

Two Distinct T Cell Subsets, CD4⁺ and CD8⁺CD60⁺, and Their Cytokines Are Required for In Vitro Induction of Human Ragweed-Specific Memory IgE Responses

Tamar A. Smith-Norowitz,^{1,*†} Jonathan Silverberg,[‡] Kevin B. Norowitz,[†] Martin H. Bluth,^{*,§} Seto Chice,[‡] Rauno Joks,^{*,¶} Maja Nowakowski,^{*,‡} and Helen G. Durkin^{*,§}

CD8⁺CD60⁺ T cells (80–98% CD45RO⁺; 20% CD23⁺) are significantly increased in the blood of serum IgE⁺ ragweed-sensitized (RS) compared with serum IgE-nonatopic humans ($p = 0.001$). CD8⁺CD60⁺ T cells of the RS patients produced IL-2, IL-4, IL-10, IL-12, IFN- α , and IFN- γ , but not IL-6 or IL-13. When their PBMC were cultured with ragweed Ag (RA), peak IgE responses occurred on day 10; none was induced with non-cross-reacting or without Ag; nonatopic PBMC did not respond to any stimulant. When either CD4⁺ or CD8⁺CD60⁺ T cells were depleted from RS PBMC before culture with RA, no IgE responses were induced. If purified CD4⁺ T cells or low numbers of CD8⁺CD60⁺ T cells were added back to the depleted PBMC, IgE responses were restored. However, higher numbers of CD8⁺CD60⁺ T cells totally suppressed IgE responses. Total suppression also was obtained when RS PBMC were cultured with RA and either anti-IL-2, IL-4, IL-10, IL-12, IFN- γ (all concentrations), or IFN- α (low concentrations), but not anti-IL-6 or IL-13. Higher concentrations of anti-IFN- α potentiated IgE responses. *The Journal of Immunology*, 2008, 181: 4761–4769.

It is well recognized that CD4⁺ T cells and their cytokines (IL-4 and IL-13) are required for induction of human and murine IgE responses in vivo and in vitro (reviewed in Refs. 1 and 2). However, there is evidence that different cell/cytokine pathways are required for induction/maintenance of memory IgE responses. Herrick et al. (3) were the first to demonstrate that in vitro induction of benzylpenicilloyl (BPO)²-specific memory IgE responses by spleen cells of BPO-keyhole limpet hemocyanin (KLH)-sensitized mice required two distinct T cell subsets: Thy 1⁺ asialo GM1 ganglioside-negative and Thy 1⁺ asialo GM1 ganglioside-positive T cells (3); in her studies, IL-4 was responsible for approximately one-half of the memory IgE response and other cytokines (IFN- α and IFN- γ) for the remainder of the memory IgE response. Studies of Auci et al. (4), in the same BPO-KLH-sensitized mouse model, found that after each booster injection of Ag, Thy 1⁺ asialo GM1 ganglioside-positive T cells transiently appeared in spleen and mesenteric lymph node before IgE responses increased in these organs (4, 5). A search for a human counterpart of murine Thy 1⁺ asialo GM1⁺ T cells proved futile because T cells of allergic patients do not express asialo GM1 ganglioside (6).

Smith-Norowitz (6) and Smith-Norowitz et al. (7) found that there is a statistically significant increase in numbers of the CD8⁺CD60⁺ T cells (CD60 also is a ganglioside) in the blood of serum IgE⁺ ragweed-sensitized (RS) humans at the peak of the ragweed allergy season. Others demonstrated that CD8⁺ T cells, like CD4⁺ T cells, help IgG responses (8, 9) and that CD60⁺ T cells also help these responses (10). However, the role of CD8⁺ or CD60⁺ T cells in IgE responses and T cells that simultaneously express both CD8 and CD60 in humoral responses, including IgE responses, has not been studied. The present studies further characterize CD8⁺CD60⁺ T cells and other lymphocytes in the blood of RS humans obtained at the peak of the ragweed allergy season and investigate the ability of CD8⁺CD60⁺ T cells to regulate human memory IgE responses.

We found that CD8⁺CD60⁺CD45RO⁺ T cell numbers are greatly increased in the blood of serum IgE⁺ RS humans at the peak of the ragweed allergy season. CD8⁺CD60⁺ T cells and their cytokines (IL-2, IL-4, IL-10, IL-12, IFN- α , and IFN- γ) are required for induction of human ragweed-specific memory IgE responses; CD4⁺ T cells also are required for memory IgE responses, as previously shown by others (reviewed in Refs. 1 and 2). CD8⁺CD60⁺ T cells and IFN- α in high numbers/concentration suppressed induction of memory IgE responses.

Materials and Methods

Subjects

All subjects ($n = 32$) were medical student volunteers (males and females, ages 22–40 years). At the time of the study, none of the subjects received allergy therapy and none was being treated with any medication. RS subjects may or may not have exhibited a past history of clinical allergic reactions/symptoms. Approval was obtained from the State University of New York (SUNY) Downstate Institutional Review Board and the procedures followed were in accordance with institutional guidelines involving human subjects.

Peripheral blood (40 ml) was obtained from subjects 2 days after they were defined as either RS, serum IgE⁺ (>100 IU/ml; $n = 20$), or nonatopic (NA) serum IgE[−] (<100 IU/ml; $n = 12$), in August–September, the peak of the ragweed allergy season in New York City. Subjects were skin tested by intradermal injection of pollen Ags (mixed ragweed (tall and short);

*Center for Allergy and Asthma Research at State University of New York Downstate and [†]Department of Pediatrics, [‡]Department of Pathology, [§]Department of Surgery, and [¶]Department of Medicine, State University of New York Downstate Medical Center, Brooklyn, NY 11203

Received for publication February 27, 2008. Accepted for publication July 29, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Address correspondence and reprint requests to Dr. Tamar A. Smith-Norowitz, Department of Pediatrics, State University of New York Downstate Medical Center, Box 49, 450 Clarkson Avenue, Brooklyn, New York 11203. E-mail address: tamar.smith-norowitz@downstate.edu

² Abbreviations used in this paper: BPO, benzylpenicilloyl; KLH, keyhole limpet hemocyanin; RS, ragweed sensitized; NA, nonatopic; ECD, PE-Texas Red; NGS, normal goat serum.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/\$2.00

Center Laboratories), standardized mite Ags (*Dermatophagoides farinae*; Miles), cat pelt (0.02 ml, 10,000 BAU/ml; Center Laboratories), Histatrol (0.1 mg/ml in 0.02 ml, histamine based; Center Laboratories), or sterile diluent for allergenic extract (saline control, 0.02 ml; Center Laboratories) at the Asthma Center of Excellence (SUNY Downstate Medical Center, Brooklyn, NY). All patients defined as RS were skin test positive by a single unequivocally positive test, where the Ag-positive injection site had a mean diameter 1 mm greater than the saline site, and the histamine-positive site had a mean diameter 2 mm greater than the saline site. The RS subjects also were skin test positive to other allergens.

Blood specimens

For flow cytometry and cell culture studies, blood was collected into EDTA Monoject tubes (Sherwood Medical) and retained for up to 2 h at room temperature. PBMC used in cell cultures were separated from whole blood on Ficoll-Paque (Pharmacia) gradients (density, 1.077). Viability was >98%, as judged by trypan blue dye exclusion.

For studies of serum IgE, blood was collected into red top Monoject tubes (Sherwood Medical) and allowed to clot for 30 min at room temperature. Sera were collected and stored at -20°C until assayed. All serum IgE determinations were conducted in the Clinical Diagnostic Laboratory at SUNY Downstate Medical Center. Serum IgE levels were determined using the UniCAP Total IgE fluoroenzyme immunoassay (Pharmacia and Upjohn Diagnostics) that was performed according to the manufacturers' recommendation. To evaluate test results, the response for a subject's sample was compared directly to the response of the IgE calibrators. Data are expressed as IU/ml, where serum IgE⁺ was >100 IU/ml and serum IgE⁻ was <100 IU/ml.

Flow cytometry

Antibodies. Mouse anti-human mAbs directly conjugated to FITC (IgG1 anti-CD3, TCR α/β , CD45RA, CD23; IgG2b anti-CD14; IgM anti-CD60); PE (IgG1 anti-TCR α/β , TCR γ/δ , CD1d, CD4, CD8, CD45RA, CD23; IgG2a anti-CD45RO), PE-Texas Red (ECD; IgG2a anti-CD45RO), PE-cyanin 5 (PC5) (IgG2a anti-CD4, CD8). Simultest (FITC/PE-conjugated) reagents (CD3/CD4, CD3/CD8, CD3/CD19, CD3/CD16⁺CD56) and appropriately matched isotype control mAbs (FITC-conjugated IgG1, FITC-conjugated IgG2b, PE-conjugated IgG1 and IgG2a, ECD-conjugated IgG2a, PC5-conjugated IgG2a, Simultest control γ_1/γ_2 , FITC-conjugated IgM). All Abs were purchased from BD Biosciences, except IgM anti-CD60, which was purchased from Ancell, IgG1 anti-CD1d, which was purchased from BD Pharmingen, and CD45RO-ECD, CD4-PC5, CD8-PC5, IgG2a ECD, and IgG2a PC5, which were purchased from Beckman Coulter (Immunotech); all were used according to the manufacturers' recommendation.

Assay. All labeling and sorting studies were conducted within 6 h after blood was obtained. Conjugated Abs (10 μl or 80 μl of titrated anti-CD60) directed against one to three markers were added to the blood (100 μl) in 12 $\times$ 75-mm (5-ml) tubes (Fisher Scientific) and incubated for 10 min at room temperature, after which erythrocytes were lysed with whole blood-lysing reagent (Immunoprep; Beckman Coulter), and the cells were counted. Lymphocyte distributions were determined with a Coulter Epics XL/MCL flow cytometer with System II software (Coulter), and CytoComp (Coulter) QC Windows (Flow Cytometry Systems) was used to ensure consistent instrument settings. Forward and side scatter were used to identify the lymphocyte population, with CD45 used to establish an optimal lymphocyte gate. The gain on the photomultiplier tube detecting fluorescence intensity was adjusted so that 99% of cells with background fluorescence staining were scored between 10^0 and 10^1 on a 4-decade log scale. Specific fluorescence was reported as the percentage of cells with relative fluorescence intensity scored above background; at least 15,000 cells were counted. Data are expressed as total lymphocytes per mm^3 and as percent CD8⁺CD60⁺ T cells.

Intracellular cytokines

Peripheral blood (3 ml) was added to RPMI 1640 (1:1 ratio) containing L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$, L-glutamine-penicillin-streptomycin solution; Sigma-Aldrich). Phytohemagglutinin P (60 $\mu\text{l}/\text{ml}$; Difco Laboratories) and GolgiPlug (1 $\mu\text{l}/\text{ml}$; BD Pharmingen) were added to blood, which was then incubated for 6 h at 37°C . Blood (50 μl) was then incubated with specific mAb (10 μl): CD8-PC5 (IO Test; Beckman Coulter) or CD4-PC5 (IO Test; Beckman Coulter) plus CD45RO-ECD (IO Test; Beckman Coulter) plus CD60-FITC (Ancell) for 10 min at room temperature in the dark, after which Intraprep Reagent 1 (100 μl , Intraprep Kit; Beckman Coulter Immunotech) was added to each tube and the tubes were vortexed and then incubated for 15 min at room temperature in the dark according to the manufacturer's recommendation.

Dulbecco's PBS (D-PBS, 4 ml; Life Technologies) was added to the tubes, which were then centrifuged at $300 \times g$ for 5 min at room temperature. Supernatants were discarded, cells were resuspended in Intraprep Reagent 2 (100 μl ; Beckman Coulter), and the tubes were incubated for 5 min at room temperature. Tubes were then gently agitated, after which Abs to individual cytokines (20 μl ; BD Pharmingen) were added: PE-conjugated mouse anti-human IL-2, IL-4, or IL-12; PE-conjugated rat-anti-human IL-6, IL-10, or IL-13; and PE-conjugated mouse anti-human IFN- γ or IFN- α . The tubes were incubated for 10 min at room temperature, after which D-PBS (4 ml) was added, and the tubes were centrifuged at $300 \times g$ for 5 min at room temperature. Supernatants were discarded, pellets were resuspended in D-PBS (400 μl) and D-PBS with 0.1% paraformaldehyde (100 μl , ImmunoPrep C; Beckman Coulter). Cells were then counted with a Coulter Epics XL/MCL Flow Cytometer using System II software (Coulter), with CytoComp (Coulter) and QC Windows (Flow Cytometry Systems) used to ensure consistent instrument settings. Data are expressed as percent total lymphocytes. Some experiments were conducted using PBMC from Ficoll-Paque gradients, with similar results obtained.

Confocal microscopy: immunofluorescence labeling

Human PBMC ($1 \times 10^5/\text{ml}$) were washed once with D-PBS, then fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature, after which they were washed with D-PBS. The cells were then incubated with 0.1% glycine buffer (Sigma-Aldrich) for 10 min at room temperature and then washed twice in D-PBS. Cells were then resuspended in D-PBS ($1.0 \times 10^5/\text{ml}$). For all cell-labeling experiments, normal goat serum (NGS) incubation steps were for 15 min and Ab incubations were for 15 min at room temperature, followed by two washing steps with D-PBS.

To label CD8⁺CD60⁺ TCR α/β T cells, PBMC were incubated in the following sequence: NGS: primary Ab (mouse IgG anti-human CD8 (2–5 $\mu\text{g}/\mu\text{l}$), clone G42-8; BD Pharmingen) and secondary Ab (Alexa Fluor 647 goat anti-rat IgG, 1/200 dilution in 0.1% BSA; Molecular Probes/Invitrogen); NGS: primary Ab (mouse IgM mAb anti-human CD60 (2–5 $\mu\text{g}/\mu\text{l}$, clone UM4D4; Ancell) and secondary Ab (Alexa Fluor 488 goat anti-mouse IgM, 1/200 dilution in 0.1% BSA; Molecular Probes); and NGS: primary Ab (mouse IgM anti-human TCR α/β (2–5 $\mu\text{g}/\mu\text{l}$, clone T10B9.1A-31; BD Pharmingen) and secondary Ab (Alexa Fluor 568 goat anti-mouse IgM, 1/200 dilution in 0.1% BSA; Molecular Probes).

To label CD8⁺CD60⁺CD45RO⁺ T cells, PBMC were incubated in the following sequence: NGS: primary Ab (rat IgG2b mAb anti-human CD8 (2–5 $\mu\text{g}/\mu\text{l}$, clone 5H10-1; Santa Cruz Biotechnology) and secondary Ab (Alexa Fluor 647 goat anti-rat IgG, 1/200 dilution in 0.1% BSA; Molecular Probes) and primary Ab (mouse IgM mAb anti-human CD60 (2–5 $\mu\text{g}/\mu\text{l}$, clone UM4D4; Ancell) and secondary Ab (Alexa Fluor 488 goat anti-mouse IgM, 1/200 dilution in 0.1% BSA; Molecular Probes) and NGS: primary Ab (mouse IgG anti-human CD45RO (2–5 $\mu\text{g}/\mu\text{l}$, clone UCHL1; BD Pharmingen) and secondary Ab (Alexa Fluor 568 goat anti-mouse IgG, 1/200 dilution in 0.1% BSA; Molecular Probes).

Cells were then resuspended in 100–200 μl of D-PBS, an aliquot was placed on each slide, then mounted using Prolong Gold (Molecular Probes), and observed under a laser-scanning confocal microscope (Leica Instruments). CD8⁺CD60⁺, CD8⁺CD60⁺CD45RO⁺, and CD8⁺CD60⁺ TCR α/β ⁺ cells were identified with a confocal laser-scanning microscope (Leica Instruments), with separate settings for excitation and detection of the one, two, or three fluorochromes. No labeled cells were detected when primary Ab was omitted during labeling.

Cell cultures

PBMC depleted or not of CD4⁺ or CD8⁺CD60⁺ T cells (2×10^6 in 1 ml of complete medium) were placed in 12 $\times$ 75-mm (5 ml) polystyrene tubes (Fisher Scientific) with or without purified ragweed Ag (1–100 μl , tall and short ragweed pollens; Center Laboratories), with or without OVA (1–100 mg), and cultured for 0–12 days at 37°C in a humidified atmosphere of 4% CO_2 in air. Tubes were centrifuged at $700 \times g$ for 10 min at room temperature, supernatants collected, and stored at -20°C until assayed for IgE. The levels of IgE in culture supernatants were determined using solid-phase sandwich ELISA (Total IgE Microplate Test Kits; Kallestad Diagnostics or HOPE) performed according to the manufacturers' recommendations. Specimens were analyzed in duplicate and a standard curve was derived from known concentrations of IgE. Plates were read using an automated microplate reader (model Elx800; Bio-Tek Instruments), with a 405-nm measurement filter and a 600 reference filter (Bio-Tek Instruments). OD were converted to IU/ml and/or ng/ml (1 IU/ml = 2.4 ng/ml). Complete RPMI 1640 medium (Fisher Scientific) consisted of HEPES (2.5 mM/100 ml; Fisher Scientific), penicillin (100 U/ml; Life Technologies),

Table I. Distributions of lymphocyte subpopulations in blood of serum IgE⁺ RS humans^a

Subject	Serum IgE (IU ^b /ml)	Skin Test ^c	Fluorescent Cells (mm ³)								CD19 ⁺	CD16/56 ⁺
			T Cells							B Cells		
			CD3 ⁺	CD4 ⁺	CD8 ⁺	CD8 ⁺ CD60 ⁺	CD8 ⁺ CD60 ⁺	TCRα/β	TCRγ/δ			
1	>1000	+	1787	1090	745	558	187	nt ^d	nt	nt	318	
2	301	+	1654	890	659	480	179	928	373	134	241	
3	230	+	994	601	372	262	110	nt	nt	154	220	
4	123	+	888	466	495	418	77	1127	352	430	277	
5	110	+	1660	764	861	610	251	1880	194	216	692	
6	105	+	1446	956	555	467	88	nt	nt	68	324	
7	320	+	1851	1014	647	485	162	nt	nt	197	235	
8	229	+	782	480	279	245	25	nt	nt	264	237	
9	377	+	1928	1184	762	696	66	nt	nt	596	116	
10	182	+	1311	989	387	365	22	1333	43	322	330	
11	525	+	1862	903	959	761	198	1777	113	536	367	
12	578	+	1183	850	314	258	56	1091	18	351	259	
13	181	+	1286	878	347	244	103	1225	61	245	171	
14	206	+	1863	1082	721	654	67	1833	60	842	90	
15	344	+	1435	902	389	327	62	1312	61	369	143	
16	732	+	1044	690	541	447	94	1082	112	392	298	
17	107	+	2134	988	988	865	123	2292	514	672	869	
18	>1000	+	952	551	432	268	164	997	60	208	253	
19	>1000	+	1199	394	335	273	62	1181	49	335	160	
20	216	+	1346	1020	304	260	44	1324	43	478	261	

^a Flow cytometry data are expressed as total cells/mm³.^b 1 IU = 2.4 ng.^c Serum IgE⁺, RS humans allergic to standardized pollen, mite, or cat Ags.^d nt, Not tested.

streptomycin (100 μg/ml; Eli Lilly) and L-glutamine (2 mM; Life Technologies) and was supplemented with 10% FCS (Life Technologies).

In some experiments, varying concentrations of Abs to cytokines were included in cultures with unfractionated PBMC and ragweed Ag (10 μl): human anti-IL-2 (1.0–100 μg/ml; 1 μg of Ab will neutralize ~1.0 biological response modifier program unit of human IL-2); anti-IL-4 (1.0–100 μg/ml; 0.1- to 1.0-μg/ml Ab will neutralize the bioactivity of a 250-pg/ml solution of IL-4); anti-IL-6 (0.2–20 μg/ml; ~0.2-μg/ml Ab will neutralize >90% of the activity of a 10-pg/ml solution of IL-6); anti-IL-10 (1.0–100 μg/ml; ~10-μg/ml Ab will neutralize 200 ng of IL-10); and anti-IFN-γ (0.5–50 μg/ml; 1 ng of Ab will neutralize 100% of antiviral activity exhibited by ~1 U of IFN-γ) Abs, all purchased from Genzyme. Human anti-IL-12 (0.2–20 μg/ml; 50% neutralization dose (ND₅₀) was ~1.0–2.0 μg/ml in the presence of 1.0 ng/ml IL-12) and anti-IL-13 (0.4–40 μg/ml; ND₅₀ was ~1–4 μg/ml in the presence of 10 ng/ml IL-13) Abs were purchased from R&D Systems. Anti-IFN-α (1.0–100 U/ml; 1 neutralization unit is the amount of antiserum required to neutralize 1 U of IFN-α) Abs were purchased from BioSource International. The ND₅₀ is defined as that concentration of Ab required to yield one-half maximal inhibition of the cytokine activity on a responsive cell line when that cytokine is present at a concentration high enough to elicit a maximum response. All concentrations of anti-cytokine Abs were used within recommended ranges provided by the suppliers. Anti-cytokine Abs were used within 1 mo of receipt.

Depletion of CD4⁺CD3⁺ T cells and CD8⁺CD60⁺ T cells

PBMC were first depleted of CD14⁺ (CD4⁺/-) monocytes by incubating PBMC (1 × 10⁷/ml) with anti-human CD14 mAb-coated magnetic beads (25 μl of beads/1 ml of cells) (Dynabeads CD14; Invitrogen/Dynal) in 5-ml tubes (Fisher Scientific) for 20 min at 2–8°C, with gentle tilting and rotation, according to the manufacturer's recommendation. Tubes were then placed on MPC-1 magnets (Invitrogen/Dynal) for 2 min to allow bead-bound cells to collect on tube walls. Supernatants were removed and stored on ice. Tubes were removed from magnets and bead-bound cells recovered and washed three to four times in buffer 1 (PBS with 0.1% BSA, pH 7.4) by centrifuging at 1000 rpm for 5 min at room temperature. Cells were resuspended in buffer 2 (1 × 10⁷/ml; RPMI 1640/1% FCS) and incubated in complete medium for up to 18 h at 37°C, when most cells had dissociated from beads. Tubes were placed on magnets for 2 min to allow beads to collect on walls, and the cell-containing supernatants were recovered and stored at room temperature. If the numbers of CD14⁺ cells in supernatants were >1%, the separation procedure was repeated. Purity of CD14⁺ cells was >99% (flow cytometry).

CD4⁺CD3⁺ T cells were depleted from PBMC which had been depleted of CD14⁺ cells by incubating cells (1 × 10⁷/ml) with anti-human CD4 mAb-coated magnetic beads (25 μl of beads/1 ml of cells, Dynal CD4-positive Isolation Kit; Invitrogen/Dynal) in 5-ml tubes for 20 min at 2–8°C, with gentle tilting and rotation, according to the manufacturer's recommendation. Tubes were then placed on MPC-1 magnets (Invitrogen/Dynal) for 2 min to allow bead-bound cells to collect on walls. Supernatants were removed and stored on ice. Tubes were removed from the magnets and bead-bound cells were recovered, washed in buffer 1 by centrifuging at 1000 rpm for 5 min at room temperature, and resuspended in buffer 2 (1 × 10⁷/ml). Bead-bound cells were detached from magnetic beads using CD4 Detachabeads (1 × 10⁷ cells/25 μl of Detachabeads; Dynal), with cells incubated for 45 min at room temperature, with gentle mixing. Tubes were then placed on the magnets for 2 min to allow beads to collect on walls, after which supernatants containing released cells were transferred to a fresh tube and cells were washed twice in buffer 2 by centrifuging at 1000 rpm for 5 min at room temperature. The pellet was resuspended in buffer 2 (1 ml) and stored at room temperature. If >1% CD4⁺CD3⁺ T cells were detected, the separation procedure was repeated. Purity of the CD4⁺ cells recovered from beads was >99% (flow cytometry).

Reconstitution of PBMC with purified CD14⁺ cells and varying numbers of purified CD4⁺CD3⁺ T cells

After depletion of CD4⁺CD3⁺ T cells from CD14⁺ cell-depleted PBMC, purified CD14⁺ cells were added back to the depleted PBMC in the same ratio as was present in each subject's PBMC before fractionation. Purified CD4⁺CD3⁺ T cells, in varying numbers (0.01–10.0 × 10⁴), were then added back to the CD14⁺ cell-reconstituted, CD4⁺CD3⁺ T cell-depleted PBMC (2 × 10⁶/ml), and cells were cultured as described. In some experiments, purified CD4⁺CD3⁺ T cells were added to unfractionated PBMC before culture.

Depletion of CD8⁺CD60⁺ T cells

CD8⁺CD3⁺ T cells were depleted from PBMC by incubating cells (1 × 10⁷/ml) with anti-human CD8 mAb-coated magnetic beads (25 μl of beads/1 ml of cells, Dynal CD8-positive Isolation Kit; Invitrogen/Dynal) in 5-ml tubes for 20 min at 2–8°C, with gentle tilting and rotation, according to the manufacturer's recommendation. Tubes were then placed on MPC-1 magnets (Invitrogen/Dynal) for 2 min to allow bead-bound cells to collect on walls. Supernatants were removed and stored on ice. Tubes were removed from the magnets and bead-bound cells were recovered, washed in

Table II. Distributions of lymphocyte subpopulations in blood of serum IgE⁻ humans^a

Fluorescent Cells (mm ³)											
Subject	Serum IgE (IU ^b /ml)	Skin Test	T Cells							B Cells	NK Precursors
			CD3 ⁺	CD4 ⁺	CD8 ⁺	CD8 ⁺ CD60 [−]	CD8 ⁺ CD60 ⁺	TCRα/β	TCRγ/δ	CD19	CD16/56 ⁺
1	76	—	704	463	370	342	28	527	302	434	274
2	59	—	1575	926	665	657	8	nt ^c	nt	213	523
3	21	—	1097	717	478	422	56	81	121	476	234
4	19	—	1028	615	430	426	4	1035	344	405	272
5	15	—	698	548	126	97	29	867	246	420	263
6	87	—	1108	759	278	249	29	1348	245	672	515
7	72	—	1386	656	640	570	70	1260	69	222	162
8	18	—	1092	626	422	352	70	1034	73	116	189
9	19	—	1035	705	255	181	74	1005	75	195	210
10	42	—	291	357	92	62	30	478	10	284	136
11	31	—	411	238	137	101	36	387	60	86	70
12	45	—	1052	802	332	293	39	1115	274	235	430

^a Flow cytometry data are expressed as total cells/mm³.^b Standard conversion: 1 IU = 2.4 ng.^c nt, Not tested.

buffer 1 by centrifuging at 1000 rpm for 5 min at room temperature, and resuspended in buffer 2 (1×10^7 /ml). CD8⁺ bead-bound cells were detached from the magnetic beads using CD8 Detachabeads (1×10^7 cells/25 μ l of Detachabeads; Dynal), with cells incubated for 45 min at room temperature, with gentle mixing. Tubes were then placed on the magnets for 2 min to allow beads to collect on walls, after which supernatants containing released cells were transferred to a fresh tube and cells were washed twice in buffer 2 by centrifuging at 1000 rpm for 5 min at room temperature. The pellet was resuspended in buffer 2 (1 ml) and stored at room temperature. If >1% CD8⁺CD3⁺ T cells were detected in supernatants, the separation procedure was repeated. Purity of CD8⁺ (CD60^{+/−}) T cells recovered from beads was >99% (flow cytometry).

To separate CD8⁺CD60[−] T cells from CD8⁺CD60⁺ T cells, CD8⁺ T cells (1×10^7 /ml) were incubated with mouse IgM anti-CD60 mAb (80 μ l of titrated anti-CD60; Ancell) in 5-ml tubes for 20 min at 2–8°C, with gentle tilting and rotation. Cells were washed twice in PBS (2–3 ml) by centrifuging at 1000 rpm for 5 min at room temperature, after which they were resuspended in buffer 1 (1.0×10^7 /ml). Rat anti-mouse IgM Dynabeads (25 μ l/ml; Invitrogen/Dynal) were added to cells, which were then incubated for 10 min at 2–8°C, with gentle tilting and rotation, according to the manufacturer's recommendation. Tubes were then placed on magnets for 2 min to allow bead-bound cells to collect on walls, after which supernatants were recovered and stored on ice. Bead-bound cells were recovered, washed three to four times in buffer 1 by centrifuging at 1000 rpm for 5 min at room temperature, resuspended in buffer 2 (1×10^7 /ml),

and incubated in complete medium for up to 18 h at 37°C, when cells had dissociated from beads. Tubes were placed on magnets for 2 min to allow beads to collect on walls and the cell-containing supernatants were recovered and stored at room temperature. Purity of CD8⁺CD60[−] (CD3⁺) T cells was >99% (flow cytometry).

Reconstitution of PBMC with purified CD8⁺CD60[−] T cells and varying numbers of purified CD8⁺CD60⁺ (CD3⁺) T cells

After depletion of CD8⁺CD60^{+/−} (CD3⁺) T cells from PBMC, purified CD8⁺CD60[−] T cells were added back to PBMC depleted of CD8⁺ T cells in the same ratio as was present in each subject's PBMC before fractionation. Purified CD8⁺CD60⁺ (CD3⁺) T cells, in varying numbers (0.01 – 10.0×10^3), were then added back to the CD8⁺CD60[−] T cell-depleted PBMC (2×10^6) that had been reconstituted with CD8⁺CD60[−] T cells and cells were cultured as described. In some experiments, purified CD8⁺CD60⁺ T cells (0.01 – 10.0×10^3) were added to unfractionated PBMC (2×10^6) before culture.

Statistical analysis

Lymphocyte distributions and serum IgE levels of serum IgE⁺ and serum IgE[−] subjects were compared on each variable. Significance between variables was determined using Student's *t* test. A value of $p < 0.05$ was considered statistically significant for all comparisons. The degree of association between these measures was assessed using Pearson's correlations. Statistical analyses were performed using SPSS for Windows, version 10.0 software.

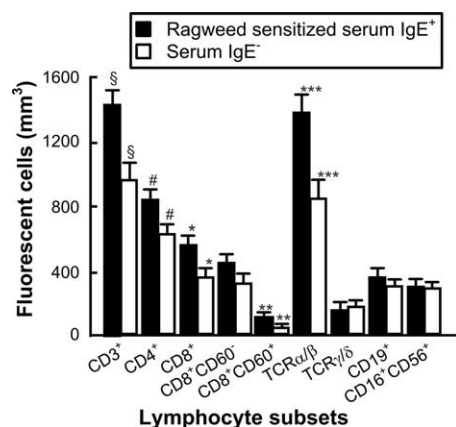


FIGURE 1. Distributions of lymphocyte subpopulations in peripheral blood of serum IgE⁺ RS (■; $n = 20$) and serum IgE[−] NA (□; $n = 12$) humans obtained at the peak of the ragweed allergy season in New York City (September) determined by flow cytometry. Data are expressed as mean total lymphocytes per mm³ $\pm$ SE. Serum IgE⁺ RS > serum IgE[−] NA humans (§, $p = 0.002$; #, 0.008; *, 0.01; **, 0.001; and ***, 0.003).

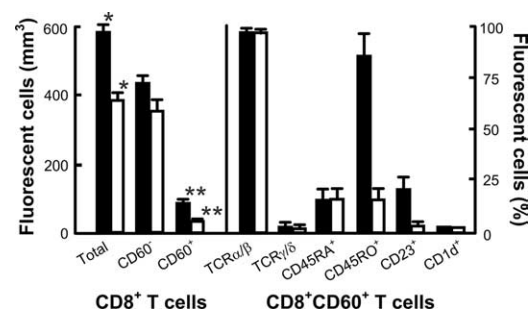


FIGURE 2. Distributions of CD8⁺CD60[−] and CD8⁺CD60⁺ T cells (left panel) and CD8⁺CD60⁺ T cell subsets coexpressing TCRα/β, TCRγ/δ, CD45RA, CD45RO, CD23, and CD1d (three-color labeling; right panel) in peripheral blood of serum IgE⁺ RS (■; $n = 6$) and serum IgE[−] NA (□; $n = 6$) humans (subjects 1–6 from Tables I and II, respectively). Data are expressed as mean total lymphocytes per mm³ and as percent CD8⁺CD60⁺ T cells $\pm$ SE. Serum IgE⁺ RS > serum IgE[−] NA humans (*, $p = 0.01$ and **, $p = 0.001$).

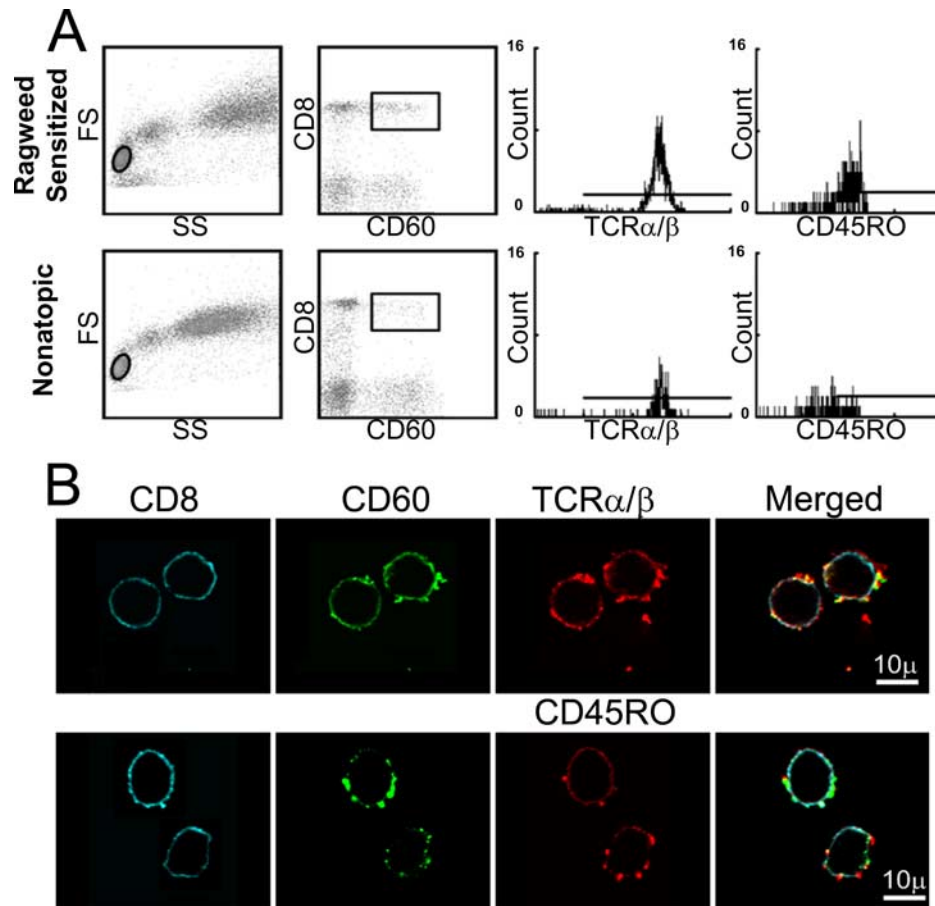


FIGURE 3. A, CD8⁺CD60⁺ TCRα/β⁺, and CD8⁺CD60⁺CD45RO⁺ T cells in peripheral blood of serum IgE⁺ RS (top panels) and serum IgE⁻ NA (bottom panels) humans (flow cytometry, three-color labeling; see *Materials and Methods*). B, CD8⁺CD60⁺TCRα/β⁺ (top panels) and CD8⁺CD60⁺CD45RO⁺ (bottom panels) T cells in PBMC of the same representative serum IgE⁺ RS human shown in A (confocal immunofluorescence). To label CD8⁺CD60⁺TCRα/β⁺ T cells, PBMC were 1) incubated with mouse IgG anti-human CD8, then with goat anti-mouse IgG (Alexa Fluor 647; blue); 2) incubated with mouse IgM anti-human CD60, then with goat anti-mouse IgM (Alexa Fluor 488; green); and 3) incubated with mouse IgM anti-human TCRα/β, then with goat anti-mouse IgM (Alexa Fluor 568; red). To label CD8⁺CD60⁺CD45RO⁺ T cells, PBMC were incubated with mouse IgG anti-human CD8, then with goat anti-mouse IgG (Alexa Fluor 647; blue); incubated with mouse IgM anti-human CD60, then incubated with goat anti-mouse IgM (Alexa Fluor 488; green); and incubated with mouse IgG anti-human CD45RO, then with goat anti-mouse IgG (Alexa Fluor 568; red). Slides were analyzed with a confocal laser-scanning microscope (Bio-Rad MRC 1024 Zeiss) with separate settings for excitation and detection of one, two, or three fluorochromes. Merged panel: CD8⁺CD60⁺TCRα/β (top) or CD8⁺CD60⁺CD45RO⁺ (bottom) T cells which were labeled with three primary Abs and secondary Alexa Fluor Abs (blue, green, red; original magnification, $\times 100$ oil immersion).

Results

Serum IgE⁺ RS and serum IgE⁻ NA subjects

All subjects defined as RS ($n = 20$) were skin test positive to ragweed Ag and to other allergens tested (see *Materials and Methods*) 2 days before their blood was obtained for the present studies, and all were serum IgE⁺ (107 to >1000 IU/ml; Table I). Subjects defined as NA ($n = 12$) were skin test negative to ragweed Ag and to the other allergens; all NA subjects were serum IgE⁻ (<100 IU/ml; Table II). Serum IgE levels were significantly increased in RS compared with NA subjects (407 ± 76 and 42 ± 7 , respectively; $p = 0.001$; individual data shown in Tables I and II).

Lymphocyte distributions. The distributions of blood T cells (CD3⁺, CD4⁺, CD8⁺, TCRα/β⁺) dramatically increased in RS compared with NA subjects (CD3: $1430/\text{mm}^3 \pm 88$ and 956 ± 107 , respectively; $p = 0.002$; CD4: $834/\text{mm}^3 \pm 52$ and 618 ± 56 , respectively; $p = 0.008$; CD8: $555/\text{mm}^3 \pm 50$ and 352 ± 54 , respectively; $p = 0.01$; TCRα/β: $1334/\text{mm}^3 \pm 81$ and 887 ± 109 , respectively; $p = 0.003$; Fig. 1 and Tables I and II). The increase in CD8⁺ T cells in RS, compared with NA subjects, in-

cluded a dramatic increase in CD8⁺ T cells that simultaneously expressed CD60 (>2 -fold) ($107/\text{mm}^3 \pm 14$ and 39 ± 7.0 , respectively; $p = 0.001$). Similar numbers of CD8⁺CD60⁻ T cells were detected in both groups ($p > 0.05$). CD4⁺CD60⁺ T cells were not studied. In contrast, the distributions of TCRγ/Δ⁺ T cells, CD19⁺ B cells, and CD16/56⁺ NK precursor cells were similar in the blood of RS and NA subjects ($p > 0.05$). No CD1d⁺ T cells were detected in the blood of either RS or NA subjects ($<1\%$; data not shown in Fig. 1).

Although the numbers of CD8⁺CD60⁺ T cells and serum IgE levels were significantly increased in RS compared with NA subjects, the correlation between the numbers of CD8⁺CD60⁺ T cells and serum IgE levels was not significant ($p > 0.40$; see Table I). **CD8⁺CD60⁺ T cell subsets.** CD8⁺CD60⁺ T cells in the blood of RS and NA subjects were virtually all TCRα/β⁺ ($>98\%$; Figs. 2 and 3). The numbers of CD8⁺CD60⁺ T cells expressing the memory marker CD45RO were dramatically increased in the RS, compared with NA, subjects (80–98%, $\sim 15\%$, respectively; see also Fig. 3). A subset of the CD8⁺CD60⁺ T cells of RS, but not NA subjects, expressed CD23 ($\sim 20\%$).

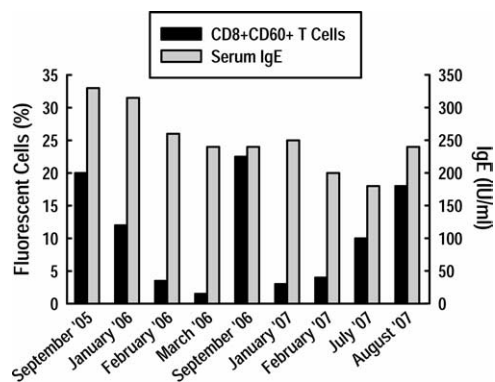


FIGURE 4. Distributions of blood $CD8^+CD60^+$ T cells and serum IgE levels of a RS human studied over a 2-year period (September 2005–August 2007), including peak ragweed allergy season in New York City (August and September) (flow cytometry, UniCAP IgE immunofluorescence assay). Data are expressed as percent total lymphocytes and as IgE (IU/ml).

Seasonal changes in $CD8^+CD60^+$ T cell numbers in the blood of a RS human. In the present studies, excluding those described in this section, blood of RS and NA subjects always was obtained at the peak of the ragweed allergy season. Fig. 4 shows data for blood from an extensively studied RS subject over a period of 25 mo, including ragweed allergy season and outside ragweed allergy season. $CD8^+CD60^+$ T cells were present in high numbers in August or September, the peak of the ragweed allergy season. However, excluding January 2006, the numbers of $CD8^+CD60^+$ blood T cells were greatly decreased at other times of the year. There was little change in serum IgE during the entire study period, and the numbers of blood $CD8^+CD60^+$ T cells did not correlate with total serum IgE levels.

In vitro induction of ragweed-specific memory IgE responses

When PBMC (2×10^6) were cultured for 0–12 days with ragweed Ag, IgE was first detected on day 5, reached peak levels on day 10, and decreased on day 12 (Fig. 5). No IgE responses were induced with non-cross-reacting Ag (OVA) or without Ag. PBMC of NA humans never produced IgE in response to any stimulant. In subsequent experiments, IgE levels were determined on day 10 of culture with ragweed Ag.

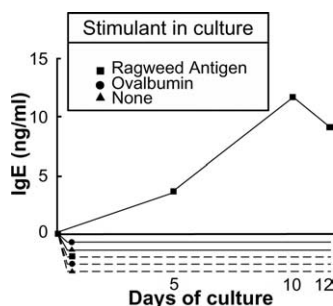


FIGURE 5. Ragweed-specific IgE responses by PBMC of RS humans on various days of culture. PBMC (2×10^6) obtained from serum IgE⁺ RS (solid lines; $n = 4$ /group) and serum IgE[−] NA (dashed lines; $n = 4$ /group) were individually cultured for 0–12 days with varying concentrations of ragweed Ag (1–100 μ l), OVA (1–100 mg), or without Ag, after which the levels of IgE in culture supernatants were determined (ELISA). Data shown are for optimal IgE responses induced by ragweed Ag (10 μ l). Data are expressed as mean ng/ml $\pm$ SE.

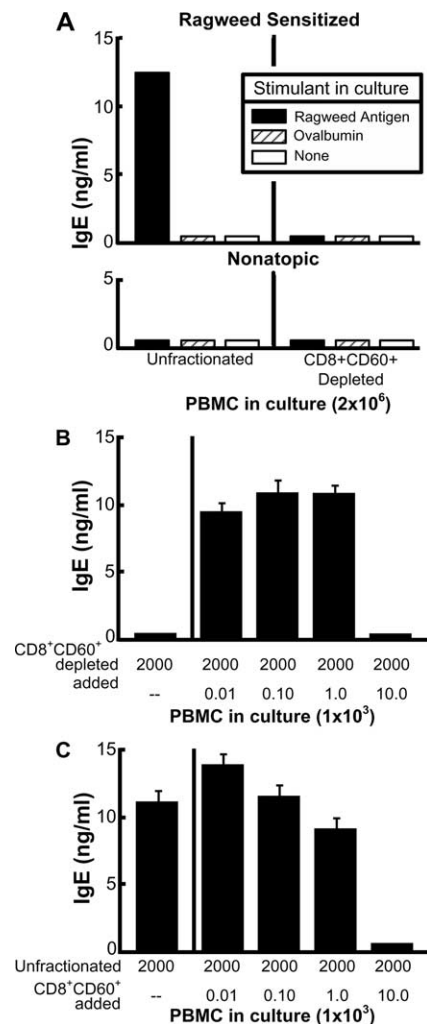


FIGURE 6. Effect of depletion of and reconstitution with $CD8^+CD60^+$ T cells on in vitro induction of ragweed-specific memory IgE responses by PBMC of serum IgE⁺ RS humans. PBMC of serum IgE⁺ RS or serum IgE[−] NA humans ($n = 4$ /group), depleted or not of $CD8^+CD60^+$ T cells (2×10^6 ; see *Materials and Methods*), were cultured with or without ragweed Ag (1, 10, 100 μ l) or with or without OVA (1–100 mg), after which levels of IgE in culture supernatants were determined on days 0–12 (ELISA). Data are shown for peak mean IgE responses on day 10, the peak of the ragweed-specific memory IgE response (A–C). The effect of adding varying numbers of purified $CD8^+CD60^+$ T cells (0.01–10.0 $\times 10^3$) back to either PBMC of serum IgE⁺ RS humans depleted of these cells (B), or to unfractionated PBMC (C), on in vitro induction of ragweed-specific memory IgE responses on day 10 of culture was determined. No IgE responses were detected on days 0–12 in cultures with OVA (1–100 μ g/ml) or without Ag (<2 ng/ml). Data represent peak mean levels of IgE in triplicate wells of cultures with ragweed Ag and are expressed as mean ng/ml $\pm$ SE.

Role of $CD8^+CD60^+$ and $CD4^+$ T cells in induction of memory IgE responses

Depletion and reconstitution studies (see *Materials and Methods*) established that both $CD8^+CD60^+$ T cells and $CD4^+$ T cells are required for in vitro induction of ragweed-specific memory IgE responses by PBMC of RS humans.

$CD8^+CD60^+$ T cells. When PBMC of RS humans were depleted of $CD8^+CD60^+$ T cells before culture with ragweed Ag, the IgE responses observed with unfractionated PBMC (Figs. 5 and 6A) were not induced on days 0–12 of culture (data for day 10 shown in Fig. 6A). When low numbers of purified

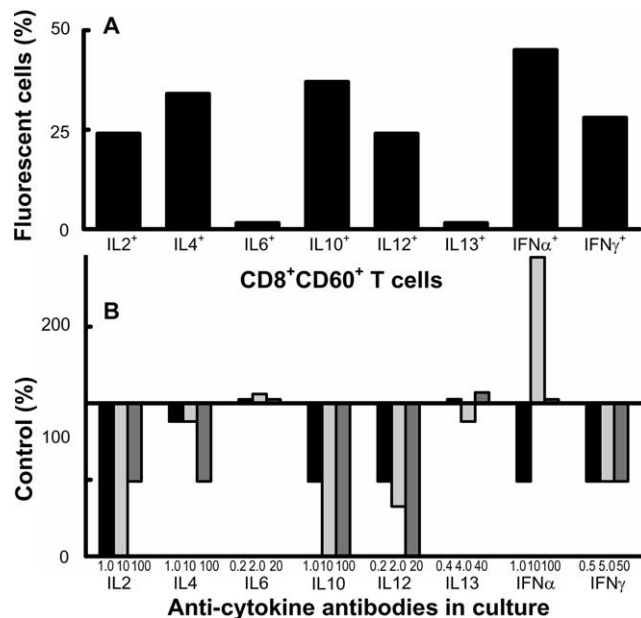


FIGURE 7. Cytokines produced by CD8⁺CD60⁺ T cells of a representative RS human at the peak of the ragweed allergy season (September), and effect of Abs to these cytokines on in vitro induction of ragweed-specific memory IgE responses by PBMC of the same donor. Distributions of CD8⁺CD60⁺ T cells producing IL-2, IL-4, IL-6, IL-10, IL-12, IFN-α, and IFN-γ in PBMC were determined (flow cytometry; A). PBMC of the same human were cultured for 10 days with ragweed Ag (10 μl) with or without varying concentrations of Abs to IL-2 (1.0–100 μg/ml), IL-4 (1.0–100 μg/ml), IL-6 (0.2–20 μg/ml), IL-10 (1.0–100 μg/ml), IL-12 (0.2–20 μg/ml), IL-13 (0.4–40 μg/ml), IFN-α (1.0–100 U/ml), and IFN-γ (0.5–50 U/ml), at which time the levels of IgE in culture supernatants were determined (ELISA; B). Data are from a representative experiment with blood obtained from one of two different donors, with similar results. Data are expressed as mean percent control in triplicate cultures with or without anti-cytokine Ab (IgE: 15 and <2 ng/ml, respectively).

CD8⁺CD60⁺ T cells ($0.01\text{--}1.0 \times 10^3$) were added back to PBMC (2×10^6) depleted of these cells and PBMC cultured for 10 days with ragweed Ag, ragweed-specific IgE responses were restored (Fig. 6B). In contrast, when higher numbers of purified CD8⁺CD60⁺ T cells (10.0×10^3) were added back to the depleted PBMC (2×10^6), IgE responses were totally suppressed. Furthermore, when low numbers of purified CD8⁺CD60⁺ T cells ($0.01\text{--}1.0 \times 10^3$) were added back to unfractionated PBMC (2×10^6) and PBMC cultured for 10 days with ragweed Ag, ragweed-specific IgE responses were induced (Fig. 6C). Again, when higher numbers of CD8⁺CD60⁺ T cells (10.0×10^3) were added back to unfractionated PBMC (2×10^6), IgE responses were totally suppressed.

CD4⁺ T cells. Previous studies by others determined that CD4⁺ T cells are required for in vitro induction of memory IgE responses (1, 2). In the present studies, no ragweed-specific IgE responses were induced when PBMC were depleted of CD4⁺ T cells and cultured for 0–12 days with ragweed Ag. These responses were completely restored when purified CD4⁺ T cells (10.0×10^4) were added back to depleted PBMC (2×10^6 ; data not shown).

Cytokine requirements for in vitro induction of ragweed-specific memory IgE responses

CD8⁺CD60⁺ blood T cells of RS humans produced IL-2, IL-4, IL-10, IL-12, IFN-α, and IFN-γ (23–40%), but not IL-6 or IL-13 (<1%; Fig. 7A); their CD4⁺ T cells also produced IL-4 (~30%; data not shown; other cytokines not studied).

When unfractionated PBMC obtained from the RS subjects were cultured for 10 days with ragweed Ag, IgE responses were induced (12–17 ng/ml; Fig. 7B, see legend). When cultures also contained either anti-IL-2, IL-10, IL-12, or IFN-γ (all concentrations tested) or IL-4 (highest concentration only), in vitro induction of ragweed-specific memory IgE responses was abrogated. Interestingly, anti-IFN-α had a bimodal effect, in that IgE responses decreased with the lowest concentration of anti-IFN-α (1.0 U/ml), whereas an intermediate concentration (10.0 U/ml) potentiated IgE responses and the highest concentration (100 U/ml) had no effect. Anti-IL-6 and anti-IL-13 had no effect on IgE responses at any concentration tested.

Discussion

The present studies are the first to report that 1) CD8⁺CD60⁺ (CD45RO⁺) T cell numbers are significantly increased in blood of serum IgE⁺ RS humans in ragweed allergy season, a subset of which expressed CD23; 2) CD8⁺CD60⁺ T cells and cytokines made by CD8⁺CD60⁺ T cells (IL-2, IL-4, IL-10, IL-12, IFN-α, and IFN-γ) are required for induction of human memory IgE responses; 3) CD4⁺ T cells also are required for induction of these responses; the requirement for CD4⁺ T cells was previously reported by others (reviewed in Refs. 1 and 2); and 4) CD8⁺CD60⁺ T cells and IFN-α, depending on their numbers/concentration, can either help or suppress induction of memory IgE responses.

The CD8⁺CD60⁺ T cells found in high numbers in blood of RS humans during peak ragweed allergy season were comprised of cells that coexpressed the memory marker CD45RO (80–98%) compared with ~15% in NA humans. From experiments in which purified CD8⁺CD60⁺ T cells were used to restore memory IgE responses by PBMC depleted of CD8⁺CD60⁺ T cells, it is known that CD8⁺CD60⁺ T cells are required for memory IgE responses. The organ(s) in which blood CD8⁺CD60⁺ (CD45RO⁺) T memory cells were generated is unknown. The number of CD8⁺CD60⁺ T cells with specificity for ragweed Ag also is unknown because the RS donors of these cells also were sensitized to other allergens (skin testing; see *Materials and Methods*). However, the CD8⁺CD60⁺ blood T cells were 1) obtained in peak ragweed allergy season and 2) required for induction of ragweed-specific memory IgE responses. Therefore, many, if not most, of the CD8⁺CD60⁺CD45RO⁺ T cells probably are associated with specificity to ragweed Ag. Nevertheless, the exact numbers of CD8⁺CD60⁺CD45RO⁺ T cells that were ragweed specific, rather than T cells recruited into the ragweed IgE response, is unknown. It seems reasonable to infer that during peak ragweed allergy season, ragweed-specific memory CD8⁺CD60⁺ T cells divided in response to this allergen in lymphoid organs (MALT?) and emigrated and entered the circulation, whereas after ragweed allergy season, when allergen-induced cloning decreased, the numbers of ragweed-specific blood CD8⁺CD60⁺ T cells decreased. This idea is supported by the fact that high numbers of CD8⁺CD60⁺CD45RO⁺ T cells were present in blood of one extensively studied RS human at the peak of two ragweed allergy seasons (Fig. 4) and dramatically decreased thereafter to levels observed in NA humans (Fig. 1 and Table II). Nevertheless, because all RS humans in our study also were sensitized to other allergens, it would follow that CD8⁺CD60⁺ (CD45RO⁺) T memory cells related to these allergens might also be present in blood.

In the present studies, the highest numbers of CD8⁺CD60⁺ T cells were identified in blood during ragweed allergy season, after which their numbers dramatically decreased, but there were no significant decreases in total serum IgE levels. The lack of correlation between numbers of CD8⁺CD60⁺ T cells and serum IgE levels might be explained by the fact that all RS humans also were

skin test positive to other allergens. Their blood was obtained at the peak of the ragweed allergy season; therefore, many, if not most, of the CD8⁺CD60⁺ (CD45RO⁺) T cells were likely to be ragweed-specific memory cells, whereas serum IgE probably was directed against several allergens, including ragweed, so that no correlation would be expected because of the multiple specificities. Nevertheless, ragweed-specific IgE remains to be quantified in and out of ragweed allergy season, and it is possible that changes in CD8⁺CD60⁺ T cell numbers will directly correlate with changes in ragweed-specific IgE.

The present studies have shown that a subset of CD8⁺CD60⁺ T cells (~20%) simultaneously expressed CD23, in contrast to <1% in serum IgE⁻ NA humans. Therefore, it is important to better define the identities of the CD8⁺CD60⁺ T cell subset (CD23^{+/+}) that help and/or suppress memory IgE responses in order that cells/cytokine pathways might be accurately determined. Because CD23 is shed from cell surfaces (11–13), it could be that in vivo, even more CD8⁺CD60⁺CD45RO⁺ T cells of RS subjects coexpressed CD23. There is substantial literature implicating CD23⁺ cells, T and B, and soluble CD23 as regulators of IgE responses (reviewed in Refs. 14–17). If so, CD23⁺ T cells that coexpress CD8 and CD60 may distinguish the ϵ -specific helper or suppressor cells from CD8⁺ T cells that are known to help IgG (8, 9) and CD60⁺ T cells that are known to help IgG and IgA (10) responses and permit their selective deletion. However, the present experiments lay the groundwork for isolation and functional studies of this potentially important T cell subset.

The present studies confirm the well-known requirement for CD4⁺ T cells and IL-4 for induction of human IgE responses (reviewed in Refs 1 and 2). However, our studies also demonstrated that two distinct T cell subsets, CD4⁺ and CD8⁺CD60⁺, are required for induction of memory IgE responses, extending the network(s) leading to memory IgE responses to include CD4⁺ (18, 19) and CD8⁺CD60⁺ T cells and cytokines produced by CD8⁺CD60⁺ T cells (IL-2, IL-4, IL-10, IL-12, IFN- α , IFN- γ ; IL-4 also was produced by CD4⁺ T cells; other cytokines were not studied). Interestingly, in the present studies, IL-13 was not required for induction of memory IgE responses. The foregoing notwithstanding, it is possible that these cytokines are also produced by other PBMC.

Although the present studies are the first to demonstrate the strict requirement for two distinct T cell subsets, CD4⁺ and CD8⁺CD60⁺ for induction of human memory IgE responses, earlier studies of Herrick et al. (3) were the first to demonstrate the requirement for two T cell subsets: Thy1⁺ asialo GM1 ganglioside-negative and Thy1⁺ asialo GM1 ganglioside-positive for induction of murine hapten-specific memory IgE responses. Furthermore, the observations with cytokines in the present human studies directly parallel those of Herrick et al. (3). Both studies demonstrated that IL-4 was responsible for ~50% of the in vitro memory IgE response and that IFN- γ and IFN- α also were required, with IFN- α either helping or suppressing these IgE responses, depending on its concentration. In the present studies, human memory IgE responses additionally require IL-2, IL-10, and IL-12, but not IL-13 (or IL-6).

In the present study, help (or potentiation) or suppression of memory IgE responses was observed when purified CD8⁺CD60⁺ T cells, which made IL-2, IL-4, IL-10, IL-12, IFN- α , and IFN- γ , interacted in different proportions with PBMC of RS donors, which had been depleted of these cells, or with unfractionated PBMC. The mechanism(s) by which addition of low numbers of CD8⁺CD60⁺ T cells back to the depleted PBMC restored the ability of ragweed Ag to induce memory IgE responses, whereas addition of higher numbers totally suppressed these responses, are

unknown. Because low numbers of CD8⁺CD60⁺ T cells and IFN- α helped memory IgE responses, whereas higher numbers and IFN- α suppressed them, an attractive possibility is that CD8⁺CD60⁺ (CD45RO⁺; CD23⁺?) IFN- α ⁺ T cells secrete IFN- α , which directly or indirectly helps and/or suppresses memory IgE responses. This hypothesis is made more attractive by the fact that two T cell subsets and IFN- α performed similar helper and suppressor functions in mice (3).

The present findings with CD8⁺CD60⁺ T cells resemble earlier findings of Haskill and Axelrad (20) and Haskill and Harbrook (21), who showed that addition of low numbers of large spleen cells from mice primed with sheep erythrocytes suppressed plaque-forming cell responses of small lymphocytes, whereas higher numbers failed to do so. Kontiainen and Feldmann (22) found carrier-specific suppression with low numbers of KLH-primed cells, as low as 1×10^3 , in a system which tested helper activity as the target of suppression. Durkin et al. (23) demonstrated that low numbers of OVA-sensitized T memory cells added to higher numbers of lymph node cells from the same OVA-sensitized rats sometimes helped or potentiated and sometimes totally suppressed proliferative responses to either OVA or phytohemagglutinin. In the present studies, as was true of the earlier studies by others (20–23), the mechanisms involved in the network(s) leading to help/potentiation or suppression of memory responses obviously are complex, involving other cells and mediators released by cells participating in the memory response and feedback loops (24, 25).

The mechanism(s) by which CD8⁺CD60⁺ T cells helped IgE responses in the present studies is unknown, including the exact cell-cell and cell-cytokine interactions involved. Unraveling these mechanisms will require studies such as use of chamber experiments (26, 27). These studies would determine whether, for example, CD4⁺ T cells interacted with CD8⁺CD60⁺ T cells, or vice versa, or with intermediary cells to induce memory IgE responses. The requirement for six cytokines (IL-2, IL-4, IL-10, IL-12, IFN- α , and IFN- γ), each of which was produced by ~25% of CD8⁺CD60⁺ T cells, with IL-4 also produced by CD4⁺ T cells, and the probability that some these cytokines also might be produced by other PBMC, points to the fact that CD8⁺CD60⁺ T cells probably acted indirectly to induce memory IgE responses.

There is substantial literature related to CD8⁺ T cell-mediated killing (28–31). In the present studies, it remains to be determined whether suppression of memory IgE responses observed with higher numbers of CD8⁺CD60⁺ T cells than those required for helper activity involved killing mechanisms. However, if CD8⁺CD60⁺ T cells prove to kill other PBMC to suppress memory IgE responses, this could be a mechanism by which IgE responses, in general, are selectively suppressed.

With respect to requirements for two distinct T cell populations for in vitro induction of specific memory IgE responses observed by Herrick et al. (3), Thy1⁺ asialo GM1 ganglioside negative and Thy1⁺ asialo GM1 ganglioside positive, and, in the present studies CD4⁺ and CD8⁺CD60⁺, it should be mentioned that CD60 also is a ganglioside (disialoganglioside II³(Neu5Ac)₂-LacCer(9-*O*-acetyl G_{D3}) (10), albeit a different ganglioside from asialo GM1 ganglioside. This, of course, raises the question of whether or not it is simply happenstance that one of the two T cell subsets required for memory IgE responses in the murine and human systems coexpress gangliosides or if the gangliosides play an important role in generation of memory IgE responses, and this is the subject of our ongoing studies.

Lastly, it should be mentioned that in studies in our laboratory, when PBMC obtained from serum IgE⁺ RS donors outside the allergy season were cultured with ragweed Ag, IgE frequently was

not induced (>60% of experiments). When blood was obtained in season, IgE was not produced in ~25% of experiments. The reason(s) for this is unknown. One explanation for the successful induction of ragweed-specific memory IgE responses reported in the present studies is the fact that a requirement for induction of ragweed-specific memory IgE responses was met because blood was obtained in ragweed allergy season and contained the requisite numbers of CD8⁺CD60⁺ T cells required for induction of these responses. This, however, does not rule out that there may be additional unknown requirements for induction of memory IgE responses, which have been met in the present studies by obtaining PBMC during peak ragweed allergy season.

Disclosures

The authors have no financial conflict of interest.

References

- Finkelman, F. D., J. Holmes, I. M. Katona, J. F. Urban, Jr., M. P. Beckman, L. S. Park, K. A. Schooley, R. Coffman, T. R. Mossman, and W. E. Paul. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* 8: 303–333.
- Zurawski, G., and J. E. deVries. 1994. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol. Today* 15: 19–26.
- Herrick, C. A., J. A. Carucci, G. I. Kleiner, and H. G. Durkin. 1993. Regulation of hapten-specific memory IgE responses induced in vitro: requirement for both Thy-1⁺AsGM1⁺ and Thy-1⁺AsGM1⁺ cells and for IFN- α and IL-4. *J. Immunol.* 151: 3440–3449.
- Auci, D. L., G. I. Kleiner, S. M. Chice, P. Dukor, and H. G. Durkin. 1993. Control of IgE responses: III. IL-6 and IFN- α are isotype-specific regulators of peak BPO-specific IgE antibody forming cell responses in mice. *Clin. Immunol. Immunopathol.* 66: 219–225.
- Auci, D. L., G. I. Kleiner, S. M. Chice, P. Dukor, and H. G. Durkin. 1993. Control of IgE responses: IV. Isotype specific suppression of peak BPO-specific IgE antibody forming cell responses and of BPO-specific IgE in serum of mice administered muramyl dipeptide or murabutide by gavage. *Int. Arch. Allergy Immunol.* 101: 167–176.
- Smith-Norowitz, T. A. 1997. "Role of CD8⁺CD60⁺ T cells in peripheral blood of atopic humans in regulation of allergic (IgE) responses." Doctoral Dissertation, State University of New York Science Center, Brooklyn, NY, pp. 1–112.
- Smith-Norowitz, T. A., D. L. Auci, K. B. Norowitz, S. M. Chice, and H. G. Durkin. 1997. CD8⁺CD60⁺ T cells as regulators of human allergic (IgE) responses. *J. Allergy Clin. Immunol.* 99: S406.
- Massague, J. 1990. The transforming growth factor-B family. *Annu. Rev. Cell Biol.* 6: 697–705.
- Swain, S. 1981. Significance of Lyt phenotypes: Lyt 2 antibodies block activities of T cells that recognize class I major histocompatibility complex antigen regardless of their function. *Proc. Natl. Acad. Sci. USA* 78: 7101–7110.
- Rieber, E. P., and G. Rank. 1994. CDw60: a marker for human CD8⁺ T helper cells. *J. Exp. Med.* 179: 1385–1390.
- Bonnefoy, J.-Y., S. Lecoanet-Henchoz, J.-F. Gauchat, P. Graber, J.-P. Aubry, P. Jeannin, and C. Plater-Zyberk. 1997. Structure and functions of CD23. *Int. Rev. Immunol.* 16: 113–128.
- Ford, J. W., M. A. Kilmon, K. M. Haas, A. E. Shelburne, Y. Chan-Li, and D. H. Conrad. 2006. In vivo murine CD23 destabilization enhances CD23 shedding and IgE synthesis. *Cell. Immunol.* 243: 107–117.
- Munoz, O., C. Brignone, N. Grenier-Brossette, J.-Y. Bonnefoy, and J. L. Cousin. 1998. Binding of anti-CD23 monoclonal antibody to the leucine zipper motif of Fc ϵ R2/CD23 on B cell membrane promotes its proteolytic cleavage: evidence for an effect on the oligomer/monomer equilibrium. *J. Biol. Chem.* 273: 31795–31800.
- Ishizaka, K. 1980. Cellular events in the IgE antibody response. *Adv. Immunol.* 23: 1–10.
- Yodoi, J., and K. Ishizaka. 1980. Induction of Fc ϵ -receptor bearing cells in vitro in human peripheral lymphocytes. *J. Immunol.* 124: 934–938.
- Carini, C., J. Margolick, J. Yodoi, and K. Ishizaka. 1988. Formation of IgE-binding factors by T cells of patients infected with human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* 85: 9214–9218.
- Conrad, D. H., J. W. Ford, J. L. Sturgill, and D. R. Gibb. 2007. CD23: an overlooked regulator of allergic disease. *Curr. Allergy Asthma Rep.* 7: 331–337.
- Ben-Sasson, S. Z., I. Zuckovsky, A. Biton, R. Vogel, G. Fourcas, N. Hayashi, and W. E. Paul. 2002. Generation and characterization of memory CD4⁺ T cells. *Adv. Exp. Med. Biol.* 512: 129–134.
- Wantanabe, T., H. Katsukura, Y. Shirai, M. Yamori, T. Chiba, T. Kita, and Y. Wakatsuki. 2003. Helper CD4⁺ T cells for IgE responses to a dietary antigen develop in the liver. *J. Allergy Clin. Immunol.* 111: 1375–1385.
- Haskill, J. S., and M. A. Axelrad. 1972. Cell-mediated control of an antibody response. *Nat. New Biol.* 237: 251–252.
- Haskill, J. S., and J. Harbrook. 1972. In vitro immunity to sheep erythrocytes by fractionated spleen cells: differentiation within the antibody-forming-cell precursor population. *Cell. Immunol.* 3: 448–460.
- Kontianen, S., and M. Feldmann. 1973. Induction of specific helper cells in vitro. *Nat. New Biol.* 245: 285–286.
- Durkin, H. G., J. A. Bash, and B. H. Waksman. 1975. Separation of T cell subpopulations capable of DNA synthesis, lymphotoxin release, and regulation of antigen and phytohemagglutinin responses on the basis of density and adherence properties. *Proc. Nat. Acad. Sci. USA* 72: 5090–5094.
- Calkins, C. E., S. Orbach-Arbouys, O. Stutman, and R. K. Gershon. 1976. Cell interactions in the suppression of in vitro antibody responses. *J. Exp. Med.* 143: 1421–1428.
- Ingenito, G. G., and C. E. Calkins. 1983. Lymphocyte subsets involved in tumor-activated nonspecific feedback suppression. *Cell. Immunol.* 79: 26–35.
- Ledgerwood, L. G., G. Lal, N. Zhang, A. Garin, S. J. Esses, F. Ginhoux, M. Merad, H. Peche, S. A. Lira, Y. Ding, et al. 2008. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. *Nat. Immunol.* 9: 42–53.
- Heuchel, R., A. Berg, M. Tallquist, K. Ahlen, R. K. Reed, K. Rubin, L. Claesson-Welsh, C.-H. Heldin, and P. Soriano. 1999. Platelet derived growth factor β receptor regulates interstitial fluid homeostasis through phosphatidylinositol-3' kinase signaling. *Proc. Natl. Acad. Sci. USA* 96: 11410–11415.
- Harms, J. S., and G. A. Splitter. 1995. CD8⁺ lymphocytes that kill allogeneic and xenogeneic major histocompatibility complex class I targets. *Hum. Immunol.* 44: 50–57.
- Medana, I. M., A. Gallimore, A. Oxenius, M. M. Martinic, H. Wekerle, and H. Neumann. 2000. MHC class I-restricted killing of neurons by virus-specific CD8⁺ T lymphocytes is effected through the Fas/FasL, but not the perforin pathway. *Eur. J. Immunol.* 30: 3623–3633.
- Ma, L. L., J. C. Spurrell, J. F. Wang, G. G. Neely, S. Epelman, A. M. Krensky, and C. H. Mody. 2002. CD8 T cell-mediated killing of *Cryptococcus neoformans* requires granulysin and is dependent on CD4 T cells and IL-15. *J. Immunol.* 169: 5787–5795.
- Li, Y., M. Bendandi, Y. Deng, C. Dunbar, N. Munshi, S. Jagannath, L. W. Kwak, and H. K. Lyerly. 2000. Tumor-specific recognition of human myeloma cells by idiotypic-induced CD8⁺ T cells. *Blood* 96: 2828–2833.