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Immunomodulatory Effects of Egg White Enzymatic Hydrolysates Containing Immunodominant Epitopes in a BALB/c Mouse Model of Egg Allergy

MARIE YANG,[†] CHENGBO YANG,[‡] FRANÇOISE NAU,[#] MARYVONNE PASCO,[#] LEKH R. JUNEJA,[§] TUTOMU OKUBO,[§] AND YOSHINORI MINE*,[†]

Department of Food Science and Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada; UMR 1253 INRA-Agrocampus Rennes Sciences et Technologie du Lait et de l'Oeuf, 65 rue de Saint Brieuc, 35042 Rennes Cedex, France; and Research Institute, Taiyo Kagaku Company Ltd., 1-3 Takaramachi, Yokkaichi, Mie 510-0844, Japan

Egg has ben documented as a rich source for the supply of biologically active peptides. This study characterizes the immunomodulatory effects of an egg white enzymatic hydrolysate (EWH) using a BALB/c mouse model of egg allergy. Mice were orally sensitized to egg white and subsequently gavaged with EWH. ELISA results indicated significant reductions of both serum histamine and specific IgE titers in EWH-fed mice, accompanied by a repression of both IL-4 and IFN-γ production in spleen cell cultures. Similarly, real-time RT-PCR analyses highlighted decreased mRNA expression of IFN-γ and IL-12 (Th1-biased), as well as lower ratios of IL-4 and IL-13 mRNA (Th2-biased). On the other hand, increased intestinal expressions of TGF-β and FOXp3 mRNA were determined in EWH-fed mice, suggesting induction of local regulatory mechanisms. The presence of immunodominant epitopes was proposed to be responsible for the immunomodulatory effects observed.

KEYWORDS: Egg allergy; enzymatic hydrolysate; immunomodulation; epitopes; regulatory T cells

INTRODUCTION

Egg components have numerous properties that make them attractive for a wide variety of utilizations in the food industry as well as for biomedical applications. Indeed, beyond their well-known nutritional and functional attributes, compounds found in eggs have generated a growing interest for their biological activities and, more particularly, for their immunomodulating effects. Recent reviews described how peptides released from enzymatic hydrolysis of certain egg proteins could exert modulatory effects on immune functions such as phagocytosis, cytokine production, or lymphocyte proliferation (*1*, *2*). These effects suggest their potential use in the management of immune disorders such as food allergy. With a reported 2-fold increase of its prevalence in the past decade (*3*), food allergy affects 6−8% of children and nearly 4% of adults in the United States (*4*), and it continues to emerge as a substantial health problem. The disorder results from an adverse immune response to food components, mainly glycoproteins, and its most common form is mediated by the class of immunoglobulin E (IgE), and is otherwise known as type I hypersensitivity.

Among the strategies explored for the management of food allergy, the present study considered the use of native proteins as template for the development of immunotherapeutic peptides. The egg itself represents a major source of allergenic proteins. The worldwide prevalence of egg allergy varies between 1.6 and 3.2% (*5*−*7*), and its cumulative prevalence from birth to age 2.5 years ranges from 2 to 6% (*5*). It was documented as the second major cause of food-induced allergic reactions within the pediatric population in the United States (*8*) and represents the most prevalent childhood food hypersensitivity in Europe and in Asian industrialized countries, surpassing that of cow's milk allergy (*9*). Due to the absence of an efficient cure, the most medically advised approach to egg allergy remains that of a preventive intervention consisting in complete exclusion of the offending food from the patient's diet (*10*). Nevertheless, major hurdles associated with elimination diets are those of compliance and possibilities for nutritional deficiencies. Therefore, alternative strategies involving, for instance, the use of recombinant molecules or bacterial components are under active investigation (*3*); however, none of them is yet available to allergic patients. More immediate and practical approaches are thus highly warranted and, for example, the use of hydrolyzed food formulas, as a preventive or curative measure, represents an attractive approach. Whereas studies on the safety and efficacy of hydrolyzed food formulas remain

* Corresponding author: Professor Yoshinori MINE, Ph.D., Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G2W1, Email: ymine@uoguelph.ca, Phone: +1-519-824-4120 x52901, Fax: +1-519-824-6631.

[†] Department of Food Science, University of Guelph.

[‡] Department of Animal and Poultry Science, University of Guelph.

[#] UMR 1253 INRA-Agrocampus Rennes Sciences et Technologie du Lait et de l'Oeuf.

[§] Taiyo Kagaku Company Ltd.

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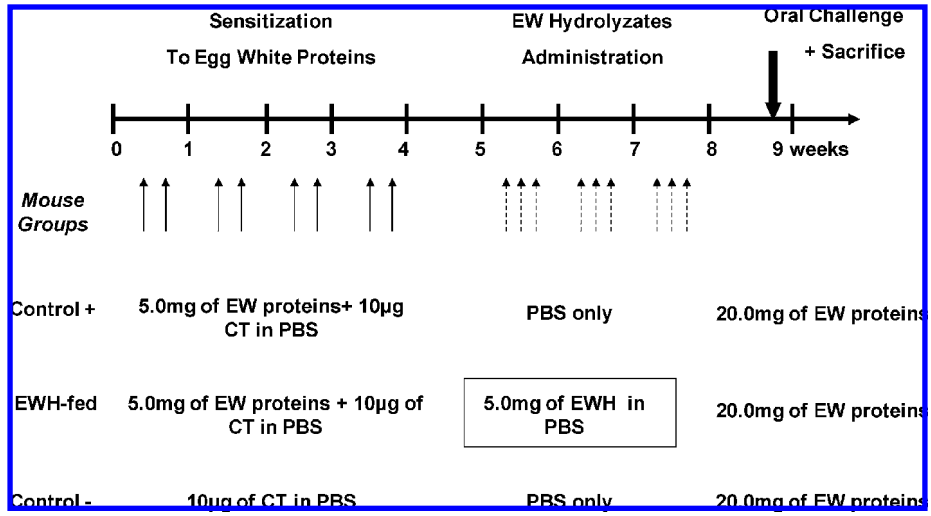

Figure 1. Experimental protocol: mouse sensitization, EWH oral administration and final oral challenge. Control +, positive control group; Control −, negative control group; EWH-fed, group orally gavaged with egg white hydrolyzate (EWH).

controversial (*11*), no report has yet proposed a rationale behind their immunomodulatory effects. Furthermore, to date, food-based hydrolyzed formulas have been primarily investigated in a preventive rather than therapeutic context. Using BALB/c mice, the present study addressed these issues and explored the mechanisms behind the immunomodulatory effects of an egg white protein-based hydrolysate (EWH) obtained via standard food-processing methods based on enzymatic hydrolysis and ultrafiltration.

EWH was orally administered to BALB/c mice primed to egg white proteins for a period of 3 weeks, three times weekly. At the experimental end point, serum histamine and specific immunoglobulin levels as well as spleen cell cytokine secretions and intestinal gene expression were analyzed. Some structural features of EWH (size and primary sequence) were also explored to identify the elements potentially responsible for the immunomodulatory effects observed.

MATERIALS AND METHODS

Preparation of Egg White Protein Hydrolysate (EWH). EWH was provided by Taiyo Kagaku Co., Ltd. (Yokkaichi, Japan) and prepared as follows: homogenates of liquid egg white were subjected to enzymatic hydrolysis in the presence of food-grade aminopeptidase of *Aspergillus* sp. origin (EC 3.4.11.1, Novozymes AS, Bagsvaerd, Denmark). The hydrolysis reaction was carried out at 55−60 °C for 24 h, stopped by heating at 90 °C for 30 min, and followed by a 10 kDa cutoff ultrafiltration of the soluble fraction. The resulting permeates were then spray-dried.

Characterization of EWH. Molecular size distribution was determined using high-performance gel permeation chromatography (HP-GPC, Superdex Peptide 10/300GL column, Amersham-Pharmacia, Piscataway, NJ) with a 0.02 M phosphate buffer supplemented with 0.25 M NaCl (pH 7.2). The Waters LC system (Milford, MA) used was composed of a Waters 717 plus autosampler, a Waters 2487 dual λ absorbance detector set at a detection wavelength of 214 nm, and a Waters 1525 binary pump, with version 3.2 Breeze software running under Microsoft Windows NT version 4.0 for system control and data handling. For calibration, the following molecular markers were used: cytochrome *c* (relative mass M_r 12500), aprotinin (M_r 6512), vitamin B12 (M_r 1355), cytidine (M_r 246), and glycine (M_r 75).

EWH amino acid sequence analysis was performed at the mass spectrometry facility, Institut National Supérieur de Formation Agro-alimentaire (INSFA, Agrocampus Rennes, Rennes, France). All mass spectra of intact oligopeptides were obtained using matrix-assisted laser ionization/desorption time-of-flight (MALDI-TOF) configuration with a Voyager DE STR spectrometer (Applied Biosystem, Courtaboeuf, France) equipped with a nitrogen laser (337 nm, 20 Hz). All nano-electrospray mass spectrometry (nES-MS) experiments for peptide mapping were conducted on a Q-TOF hybrid quadrupole/time-of-flight instrument (Micromass, Manchester, U.K.), for high resolution and online liquid chromatography−tandem mass spectrometry (LC-MS/MS) analyses. An external calibration was first performed in the range of 500−3000 Da. The monoisotopic mass lists were compared to the Swiss-Prot and TrEMBL protein databanks available on the ExPASy proteomic server (http://us.expasy.org/) using Protein prospector software (http://prospector.ucsf.edu/) for peptide mass fingerprinting (PMF) analysis. The list of peptide masses was searched against the nonredundant protein sequence database provided by the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov).

Animal Sensitization, EWH Administration, and Challenge Protocols. BALB/c mice (6−7 weeks old) were purchased from Charles River laboratories (Montreal, QC, Canada) and randomly divided into three groups of five mice, that is, positive control (control +), negative control (control −), and EWH-fed mice (EWH-fed group). All animals were housed in the campus animal facility at the University of Guelph in a 12-h lighting cycle and fed an egg-free diet (Harlan Teklad global diet, 14% protein and 3.5% fat, Madison, WI). Food and water were available ad libitum. All procedures were performed in accordance with the guidelines established by the Canadian Council of Animal Care (CCAC) and approved by the Animal Care Committee at the University of Guelph.

Following a 1-week acclimatization period, the positive control group (control +) and the EWH-fed group (EWH-fed) were orally gavaged with 100 μL/mouse of a PBS solution containing 5.0 mg of freeze-dried whole egg white (EW) prepared in-house and 10 μg of cholera toxin (CT) (List Biologicals Laboratories, Denver, CO), twice per week for a duration of 4 weeks. The third group (control −) was orally gavaged according to an identical schedule, with 100 μL/mouse of a placebo solution consisting of PBS buffer containing 10 μg of CT, but devoid of EW proteins. Blood samples were collected (saphenous vein) at the end of the 4-week sensitization period, and EW specific IgE levels were determined to confirm induction of significant IgE titers specific to the four major egg white proteins, commonly known as ovalbumin (OVA), ovomucoid (OVM), lysozyme (LYS), and ovotransferrin (OVT) (*9*). Following the 4-week sensitization period, mice in the EWH-fed group received 5.0 mg of EWH in 100 μL of PBS solution (oral gavage), three times per week for duration of 3 weeks. During the same period, the other two groups (control + and control −) were orally gavaged with 100 μL of PBS placebo solution. At the experimental end point, all mice were orally challenged with 20.0 mg/mouse of EW proteins dissolved in 100 μL of PBS solution (**Figure 1**).

Measurement of Serum Histamine Levels. At the end point, whole blood was collected by cardiac puncture, and serum samples were

pooled in equal volumes within each group. Histamine concentrations were determined by ELISA assay using a commercial kit (Histamine EIA, LDN Labor Diagnostika Nord, Nordhon, Germany). The test is based on the prederivatization of histamine by acylation, followed by a competitive ELISA in a microtiter plate format. Quantification of unknown samples was achieved by comparison of their absorbance with a reference curve prepared with histamine standards of known concentration (0.3−30 ng/mL) provided by the manufacturer. Serum samples were diluted (1:10) with diluent buffer (provided in the kit) prior to their application onto the plate and were run in triplicate wells along with the histamine standards.

Determination of Egg White Specific IgE and IgG. Egg white specific IgE and IgG levels were determined by sandwich ELISA according to a previously described procedure (*12, 13*) using a coating level of 5 μg/well of EW proteins in 100 mM of sodium bicarbonate buffer (pH 9.5). Mouse sera were diluted in 1% BSA in PBST (1:5 for EW specific IgE and 1:100 for EW specific IgG) and assayed in triplicate wells. For determination of egg white specific IgE, monoclonal anti-mouse IgE conjugated to biotin (Caltag Laboratories Inc., Burlingame, CA) was used in combination with avidin−horse radish peroxidase (Av-HRP) conjugate (BD Pharmingen, Mississauga, Canada), and color development was revealed by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) chromogenic substrate (Sigma, St. Louis, MO). For determination of egg white specific IgG, polyclonal goat anti-mouse IgG (Fab specific) conjugated to alkaline phosphatase (Sigma) was employed followed by the addition of *p*-nitrophenyl phosphate (*p*NPP, Sigma).

Determination of Egg White Specific IgG1 and IgG2a. Subisotypes of mouse IgG, namely, IgG2a and IgG1, reflect the murine immune balance existing between Th1- and Th2-mediated responses, respectively (*14*). Each isotype was determined with use of an indirect ELISA microplate assay according to a previously described procedure (*12,13*) using a coating level of 5 μg/well of EW proteins in 100 mM sodium bicarbonate buffer (pH 9.5). Sera within each group ($n = 5$) were pooled in equal volumes prior to analyses and diluted 1:10 for analysis of specific IgG2a and 1:500 for specific IgG1. For determination of bound IgG1 and IgG2a, monoclonal rat anti-mouse IgG1 antibody (1:2000) or anti-mouse IgG2a (1:2000) (BD Pharmingen) was added to the plates followed by incubation with polyclonal rat anti-mouse Ig conjugated to HRP (1:3000; BD Pharmingen). Color development was revealed by the addition of TMB substrate (Sigma).

Allergen-Induced Cytokine Secretion in Spleen Cell Cultures. At the end point, spleens from each individual mouse were aseptically removed into ice-cold RPMI 1640 medium (Gibco Invitrogen, Grand Island, NY), containing sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 units/mL), and streptomycin (50 μg/mL), and whole spleens were pooled within each group. Single cell suspensions were prepared by gently grinding spleens using a syringe plunger and passing through a 100 μM nylon membrane cell strainer (BD Biosciences). The spleen cell suspension was transferred onto a 15 mL tube and subjected to centrifugation for 5 min at 100*g*. The supernatant was discarded, erythrocytes were depleted by the addition of 5 mL of red blood cell lysing buffer (Sigma), and the reaction was stopped by adding 5 mL of sterile RPMI 1640 medium. After resuspension, the preparation was centrifuged for another 5 min at 100*g* and washed twice with 10 mL of RPMI 1640 medium. The splenocytes were resuspended in RPMI 1640 medium supplemented with 8% fetal bovine serum (FBS) (Hyclone, Fisher Scientific, Ottawa, ON, Canada), and cell viability was assessed by trypan blue exclusion. Cells were cultured in 24-well plates (Corning) at a density of 2.5×10^6/mL in the absence (negative control wells) or presence of EW proteins (100 μg/mL). Supernatants were collected after 72 h of culture in a 5% CO_2 humidified incubator and assayed for the presence of cytokines IFN-γ (Th1-biased) and IL-4 (Th2-biased), in triplicate wells, using antibody reagents purchased from commercial kits (BD Pharmingen, Mississauga, Canada) following the manufacturer's instructions (standard ranges: IFN-γ, 1000−31.250 pg/mL; IL-4, 500−7.8 pg/mL).

Real-Time RT-PCR Analysis of Gene Intestinal Expression. Mouse ileums from all three groups were freshly and individually isolated ($n = 5$ samples collected per group) for relative quantification of gene mRNA expression by real-time RT-PCR analyses. The ileal samples were immediately placed in ice-cold RNAlater solution (Sigma) and stored at −30 °C until RNA isolation. Total ileum RNA was extracted from individual mice (30 mg of homogenized tissue/mouse, $n = 5$ per group) using the spin column procedure from RNeasy kits (Qiagen, Mississauga, Canada). The total RNA quality was assessed on 1% agarose gels, and respective concentrations and purity were determined by measuring absorbance ratios A_{260}/A_{280}. Complementary DNA (cDNA) was synthesized from 1.0 μg/mouse of total RNA using Quantitect Rev kit (Qiagen), following the manufacturer's instructions. The real-time fluorescence-monitored PCR reactions were performed using an iCycler (Bio-Rad Laboratories) detection system. The temperature profile was 95 °C for 15 min, then 15 s at 95 °C (denaturation), 56 °C for 15 s (annealing), and 72 °C for 30 s (extension), repeated for 45−50 cycles. Melting curve analysis was used to confirm specific replicon formation. Negative controls without cDNA were assayed along with the mouse samples, and each mouse sample was analyzed in duplicate for each target gene. Relative mRNA expression ratios were determined using the $2^{-\Delta Ct}$ (threshold cycle) method (*15*), and all data were normalized to the housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of the primers used for real-time RT-PCR analyses are presented in **Table 1**.

Table 1. Sequences of Primers Used for Real-Time RT-PCR Analyses[a]

mouse gene	sequence (5′−3′)	T_m value (°C)	PCR product size (bp)	accession no.
IL-4	FP: CCTCACAGCAACGAAGAACA	60.02	155	NM_021283
	RP: ATCGAAAAGCCCGAAAGAGT	60.21		
IL-5	FP: GAAGTGTGGCGAGGAGAGAC	59.99	177	NM_010558
	RP: GCACAGTTTTGTGGGGTTTT	59.88		
IL-13	FP: CAAGACCAGACTCCCCTGTG	60.70	151	NM_008355
	RP: GGTTACAGAGGCCATGCAAT	59.96		
IL-12p40	FP: TCAGGGACATCATCAAACCA	59.96	160	NM_008352
	RP: TTTTCTTTCTTGCGCTGGAT	59.99		
IL-10	FP: GCCTTATCGGAAATGATCCA	59.86	151	NM_010548
	RP: AGGGGAGAAATCGATGACAG	59.09		
IL-18	FP: TTGCTTGCCAAAAGGAAGAT	59.82	121	NM_008360
	RP: CAAACCCTCCCCACCTAACT	60.22		
IFNγ	FP: GCTCTTCCTCATGGCTGTTT	59.43	154	NM_008337
	RP: GTCACCATCCTTTTGCCAGT	59.97		
TGFβ1	FP: TTGCTTCAGCTCCACAGAGA	59.86	156	NM_011577
	RP: TACTGTGTGTCCAGGCTCCA	60.31		
FOXp3	FP: TCCTTCCCAGAGTTCTTCCA	59.77	157	NM_054039
	RP: CGAACATGCGAGTAAACCAA	59.73		
GAPDH	FP: ACTGGGACGACATGGAGAAG	60.11	132	NM_008084
	P: GGATGCAGGGATGATGTTCT	59.89		

[a] FP, forward primer; RP, reverse primer; IL-4, interleukin-4; IL-5, interleukin-5; IL-13, interleukin-13; IL-12p40, interleukin-12 unit p40; IL-10, interleukin-10; IL-18, interleukin 18; IFN-γ, interferon gamma; TGF-β1, transforming growth factor beta 1; FOXp3, forkhead box transcription factor 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T_m, melting temperature.

Statistical Analysis. Histamine, immunoglobulin, and cytokine concentrations measured by ELISA assays were subjected to ANOVA analyses followed by post hoc multiple comparison using Tukey's test. Statistical differences in the expression ratios determined by real-time RT-PCR analyses were assessed using the Kruskall−Wallis test, followed by Dunn's post hoc test. In all cases, p values of ≤ 0.05 were considered to be statistically significant. Statistical calculations were performed using the GraphPad Prism 5.0 package (GraphPad Software Inc., San Diego, CA).

RESULTS

Characterization of EWH. Analyses by GPC determined that >85% (46.81 + 35.2 + 4.86 = 86.87%) of the fragments

Table 2. Molecular Mass Distribution of Egg White Hydrolysate (EWH) As Determined by HP-GPC Analyses[a]

	peptide approx M_r				
	12.5−6.5	6.5−1.3	1.3−0.25	0.25−0.07	<0.07
percentage of total EWH	6.48	6.65	46.81	35.20	4.86

[a] Molecular markers used: cytochrome *c* (M_r 12500), aprotinin (M_r 6512), vitamin B12 (M_r 1355), cytidine (M_r 246), glycine (M_r 75). M_r, relative molecular mass.

Table 3. Amino Acid Sequences of Oligopeptide Fragments Identified in Egg White Hydrolysates (EWH)[a]

EWH peptide amino acid sequence	OVA B-cell epitope sequence identified	OVA T-cell epitope sequence identified
SIGAASMEF		
CFDVFKELK		
NSWVESQTNGI		^{147}SWVESQTNGI^{156}
RFDKLPGFGDSIEAQC		
GFGDSIEAQCGTSVNVHS	^{77}VNVHS^{81}	
RDILNQI		
TKPNDVYSFS		
LYAEERYPI		
PILPEYL		
ELYRGGLEPINFQTAADQAREL	^{126}GGLEPINFQT^{136}	
RFDKLPGFGDSIEAQ		
WVESQTNGIIRNV		
AIVFKGL		
GLWEKAFKDEDTQAMPF		
KAFKDEDTQAMPFR		
VTEQESKPVQMM		
SIINFEKLTEWTSSNVMEERKI		^{263}KLTEWTSSNVME^{274}
MLVLLPDEVSGLEQLESI		
LPDEVSGLEQLESIINF		
KILELPF		
LELPFASGTMSML		
KLTEWTSSNVMEERKI		
NLTSVLMAMGI		
MGITDVFS	^{301}GITDVF^{306}	
GITDVFSSSA	^{301}GITDVF^{306}	
HIATNAVLFF		
EVVGSAEAGVDAASV		
GVDAASVSEEFRADHPFL		
EINEAGREVVGSAEAGV		^{333}EINEAGRE^{340}

[a] Analyses were conducted at the Institut National Superieur de Formation Agro-alimentaire (INSFA, Agrocampus Rennes, Rennes, France) and were performed using mass spectrometry in a MALDI-TOF-q (matrix-assisted laser desorption/ionization time-of-flight) configuration with online LC-MS/MS analytical equipment. Superscript numbers indicate the position of the amino acid residue in the native sequence of ovalbumin.

contained in the EWH preparation have molecular masses lower than approximately 1.3 kDa (**Table 2**), a molecular size that was documented to have a low probability of displaying IgE cross-linking activity when used in allergic individuals (*16*, *17*).

Amino acid sequence analyses of EWH using LC-MS/MS (MALDI-TOF-q) are presented in **Table 3**. B- and T-cell epitope regions of the two major egg allergens, that is, ovalbumin (OVA) and ovomucoid (OVM), have recently been reported in the BALB/c mouse strain (*9*, *18*) and were compared to the sequences identified in EWH. Interestingly, results revealed the presence of three sequences corresponding to OVA B-cell epitopes (i.e., V77S81, G127T136, and G301F306) as well as three sequences matching OVA T-cell epitopes (i.e., S147I156, K263E274, and E333E340). The presence of peptides corresponding to OVA B- and T-cell epitopes suggests that these particular sequences are resistant to the action of the food-grade microbial enzyme used during the preparation of EWH. On the other hand, no peptide sequences matching OVM epitopes were identified. The lack of information on OVT and LYS epitopes in BALB/c mice did not allow for the determination of the absence or presence of other major egg allergen epitopes.

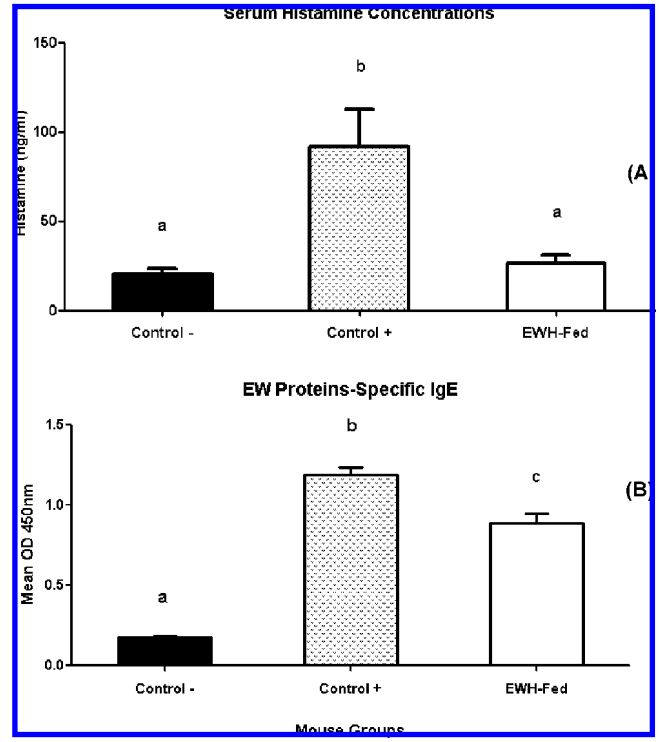

Figure 2. Serum histamine (**A**) and EW specific IgE (**B**) levels in EW-sensitized BALB/c mice after repeated oral administration of EWH. Data are represented as mean ± standard deviation ($n = 3$ test replicates). Different letters indicate significant differences ($p < 0.05$) between groups of mice.

However, on the basis of the observation that native sequences of OVM were absent from the EWH preparation (potentially due to its resistance to the enzymatic hydrolysis) and the knowledge that OVA represents >50% (w/w) of the total protein content in egg white, we suggest that the presence of OVA B-cell and T-cell epitopes may account for the immunomodulatory effects observed in the study.

Reduction of both Serum Histamine and EW-Specific IgE Levels. Upon binding with the food allergen, the cross-linking of specific IgE present on the surface of mast cells triggers the release of vasoactive inflammatory mediators such as histamine. Serum histamine concentration represents, therefore, a good indicator of the extent of immediate-type hypersensitivity in the BALB/c mouse model. Upon final oral challenge with an excess amount of EW proteins, EWH-fed mice showed significantly lower histamine titers, with concentration levels close to those measured in the negative control group (**Figure 2A**). This indicates a suppressive influence of EWH on the development of allergic clinical manifestations.

Serum specific IgE raised against egg white proteins was determined by ELISA in pooled sera obtained from all three groups of mice (**Figure 2B**). No or very little IgE was detected in the serum of mice challenged with placebo solution (control −). Compared to the positive control group, significantly lower levels were observed in EWH-fed mice. This suggests a repressive effect of the EWH on the production of EW specific IgE, which is consistent with the serum histamine levels.

EW-Specific IgG Levels: Down-regulation of Specific IgG2a, but Absence of Modulation on Specific IgG1. Serum EW-specific IgG were determined by ELISA in pooled sera obtained from all three groups of mice (**Figure 3A**). No or very little specific IgG was detected in the serum of mice challenged with placebo solution (control −). Compared to the positive

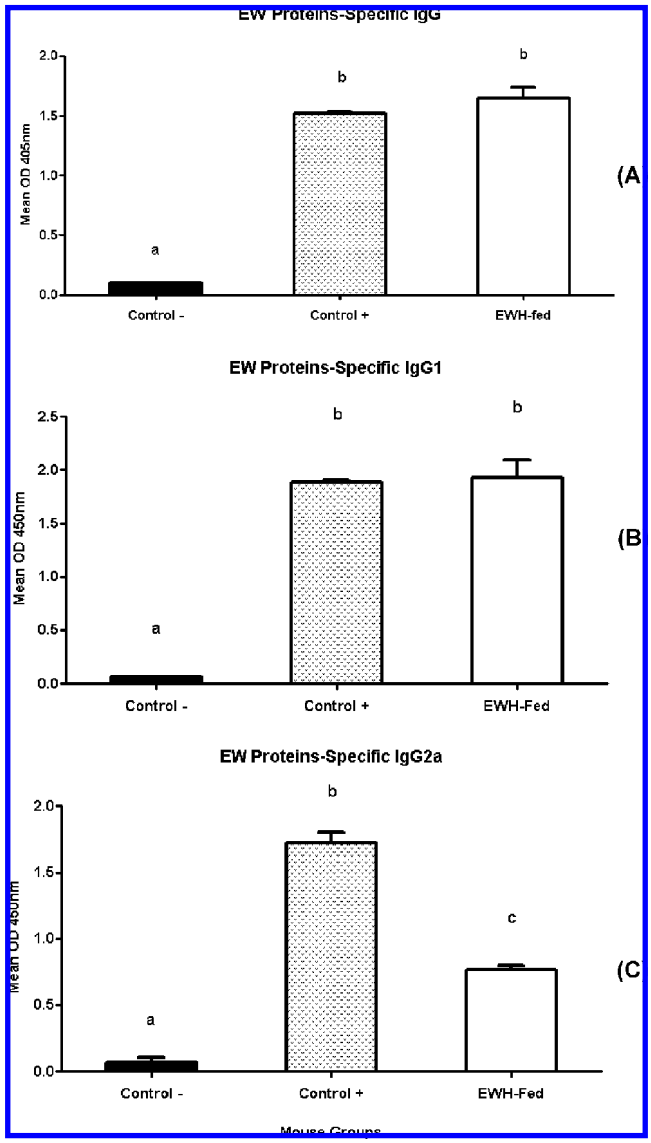

Figure 3. Serum EW specific IgG (**A**), EW specific IgG1 (**B**), and EW specific IgG2a (**C**) levels in EW-sensitized BALB/c mice after repeated oral administration of EWH. Data are represented as mean ± standard deviation ($n = 3$ of replicate tests). Different letters indicate significant differences ($p < 0.05$) between groups of mice.

control group, no statistically significant difference was determined with EWH-fed mice. On the basis of the Th1/Th2 balance, the levels of EW specific IgG1 (Th2-bias) and IgG2a (Th1-bias) were analyzed by ELISA (**Figure 3B**,**C**). There was no detectable level of either subisotype in the sera of mice challenged with PBS (control −). Compared to the positive control group, no statistically significant difference in specific IgG1 titers was determined in EWH-fed mice, whereas there was a significant decline in specific IgG2a. This suggests that inhibitory effects were exerted at least on the Th1-biased specific immune response.

Repressive Effects on both Type-1 and Type-2 Cytokine Responses. To investigate the effects of EWH oral administration on EW-induced cytokine responses, Th1-biased (IFN-γ) and Th2-biased (IL-4) cytokine production were analyzed in the culture supernatant of mouse splenocytes incubated in the presence of EW proteins (**Figure 4**). Cytokines such as IL-4 drive the differentiation of naïve T cells toward a Th2 phenotype, thus promoting a switch toward the production of IgE isotype, whereas IFN-γ is critical for the development of Th1 cells. No

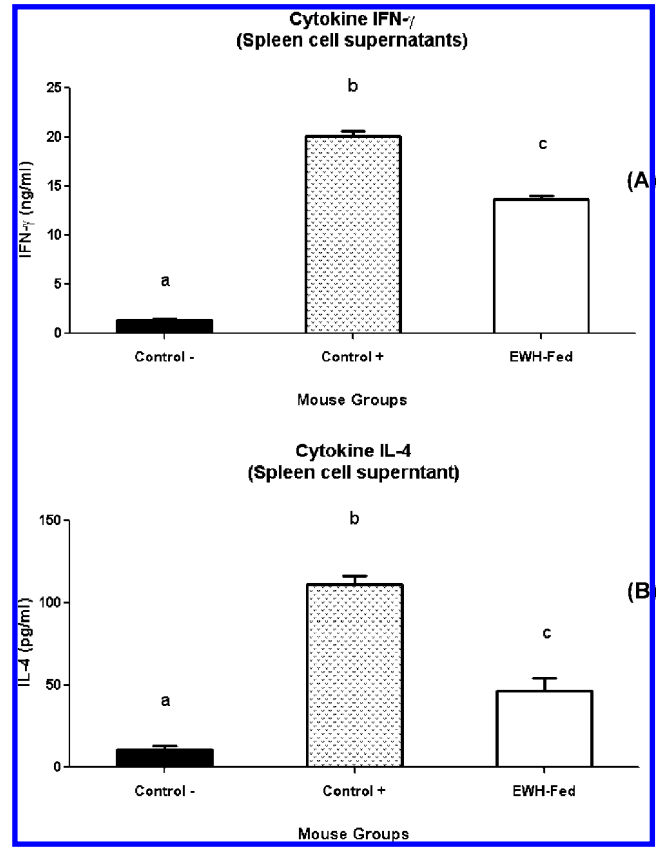

Figure 4. Determination of cytokine concentrations of IFN-γ (**A**) and IL-4 (**B**) following in vitro restimulation of spleen cell cultures with EW proteins. Data are represented as mean ± standard deviation ($n = 3$ of replicate tests). Different letters indicate significant differences ($p < 0.05$) between groups of mice.

or very little cytokine release was detected in the cell cultures prepared from mice challenged with placebo solution (control −). On the other hand, a marked down-regulation of both IL-4 and IFN-γ secretion was detected in EWH-fed mice, compared to the positive control mice. The reduction in IL-4 (Th2-bias) production may be correlated with the decreased levels of serum histamine and EW-specific IgE levels observed earlier (**Figure 2**), whereas the decline in IFN-γ (Th1-bias) may be associated with the diminution of EW-specific IgG2a (**Figure 3**).

Further insight into the immunomodulatory effects of EWH were sought by analyses of gene expression levels in mouse ileum tissues collected at the experimental end point. Expression ratios for mouse Th1-biased cytokines (IFN-γ, IL-12, and IL-18), Th2-biased cytokines (IL-4, IL-5, and IL-13), and regulatory molecules (TGF-β, IL-10, and transcription factor FOXp3) were determined and are presented in **Figures 5**−**7**. Consistent with results obtained in spleen cell cultures, the first observation is that expression ratios for cytokines IFN-γ and IL-4 were significantly lower in the EWH-fed mice (**Figures 5A** and **6A**). Expression ratios for both cytokines IL-13 (which acts in synergy with IL-4 to promote the production of allergen specific IgE) and IL-12 (which favors the production of IFN-γ and the differentiation and proliferation of Th1 cells) are consistent with these variations (**Figures 5B** and **6C**). On the other hand, higher mRNA expression ratios for IL-5 and IL-18 in EWH-fed mice (**Figures 5C** and **6B**) appear to be incompatible with these results, suggesting that mechanisms more complex than a simple inhibition of both Th1- and Th2-mediated responses are occurring at the intestinal level and merit further investigations.

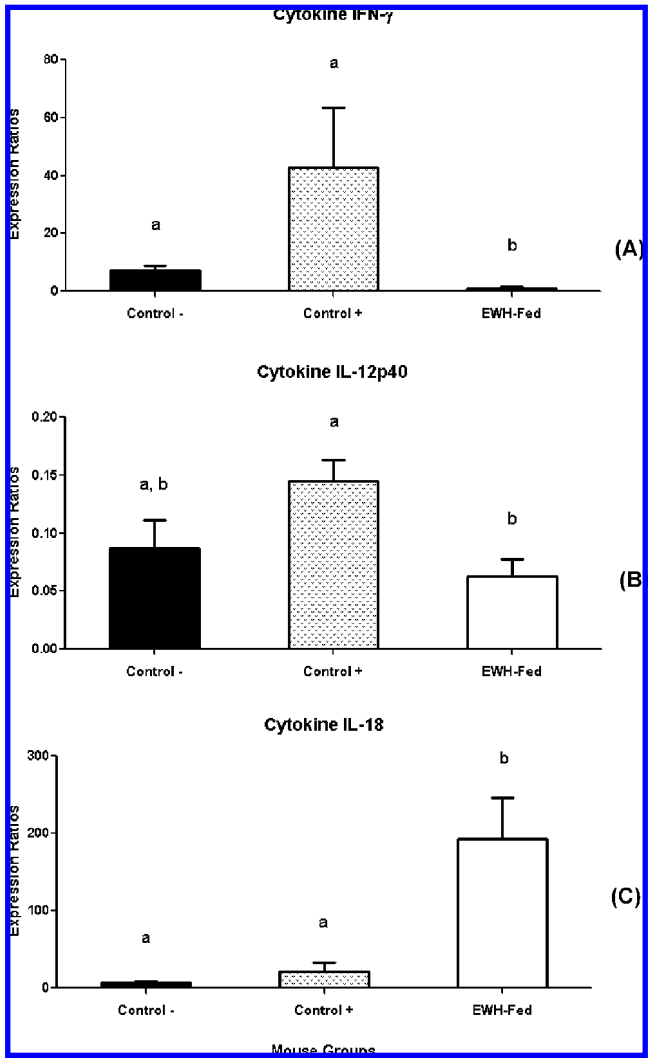

Figure 5. Modulation of intestinal cytokine expression patterns in EWP-treated mice: expression ratios determined by real-time RT-PCR analyses for IFN-γ (**A**), IL-12p40 (**B**), and IL-18 (**C**). Data are represented as mean $\pm$ SEM ($n = 5$ of individual mouse ratios). Different letters indicate significant differences ($p < 0.05$) between groups of mice.

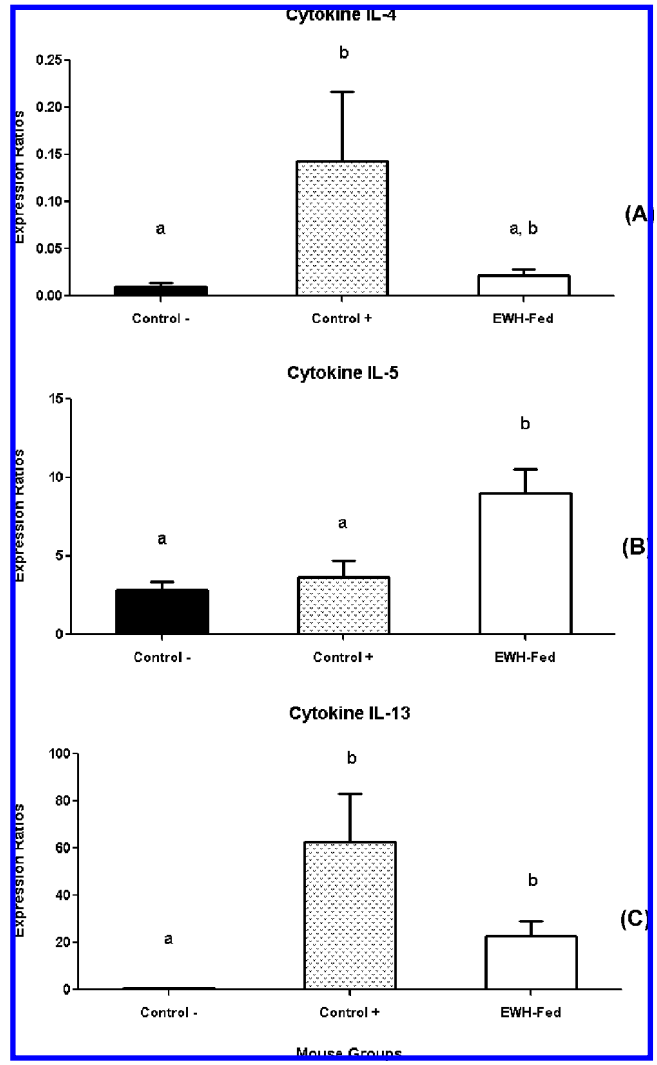

Figure 6. Modulation of intestinal cytokine expression patterns in EWP-treated mice: expression ratios determined by real-time RT-PCR analyses for IL-4 (**A**), IL-5 (**B**), and IL-13 (**C**). Data are represented as mean $\pm$ SEM ($n = 5$ of individual mouse ratios). Different letters indicate significant differences ($p < 0.05$) between groups of mice.

The most noteworthy observation may be the enhanced expression observed for both TGF-β and FOXp3 mRNA expressions (**Figure 7**) in the EWH-fed group. The concomitant increase of both molecules indicates that the gene expression of FOXp3 may be TGF-β-induced, as suggested by recent studies (*19*). Inhibition of the activity of both Th1 and Th2 responses (in spleen cell cultures and in ileal tissues) and the higher expression of regulatory molecules that are TGF-β and FOXp3 are two classes of events which might be intertwined and will be the object of the discussion.

DISCUSSION

Various foods were identified as a source of biologically active peptides enclosed within the sequence of the parent proteins and often released by enzymatic proteolysis (*1*), which occurs during gastrointestinal digestion or food processing. In the present study, we characterized the immumodulatory effects of a preparation of EWH, at both the protein and gene expression levels, using a BALB/c mouse model of food allergy. We showed that repeated oral administration of EWH with peptic fragments of <1.4 kDa led to a specific immune hyporesponsiveness in EW-primed BALB/c mice. The suppressive effects were observed at the protein levels with significantly reduced concentrations of both serum histamine and specific IgE, as well as inhibition of both Th1- and Th2-cytokine secretion (**Figures 1**−**3**). Although the allergy-suppressive effects were not total using the current experimental conditions, for example, histamine and specific IgE levels were significantly decreased, but not fully abolished, our results suggest that further optimization of dose and duration of EWH administration may lead to a complete inhibition.

To further elucidate the immunomodulatory effects of EWH, we determined the cytokine expression profiles of intestinal tissues (ileum) collected from individual BALB/c mice. In line with the cytokine secretion pattern obtained in spleen cell cultures, intestinal expressions of both Th1-biased (IFN-γ and IL-12) and Th2-biased (IL-4 and IL-13) cytokines were lower in the EWH-fed group, compared to positive control mice. In line with our results, previous investigations on allergen immunotherapy have reported inhibition of both allergen-induced Th1- and Th2- cytokine production in blood samples and mucosal tissues (*20*). A majority of papers have attributed this double-inhibition effect to the suppressive activity of IL-10-producing regulatory T cells (*21*). Our study failed to indicate any significant variation of IL-10 intestinal mRNA expression

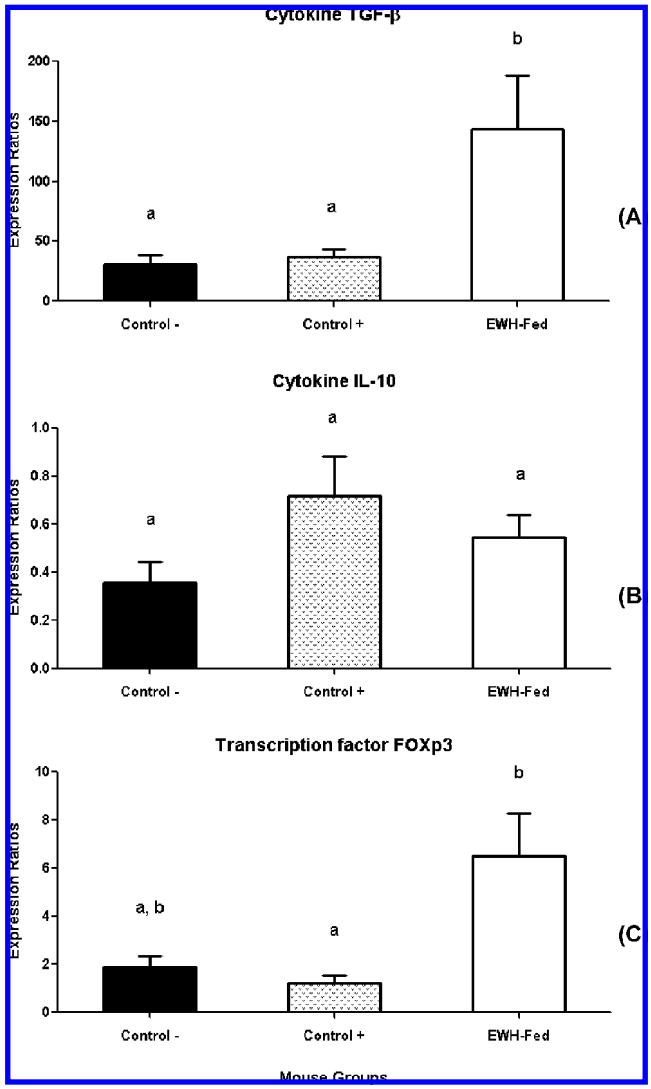

Figure 7. Modulation of intestinal cytokine expression patterns in EWP-treated mice: expression ratios determined by real-time RT-PCR analyses for TGF-β (**A**), IL-10 (**B**), and (**C**) FOXp3. Data are represented as mean $\pm$ SEM ($n = 5$ of individual mouse ratios). Different letters indicate significant differences ($p < 0.05$) between groups of mice.

in the EWH-fed mice (**Figure 7**B). This suggests either that although no higher IL-10 expression was established in the ileum, an enhanced production may have occurred elsewhere, for example, further along the intestine or in peripheral blood, but failed to be detected, or that mechanisms other than IL-10-mediated signals may be responsible for the inhibition on both Th1- and Th2-type responses. We support the latter hypothesis on the basis of the observation that greater intestinal expressions of both TGF-β and FOXp3 were observed in the EWH-fed group (**Figure 7**). The pleiotropic activity of TGF-β cytokine has been well-documented (*22*). Using BALB/c mouse experimental models, TGF-β was found to block the proliferation and development of Th1 and Th2 cells by modifying expression of the transcription factors T-bet and GATA-3, respectively (*23*, *24*). Similarly, a number of studies have demonstrated the inhibitory effects of FOXp3-expressing T cells on overt immune responses mediated by both Th1- and Th2-biased cytokines, particularly in autoimmune and allergic disorders (*25*, *26*). Supplementary analyses aimed at verifying the production of functional proteins at the intestinal mucosa (in particular, TGF-β and Foxp3) are ongoing to confirm our findings with use of immunohistochemistry and in situ hybridization methods.

Numerous health-promoting effects have been attributed to food-derived peptides; however, the relationship between their structural properties and their physiological effects remains obscure. It seems obvious that the nature of the enzymatic treatment will have an influence on their immunomodulatory properties, which may explain the conflicting data reported so far with regard to the efficacy of food-based hydrolysates. In an attempt to clarify the immunomodulatory nature of the EWH used in our study, we analyzed the amino acid sequences of the oligopeptides present in the EWH preparation and determined the presence of OVA immunodominant T- and B-cell epitopes (**Table 3**). We thus suggest that these immunologically active sequences are responsible for the allergy-suppressive effects observed in the EWH-fed mice. In accordance with this view, a recent monograph reported the single clinical case of a 10-year-old patient who was subjected to ingestion of wheat flour hydrolysate, containing T-cell reactive peptides, for a duration of 18 months and failed to develop any severe side effects upon subsequent exposure to nonhydrolyzed wheat flour (*27*). Similarly, the use of peptides containing allergen B-cell epitopes has also been suggested as an immunotherapeutic rationale (*28*), and proof-of-principle has been provided with grass and birch pollen allergens using mice models (*29*, *30*). Fractionation of the EWH preparation, for example, by preparative liquid chromatography, and isolation of the peptidic fractions responsible for the immunosuppressive effects observed would be the next logical step to confirm our findings and is currently being addressed.

In this study, we finely characterized the allergy-modulating effect of egg white-derived peptides obtained by standard food-processing methods and proposed that the presence of OVA B-cell and T-cell epitopes account for these effects. As opposed to synthetic bioactive materials, the safer nature of food-derived peptides promotes their consumption in the form of nutraceuticals. If considered for human applications, further developmental stages would be required and involve, for instance, the sensorial characterization of the EWH preparation, as well as safety and efficacy assessments (e.g., hypoallergenicity and dose−response study) in order to determine the health impacts of such an EWH-based product on the human immune response.

ABBREVIATIONS USED

CT, cholera toxin; ELISA, enzyme-linked immunosorbent assay; EW, egg white; EWH, egg white hydrolysate; FOXp3, forkhead box protein 3; Ig, immunoglobulins; IL, interleukin; IFN, interferon; LC-MS/MS, liquid chromatography tandem mass spectrometry; LYS, lysozyme; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; OVA, ovalbumin; OVM, ovomucoid; OVT, ovotransferrin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline Tween 20 0.05%; PCR, Polymerase Chain Reaction; PMF, peptide mass fingerprinting; RT-PCR, reverse-transcription Polymerase Chain Reaction; TGF, transforming growth factor; Th, T helper cell.

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