Simplify Your Multiomics Workflow
Pre-Optimized Antibody Cocktails for Mouse and Human Targets
BioLegend®


The Journal of Immunology

Antigenic Epitopes Regulate the Phenotype of CD8$^+$ CTL Primed by Exogenous Antigens

Hakling Ma and Judith A. Kapp

This information is current as of March 7, 2022.

J Immunol 2000; 164:5698-5703; ;
doi: 10.4049/jimmunol.164.11.5698
http://www.jimmunol.org/content/164/11/5698

References This article **cites 55 articles**, 36 of which you can access for free at:
http://www.jimmunol.org/content/164/11/5698.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2000 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on March 7, 2022

Antigenic Epitopes Regulate the Phenotype of CD8$^+$ CTL Primed by Exogenous Antigens[1]

Hakling Ma and Judith A. Kapp[2]

We previously reported that insulin-specific, MHC class I-restricted CTL precursors can be primed by injecting C57BL/6 mice with bovine insulin in CFA. These bovine insulin-primed CTL displayed a type 0 CTL phenotype, producing IL-4, IL-5, IL-10, low levels of IFN-γ, but no TNF-α. By contrast, CTL generated from C57BL/6 mice primed with OVA in CFA produced IFN-γ and TNF-α but no IL-4, IL-5, or IL-10 and therefore were classified as type 1 CTL. Although CD4$^+$ T cell subsets have been compared extensively in the literature, CTL subsets are less well characterized. Here, the phenotype, function, and requirements for the in vivo activation of type 1 and type 0 CTL cells were studied. Although both types of CTL express many of the same cell-surface Ags, OVA-specific CTL but not bovine insulin-primed CTL expressed CT-1, a carbohydrate epitope of CD45, and bovine insulin-primed CTL but not OVA-specific CTL expressed Fas constitutively. Priming of CTL was abrogated by depletion of phagocytic cells but not CD4$^+$ T cells, whereas depletion of CD4$^+$ T cells but not phagocytic cells inhibited Ab responses in the same mice. Neither endogenous IL-4 nor the dose of priming Ag altered the CTL phenotypes, but the antigenic peptides of OVA and bovine insulin were key to determining the differentiation of either type 1 or type 0 CTL. To our knowledge, this is the first time that antigenic epitopes have been demonstrated to influence the phenotype of Ag-specific CTL responses. These results may be relevant to the development of peptide vaccines in which a particular type of CTL response is desired. ***The Journal of Immunology*****, 2000, 164: 5698–5703.**

Although virus- and alloantigen-specific CD8$^+$ CTL generally produce type 1 cytokines (IFN-γ and TNF-α) upon activation, CD8$^+$ T cells have been shown to secrete other patterns of cytokines in vivo. Borrowing from a CD4$^+$ T cell nomenclature, type 1 CD8$^+$ CTL (Tc1)[3] are so named because they produce IFN-γ, TNF-α, and IL-2. Type 2 CD8$^+$ CTL (Tc2) produce IL-4, IL-5, IL-6, and IL-10, whereas type 0 CD8$^+$ CTL (Tc0) produce a mixture of both types of cytokines (1–3). Tc0 cells have been isolated from lesions of patients with lepromatous leprosy (4) and from HIV-infected individuals with a Job's-like syndrome (5). In murine systems, CD8$^+$ T cells that produce either type 1 or type 2 cytokines have been found in gut-associated tissues in mice (6). In addition, type 0 CD8$^+$ T cells were found in mice infected with lymphocytic choriomeningitis virus (7) and influenza virus (8–10).

Detailed characterization of the subsets of CD8$^+$ T cells have been primarily limited to in vitro-derived CD8$^+$ T cells (11–13). Therefore, a systematic comparison of Tc1 and Tc2 cells in terms of their surface marker expression, lytic mechanisms, and requirements for their generation is indicated to elucidate the differences in their functions. A major question to be addressed is what determines the differentiation of polarized subsets of CD8$^+$ T cells. CD8$^+$ TCR transgenic or allogeneic CD8$^+$ T cells can differentiate into Tc1 and Tc2 subsets when directing cytokines are present during primary stimulation in vitro (1–3). However, a more physiologically relevant approach is to assay the generation requirements of polarized CD8$^+$ T cells in vivo in the absence of directing cytokines. Understanding the factors that affect the phenotype of CD8$^+$ T cells may provide insight that will be useful for the design of peptide vaccines.

Previously, we reported that CTL specific for exogenous proteins were primed in C57BL/6 (B6) mice by OVA (14) or bovine insulin (BINS) (15) in CFA. No CTL were elicited in mice primed with soluble proteins or proteins emulsified in IFA (14). Splenocytes from immunized mice were restimulated in vitro with transfected target cell lines that express either the OVA or the insulin gene. OVA-specific CTL (OVA-CTL) produced IFN-γ and TNF-α upon stimulation, whereas BINS-specific CTL (INS-CTL) produced IL-4, IL-5, IL-10, and IFN-γ. Here, OVA-CTL and INS-CTL are systematically compared in terms of their phenotype, function, and requirements for their generation.

Materials and Methods

Mice

Female 8- to 12-wk-old B6 mice were purchased from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). IL-4-deficient, IL-4^{tm1Nnt} (16) (IL-4$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All procedures on animals were conducted according to the principles outlined in the guidelines of the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council.

Reagents

Purified OVA (grade VI) and crystalline BINS were purchased from Sigma (St. Louis, MO). CFA containing *Mycobacterium tuberculosis* strain H37Ra and IFA was obtained from Difco (Detroit, MI). Emulsions of OVA, BINS, or their peptides in CFA or IFA were prepared by mixing aqueous Ag solution with oil phase at a 1:1 ratio and achieved a final concentration of 1 mg/ml as described previously (14). Dr. Maurice Gately

Department of Ophthalmology, Pathology, and Winship Cancer Center, Emory University School of Medicine, Atlanta, GA 30322

Received for publication December 20, 1999. Accepted for publication March 22, 2000.

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] This work was supported by the National Institutes of Health Grant CA-70372 and a gift from Malcolm E. and Musette Powell.

[2] Address correspondence and reprint requests to Dr. Judith A. Kapp, Department of Ophthalmology, Emory University School of Medicine, Building B, Room 2623, 1365 Clifton Road Northeast, Atlanta, GA 30322. E-mail address: jkapp@emory.edu

[3] Abbreviations used in this paper: Tc1, type 1 cytokine-producing cytotoxic T cell; BINS, bovine insulin; OVA-CTL, OVA-specific CTL; INS-CTL, bovine insulin-primed CTL, Tc0, type 0 cytokine-producing cytotoxic T cell; Tc2, type 2 cytokine-producing T cell; B6, C57BL/6; FasL, Fas ligand.

Copyright © 2000 by The American Association of Immunologists

0022-1767/00/$02.00

Downloaded from http://www.jimmunol.org/ by guest on March 7, 2022

(Hoffman-La Roche, Nutley, NJ) generously provided recombinant human IL-2. The $OVA_{257–264}$ (SIINFEKL) and INS $A_{12–21}$ (SLYQLENYCN) peptides used in this paper were synthesized at the Emory University Microchemical Facility or provided by Dr. Brian Evavold (Emory University, Atlanta, GA).

Tumor cells and cell cultures

H-2^b thymoma cells (EL4) were purchased from American Type Culture Collection (Manassas, VA). EG7-OVA, generated by transfection of EL4 cells with the OVA cDNA gene (17), were kindly provided by Dr. M. J. Bevan (University of Washington, Seattle, WA). EL4-INS cells were generated by tranfection of EL4 cells with human proinsulin DNA (18). RMA-S (H-2^b) cells (19) were generously provided by Dr. K. Karre (Stockholm, Sweden). All cell cultures were maintained in RPMI 1640 medium supplemented with 5% FBS, 1 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, and antibiotics at 37°C in 6% CO_2 in air.

Immunization and establishment of T cell lines

Unless otherwise noted, B6 mice were immunized in the hind footpad with 100 μg of OVA or BINS in CFA as previously described (14). In some experiments, 100 μg each of OVA and BINS were mixed together in CFA. After 10–14 days, splenocytes were cultured with irradiated (20,000 rad) stimulator cells, E.G7-OVA, or EL4-INS. Thereafter, T cells were restimulated weekly with irradiated syngeneic splenocytes and stimulator cells plus 20 U/ml recombinant human IL-2. For peptide priming, B6 mice were immunized with 50 μg of the indicated peptides in CFA at the hind footpad. After 12 days, these mice were boosted with 50 μg of the same peptide in IFA. One week later, splenocytes from mice immunized with peptides were stimulated with RMA-S cells pulsed with the corresponding peptides rather than E.G7-OVA or EL4-INS in vitro.

Cr ^{51} release assay

Cytotoxicity was quantified by a 4-h ^{51}Cr release assay (14). Syngeneic targets (E.G7-OVA, EL4-INS, or peptide-pulsed RMA-S cells) were labeled with $Na_2{}^{51}CrO_4$ (DuPont, Boston, MA) at 37°C for 1 h. After washing, ^{51}Cr-labeled target cells were incubated with INS-CTL or OVA-CTL at different E:T ratios in 96-well round-bottom plates. After 4-h incubation at 37°C, supernatants were collected and radioactivity was detected in a scintillation counter (Wallac, Turku, Finland). Percent specific lysis was calculated as 100 × ([release by CTL − spontaneous release]/[maximal release − spontaneous release]). Maximal release was determined by the addition of 1% Triton X-100 (EM Science, Gibbstown, NJ). The spontaneous release, in the absence of CTL, was generally <15% of the maximal release.

Cytokine assay

Effector T cells (10^6) were incubated for 24 h with 5×10^5 stimulator cells (unless otherwise noted) in a final volume of 1 ml/well in 24-well plates. Supernatants were collected and tested for lymphokines using paired mAbs specific for cytokines IL-4, IL-5, IL-10, IFN-γ, or TNF-α (PharMingen, San Diego, CA). Biotinylated Ab were added and detected with avidin-peroxidase (Vector Laboratories, Burlingame, CA) plus 2.2-azino-di[3-ethyl-benzthiazoline sulfonate] substrate containing H_2O_2 (Kirkegaard & Perry, Gaithersburg, MD). The colorimetric reaction was read at 450 nm using an automatic microplate reader (Molecular Devices, Menlo Park, CA). The concentrations of the cytokines were calculated according to the standard curves of the appropriate recombinant cytokines.

Abs

The Abs used in this study are listed in Table I.

Flow cytometry

Viable T cells were isolated by Ficoll-Hypaque gradient centrifugation (26) and then incubated for 30 min on ice in 50 μl of staining buffer (PBS, pH 7.4, containing 1% BSA and 0.1% sodium azide) containing 1 μg of fluorochrome-conjugated or unconjugated Ab. Fluorochrome-conjugated rat or hamster IgG was used as the isotype control. The cells were fixed in 0.5% paraformaldehyde after washing three times with staining buffer. In the case of CT-1, a secondary FITC-conjugated goat anti-mouse Ig (A,M,G) (Cappel, Durham, NC) was used. For Fas and Fas ligand (FasL) staining, CTL were stimulated with Con A (5 μg/ml) for 4 h and stained with hamster anti-mouse Fas or rabbit anti-mouse FasL Abs followed by either biotinylated anti-hamster IgG mixture or biotinylated anti-rabbit IgG, respectively. Biotinylated Abs were detected with streptavidin-PE.

The stained cells were examined on a FACScan Cytofluorimeter using LYSIS-II software (Becton Dickinson, San Jose, CA). Forward angle, light scatter was used to exclude dead and aggregated cells. The results are presented as fluorescence histograms with the relative number of cells on a linear scale plotted vs the relative fluorescence intensity on a log scale.

Table I. *Abs used in this study*

	Reference	Isotype Controls
Conjugated mAbs		
Anti-CD4 (GK1.5)	20	Rat IgG1
Anti-CD8α (53.6.72)	21	Rat IgG2a
Anti-CD3 (145-2C11)	22	Hamster IgG
Anti-$\alpha\beta$ TCR (H57)	23	Hamster IgG
Anti-CD28	24	Hamster IgG
Anti-CTLA4	24	Hamster IgG
Unconjugated Abs		
Anti-T200 (CT-1)	25	Mouse IgM
Anti-Fas (Jo2)	26	Hamster IgG
Anti-Fas-L (Q-20)	27	Rabbit IgG

Depletion of CD4$^+$ and phagocytic cells

B6 mice were injected i.p. with 200 μg of anti-CD4 (GK1.5) mAb or isotype controls on days −3, −2, and −1 (28). For depletion of phagocytic cells, mice were injected i.v. on day −2 and i.p. on day −1 with 0.5 mg silica (29, 30) (kindly provided by Dr. R. L. Hunter, University of Texas, Houston, TX) before immunization.

Serum Ab detection

Fourteen days postimmunization, mice were bled individually and the sera were tested for OVA- and INS-specific Abs by ELISA. Microtiter plates were coated with 100 μl of either OVA or BINS (10 μg/ml) in borate-buffered saline. Sera from individual mice were first diluted 1:100 followed by serial 2-fold dilutions. Ag-specific IgG was detected with alkaline phosphatase-conjugated goat anti-mouse IgG. Subsequent addition of the substrate ρ-nitrophenyl phosphate in buffer diethanolamine and $MgCl_2$ caused a colorimetric reaction, which was detected by an ELISA reader (Molecular Devices, Menlo Park, CA).

A minimum of three mice per group was used in each experiment in this manuscript. Representative results are shown for experiments that were repeated at least three times with similar results.

Results

Phenotypic and functional differences between OVA-CTL and INS-CTL

OVA-CTL and INS-CTL cell lines were generated by priming B6 mice with 100 μg of either OVA or BINS in CFA. Splenocytes harvested from primed mice were cultured in vitro with irradiated stimulator cells (E.G7-OVA or EL4-INS) for 1 wk. The cultures were then restimulated weekly with the same irradiated target cells plus spleen cells and exogenous IL-2. Such CTL generated are specifically lytic toward their corresponding target cells. However, no CTL were detected without in vivo Ag priming (14, 15, 17), after priming with soluble protein, or protein in IFA (30)

Although OVA-specific and insulin-specific CD8$^+$ lines are cytolytic, they secreted different cytokines upon stimulation. OVA-CTL produced significant amounts of IFN-γ and TNF-α but no IL-4, IL-5, or IL-10, which represents a Tc1 cytokine profile (Fig. 1). By contrast, INS-CTL produced IL-5, IL-10, IFN-γ, and some IL-4 but no detectable TNF-α (Fig. 1). It should be noted that independently derived OVA-CTL lines always produced more IFN-γ than did INS-CTL lines. Because the CTL clones generated from OVA-CTL and INS-CTL lines expressed the same phenotypes as the bulk lines (data not shown), we hereafter refer to OVA-CTL and INS-CTL as Tc1 and Tc0 cells, respectively. Neither OVA-CTL nor INS-CTL produced IL-2 upon activation (data not shown), which is not unusual for cells that have been restimulated with supplemental IL-2. OVA-CTL and INS-CTL represent

Downloaded from http://www.jimmunol.org/ by guest on March 7, 2022

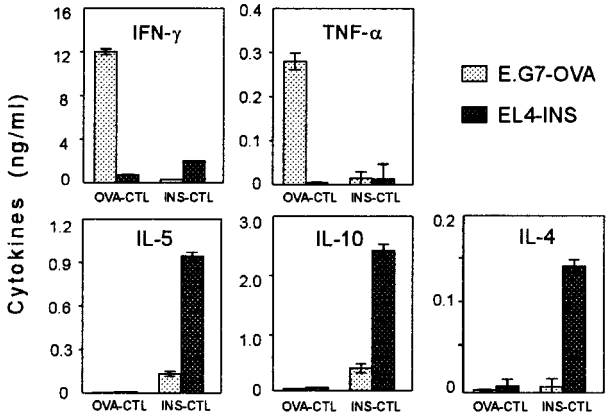

FIGURE 1. Lymphokine production by OVA-CTL and INS-CTL. OVA-CTL or INS-CTL were incubated for 24 h at 10^6 cells per well with 5×10^5 EG7-OVA or EL4-INS stimulator cells, respectively. Supernatants were then assayed for IL-4, IL-5, IL-10, IFN-γ, and TNF-α by ELISA using recombinant lymphokines as standards. Results are shown as cytokine concentrations ± SD.

two polarized subsets of $CD8^+$ T cells, as their phenotypes have been stable over long-term culture.

Cell-surface markers were determined for INS-CTL and OVA-CTL (Fig. 2). Both CTL are $CD8^+$, $CD3^+$, $\alpha\beta TCR^+$, and $CD28^+$ but CTLA-4^-. OVA-CTL but not INS-CTL express the CT-1 (T200), a carbohydrate epitope of CD45 expressed by activated CTL (25). Fas was expressed by both resting (not shown) and activated INS-CTL but not OVA-CTL, while both CTL expressed FasL upon activation. Thus, INS-CTL and OVA-CTL express similar, but not identical, phenotypic features.

Requirements for priming CTL precursors

In some systems, priming of Ag-specific $CD8^+$ T cells has been shown to require CD4 T cell help (31–33). Thus, we asked whether $CD4^+$ T cells were required for the priming of Tc1 or Tc2 cells.

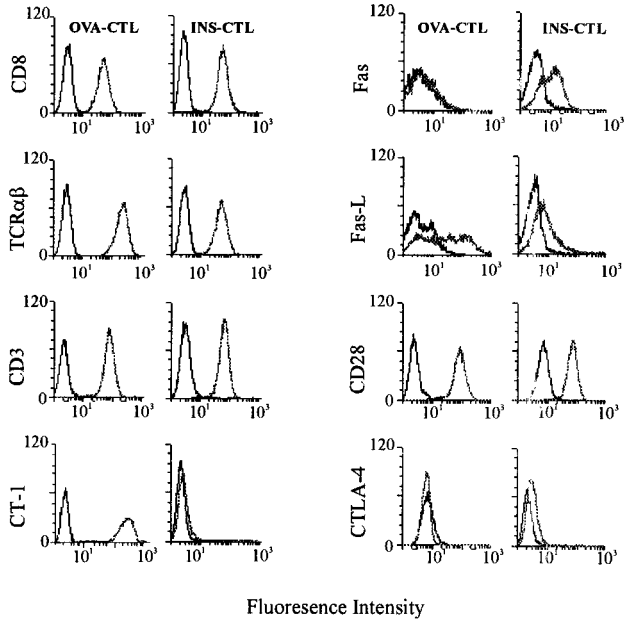

FIGURE 2. Cell-surface phenotype of OVA-CTL and INS-CTL cell lines. OVA-CTL and INS-CTL were stained with the indicated Abs. Isotype controls are the left most peak in each panel.

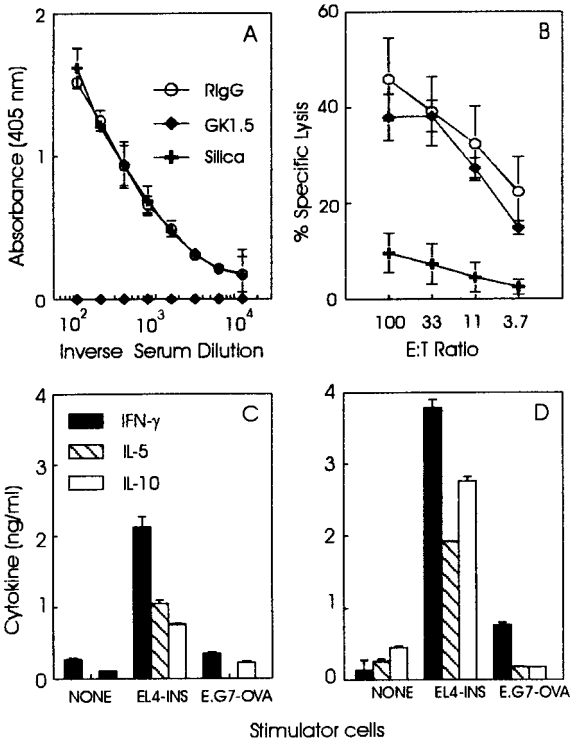

FIGURE 3. Cellular requirements for Ab production and generation of INS-CTL in vivo. B6 mice were treated with either GK1.5, rat IgG1 as the isotype control, or silica and then immunized with BINS in CFA. Mice were bled after 14 days and tested for insulin-specific IgG by ELISA (*A*) and priming for CTL using either EL4-INS as target cells at the indicated E:T ratios (*B*). CTL generated from mice treated with either rIgG (*C*) or anti-CD4 Ab (*D*) were also tested for cytokine production with or without stimulation.

Priming of OVA-CTL by OVA in CFA was previously shown to be independent of CD4 helper cells but critically dependent on phagocytic cells (14). To determine whether INS-CTL require CD4 cells for priming, B6 mice were depleted with anti-CD4 Ab before priming. Production of insulin-specific Ab was attenuated by anti-CD4 Ab, as expected (Fig. 3*A*). By contrast, INS-CTL with similar lytic activities were primed in both the mice treated with GK1.5 and the isotype control (Fig. 3*B*). Inactivation of phagocytic cells by injection of silica did not inhibit the Ab responses (Fig. 3*A*), but completely abrogated the induction of INS-CTL in the same mice (Fig. 3*B*). Thus, priming of INS-CTL, like priming of OVA-CTL (14), is relatively independent of $CD4^+$ T cells but dependent on phagocytic cells. In addition, the cytokine profiles of INS-CTL generated from mice treated with either rIgG (Fig. 3*C*) and anti-CD4 Ab (Fig. 3*D*) were similar, suggesting that Th cells exerted little effect on the phenotype of CTL primed with exogenous Ags in CFA.

IL-4 plays a very important role in skewing both $CD4^+$ (34) and $CD8^+$ (35) T cells to produce type 2 cytokines. Although $CD4^+$ T cells were not required for the activation of $CD8^+$ T cells in mice primed with Ag in CFA, other cells might provide sufficient IL-4 to active $CD8^+$ T cells in our system. To test whether endogenous IL-4 in B6 mice was responsible for priming Tc0 by BINS in CFA, $CD8^+$ T cells from insulin immunized B6 or B6 IL-$4^{-/-}$ mice (36) were compared. Fig. 4 shows that INS-CTL generated from IL-$4^{-/-}$ mice were as lytic as those from the normal B6 mice (Fig. 4*A*). Even without IL-4 in the priming microenvironment, INS-CTL produced the other type 0 cytokines (IFN-γ, IL-5, and IL-10),

Downloaded from http://www.jimmunol.org/ by guest on March 7, 2022

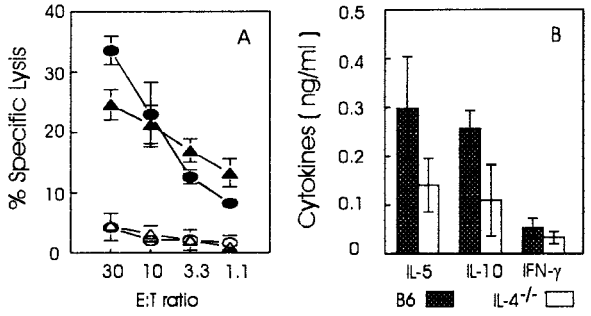

FIGURE 4. Priming of INS-CTL in IL-4$^{-/-}$ mice. Wild-type B6 or IL-4$^{-/-}$ mice were immunized with 100 μg of BINS in CFA. Cytolytic activities by B6 mice for EL4-INS (●) or E.G7-OVA (○) targets and IL-4$^{-/-}$ mice for EL4-INS (▲) or E.G7-OVA (△) were measured (*A*). Cytokines produced by B6 (▨) and IL-4$^{-/-}$ mice (□) after stimulation with EL4-INS cells are shown (*B*).

albeit at levels lower than those from the wild-type B6 mice (Fig. 4*B*). Thus, IL-4 was not required for the differentiation of naive CD8$^+$ T cells into type 2 cytokine-producing cells in vivo.

The same immunization protocols and subsequent culturing conditions were used for generating INS-CTL and OVA-CTL, suggesting either that Ag itself might regulate the phenotypes of CTL or that the hormonal activity of insulin might be responsible for the induction of type 0 INS-CTL differentiation. To assess whether the Ag itself induced changes in the APC or cytokine microenvironment, B6 mice were immunized with a mixture of OVA and BINS in CFA. Mouse splenocytes were then divided in half and cultured separately with either E.G7-OVA or EL4-INS. We reasoned that if the Ags themselves were the determining factors that regulated CTL differentiation then INS-CTL and OVA-CTL generated from mixed Ag immunization should maintain their respective cytokine profiles. In contrast, if the OVA or BINS induced changes in the cytokine environments or the properties of APC, then both CTL would assume the same (Tc1 or Tc0) phenotype. Mixed Ags induced serum Abs against both OVA (Fig. 5*A*) and BINS (Fig. 5*B*) in B6 mice. The CTL that were cultured in the presence of either E.G7-OVA (Fig. 5*C*) or EL4-INS (Fig. 5*D*) were lytic toward their corresponding stimulator cells, although some low reactivity toward the other Ag was detected in these early cultures. More importantly, CTL stimulated with E.G7-OVA produced only IFN-γ (Fig. 5*E*), whereas CTL stimulated with EL4-INS produced IL-5, IL-10, and some IFN-γ (Fig. 5*F*). The cytokine profiles induced by priming with a mixture of OVA and BINS were similar to those produced by mice primed with a single Ag. Thus, we conclude that the generation of Tc1 or Tc0 is determined by the intrinsic property of the Ag rather than a nonspecific effect.

There are several potential explanations for how Ag might regulate the phenotype of CD8$^+$ T cells. Because OVA and BINS are two very different protein Ags, differences in their processing and in presentation by APC might influence the cytokines produced by these CTL. To eliminate any potential bias in the processing and presentation of the OVA and BINS Ags, B6 mice were immunized with the dominant antigenic epitopes of OVA or BINS. $OVA_{257–264}$ induced CTL that were lytic toward $OVA_{257–264}$-pulsed RMA-S cells but not RMA-S cells pulsed with irrelevant $INS_{12–21}$ peptide (Fig. 6*A*). Similarly, BINS $A_{12–21}$ induced CTL that were lytic toward insulin peptide-pulsed RMA-S cells but not RMA-S cells pulsed with OVA peptide (Fig. 6*B*). More importantly, $OVA_{257–264}$ induced OVA-CTL to produce only IFN-γ (Fig. 6*C*), whereas INS-CTL primed by $BINSA_{12–21}$ produced IFN-γ, IL-5, and IL-10 upon activation (Fig. 6*D*). These data demonstrated that the antigenic peptides were sufficient to prime Ag-specific CTL that produce either type 1 or type 0 cytokines. Thus, the phenotypes of the peptide-primed CTL were identical with those of the CTL primed by native protein Ags, which rules out a processing difference between OVA and BINS Ags.

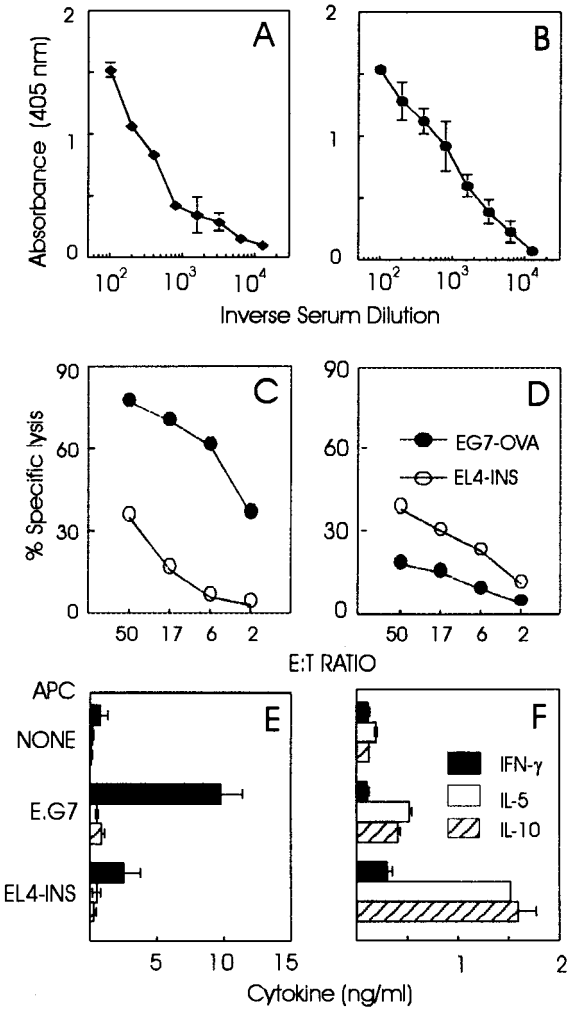

FIGURE 5. Priming of CTL with a mixture of OVA and BINS. B6 mice were primed with 100 μg of OVA and BINS mixed in CFA. Serum Abs against OVA (*A*) and BINS (*B*) were determined by ELISA. Cytolytic activities of OVA-CTL (*C*) or INS-CTL (*D*) were assayed using OVA- or INS-transfected cells as targets. The cytokines produced by OVA-CTL (*E*) and INS-CTL (*F*) are shown after activation by different stimulator cells.

Discussion

Previously, we reported that priming B6 mice with exogenous Ags induced Ag-specific OVA-CTL (14) and INS-CTL (15) that are classified as Tc1 and Tc0 cells, respectively. Like Tc1 cells, the Tc0 cells described here are a stable phenotype that does not undergo further differentiation in vitro. Although type 0 cells are thought to give rise to type 1 and type 2 clones (37), many investigators have reported that stable type 0 clones persist in vitro (38–41). Why such type 0 cells don't mature into type 1 or type 2 cells, under the influence of their own cytokines, is not clear. One possibility is that these Tc0 cells may down-modulate cytokine receptors, rendering them resistant to the polarizing effects of IL-4,

Downloaded from http://www.jimmunol.org/ by guest on March 7, 2022

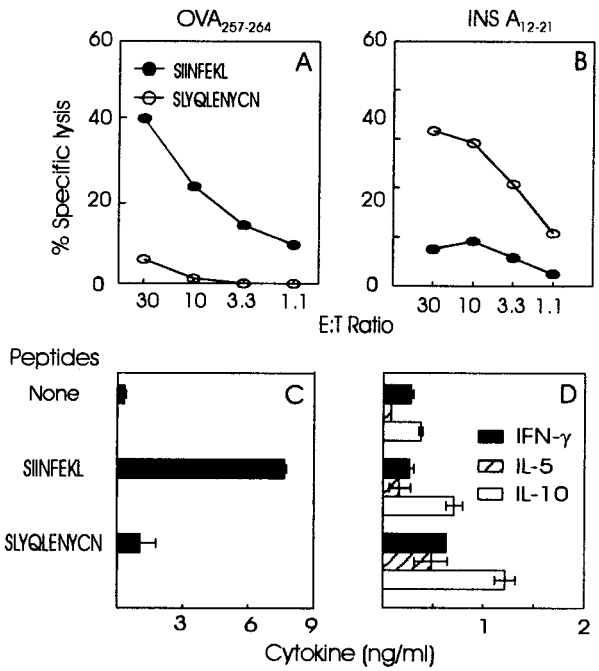

FIGURE 6. Phenotype of CTL induced by priming with peptides. B6 mice were primed with 50 μg of $OVA_{257–264}$ or $BINS_{12–21}$ in CFA on day 0 and boosted with 50 μg of corresponding peptides in IFA on day 12. Splenocytes were harvested on day 19 and cultured with irradiated, peptide-pulsed RMA-S cells. Specific lytic activity of OVA-CTL (*A*) and INS-CTL (*B*) were examined after the third week of culture. Cytokine production for the CTL was determined after activation with the corresponding peptide-pulsed APC (*C* and *D*).

IL-10, and IFN-γ. Support for this idea is provided by the observations showing that Th2 cells are resistant to the effects of IL-12 because they had down-modulated their IL-12 receptors (42, 43).

Both $CD8^+$ Tc1 and Tc0 subsets in our studies expressed similar surface Ags except CT-1, a carbohydrate determinant of CD45 (44) and Fas. CT-1 was expressed on OVA-CTL but not INS-CTL, whereas INS-CTL but not OVA-CTL expressed Fas constitutively. However, it remains to be seen whether these are random differences or functionally related to the CTL phenotype.

In this report, the factors that might regulate $CD8^+$ T cell differentiation were evaluated. $CD4^+$ Th cells are required for the activation of naive $CD8^+$ T cells through IL-2 production and activation of professional APC by up-regulating costimulatory molecules (31–33). However, in our experiments, phagocytic cells but not $CD4^+$ Th cells proved to be required for the priming of both OVA-CTL (14) and INS-CTL in vivo. $CD4^+$ cells were neither required for activation of $CD8^+$ T cells nor did they regulate the cytokines produced by the CTL. The lack of requirement for $CD4^+$ T cell is probably related to the use of CFA as an adjuvant. Mycobacteria are known to activate phagocytic cells resulting in the up-regulation of costimulatory molecules (e.g., B7 and CD40) and cytokine production (45, 46). Consequently, the activated phagocytic cells can act as APC for presentation and activation of naive CTL. Neither Ab nor CD4 responses were attenuated by the absence of phagocytic cells, possibly because other professional APC such as mature dendritic cells, which lack phagocytic activity, can activate $CD4^+$ T cells (47–49).

Because IL-4 has been shown to induce type 2 CTL in vitro (35), its role in priming Tc0 CTL was examined by immunizing B6 IL-$4^{-/-}$ mice with BINS. The resultant INS-CTL produced IFN-γ, IL-5, and IL-10, which is a similar pattern as the cytokines produced by CTL generated from normal B6 mice. However, INS-CTL from the IL-$4^{-/-}$ mice produced lower levels of IL-5 and IL-10 and did not grow as well as the normal B6 CTL, suggesting that endogenous IL-4 was not necessary for the priming of Tc0 cells but that it may be important for sustained function in vitro. Our results seem to contradict the in vitro data showing that IL-4, in the presence of anti-IFN-γ induces Tc2 differentiation. However, it is possible that endogenous cytokines (such as IL-13), which share many immune functions with IL-4 (50), might substitute for IL-4 and drive differentiation of Tc0 cells in a IL-4-deficient background (51).

Extensive studies of $CD4^+$ T cells demonstrate that Ag dose has a significant impact on the outcome of T cell phenotypes (52–54). To test whether the same was true for $CD8^+$ T cells, B6 mice were primed with different concentrations of OVA and BINS in CFA. OVA induced CTL that produced IFN-γ but no IL-5 and IL-10, whereas BINS induced CTL that secreted IFN-γ, IL-5, and IL-10 at all doses that were immunogenic (data not shown). Failure of different priming doses to alter the $CD8^+$ T cell phenotype suggests that Ag dose may not have as profound an impact on the phenotype of $CD8^+$ cells as it does on $CD4^+$ cells. Alternatively, other factors such as the Ag itself might play a pivotal role in regulating the phenotypes of the CTL.

Because OVA-CTL and INS-CTL were primed with different Ags but under identical conditions, their phenotypes (Tc1 vs Tc0) may have been influenced by the intrinsic properties of the priming Ags themselves. This hypothesis was confirmed when mixed Ags primed CTL that maintained the same phenotypes as induced by separate Ags. This mixing experiment also indicates that the Tc0 phenotype of the insulin-specific CTL persisted even under priming conditions in which the Tc1 cytokines of the OVA-specific CTL might be present. When OVA or BINS peptides were used for immunization, the resultant OVA-CTL and INS-CTL again maintained Tc1 and Tc0 phenotype, respectively, suggesting that CTL phenotypes may be determined by priming Ags at the peptide level. OVA and BINS peptides may have different binding affinities to MHC or TCR, either one of which could regulate the phenotype.

Studies in $CD4^+$ T cells have demonstrated that peptides with substituted MHC anchor residues are capable of switching the phenotypes of $CD4^+$ T cells as a result of different signals being transduced through the TCR (55, 56). Here, we provide the first evidence that antigenic peptides are also pivotal in determining the cytokine phenotypes of $CD8^+$ T cells. Experiments to examine the effect of altered peptide ligands on the phenotypes of OVA-CTL and INS-CTL are currently under investigation.

Collectively, our results demonstrate that OVA-CTL (Tc1) and INS-CTL (Tc0) primed by exogenous Ags in CFA differed in their cell-surface and functional phenotypes. However, the functional differences of CTL subsets in diseases are not yet well understood. The ability of CTL to cause inflammation or protection against infection may or may not correlate with their phenotype (12, 13). Thus, systematic comparison of Tc1 vs Tc2 cells in terms of their function and requirements for generation may in the future allow for better understanding the role they play in the immune response. Consequently, this knowledge may also assist in designing vaccines with the capacity to prime CTL that produce protective cytokine profiles.

Acknowledgments

We thank Dr. Michael J. Bevan for providing E.G7-OVA cell line and Dr. K. Karre for providing RMA-S cell line. We also thank Dr. Maurice Gately for supplying human recombinant IL-2 and Dr. Brian Evavold and Emory University Microchemical facility for providing insulin peptides

Downloaded from http://www.jimmunol.org/ by guest on March 7, 2022

and OVA peptides. We are grateful to Linda M. Kapp for her excellent technical assistance.

References

1. Croft, M., L. Carter, S. L. Swain, and R. W. Dutton. 1994. Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J. Exp. Med. 180:1715.*
2. Sad, S., R. Marcotte, and T. R. Mosmann. 1995. Cytokine-induced differentiation of precursor mouse $CD8^+$ T cells into cytotoxic $CD8^+$ T cells secreting Th1 or Th2 cytokines. *Immunity 2:271.*
3. Cerwenka, A., L. L. Carter, J. B. Reome, S. L. Swain, and R. W. Dutton. 1998. In vivo persistence of CD8 polarized T cell subsets producing type 1 or type 2 cytokines. *J. Immunol. 161:97.*
4. Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science 254:279.*
5. Maggi, E., M. G. Giudizi, R. Biaguittu, F. Annunsiato, R. Manetti, M. Piccinni, and S. Romagani. 1995. Th-2 like $CD8^+$ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type I infection. *J. Exp. Med. 180:489.*
6. Taguchi, T., J. R. McGhee, R. L. Coffman, K. W. Beagley, J. H. Eldridge, K. Takatsu, and H. Kiyono. 1990. Analysis of Th1 and Th2 cells in murine gut-associated tissues: frequencies of $CD4^+$ and $CD8^+$ T cells that secrete IFN-γ and IL-5. *J. Immunol. 145:68.*
7. Coyle, A. J., F. Erard, C. Bertrand, S. Walti, H. Pircher, and G. Le Gros. 1995. Virus-specific $CD8^+$ cells can switch to interleukin 5 production and induce airway eosinophilia. *J. Exp. Med. 181:1229.*
8. Sarawar, S. R., and P. C. Doherty. 1994. Concurrent production of interleukin-2, interleukin-10, and γ interferon in the regional lymph nodes of mice with influenza pneumonia. *J. Virol. 68:3112.*
9. Baumgarth, N., L. Brown, D. Jackson, and A. Kelso. 1994. Novel features of the respiratory tract T-cell response to influenza virus infection: lung T cells increase expression of γ interferon mRNA in vivo and maintain high levels of mRNA expression for interleukin-5 (IL-5) and IL-10. *J. Virol. 68:7575.*
10. Falchetti, R., G. Lanzilli, I. A. Casalinuovo, R. Gaziano, A. T. Palamara, P. Di Francesco, G. Ravagnan, and E. Garaci. 1996. Splenic $CD4^+$ and $CD8^+$ T cells from influenza immune mice concurrently produce in vitro IL2, IL4, and IFN-γ. *Cell. Immunol. 170:222.*
11. Carter, L. L., and R. W. Dutton. 1995. Relative perforin- and Fas-mediated lysis in T1 and T2 CD8 effector populations. *J. Immunol. 155:1028.*
12. Li, L., S. Sad, D. Kagi, and T. R. Mosmann. 1997. CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reaction. *J. Immunol. 158:4152.*
13. Cerwenka, A., T. M. Morgan, A. G. Harmsen, and R. W. Dutton. 1999. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. *J. Exp. Med. 189:423.*
14. Ke, Y., Y. Li, and J. A. Kapp. 1995. Ovalbumin injected with complete Freund's adjuvant stimulates cytolytic responses. *Eur. J. Immunol. 25:549.*
15. Ma, H., Y. Ke, Q. Li, and J. A. Kapp. 2000. Bovine and human insulin activate $CD8^+$-autoreactive CTL expressing both type 1 and type 2 cytokines in C57BL/6 mice. *J. Immunol. 164:86.*
16. Noben-Trauth, N., P. Kropf, and I. Muller. 1996. Susceptibility to *Leishmania major* infection in interleukin-4-deficient mice. *Science 271:987.*
17. Moore, M. W., F. R. Carbone, and M. J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell 54:777.*
18. Ke, Y., and J. A. Kapp. 1996. Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells by receptor-mediated uptake. *J. Exp. Med. 184:1179.*
19. Kärre, K., H. G. Ljunggren, G. Pionteck, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defense strategy. *Nature 319:675.*
20. Dialynas, D. P., Z. S. Quan, K. A. Wall, A. Pierres, J. Quintans, M. R. Loken, M. Pierres, and F. W. Fitch. 1983. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J. Immunol. 131:2445.*
21. Ledbetter, J. A., R. V. Rouse, H. S. Micklem, and L. A. Herzenberg. 1980. T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens: two-parameter immunofluorescence and cytotoxity analysis with monoclonal antibodies modifies current views. *J. Exp. Med. 152:280.*
22. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for murine T3 polypeptides. *Proc. Natl. Acad. Sci. USA 84:1374.*
23. Kubo, R. T., W. Born, J. W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine $\alpha\beta$ T cell receptors. *J. Immunol. 142:2736.*
24. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity 1:405.*
25. Lefrancois, L., and M. J. Bevan. 1985. Functional modifications of cytotoxic T-lymphocyte T200 glycoprotein recognized by monoclonal antibodies. *Nature 314:449.*
26. Ogasawara, J., R. Watanabe-Fukunaga, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itaoh, T. Suda, and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature 364:806.*
27. Dowling, P., G. F. Shang, S. Raval, J. Menonna, S. Cook, and W. Husar. 1996. Involvement of the CD95 (APO-1/Fas) receptor/ligand system in multiple sclerosis brain. *J. Exp. Med. 184:1513.*
28. Kruisbeek, A. M. 2000. In vivo depletion of CD4- and CD8-specific T cells. In *Current Protocols in Immunology*. J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds. John Wiley and Sons, New York.
29. Debrick, J. E., P. A. Campbell, and U. D. Staerz. 1991. Macrophages as accessory cells for class I MHC-restricted immune responses. *J. Immunol. 147:2846.*
30. Ke, Y., Y. Li, and J. A. Kapp. 1995. Ovalbumin injected with complete Freund's adjuvant stimulates cytolytic responses. *Eur. J. Immunol. 25:549.*
31. Keene, J. A., and J. Forman. 1982. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *J. Exp. Med. 155:768.*
32. Ridge, J. P., F. D. Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. *Nature 393:474.*
33. Bennett, S. R. M., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. A. P. Miller, and W. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478.*
34. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol. 136:2348.*
35. Mosmann, T. R., L. Li, and S. Sad. 1997. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Semin. Immunol. 9:87.*
36. Kopf, M., G. Le Gros, M. Bachmann, M. C. Lamers, H. Bluethmann, and G. Kohler. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature 362:245.*
37. Kamogawa, Y., L. A. Minasi, S. R. Carding, K. Bottomly, and R. A. Flavell. 1993. The relationship of IL-4- and IFN-γ-producing T cells studied by lineage ablation of IL-4-producing cells. *Cell 75:985.*
38. Firestein, G. S., W. D. Roeder, J. A. Laxer, K. S. Townsend, C. T. Weaver, J. T. Hom, J. Linton, B. E. Torbett, and A. L. Glasebrook. 1989. A new murine $CD4^+$ T cell subset with an unrestricted cytokine profile. *J. Immunol. 143:518.*
39. Miner, K. T., and M. Croft. 1998. Generation, persistence, and modulation of Th0 effector cells: role of autocrine IL-4 and IFN-γ. *J. Immunol. 160:5280.*
40. Paliard, X., M. de Waal, H. Yssel, D. Blanchard, I. Chretien, J. Abrams, J. de Vries, and H. Spits. 1988. Simultaneous production of IL-2, IL-4, and IFN-γ by activated human $CD4^+$ and $CD8^+$ T cell clones. *J. Immunol. 141:849.*
41. Bucy, R. P., A. Panoskaltsis-Mortari, G. Huang, J. Li, L. Karr, M. Ross, J. H. Russell, K. M. Murphey, and C. T. Weaver. 1994. Heterogeneity of single cell cytokine gene expression in clonal T cell populations. *J. Exp. Med. 180:1251.*
42. Rogge, L., L. Barberis-Maino, M. Biffi, N. Passini, D. H. Presky, U. Gubler, and F. Sinigaglia. 1997. Selective expression of an interlukin-12 receptor component by human T helper cells. *J. Exp. Med. 185:825.*
43. Szabo, S. J., A. S. Dighe, U. Gubler, and K. M. Murphy. 1997. Regulation of the interleukin (IL)-12R β2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J. Exp. Med. 185:817.*
44. Lefrancois, L., L. Puddington, L. Machamer, and M. J. Bevan. 1985. Acquisition of cytotoxic T lymphocyte-specific carbohydrate differentiation antigens. *J. Exp. Med. 162:1275.*
45. Fearon, D. T., and R. M. Locksley. 1996. The instructive role of innate immunity in the acquired immune response. *Science 272:50.*
46. Medzhitov, R., and C. A. Janeway, Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell 91:295.*
47. Steinman, R. M., and J. Swanson. 1995. The endocytic activity of dendritic cells. *J. Exp. Med. 182:283.*
48. Witmer-Pack, M. D., M. T. Crowley, K. Inaba, and R. M. Steinman. 1993. Macrophages, but not dendritic cells, accumulate colloidal carbon following administration in situ. *J. Cell Sci. 105:965.*
49. Mitchell, D. A., S. K. Nair, and E. Gilboa. 1998. Dendritic cell/macrophage precursors capture exogenous antigen for MHC class I presentation by dendritic cells. *Eur. J. Immunol. 28:1923.*
50. Chomarat, P., and J. Banchereau. 1998. Interleukin-4 and interleukin-13: their similarities and discrepancies. *Int. Rev. Immunol. 17:1.*
51. McKenzie, G. J., C. L. Emson, S. E. Bell, S. Anderson, P. Fallon, G. Zurawski, R. Murray, R. Grencis, and A. N. McKenzie. 1998. Impaired development of Th2 cells in IL-13-deficient mice. *Immunity 9:423.*
52. Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, and K. Bottomly. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med. 182:1591.*
53. Hosken, N. A., K. Shibuya, A. W. Heath, K. M. Murphay, and A. O'Garra. 1995. The effect of antigen dose on $CD4^+$ T helper cell phenotype development in a T cell receptor transgenic model. *J. Exp. Med. 182:1579.*
54. Secrist, H., R. H. DeKruyff, and D. T. Umetsu. 1995. Interleukin 4 production by $CD4^+$ T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. *J. Exp. Med. 181:1081.*
55. Chaturvedi, P., Q. Yu, S. Southwood, A. Sette, and B. Singh. 1996. Peptide analogs with different affinites for MHC alter the cytokine profile of T helper cells. *Int. Immunol. 8:745.*
56. Kumar, V., V. Bhardwaj, L. Soares, J. Alexander, A. Sette, and E. Sercarz. 1995. Major histocompatibility complex binding affinity of an antigenic determinant is crucial for the differential secretion of interleukin 4/5 or interferon γ by T cells. *Proc. Natl. Acad. Sci. USA 92:9510.*

Downloaded from http://www.jimmunol.org/ by guest on March 7, 2022