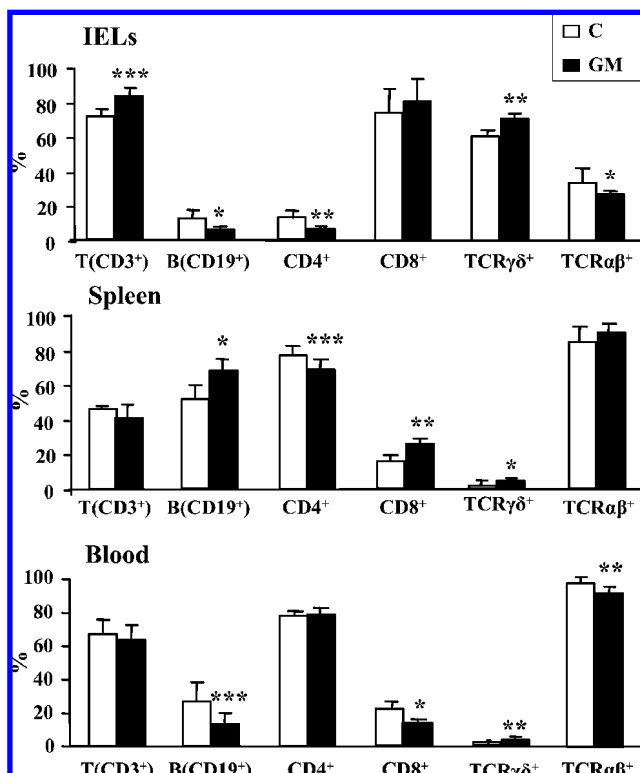
**Proliferative Response.** To verify whether the lymphocytes maintained the ability to proliferate in response to aspecific or specific stimulus, we measured the proliferation of spleen lymphocytes of weaning and elderly mice fed the MON810 or parental control maize after in vitro stimulation with the polyclonal mitogen ConA or with the purified Cry1Ab. No statistically significant differences were found in the proliferative response to ConA or Cry1Ab in any group of animals (Figure 1). However, the stimulation index was low after Cry1Ab stimulation, suggesting a low immunogenicity of Cry1Ab.

**Effect of the Transgenic and Nontransgenic Maize Consumption on Lymphocyte Populations.** To assess whether the MON810 maize consumption could have immunological consequences, we performed the phenotypic analysis of lymphocytes isolated from the intestinal and peripheral sites of mice fed MON810 or its parental control maize. To exclude any other influence than that caused by the Cry1Ab coding sequence, we also analyzed the lymphocyte subsets of mice fed a standard pellet diet containing a commercial non-GM maize. The immunophenotype of intestinal intraepithelial, spleen, and blood lymphocytes of mice fed the control maize was similar to that of pellet fed mice (data not shown). No difference in the total

**Table 2.** Total Number of CD45<sup>+</sup> Cells from Small Intestine, Spleen, and Blood of C and GM Weaning and Old Mice ( $\times 10^6$ )<sup>a</sup>

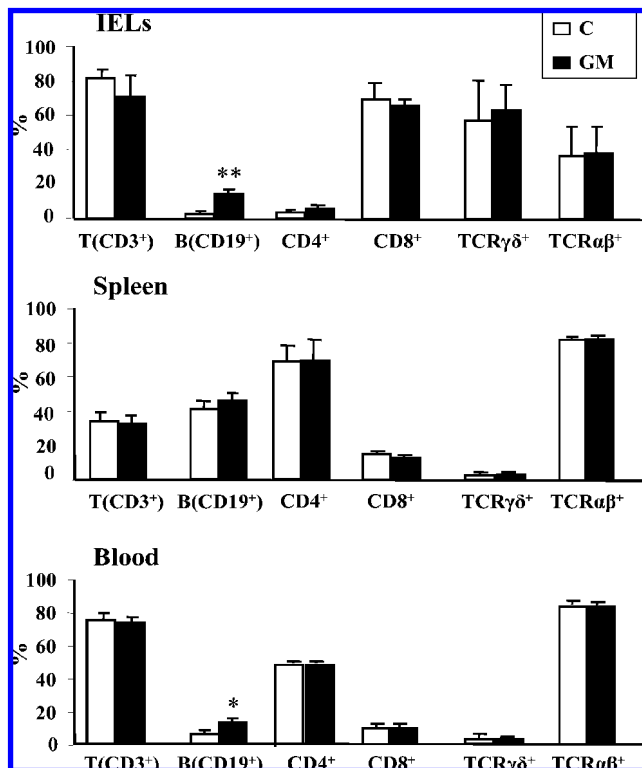
treatment	intestine ( $\times 10^6$ )	spleen ( $\times 10^6$ )	blood ( $\times 10^9$ /mL)
weaning, 30 days			
C	4.4 ± 0.8	27.9 ± 6.1	6.8 ± 1.3
GM	4.1 ± 1.3	24.2 ± 5.8	6.6 ± 2.1
weaning, 90 days			
C	5.2 ± 0.6	28.1 ± 8.4	7.1 ± 1.8
GM	4.9 ± 0.5	29.1 ± 4.9	7.4 ± 1.1
old, 90 days			
C	5.7 ± 0.8	39.2 ± 8.2	7.4 ± 0.9
GM	4.4 ± 1.1	31.7 ± 6.9	7.1 ± 1.4

<sup>a</sup>Data are the means ± SD from at least 10 mice.



**Figure 2.** Effect of feeding weaning mice with MON810 (GM) or its parental control maize (C) for 30 days on percentage of lymphocyte populations. The various cell populations of intestinal intraepithelial lymphocytes (IELs) and of spleen and blood lymphocytes were analyzed by flow cytometry. Data represent means ± SD from at least 10 mice. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , as compared to C.

number of CD45<sup>+</sup> cells of the small intestine, spleen, and blood between mice fed MON810 or its parental control maize was found (Table 2). Several changes were induced by MON810 maize in the various sites depending on the age of the animals. Indeed, in the weaning mice fed the MON810 maize for 30 days, the amount of T cells was higher in the IELs, whereas the B cells were lower in the IELs and blood and higher in the spleen (Figure 2). In addition, the CD4<sup>+</sup> subpopulation decreased in the IELs and spleen, whereas the CD8<sup>+</sup> subset was higher in the spleen but lower in the blood. The TCRγδ<sup>+</sup> subset was higher in the IELs, spleen, and blood, whereas the TCRαβ<sup>+</sup> subset was lower in the IELs and blood. After 90 days of MON810 maize feeding of weaning mice, only alterations in the percentage of B cells were found, being higher in the IELs and blood (Figure 3). In the old mice after 90 days of MON810 maize consumption, the percentage of B cells was lower in the IELs and blood and the CD4<sup>+</sup> subset was lower in the IELs



**Figure 3.** Effect of feeding weaning mice with MON810 (GM) or its parental control maize (C) for 90 days on percentage of lymphocyte populations. The various cell populations of intestinal intraepithelial lymphocytes (IELs) and of spleen and blood lymphocytes were analyzed by flow cytometry. Data represent means  $\pm$  SD from at least 10 mice. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , as compared to C.

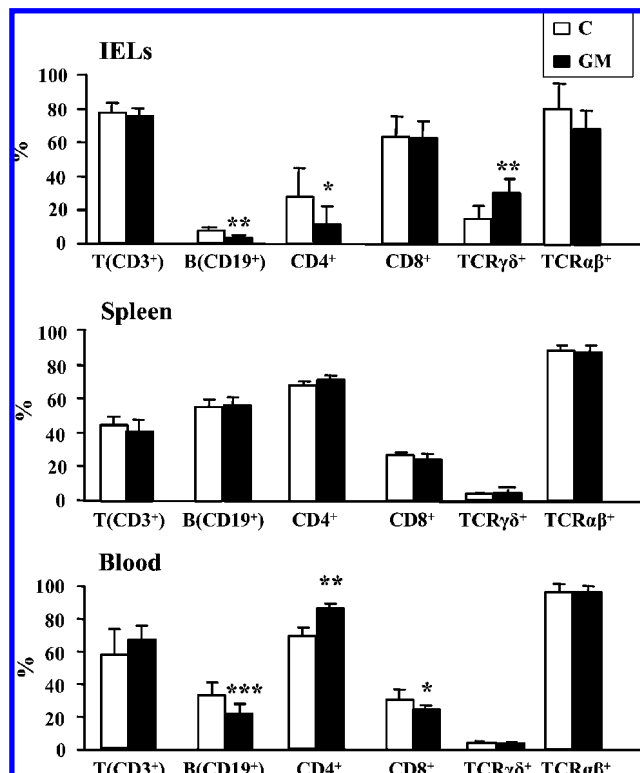
and higher in the blood, whereas the CD8<sup>+</sup> subset was lower in the blood and the TCRγδ<sup>+</sup> subset was higher in the IELs (Figure 4).

**Cytokine Profiling.** To test whether the MON810 maize consumption induced changes in cytokine pattern, we have evaluated several cytokines in serum of weaning and old mice fed with MON810 or control maize for 30 or 90 days. The results showed an increase in IL-6, IL-13, IL-12p70, and MIP-1β in weaning mice fed MON810 for 30 days, an increase of MIP-1β in weaning mice fed MON810 for 90 days and in old mice, and a small but not significant increase in IL-12p70 in old mice (Table 3).

## DISCUSSION

In this study we evaluated the immunomodulatory effects of whole transgenic MON810 maize consumption as compared to its parental control and a commercial maize, by considering the gut and peripheral immune response of mice in vulnerable conditions. We report that the MON810 maize used in this study, when given to both weaning and old mice for 30 and 90 days, induced several changes to the immunophenotype of the gut, spleen, and circulating lymphocytes and to the level of serum cytokines.

The MON810 and its parental control maize given to the animals were grown simultaneously in neighboring fields, using the same agricultural techniques and had therefore the same external climatic conditions, which eliminates or reduces environmental variables. In addition, the compositional analysis indicated that both the transgenic and nontransgenic maize had similar nutritional composition, and thus the diets given to the animals were similarly balanced, excluding that the observed



**Figure 4.** Effect of feeding old mice with MON810 (GM) or its parental control maize (C) for 90 days on percentage of lymphocyte populations. The various cell populations of intestinal intraepithelial lymphocytes (IELs) and of spleen and blood lymphocytes were analyzed by flow cytometry. Data represent means  $\pm$  SD from at least 10 mice. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , as compared to C.

effects were caused by improper nutrition. The amount of DON was higher in the transgenic than control parental maize, whereas the amount of FB1 in the control maize was almost double that of the transgenic maize. These mycotoxins are frequent contaminants of maize and may exert immunotoxic activity, depending on dose, exposure, and timing of administration (20, 21). Nevertheless, in agreement with previous studies (22), the increases of DON and FB1 were modest, their levels being slightly higher than the maximum allowable concentration and much lower than those known to affect the immune response (23–25). In addition, the immune markers of the animals fed control maize did not differ from those of animals fed the commercial nontransgenic maize. Thus, all of these data indicate that the observed immunophenotype changes were likely due to the insertion of the Cry1Ab coding sequence.

Several and different perturbations were observed in lymphocyte subsets after MON810 maize consumption, depending on the age of the animals. The most affected were the weaning mice fed for 30 days the transgenic maize, showing several alterations in immunophenotype of IELs, spleen, and blood lymphocytes. Only an increase of B cells was present after MON810 maize consumption in the weaning mice fed for 90 days, which were 2 months older than the weaning mice fed for 30 days. Also, in the old mice the consumption of MON810 maize induced several alterations in the IELs and blood, which resembled those of the weaning mice fed the transgenic maize for 30 days. These data suggest that age was an important factor in the immune response to MON810 maize. This fact is not surprising, considering that the immune system during weaning and aging can less efficiently or inappropriately respond to external stimuli than during adult age. The weaning represents

**Table 3.** Serum Cytokine Levels of Weaning and Old Mice Fed MON810 (GM) or Parental Control (C) Maize for 30 or 90 Days<sup>a</sup>

treatment	pg/mL										
	IL-4	IL-5	IL-6	IL-10	IL-13	IL-12p70	IL-21	TNF- $\alpha$	IFN- $\gamma$	MIP-1 $\beta$	MCP1
weaning, 30 days											
C	2.6 (0.3)	2.9 (0.3)	4.3 (1.7)	9.1 (2.3)	2.9 (1.2)	7.0 (0.8)	9.3 (0.7)	14.3 (2.3)	2.1 (0.2)	24.9 (5.1)	39.4 (6.7)
GM	2.8 (0.5)	3.3 (0.5)	20.6 (8.1)*	12.0 (3.9)	6.5 (0.8)*	9.7 (2.9)*	8.1 (3.0)	17.9 (6.6)	2.2 (0.4)	33.0 (6.2)*	60.5 (29.5)
weaning, 90 days											
C	2.6 (0.5)	3.2 (0.5)	3.8 (0.6)	10.2 (3.2)	6.9 (2.7)	8.4 (2.2)	8.6 (0.5)	15.6 (5.2)	2.3 (0.5)	23.3 (3.2)	42.5 (13.4)
GM	2.7 (0.3)	2.9 (0.5)	6.7 (4.7)	9.1 (2.0)	5.6 (1.9)	9.2 (4.0)	10.5 (7.4)	16.8 (2.6)	2.3 (0.6)	32.2 (6.7)*	38.3 (3.2)
old, 90 days											
C	3.2 (0.5)	3.3 (0.6)	5.7 (1.1)	13.1 (1.9)	6.5 (4.1)	10.7 (2.8)	9.4 (3.5)	17.6 (4.1)	2.4 (0.5)	27.0 (5.3)	49.4 (14.5)
GM	2.6 (0.9)	3.4 (0.8)	5.3 (2.8)	11.6 (4.4)	6.3 (1.1)	12.1 (3.1)	7.9 (2.2)	20.6 (5.7)	2.2 (0.7)	39.7 (13.4)*	41.0 (15.9)

<sup>a</sup>Data are the means  $\pm$  SD (in parentheses). For each column,  $P < 0.05$  as compared to C.

a critical point in the development of a balanced immune response to external antigens, because a maximum exposure to novel food antigens together with removal of milk maternal protective factors occurs (26–28). Nutrition at weaning may also provide new factors that influence intestinal flora, which in turn will affect antigen exposure, immune maturation, and immune responses (29–31). Problems may arise when the immune system develops and functions inappropriately, resulting in inefficacy to develop tolerance toward harmless food proteins with consequent immunologic disorders (27, 32). In the case of weaning mice fed for 90 days, the low responsiveness to MON810 maize can be due to an acquired ability to tolerate the transgenic food during the longer treatment. With regard to aging, age-associated dysregulations of the immune system are well documented (33), and alterations in antigen-specific antibody responses, impairment of oral tolerance, and reduction of natural killer cells are frequently observed (34, 35). In addition, as for weaning, changes in microflora composition occur during aging in a way that may impair the correct immune response (36, 37). In conclusion, our results suggest that age is an important factor to be taken into account in the evaluation of transgenic food safety.

One of the more recurrent alterations in lymphocyte phenotypes observed in this study was an increase in the TCR $\gamma\delta^+$  population. A high percentage of these lymphocytes are localized in the gut and in the mouse, a substantial proportion of  $\gamma\delta$ T cells resides in the IELs (38).  $\gamma\delta$ T cells seem to be important regulatory elements of the immune system, being capable of modulating inflammatory response associated with infectious agents and autoantigens (39–41). Higher numbers of  $\gamma\delta$ T cells have been observed in humans with asthma (42), in IELs of children with untreated food allergy (43), and in the duodenum of children with juvenile idiopathic arthritis or connective tissue disease with gastrointestinal symptoms (44). In addition, murine  $\gamma\delta$ T cells have been shown to abrogate the oral tolerance (45). However, an inhibition of late allergic airway responses and eosinophilia by  $\gamma\delta$ T cells has also been found (46). Besides the exact function of  $\gamma\delta$ T cells, the significance of the increase of this subpopulation observed in the present study deserves further evaluation. This is certainly true also for the other phenotypic lymphocyte alterations, the meaning of which remains to be defined. For example, the decrease of B cells does not necessarily mean a reduction of their secreted antibodies amount, and it would be interesting to evaluate the impact of MON810 maize on the different classes of antibodies. In this regard, studies are currently evaluating the amount of different antibodies in serum of mice used in the present study, and preliminary results indicate an increase of total IgG and IgE in both weaning and old mice fed MON810 maize as compared to its parental control maize (Ortolani et al., personal publication). A previous study reported no allergenicity of

MON810 maize as evaluated by skin prick test in sensitive subjects suffering for asthma-rhinitis or by IgE antibodies secretion against pure Cry1Ab protein in individuals with food allergy (10). However, these tests were not performed in vulnerable subjects such as children and elderly people. On the other hand, an anti-Cry1Ab-specific IgG2 response in rats fed transgenic rice expressing Cry1Ab protein for 90 days and increased antigen-specific IgG1 in rats fed for 28 days the same rice but spiked with Bt toxin have been found (13). In addition, a study conducted in farm workers exposed to Bt pesticides indicated elevated Bt-specific IgE and IgG antibodies in more high- than low-exposure workers, associated with positive skin prick tests to Bt spore (11). Similarly, greenhouse workers exposed to Bt pesticides reported an increase of Bt-specific IgE (12).

Alterations of the immunophenotypes induced by the transgenic maize were associated with increased levels in some of the considered cytokines, especially in the weaning mice fed for 30 days the MON810 maize. These cytokines (IL-6, IL-13, IL12p70, MIP-1 $\beta$ ) are involved in allergic and inflammatory responses (47–49), and although they were not strongly elevated by MON810 maize consumption, their increase is a further indicator of immune perturbations induced by MON810 maize.

The recent results obtained by some authors may offer a rationale for the alterations found in the present study, beyond the presence of Cry1Ab protein. Indeed, they have analyzed the seeds of MON810 and its parental control maize utilized in the present study by differential proteomic analysis to evaluate possible unintended side effects. They have found that 43 proteins were up- or down-regulated in transgenic as compared to control seeds, likely as a result of the Cry1Ab gene insertion (50). Interestingly, among them a newly expressed 50 kDa  $\gamma$ -zein, a well-known allergenic protein (51), was detected. Post-translational modifications in GM crops were also observed in a previous study demonstrating that the transgenic expression of bean  $\alpha$ -amylase inhibitor ( $\alpha$ A1) in peas led to the synthesis of a modified form of the protein that showed altered antigenic properties (7). In addition, consumption of this protein by mice predisposed  $\alpha$ A1-specific CD4<sup>+</sup>Th2-type inflammation.

In conclusion, the results obtained indicate that the consumption of MON810 maize used in the present study induced alterations in intestinal and peripheral immune response of weaning and old mice. Although the significance of these data remains to be clarified to establish whether these alterations reflect significant immune dysfunctions, these results suggest the importance of considering the gut and peripheral immune response to the whole GM crop, as well as the age, in the GMO safety evaluation.

## ABBREVIATIONS USED

GM, genetically modified; IELs, intraepithelial lymphocytes, MON810 maize, transgenic maize expressing Cry1Ab protein.

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