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*J Immunol* 2006; 176:5006-5014; doi: 10.4049/jimmunol.176.8.5006

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In Vitro Activation of CD8 Interphotoreceptor Retinoid-Binding Protein-Specific T Cells Requires not only Antigenic Stimulation but also Exogenous Growth Factors

Yong Peng,* Hui Shao,* Yan Ke,* Ping Zhang,* Jim Xiang,† Henry J. Kaplan,* and Deming Sun2*

In a previous study, we demonstrated that immunization with the uveitogenic peptide interphotoreceptor retinoid-binding protein (IRBP) 1–20 induces both CD4 and CD8 uveitogenic T cells in the B6 mouse. In the current study, we determined the role of the CD8 IRBP-specific T cells in the pathogenesis of experimental autoimmune uveitis. We also determined the conditions that facilitated the activation of CD8 autoreactive T cells. Our results showed that the β2-microglobulin−/− mouse had a greatly decreased susceptibility to induction of experimental autoimmune uveitis by adoptive transfer of IRBP-specific T cells from B6 mice. We also showed that unlike CD4 autoreactive T cells, activated CD8 autoreactive T cells produced only a limited number and amounts of growth factors. As a result, in the absence of exogenously supplied growth factor(s), CD8 T cell activation and expansion were aborted. However, the growth and expansion of triggered CD8 autoreactive T cells could be supported by various cytokines. In addition to factors produced by activated CD4 autoreactive T cells, factors produced by nonlymphoid cells, such as IL-7 and IL-15, and unidentified factors in the culture supernatants of astrocytes and retinal pigment epithelial cells support the CD8 autoreactive T cells as well. Finally, we showed that, although several cytokines augmented the CD8 T cell response in vitro, different cytokines appeared to act on different CD8 subsets or on different activation/differentiation phases of CD8 autoreactive T cells. As a result, cytokines, such as IL-7, supported the proliferation and survival of CD8 IRBP-specific T cells, while others had only a growth-promoting effect. The Journal of Immunology, 2006, 176: 5006–5014.

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1 Abbreviations used in this paper: EAU, experimental autoimmune uveitis; β2m, β2-microglobulin; EAE, experimental autoimmune encephalomyelitis; IRBP, interphotoreceptor retinoid-binding protein; MOG, myelin oligodendrocyte glycoprotein; p.I., postimmunization; RPE, retinal pigment epithelial.

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studied extensively, there is little data regarding similar cellular events in the autoimmune response. To determine whether increased activation of CD8 autoreactive T cells contributes to disease progression, we have determined the conditions that favor the recruitment and activation of CD8 autoreactive IRBP-specific T cells and determined the differences between the activation requirements of CD4 and CD8 autoreactive T cells. We also investigated the pathogenic activity of CD4 IRBP-specific T cells in the absence of CD8 IRBP-specific T cells, compared the in vitro activation response of highly purified CD4 and CD8 autoreactive T cells, and determined the survival of CD4 and CD8 autoreactive T cells in vitro and in vivo. We found that exposure to a combination of a high dose of autoantigen and an optimal number of APCs resulted in only partial activation of purified in vivo primed CD8 IRBP-specific T cells, whereas their CD4 counterparts were optimally activated under the same conditions. Unlike CD4 autoreactive T cells, activated CD8 autoreactive T cells produced only a limited number and amounts of growth factors. CD8 T cell activation depended both on antigenic stimulation and on the availability of various growth factors, which can be produced by either activated lymphocytes or nonlymphocytes. Following injection into a naive mouse, CD8 T cells persisted in vivo for at least 3 mo. A possible mechanism by which CD8 autoreactive T cells only become apparent during the late phase of chronic disease is discussed. The results of such studies should determine whether both autoreactive CD4 and CD8 T cells, rather than CD4 T cells alone, should be targeted for the treatment of chronic and progressive disease.

Materials and Methods

Animals and reagents

Pathogenic-free female C57BL/6 and B6-β2m<sup>−/−</sup> mice (8–10 wk old) were purchased from The Jackson Laboratory, and were housed and maintained in the animal facilities of the University of Louisville. Institutional approval was obtained, and institutional guidelines regarding animal experimentation were followed. The recombinant cytokines IL-2, IL-4, IL-6, IL-7, IL-12, IL-15, and IFN-αβ were purchased from R&D Systems. They were tested at the final concentration of 10 ng/ml. All Abs were purchased from BD Biosciences. Anti-IL-7 or anti-IL-15 Abs were purchased from Miltenyi Biotec. The lymph node and spleen cells were first incubated for 10 min at 4°C with a mixture of biotin-conjugated Abs against CD8 (CD8α, Ly-2) or CD4 (CD4, L3T4) T cells. B cells (CD19, B220), NK cells (CD49b, DX5), hematopoietic cells (CD11b, Mac-1), and erythrocytes (Ter<sup>119</sup>) were then washed with an anti-MAcs separator column (Miltenyi Biotec), and washed with 15 ml of medium, according to the manufacturer’s protocol. The flow-through fraction containing CD4- or CD8-enriched cells was collected. The purity of the isolated cell fraction was determined by flow cytometric analysis using FITC-conjugated anti-TCR Abs and PE-conjugated Abs directed against CD4 or CD8 (BD Biosciences). Data collection and analysis were performed on FACSCalibur flow cytometer using CellQuest software.

Preparation of IRBP1–20-specific T cells

Brieﬂy, to prepare T cells, donor mice were immunized s.c. with 200 μl of an emulsion containing 200 μg of IRBP1–20 (aa 1–20 of IRBP) (Sigma-Aldrich) and 500 μg of Mycobacterium tuberculosis H37Ra (Difco) in IFA (Sigma-Aldrich), distributed over six spots at the tail base and on the flank, and T cells were isolated at 13 days postimmunization (p.i.) from lymph node cells or spleen cells by passage through a nylon wool column; then the lymph node cells or spleen cells were stimulated with 20 μg/ml IRBP1–20 in the presence of 1 × 10<sup>6</sup> irradiated syngeneic spleen cells as APCs. After 2 days, the activated lymphoblasts were isolated by gradient centrifugation on Lymphoprep (Robbins Scientific) and cultured in RPMI 1640 medium supplemented with 15% IL-2-containing medium (supernatant from Con A-stimulated rat spleen cells).

Adoptive transfer of EAU

Uveitis was induced in naive B6 or B6-β2m<sup>−/−</sup> mice by adoptive transfer of 5 × 10<sup>5</sup> IRBP1–20-specific T cells, as described previously (30–32). The severity of EAU was examined three times per week for clinical signs of uveitis by fundoscopy, starting at week 2 posttransfer. Fundoscopy evaluation for longitudinal follow-up of disease was performed using a binocular microscope after pupil dilation using 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions. The incidence and severity of EAU were graded on a scale of 0–4 in half-point increments using previously described criteria (33), which are based on the type, number, and size of lesions present.

Proliferation assay

T cells from IRBP1–20-immunized wild-type B6 or B6-β2m<sup>−/−</sup> mice were prepared and seeded at 4 × 10<sup>5</sup> cells/well in 96-well plates, then cultured at 37°C for 48 h in a total volume of 200 μl of medium with or without IRBP1–20 in the presence of irradiated syngeneic spleen APCs (1 × 10<sup>6</sup>), and [3H]thymidine incorporation during the last 8 h was assessed using a microplate scintillation counter (Packard Instrument). The proliferative response was expressed as the mean cpm ± SD of triplicate determinations.

Purification of CD4 and CD8 T cells using autoMACS columns

Purified CD4 and CD8 T cells were prepared from the draining lymph nodes and spleen using a CD4 and CD8 isolation kit (Miltenyi Biotec). The lymph node and spleen cells were first incubated for 10 min at 4°C with a mixture of biotin-conjugated Abs against CD8 (CD8α, Ly-2) or CD4 (CD4, L3T4) T cells. B cells (CD19, B220), NK cells (CD49b, DX5), hematopoietic cells (CD11b, Mac-1), and erythrocytes (Ter<sup>119</sup>) were then washed with 15 ml of medium, according to the manufacturer’s protocol. The flow-through fraction containing CD4- or CD8-enriched cells was collected. The purity of the isolated cell fraction was determined by flow cytometric analysis using FITC-conjugated anti-TCR Abs and PE-conjugated Abs directed against CD8 or CD4 (BD Biosciences). Data collection and analysis were performed on FACSCalibur flow cytometer using CellQuest software.

CFSE staining

T cells from the draining lymph nodes and spleen from immunized mice were prepared by passage through a nylon wool column and stained with the vital dye, CFSE (Molecular Probes), as described previously (34). Brieﬂy, the cells were washed and resuspended at 50 × 10<sup>6</sup> cells/ml in serum-free RPMI 1640 medium, then incubated at 37°C for 10 min with gentle shaking with a final concentration of 10 μM CFSE, washed twice with, and resuspended in, RPMI 1640 medium containing 10% FCS, stimulated with IRBP1–20 and irradiated APCs, and analyzed by flow cytometry.

Immunofluorescence flow cytometry

Aliquots of 2 × 10<sup>5</sup> cells were double stained with combinations of FITC- or PE-conjugated mAbs against mouse αβTCR (H57-597), CD4, or CD8. Data collection and analysis were performed on a FACSCalibur flow cytometer using CellQuest software.

Flow cytometric detection of T cells binding IRBP1–20 complexed with rMHC class I (H-2K<sup>b</sup>) dimers

The MHC class I (H-2K<sup>b</sup>) molecule used was a fusion protein consisting of mouse H-2K<sup>b</sup> and mouse IgG1 obtained from BD Pharmingen (18). To produce the dimeric form, it was incubated at 4°C for 12–24 h with human β2m (BD Biosciences) (both at a final concentration of 0.15 mg/ml) and an excess of the test peptide (1 mg/ml). Double staining was performed by incubating 5 × 10<sup>5</sup> cells at 4°C for 30 min with 0.5 μg of peptide-dimer complex in a volume of 0.5 ml. The cells were then washed twice in PBS containing 1% BSA and 0.1% sodium azide, and stained with a PE-labeled anti-mouse IgG1 Ab, followed by a FITC-conjugated Ab against either CD4 or CD8 (BD Biosciences). The results are presented as PE staining vs FITC staining.

Pathological examination

Inflammation of the eye was confirmed by histopathology. Whole eyes were collected, immersed for 1 h in 4% phosphate-buffered glutaraldehyde, and transferred to 10% phosphate-buffered formaldehyde until processed. The fixed and dehydrated tissue was embedded in methacrylate, and 5-μm sections were cut through the pupillary-optic nerve plane and stained with H&E. Presence or absence of disease was evaluated blind by examining six sections cut at different levels for each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, as described previously (32).

ELISA

IL-2, IL-4, IL-7, IL-15, and IFN-γ were measured by commercially available ELISA kits (R&D Systems).

Astrocytes and retinal pigment epithelial (RPE) cells

Astrocytes and microglia were prepared from the mouse brains, as previously described (35–37). To isolate RPE cells, the eyes were enucleated from 6- to 8-wk-old B6 mice and incubated with Cu/Mg-free PBS for 3 h at 4°C. Under a dissection microscope, the anterior segment, vitreous, and neural retina were discarded. The rest of postsegment of eye was incubated at 37°C for 48 h in a total volume of 200 μl of medium with or without IRBP1–20 in the presence of irradiated syngeneic spleen APCs (1 × 10<sup>6</sup>), and [3H]thymidine incorporation during the last 8 h was assessed using a microplate scintillation counter (Packard Instrument). The proliferative response was expressed as the mean cpm ± SD of triplicate determinations.
with 0.25% trypsin/1 mM EDTA at 37°C for 30 min, and then the RPE cell layer was carefully removed from choroids, followed by trituration through 21- and 23-gauge needles. Single RPE cells were washed twice with RPMI 1640 complete medium and then cultured in cell culture dishes at 37°C in 5% CO₂ atmosphere. After two to three passages, cells were used for experiments. Before use, >95% of the cells in the culture stained positive with FITC-labeled anti-pan keratin Ab (clone PCK-26; Sigma-Aldrich), indicating that they were virtually all pigment epithelial cells.

Statistical analysis

The data are expressed as the mean ± SD. Each experiment was repeated at least three times.

Results

Adoptive transfer of IRBP-specific T cells from IRBP1–20-immunized B6-β₂m⁻/⁻ mice

To determine the role of CD8 IRBP-specific T cells in the pathogenesis of EAU, attempts were made to induce disease in wild-type B6 and B6-β₂m⁻/⁻ mice by reciprocal transfer of T cells from B6 to B6-β₂m⁻/⁻ mice and vice versa, and measurement of clinical signs of inflammation was assessed by fundoscopy and histopathology. Groups (n = 10) of B6 and B6-β₂m⁻/⁻ mice were immunized with IRBP1–20 emulsified in CFA, and then enriched IRBP1–20-specific T cells were prepared and stimulated in vitro using syngeneic APCs, as described in Materials and Methods; then 5 × 10⁶ activated T cell blasts were injected into each recipient mouse.

As shown in Fig. 1A, transfer of T cells from immunized B6 mice to naive B6 mice induced severe uveitis in recipient mice. On average, disease was first seen 10 days after injection, reached a peak at 15–20 days, and persisted for ~30–60 days (Fig. 1A). Pathologic examination as shown in Fig. 1, B–D, demonstrated that ocular inflammation started at day 10 postinjection and persisted until 45 days postinjection. In addition, the photoreceptor layer of the eye became thin examined at 45 days postinjection. The same number of B6 T cells induced mild and monophasic uveitis in B6-β₂m⁻/⁻ mice (Fig. 1F) that do not express an intact MHC class I molecule (38, 39), which prevents the reactivation of CD8 effector T cells. Moreover, the disease induced in the B6-β₂m⁻/⁻ mouse had a delayed onset and lasted only a few days before complete recovery (Fig. 1, E–G). In addition, IRBP-specific T cells prepared from B6-β₂m⁻/⁻ mice induced only acute or monophasic disease in either B6-β₂m⁻/⁻ or B6 recipients (data not shown).

![FIGURE 1. Unfractionated CD4 and CD8 IRBP1–20-specific T cells derived from wild-type B6 mice are less pathogenic in the B6-β₂m⁻/⁻ mouse. Nylon wool-enriched T cells, prepared from draining lymph nodes and spleens of IRBP1–20-immunized B6 mice at 13 days p.i., were stimulated in vitro for 48 h with IRBP1–20 (20 μg/ml) and APCs (irradiated syngeneic spleen cells); then the activated T cell blasts were separated by Ficoll gradient centrifugation and 5 × 10⁶ blasts were injected into each recipient mouse. A, Fundoscopy showing that B6-β₂m⁻/⁻ recipients developed acute, mild disease with late onset and early termination. Pathology examination showed that 10 days after adoptive transfer of syngeneic IRBP1–20-specific T cells, massive inflammation can be identified in the inflamed eyes of B6 recipients (B), but not β₂m⁻/⁻ recipients (E). The inflammation persisted in B6 recipients (C and D), whereas β₂m⁻/⁻ recipients showed a delayed inflammation (F) and recovered thereafter (G).](http://www.jimmunol.org/)

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To test this, enriched IRBP-reactive T cells from the spleens and draining lymph nodes of immunized B6 mice were stimulated for 2 days with APCs derived from either B6 or B6-β2m−/− mice; then 5 × 10^6 activated T cell blasts were injected into naive B6 recipients. The T cells stimulated by APCs from B6-β2m−/− mice induced much milder disease than the same T cells stimulated with APCs derived from B6 mice (data not shown).

**Modest activation and aborted expansion of CD8 IRBP-specific T cells in the absence of exogenously supplied growth factors**

To determine whether the activation of CD8 IRBP-specific T cells relies on help from CD4 IRBP-specific T cells, we purified CD4 and CD8 T cells from IRBP1–20-immunized B6 mice 13 days p.i. using MACS magnetic columns (Fig. 2A), and tested their proliferative response to IRBP1–20 in the presence of syngeneic APCs. As shown in Fig. 2B, the CD4 T cells mounted a strong proliferative response, whereas the response of the CD8 T cells was modest, but significant (stimulatory index ~10). The average intensity of the CD8 T cell response was only 20% that of the CD4 T cells when an optimal dose of Ag (20 µg/ml) and an appropriate number of APCs (1 × 10^5/well) were used. Importantly, an increase in Ag dose did not enhance the response (Fig. 2B), excluding the possibility that CD8 cells require a higher dose of Ag. These results show that activation of CD8 IRBP-specific T cells did not always depend on help from CD4 T cells or other cells, but, as shown below, CD8 T cell activation was only partial in the absence of exogenous cytokines, which indicates high degree activation of CD8 cells requires help from CD4 or other cells.

We then examined whether specific growth factors enhanced the Ag-dependent CD8 T cell response. First, we tested the effect of IL-12 or IFN-α/β, which have been reported to increase CD8 T cell responses against tumor or viral Ags (28, 29, 40, 41). As shown in Fig. 2C, the enhancing effect of IL-12 on the CD8 IRBP-specific T cell response was minimal and IFN-α/β had no significant effect. Interestingly, screening of a panel of common cytokines (10–20 ng/ml) showed that IL-2, IL-4, IL-7, and IL-15 significantly enhanced the Ag-dependent response of the CD8 IRBP1–20-specific T cells, in sharp contrast to the CD4 cell response the same cytokines had no significant effect.

To determine whether the distinctive cytokine dependency of the activation of CD4 and CD8 autoreactive T cells was caused by their intrinsic cytokine-producing ability, we used ELISA to measure cytokine levels in the culture medium of activated CD4 and CD8 T cells at 24–48 h poststimulation in vitro. As shown in Fig. 2D, the CD4 T cell supernatants contained much higher levels of IL-2, IL-4, and IFN-γ than the CD8 T cell supernatants. Neither cell type produced IL-7 or IL-15.

**CD8 IRBP-specific T cells can be activated by nonlymphoid cell-produced growth factors**

Given that the primary source of IL-7 and IL-15 is not the T cells (42–45), we wished to determine whether factors produced by nonlymphoid cells, specially in the target organs, also supported CD8 growth. Purified CD8 T cells from in vivo primed B6 mice were tested for proliferation using an optimal combination of IRBP1–20 and APCs in the presence or absence of culture supernatants collected from astrocytes, microglia, RPEs, or bone marrow-derived
dendritic cells. Interestingly, as shown in Fig. 3A, culture supernatants from astrocytes and RPEs significantly increased proliferation, those from dendritic cells had a modest effect, whereas microglia supernatants had no effect. When we attempted to block the stimulatory activity of astrocyte culture supernatants by preincubating them for 20 h with Abs against IL-7 and/or IL-15, little loss of activity was seen (Fig. 3B).

Synergistic effect of several cytokines

We also tested whether different cytokines had a synergistic effect in promoting CD8 activation in vitro. As shown in Fig. 4A, at a dose of 10 ng/ml, IL-2, IL-7, and IL-15, each had a significant promoting effect on CD8 T cell activation when tested individually. However, when all three were added together, the effect was much greater. Moreover, only the CD8 cells from immunized mouse respond to the cytokines, but not the same CD8 cells from naive mouse (Fig. 4, A and B).

As shown in Fig. 4C, binding tests using IRBP1-20/recombinant H-2Kb molecule complexes showed that these three growth factors promoted the expansion of Ag-specific T cells. Moreover, both the number of total proliferating CD8 cells and Ag-specific CD8 T cells increased greatly under the effect of three cytokines in combination (Fig. 4D). In addition, as shown in Fig. 4E, when unfraccionated in vivo primed splenic T cells were stimulated with IRBP1–20 in vitro in the presence of the indicated cytokines, the addition of a combination of IL-2, IL-7, and IL-15 preferentially promoted the activation of CD8 T cells and, as a result, the CD8/CD4 ratio was significantly increased in the activated cells. In cultures incubated separately with IL-2, IL-7, or IL-15, CD8 T cells accounted for, respectively, 31, 33, or 26% of the total T cells, while those treated with all three contained 80% CD8 T cells.

Survival of CD8 autoreactive T cells

We also determined the ability of specific cytokines to support the survival of CD8 autoreactive T cells. In this study, in vivo primed CD8 autoreactive T cells isolated 10–13 days p.i. were cultured in 48-well plates (5 × 10^6/well) in the presence of 10 ng/ml IL-2, IL-4, IL-7, or IL-15 for up to 5 days, and the surviving cells at each time point were measured and the ratio of live and dead cells was assessed by FACS analysis. As shown in Fig. 5, in the absence of cytokine, most of the T cells died during the first 3–4 days of culture. Addition of IL-7 resulted in much better cell survival, IL-4 had an effect in the first 3 days, while the other cytokines had no effect. After 5 days in culture, ~40% of the CD8 T cells were still alive when cultured in IL-7-containing medium, whereas >95% of those cultured in IL-2, IL-15, or IFN-αβ were dead. Thus, the ability of a specific cytokine to promote T cell proliferation, as assessed by the 48-h thymidine incorporation assay, does not always parallel its ability to support T cell survival (Fig. 5 vs Fig. 4A).

CD8 IRBP1–20-specific T cells show increased survival in vivo compared with CD4 T cells

We then compared the in vivo survival of adoptively transferred CD4 and CD8 IRBP-specific T cells. In the first set of experiments, IRBP1–20-specific T cells were prepared from IRBP1–20-immunized B6 mice and stimulated for 2 days with immunizing peptide and APCs; then the activated T cell blasts were separated by Ficoll-gradient centrifugation and labeled with CFSE. Each recipient mouse was injected i.p. with 5 × 10^6 T cells (containing both CD4 and CD8 cells); then, at various days (7–30 days) postinjection, the recipient mice were sacrificed and nylon wool nonadherent cells were collected for FACS analysis to examine the CFSE-labeled cells. As shown in Fig. 6A, CFSE-labeled T cells were detected for at least 30 days postinjection in the spleen and lymph nodes, in which both CD4 and CD8 cells could be identified. To identify the IRBP-specific T cells in the total surviving cells, we performed an IRBP1–20-stimulated proliferation assay on the spleen cells at 30 days postinjection. As shown in Fig. 6B, a strong Ag-specific response was readily detected. Phenotypic analysis of the proliferating cells before and after in vitro stimulation showed that the dominant fraction of the cells expressed CD4 before in vitro stimulation, whereas CD8 cells overwhelmed the CD4 cell after antigenic stimulation in vitro (Fig. 6C).

Discussion

The goals of the current study were to determine the pathogenic interrelationship between CD4 and CD8 autoreactive T cells and to determine the conditions that favor the activation of CD8 autoreactive T cells, allowing these cells to actively participate in disease pathogenesis. Because the pathogenic activity of the autoreactive T cell is closely associated with its degree of activation, we focused our efforts on identifying factors that would favor activation and expansion of CD8 autoreactive T cells.

Previous studies have shown that viral-specific CD8 T cells rely on IFN-αβ for their activation and expansion (28, 29, 46), whereas

FIGURE 3. Astrocyte and RPE culture supernatants increase the proliferation of CD8 IRBP1-20-specific T cells. A, Supernatants of the confluent monolayers were collected for testing. In the thymidine incorporation assay, 4 × 10^5/well column-purified CD8 T cells from IRBP1–20-immunized B6 mice were incubated with immunizing peptide (20 μg/ml) and APCs in the absence or presence of 50 μl of the indicated cell supernatant in a total volume of 200 μl. B, Anti-IL-7 or anti-IL-15 Ab minimally blocks the stimulatory effect of astrocytes. A 96-well plate was coated overnight with anti-IL-7 and/or anti-IL-15 Abs at a concentration of 20 and 100 μg/ml, respectively; a control well was incubated with PBS only. Next day, the nonbound Abs were removed and 50 μl of astrocyte culture supernatant was added. After 10–20 h, the stimulatory effect of the treated culture supernatant was assessed, as described in A.
IL-12 is an important stimulatory factor for the growth of tumor-specific CD8 T cells (40, 41). Our present study showed that IL-12 had only a minimal effect on the activation of CD8 IRBP-specific T cells, while IFN-γ had no effect. More interestingly, when we compared the responses to various growth factors of encephalitogenic (MOG-specific) or uveitogenic (IRBP-specific) CD8 T cells, our results showed that IL-7 had a more marked promoting effect on IRBP-specific CD8 T cells, while IL-15 has a stronger effect on MOG-specific CD8 T cells (data not shown), suggesting that different growth factors may preferentially promote the growth of

FIGURE 4. Synergistic effect of multiple cytokines on the activation of CD8 IRBP1–20-specific T cells. A, Thymidine incorporation assay in which column-purified CD8 T cells from IRBP1–20-immunized B6 mice were incubated for 48 h in 96-well plates (4 × 10^5 cells/well) with immunizing peptide (20 μg/ml) and APCs in the absence or presence of the indicated cytokine (10 ng/ml), and [3H]thymidine incorporation during the last 8 h was assessed. The proliferative response is expressed as the mean cpm ± SD for triplicate wells. The result shown is representative of those obtained in more than five experiments. B, Thymidine incorporation assay tests the response of CD8 T cell from naïve B6 mice. C, Binding assay using IRBP1–20/H-2Kb complexes. Column-purified CD8 T cells prepared from IRBP1–20-immunized B6 mice (3 × 10^6/well) were stimulated in vitro for 48 h with 20 μg/ml IRBP1–20 and APCs in the presence of the indicated cytokines; then T cell blasts were separated by Ficoll gradient centrifugation and cultured with the indicated cytokine for 4 days. The cells were then incubated with IRBP1–20/H-2Kb complexes (y-axis) and FITC-labeled anti-mouse CD8 (x-axis). D, A total of 5 × 10^5 column-purified immune CD8 cells was cultured in 48-well plate in medium containing indicated cytokine(s) for 4 days. Total proliferating cells were counted by FACS, and the Ag-binding cells were calculated based on the percentage of Ag-binding cells assessed in C. E, A combination of cytokines preferentially promotes the activation of CD8 IRBP-specific T cells in unfractionated, in vivo primed IRBP-specific T cells. Unfractionated in vivo primed splenic T cells were stimulated in vitro for 3 days with IRBP1–20 and APCs in the presence of the indicated cytokines; then T cell blasts were separated by Ficoll gradient centrifugation, stained with Abs specific for mouse TCR (PE) and CD8 (FITC), and subjected to FACS analysis.
distinct CD8 subsets. In this study, we found that CD8 IRBP-specific T cells have a number of unique characteristics that are significantly different from those of their CD4 T cell counterparts, which probably explains why the CD4 T cell response is stronger than the CD8 T cell response during the acute phase of the autoimmune response, but CD8 T cells may easily become dominant in chronic disease. Based on our observations, we conclude that activation of CD8 autoreactive T cells relies not only on Ag and APCs, but also on the availability of numerous cytokines produced by immune or nonimmune cells.

Many studies have examined the role of the CD4 and CD8 T cell interaction, but the results have not been conclusive. Although some studies showed that CD4 T cells are mandatory for CD8 activation (47, 48), others did not support this conclusion (49). In this study, we showed that the distinction between the activation requirements of CD4 and CD8 IRBP-specific T cells was more easily demonstrated when highly purified, in vivo primed T cells were used as responder cells. It is likely that coactivated CD4 T cells produce various growth factors that are required for CD8 T cell activation, and growth factor-dependent CD8 T cell activation is therefore more readily detected when CD4 T cells are completely removed. This was supported by our screening tests determining the promoting effect of varying cytokines, which showed that addition of exogenous cytokines greatly promoted activation of CD8, but not CD4 or unfractionated, T cells.

FIGURE 5. IL-7 supports the survival of CD8 IRBP1–20-specific T cells in vitro. Column-purified CD8 T cells from IRBP1–20-immunized B6 mice (5 × 10^3/well) were incubated in the presence or absence of the indicated cytokine (10 ng/ml) for 1–5 days; at each time point, representative cultures from each group were subjected to FACS analysis, and the percentage of live cells was determined.

FIGURE 6. Persistence of adoptively transferred IRBP1–20-specific T cells in vivo. A, Unfractionated (CD4 and CD8) T cells prepared from IRBP1–20-immunized B6 mice were subjected to in vitro stimulation with immunizing peptide and APCs for 2 days. After separation by Ficoll gradient centrifugation, the activated T cells were labeled with 10 μM CFSE; then 5 × 10^6 labeled T cells were injected into each recipient mouse. After 30 days, the recipient mice were sacrificed, and splenic, thymic, and lymph node (LN) T cells were prepared by passage through nylon wool. For FACS analysis, the cells were stained with PE-labeled anti-mouse CD4 or CD8 Abs. B, The same splenic T cells were subjected to a proliferation test, in which 4 × 10^5 T cells/well were incubated with 10^5 APCs and IRBP1–20 (20 μg/ml). C, To determine the phenotype of the proliferating T cells in B, the T cells before and after the proliferation test were stained with Abs specific for mouse CD4 (PE labeled) and CD8 (FITC labeled) and analyzed by FACS.

Both the number of cells and their activation status are crucial for autoreactive T cells to have (50, 51) pathogenic activity. Indeed, in both EAE and EAU, disease is readily induced by injection of as few as 1 × 10^6 newly activated autoreactive T cells, but not by 100 times more nonactivated T cells (our unpublished observations). Because Ag-specific T cells can be activated to varying degrees and display some, but not all, of their activated functions (52, 53), we hypothesize that CD8 uveitogenic T cells acquire a stronger pathogenic activity only when they gain a high degree of activation.

The activation of CD4 autoreactive T cells is completely dependent on Ag and APC (54, 55), and we showed that addition of exogenous cytokine did not significantly augment the total response. In contrast, a high dose of autoantigen and optimal numbers of APCs only induced partial activation of CD8 T cells, and enhanced activation was seen when exogenous cytokines were provided. Cytokine assays showed that partially activated CD8 T cells produced very low amounts of growth-supporting cytokines, such as IL-2 and IL-4, indicating that, in the absence of exogenous cytokine, CD8 autoreactive T cells were not able to complete the entire process of activation and subsequent expansion. Conceivably, the fundamental difference in the activation requirements of CD4 and CD8 autoreactive T cells is their ability to produce cytokines. A related conclusion drawn from these observations is that minimal activation of CD8 autoreactive T cells does not always rely on CD4 T cell help, whereas a higher degree of CD8 T cell activation does.
We showed that a number of cytokines could increase the activation of primed, but not naive (data not shown), CD8 T cells. Interestingly, a higher degree of activation of CD8 autoreactive T cells could also be produced by non-T cell-derived cytokines, such as IL-7 and IL-15. For example, culture supernatants of astrocytes and RPE significantly promoted the activation of CD8 autoreactive T cells. Parenchymal cells in the autoimmune organs have been shown in several systems to actively participate in local regulation of the inflammatory process. Many types of parenchymal cells are reported to have immune regulatory potential; these include retinal glial Müller cells (56), keratinocytes (57), pancreatic B cells (58), and CNS astrocytes (36, 59). In this study, we showed that astrocytes and RPE cells were capable of promoting activation of CD8 autoreactive T cells. Such an observation might be related to the early finding that CD8 cells are more abundant in the CNS during multiple sclerosis (21). Conceivably, the factors produced by local cells contribute to the survival and expansion of the CD8 autoreactive T cells. The fact that Abs against IL-7 or IL-15 only marginally blocked the stimulatory potential of the cultured cell supernatants suggests that some unidentified cytokines produced by astrocytes and RPE may be responsible for promoting CD8 T cell activation.

An important point we would like to make is that, in the present study, we used a number of experimental methods to assess the activation of CD8 autoreactive T cells and found that different growth-promoting factors act differently in the activation and expansion of CD8 autoreactive T cells. For example, we assessed CD8 activation using thymidine incorporation and by monitoring the activation of T cells prelabelled with CFSE. In addition, we also identified Ag-specific T cells by the binding of oligomeric MHC molecules to distinguish autoreactive CD8 T cells from non-specific bystander CD8 T cells at 7–10 days after cell activation and expansion. The rationale for this experimental design is that thymidine incorporation assesses only T cell activation during the first 48 h after exposure to stimulus, whereas the CFSE assay optically measures the in vitro expansion of T cells at 4–6 days after Ag exposure. Furthermore, the best time for distinguishing activation/expansion of Ag-specific T cells from non-specific primed T cells is at 7–10 days postactivation. The use of a combination of these experimental methods should determine the short-term, as well as the long-term, effects of a specific growth factor. In addition, we also assessed the in vitro survival of in vivo primed CD8 autoreactive T cells in the presence of different growth factors and showed that the activation and expansion of CD8 autoreactive T cells were regulated by a number of factors derived from activated T cells or nonlymphoid cells (such as IL-7 and IL-15) and that, while some factors (such as IL-7) support the activation/expansion and survival of the cell, others only facilitate activation.

In summary, our study demonstrated that, unlike CD4 autoreactive T cells, the activation and expansion of which are totally dependent on the availability of Ag and APCs, full activation of CD8 autoreactive T cells is only achieved when various growth factors are available. It is therefore likely that CD8 autoreactive T cells are less easily activated during the early phase of disease, but become activated when large amounts of inflammatory cytokines become available. Once activated, they are better able to survival in vivo, possibly because their expansion and survival can be enhanced by several growth factors, rather than a single growth factor, produced by activated lymphoid or nonlymphoid cells. As a result, when the disease progresses to the chronic phase, the pathogenic effects of CD8 autoreactive T cells become more prominent.

Acknowledgments

The editorial assistance of Dr. Tom Barkas is greatly appreciated.

Disclosures

The authors have no financial conflict of interest.

References

ACTIVATION OF CD8 AUTOREACTIVE T CELLS

5022–5526


