Peripheral Tolerance and the Qualitative Characteristics of Autoreactive T Cell Clones in Primary Biliary Cirrhosis

Akira Kawano, Shinji Shimoda, Takashi Kamihira, Fumihiko Ishikawa, Hiroaki Niiro, Yuji Soejima, Akinobu Taketomi, Yoshihiko Maehara, Minoru Nakamura, Atsumasa Komori, Kiyoshi Migita, Hiromi Ishibashi, Miyuki Azuma, M. Eric Gershwin and Mine Harada

*J Immunol* 2007; 179:3315-3324; doi: 10.4049/jimmunol.179.5.3315

http://www.jimmunol.org/content/179/5/3315

---

### References

This article cites 39 articles, 16 of which you can access for free at: [http://www.jimmunol.org/content/179/5/3315.full#ref-list-1](http://www.jimmunol.org/content/179/5/3315.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

---

### Subscription

Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

### Permissions

Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](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](http://jimmunol.org/alerts)
Peripheral Tolerance and the Qualitative Characteristics of Autoreactive T Cell Clones in Primary Biliary Cirrhosis

Akira Kawano,* Shinji Shimoda, Takashi Kamihira, Fumihiko Ishikawa, Hiroaki Niiro, Yuji Soejima, Akinobu Taketomi, Yoshihiko Maehara, Minoru Nakamura, Atsumasu Komori, Kiyoshi Migita, Hiromi Ishibashi, Miyuki Azuma, M. Eric Gershwin, and Mine Harada*

Primary biliary cirrhosis is characterized by autoreactive T cells specific for the mitochondrial Ag PDC-E2163–176. We studied the ability of eight T cell clones (TCC) specific for PDC-E2163–176 to proliferate or become anergic in the presence of costimulation signals. TCC were stimulated with either human PDC-E2163–176, an Escherichia coli 2-oxoglutarate dehydrogenase mimic (OGDC-E234–47), or analogs with amino acid substitutions using HLA-matched allogeneic PBMC or mouse L-DR53 fibroblasts as APC. Based on their differential responses to these peptides (human PDC-E2163–176, E. coli OGDC-E234–47) in the different APC systems, TCC were classified as costimulation dependent or independent. Only costimulation-dependent TCC could become anergic. TCC with costimulation-dependent responses to OGDC-E2 become anergic to PDC-E2 when preincubated with mimic, even if costimulation is independent for PDC-E2163–176. Anergic TCC produced IL-10. One selected TCC could not become anergic after preincubation with PDC-E2163–176-pulsed L-DR53 but became anergic using L-DR53 pulsed with PDC-E2 peptide analogs with a substitution at a critical TCR binding site. TCC that only respond to peptide-pulsed PBMC, but not L-DR53, proliferate with peptide-pulsed CD80/CD86-transfected L-DR53; however, anergy was not induced with peptide-pulsed L-DR53 transfected with only CD80 or CD86. These data highlight that costimulation plays a dominant role in maintaining peripheral tolerance to PBC-specific Ags. They further suggest that, under specific circumstances, molecular mimicry of an autoantigen may restore rather than break peripheral tolerance. The Journal of Immunology, 2007, 179: 3315–3324.

*Department of Surgery and Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan; †Research Unit for Human Disease Model, RIKEN Center for Allergy and Immunology, Yokohama, Japan; ‡Center for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka, Japan; †National Nagasaki Medical Center, Omura, Japan; ‡Department of Molecular Immunology, Tokyo Medical and Dental University, Tokyo, Japan; and ¶Division of Rheumatology, Allergy and Clinical Immunology, School of Medicine, University of California, Davis, CA 95616

Received for publication February 28, 2007. Accepted for publication June 11, 2007.

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 study was partly supported by Grant-in-Aid 17590657 for Scientific Research (C) of Japan and National Institutes of Health Grant DK39588.

2 Address correspondence and reprint requests to Dr. Shinji Shimoda, Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan. E-mail address: sshimoda@intmed1.med.kyushu-u.ac.jp

3 Abbreviations used in this paper: PBC, primary biliary cirrhosis; Treg, regulatory T cell; PDCpep, human PDC-E2163–176 peptide; OGDCpep, E. coli OGDC-E234–47 peptide.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00
Herein, we have addressed both the mimicry issue and the co-stimulation requirements in peripheral tolerance in more depth by studying the role of CD80 and CD86 in the differential responses of a panel of eight autoreactive TCC to human PDC-E2\textsubscript{163–176} and its putative mimic E. coli OGDC-E2\textsubscript{36–47}, both restricted by HLA-DR53. To dissect the role of co-stimulation molecules in the proliferative response and the susceptibility to the induction of anergy of these TCC, we used “complete” or costimulation-competent and “incomplete” or costimulation-incompetent Ag presentation systems, i.e., systems with or without the costimulation molecules CD80 and CD86. PBMC represent the complete system capable of presenting Ag and delivering costimulatory signals, whereas mouse fibroblasts transfected with HLA-DR53 (L-DR53), CD80-transfected L-DR53 (CD80-L-DR53), and CD86-transfected L-DR53 (CD86-L-DR53) constitute incomplete systems capable of presenting Ag while providing no or only partial costimulation. We report herein that TCC found in PBC exhibit distinct functional characteristics, and we suggest that these data are critical to understanding loss of peripheral tolerance.

Materials and Methods

TCC and other cells

The development of eight TCC derived from PBMC of six patients with PBC has previously been described (15). In all cases, the diagnosis of PBC was based on international criteria and patients were positive for antimitochondrial Abs and had previously undergone a liver biopsy (1). All patients were HLA-DRB4 0101 positive (4). The HLA-DR53-transfected mouse fibroblast cell line L-DR53 was a gift from Prof. Y. Nishimura (Kumamoto University, Kumamoto, Japan). L-DR53 transfected with CD80 or CD86 were prepared as described below.

Preparation of CD80-transfected L-DR53 (CD80-L-DR53) and CD86-transfected L-DR53 (CD86-L-DR53)

To transfect CD80 or CD86 into L-DR53 cells, we used lipofection of the BCMGShyg and human CD80 or CD86 cDNA) (24). After selection in medium containing hygromycin (Sigma-Aldrich) at 400 \mu g/ml, the cells were cloned by limiting dilution. Transfectants expressing CD80 or CD86 were monitored for stable expression over a period of 4 wk. Representative stable transfectants were selected for further work. Expression of CD80 or CD86 on these transfected L-DR53 cells (CD80-L-DR53 or CD86-L-DR53) was confirmed by flow cytometric analysis. Briefly, PBMC, L-DR53, CD80-L-DR53, or CD86-L-DR53 were stained by immunofluorescence using optimal dilutions of anti-HLA-DR, anti-CD80, or anti-CD86 Abs (BD Biosciences); the optimal dilution of each Ab was 10 \mu g/ml and stained cells were analyzed on a FACScan (BD Biosciences).

Preparation of synthetic peptides

Human PDC-E2\textsubscript{163–176} (GDLLAEIETDKATI) peptide and E. coli OGDC-E2\textsubscript{34–47} (DEVLVEIETDKVVL) peptide were synthesized by F-moc chemistry using a peptide synthesizer (Model Synergy; Applied Biosystems); the optimal dilution of each Ab was 10 \mu g/ml of H9262. Counter.

Statistics

All experiments were performed in triplicate and mean results are presented graphically, with the error bars showing the SD. Due to the great variability in the magnitude of the responses, results are shown separately for each of the clones, but grouped according to the response pattern. Because the differences in the responses of the TCC were distinctive, statistical analyses were not performed.

Results

Proliferation patterns of TCC using human PDC-E2\textsubscript{163–176} or E. coli OGDC-E2\textsubscript{34–47} as Ag and using L-DR53 or HLA-DR53-matched PBMC as APC

We previously established eight TCC from six patients with PBC who were HLA-DRB4 0101 following repeated stimulation of the human PDC-E2 peptide (15). The names and characteristics of these clones are shown in Fig. 1. As noted in Fig. 1, TCC-1, -2, -3, -4, -5, -6 cross-reacted with E. coli OGDC-E2\textsubscript{34–47}, whereas TCC-7 and -8 responded only to human PDC-E2\textsubscript{163–176}. HLA restriction of human PDC-E2\textsubscript{163–176} and E. coli OGDC-E2 were HLA-DRB4 0101 (HLA-DR53) (data not shown).

To assess the co-stimulation requirement of the eight TCC, we examined their ability to proliferate in response to human PDC-E2\textsubscript{163–176} peptide and E. coli OGDC-E2\textsubscript{34–47} peptide using either a complete or an incomplete Ag presentation system (HLA-DR53-matched allogeneic PBMC or L-DR53, respectively). Four proliferative response patterns emerged (Fig. 1). TCC-1 and TCC-2 proliferated in response to human PDC-E2 and E. coli OGDC-E2 regardless of whether PBMC or L-DR53 was used as APC. Hence, these TCC are costimulation independent when either human PDC-E2\textsubscript{163–176} or E. coli OGDC-E2\textsubscript{34–47} are used as Ags. We designated TCC-1 and TCC-2 as group A. In contrast, TCC-3, -4, and -5 proliferated in response to human PDC-E2 presented by
PBMC or L-DR53 and to *E. coli* OGDC-E2 presented by PBMC, but not to *E. coli* OGDC-E2 when L-DR53 were used as APC. Hence, these TCC are costimulation independent for human PDC-E2163–176, but dependent for *E. coli* OGDC-E2 when presented by PBMC but not when presented by L-DR53. Thus, TCC-6 is costimulation dependent for human PDC-E2163–176 and *E. coli* OGDC-E2. TCC-6 is the only member of group C. Finally, TCC-7 and TCC-8 proliferated when cocultured with human PDC-E2-pulsed PBMC, but not with human PDC-E2-pulsed L-DR53, and did not respond to *E. coli* OGDC-E2 regardless of whether it was presented by PBMC or L-DR53. This indicates that TCC-7 and TCC-8 are costimulation dependent for human PDC-E2163–176 but ignorant of OGDC-E2. TCC-7 and TCC-8 were classified as group D.

**Proliferation assay of TCC preincubated with Ag-pulsed L-DR53**

We next examined whether presentation of human PDC-E2163–176 or *E. coli* OGDC-E2 by an incomplete Ag presentation system could render TCC anergic and whether susceptibility to anergy induction correlated with costimulation dependence. For this purpose, we preincubated the eight TCC with L-DR53 that had been pulsed with either of these peptides, then used irradiated allogeneic PBMC for a second round of stimulation. When preincubated with L-DR53 pulsed with human PDC-E2163–176 or *E. coli* OGDC-E2, TCC-1 and TCC-2 showed a strong proliferative response to human PDC-E2163–176 presented by allogeneic PBMC (Fig. 2, group A). TCC-3, -4, and -5 preincubated with PDC-E2-pulsed L-DR53 also proliferated when cocultured with allogeneic PBMC. In contrast, however, TCC-3, -4, and -5 preincubated with L-DR53 pulsed with allogeneic PDC-E2 did not proliferate when restimulated with human PDC-E2 using allogeneic PBMC as APC (Fig. 2, group B). Yet another pattern was shown by TCC-6. When this clone was preincubated with L-DR53 pulsed with human PDC-E2163–176 or *E. coli* OGDC-E2, it did not show any proliferative response to either peptide presented by allogeneic PBMC (Fig. 2, group C). Finally, TCC-7 and TCC-8 preincubated with PDC-E2-pulsed L-DR53 did not proliferate when restimulated with *E. coli* OGDC-E2 using allogeneic PBMC as APC (Fig. 2, group D). These results indicate that TCC whose response to a specific peptide is costimulation independent are more likely to be anergic when preincubated with an incomplete Ag presentation system.
peptide is dependent on costimulation become anergic when preincubated with L-DR53 pulsed with this Ag, whereas TCC whose response is costimulation independent do not become anergic under these conditions. The responses of all of these TCC are summarized in Table I.

Cytokine production of TCC before or after prestimulation with Ag-pulsed L-DR53

We then examined whether the induction of anergy was associated with changes in the production of IL-10 and IFN-γ. For this purpose, we first stimulated the eight TCC with human PDC-E2163-176 using irradiated HLA-DR53-matched allogeneic PBMC as APC. All TCC produced significant amounts of IFN-γ and IL-10 under these conditions (Fig. 3). We next used TCC that had been prestimulated with L-DR53 pulsed with human PDC-E2163-176 or E. coli OGDC-E234-47 and restimulated them with irradiated allogeneic PBMC that were or were not pulsed with human PDC-E2163-176 and again measured cytokine production. There were again four different patterns of cytokine production. TCC-1 and TCC-2 produced both IL-10 and IFN-γ in response to human PDC-E2 regardless of whether they were prestimulated with human PDC-E2 or E. coli OGDC-E2 presented by L-DR53 (Fig. 3, group A). TCC-3, -4, and -5 that were preincubated with L-DR53 pulsed with human PDC-E2 secreted both IL-10 and IFN-γ after restimulation with human PDC-E2 (Fig. 3, group B). In contrast, when preincubated with E. coli OGDC-E2, all three TCC produced only IL-10 after restimulation. Preincubation of TCC-6 with either of the peptides using L-DR53 as APC resulted in production of only IL-10 after restimulation with human PDC-E2 presented by allogeneic PBMC (Fig. 3, group C). Finally, TCC-7 and -8 synthesized only IL-10 after preincubation with human PDC-E2, but both IL-10 and IFN-γ after preincubation with E. coli OGDC-E2 (Fig. 3, group D).

These data indicate that all eight TCC produce both IFN-γ and IL-10 in response to stimulation with human PDC-E2. The induction of anergy is associated with suppression of IFN-γ synthesis, while IL-10 production is not markedly affected. When anergy is not induced, TCC continue to produce IFN-γ and IL-10.

Regulatory activity of T cells following preincubation with Ag-pulsed L-DR53

We next examined the effect of preincubation with L-DR53 pulsed with human PDC-E2163-176 or E. coli OGDC-E234-47 on the ability of the eight TCC to regulate the proliferative response of their corresponding TCC in the presence of human PDC-E2163-176 peptide presented by PBMC. The proliferative response of TCC-1 and TCC-2 to PDC-E2 was not affected by coculture with corresponding TCC that had been preincubated with L-DR53 pulsed with human PDC-E2163-176 or E. coli OGDC-E234-47 (see Fig. 4A for
results of TCC-1, representative of group A). Preincubation of TCC-3, -4, and -5 with human PDC-E2/L-DR53 also had no effect on the proliferation of their corresponding TCC in the presence of PBMC pulsed with human PDC-E2 peptide. However, when preincubated with L-DR53 pulsed with E. coli OGDC-E2, all three TCC completely inhibited the proliferation of their corresponding PBMC pulsed with human PDC-E2 peptide. However, when preincubated with L-DR53 pulsed with E. coli OGDC-E2, all three TCC completely inhibited the proliferation of their corresponding

Table I. Profile of the TCC

|                | Proliferation APC | Anergy Induction by Preincubation with Ag-Pulsed L-DR53 | Breaking Tolerance by Preincubation with Ag-pulsed CD80-L-DR53 or CD86-L-DR53 |
|----------------|-------------------|--------------------------------------------------------|--------------------------------------------------------------------------|
|                | PBMC L-DR53       | PBMC L-DR53                                           | PBMC L-DR53                                                              |
|                | PDCpep OGDCpep PDCpep OGDCpep | PDCpep OGDCpep PDCpep OGDCpep | PDCpep OGDCpep PDCpep OGDCpep | PDCpep OGDCpep PDCpep OGDCpep |
| Group A        |                   |                                                        |                                                                         |
| TCC-1          | Yes               | Yes                                                    | No                                                                       | No                                                                       | NT     | NT     |
| TCC-2          | Yes               | Yes                                                    | No                                                                       | No                                                                       | NT     | NT     |
| Group B        |                   |                                                        |                                                                         |
| TCC-3          | Yes               | Yes                                                    | No                                                                       | No                                                                       | Yes    | NT     |
| TCC-4          | Yes               | Yes                                                    | No                                                                       | No                                                                       | Yes    | NT     |
| TCC-5          | Yes               | Yes                                                    | No                                                                       | No                                                                       | Yes    | NT     |
| Group C        |                   |                                                        |                                                                         |
| TCC-6          | Yes               | No                                                     | Yes                                                                      | Yes                                                                      | Yes    | Yes    |
| Group D        |                   |                                                        |                                                                         |
| TCC-7          | Yes               | No                                                     | No                                                                       | Yes                                                                      | Yes    | NT     |
| TCC-8          | Yes               | No                                                     | No                                                                       | Yes                                                                      | Yes    | NT     |

The proliferative response was deemed positive if the cpm count in the presence of APC (PBMC or L-DR53) pulsed with Ag (PDCpep or OGDCpep) was at least two times greater than in the presence of APC alone. TCC were considered anergic if, after preincubation with peptide-pulsed L-DR53, their proliferative response to peptide-pulsed PBMC was no more than twice as high as the response to PBMC alone. Tolerance was considered to be broken when TCC, after preincubation with peptide-pulsed CD80-L-DR53 or CD86-L-DR53, showed a proliferative response to peptide-pulsed PBMC that was at least two times greater than in the presence of PBMC alone.

NT, Not tested.

FIGURE 3. Cytokine production of TCC stimulated with PDC-E2 presented by irradiated HLA-DR53-matched allogeneic PBMC with or without preincubation in the presence of L-DR53 pulsed with human PDC-E2 or E. coli OGDC-E2 peptide. All TCC produce IFN-γ and IL-10 in response to stimulation with PDC-E2 and PBMC. When anergy is induced in costimulation-dependent TCC by preincubation with peptide-pulsed L-DR53, restimulated TCC produce IL-10 but not IFN-γ. The failure of costimulation-independent TCC to become anergic is accompanied by continued secretion of IFN-γ and IL-10 after restimulation.
unstimulated TCC (see Fig. 4B for results of TCC-3, representative of group B). In the case of TCC-6, preincubation with L-DR53 pulsed with either human PDC-E2\textsubscript{163–176} or \textit{E. coli} OGDC-E2\textsubscript{4–47} abolished the proliferative response of its corresponding nonanergic TCC. A, TCC-1, which does not become anergic when preincubated with PDCpep- or OGDCpep-pulsed L-DR53 (see Fig. 2), does not have regulatory function. B, TCC-3 preincubated with PDCpep-pulsed L-DR53 does not have regulatory function while this TCC preincubated with OGDCpep-pulsed L-DR53 exhibits regulatory activity. C, TCC-6 preincubated with PDCpep- or OGDCpep-pulsed L-DR53 has regulatory function. D, TCC-7 exhibits regulatory activity after preincubation with PDCpep-pulsed L-DR53, but not OGDCpep-pulsed L-DR53.

These data suggest that TCC with co-stimulation-independent responses to a specific Ag do not have a regulatory role after contact with the corresponding Ag in a costimulation-deficient manner. In contrast, TCC with co-stimulation-dependent responses to a specific Ag acquire regulatory characteristics when presented with this Ag in a costimulation-deficient manner. TCC that do not respond to a specific Ag have no effect on regulation after contact with the corresponding Ag in a costimulation-deficient manner.

FIGURE 5. Proliferation assay of TCC-1 to analog peptides substituted at position 168E of human PDC-E2\textsubscript{163–176} using L-DR53 or PBMC as APC. TCC-1 proliferated in response to all substituted peptides when PBMC were used as APC. This clone also proliferated to most of these peptides when presented by L-DR53, but not when amino acids with a negative or positive charge, aromatic amino acids, or G were substituted at position 168E of human PDC-E2\textsubscript{163–176}. WT, Wild type.
Given the importance of the E residue at position 168 of PDC-E2 as the first TCR recognition site (25), we then evaluated the proliferation of TCC-1 in response to a panel of human PDC-E2163–176 peptides containing single amino acid substitutions at this position. As shown in Fig. 5, TCC-1 proliferated after stimulation with all substituted human PDC-E2163–176 peptides presented by L-DR53.

**FIGURE 6.** Proliferation and cytokine production in response to PDC-E2 of TCC-1 preincubated with L-DR53 pulsed with 168K or 168W and regulatory function of anergic TCC-1. A, TCC-1, which responds to 168K or 168W in a costimulation-dependent manner, becomes anergic when preincubated with L-DR53 pulsed with 168K or 168W. B, TCC-1 initially produces IFN-γ and IL-10, but only IL-10 once anergy is induced. C, TCC-1 regulates the proliferation of the corresponding TCC. Please note that in Fig. 2, TCC-1 could not be rendered anergic by pre-incubation with PDC-E2/L-DR53.

**FIGURE 7.** Proliferative response of TCC to human PDC-E2163–176 or E. coli OGDC-E234–47 using L-DR53, PBMC, CD80-L-DR53, or CD86-L-DR53 as APC. A, TCC-3 is costimulation dependent in its recognition of E. coli OGDC-E2 peptide. B, TCC-6 shows costimulation-dependent responses to both human PDC-E2 and E. coli OGDC-E2 peptide. C, The response of TCC-7 to human PDC-E2 peptide is costimulation dependent. CD80 or CD86 stimulation partially supports these proliferative responses in all cases.
HLA-DR53-matched allogeneic PBMC. When L-DR53 was used as an APC, however, TCC-1 did not show a proliferative response to 168D, 168K, 168R, 168W, and 168G. These results indicate that TCC-1 is costimulation dependent for peptides 168D, 168K, 168R, 168W, and 168G.

Proliferation, cytokine production, and regulatory assay of TCC-1 preincubated with 168K- or 168W-pulsed L-DR53

Since altered peptide ligands with single residue substitutions in the antigenic peptide can induce agonism, antagonism, or antagonism with partial activation (26), it was of particular interest to further characterize the response of TCC-1 to some of the costimulation-dependent peptide analogs. TCC-1 preincubated with 168K- or 168W-pulsed L-DR53 did not proliferate when cocultured with PDC-E2-pulsed allogeneic PBMC (Fig. 6A). We next prestimulated TCC-1 with 168K- or 168W-pulsed L-DR53 and then re-stimulated this TCC with PBMC that had or had not been pulsed with human PDC-E2163–176. TCC-1 prestimulated with 168K- or 168W-pulsed L-DR53 produced IL-10 but not IFN-γ in the presence of PDC-E2-pulsed PBMC (Fig. 6B). They also regulated the proliferation of their corresponding TCC in the presence of PDC-E2 peptide-pulsed PBMC (Fig. 6C). These findings are of particular interest since TCC-1 is one of the clones that could not be rendered anergic by preincubation with PDC-E2/L-DR53.

Role of costimulation molecules and anergy

We next focused on whether anergy is induced when only a specific HLA molecule is expressed on the APC and presents the peptide of interest. We also examined whether anergy is maintained when the costimulation molecules CD80 or CD86 are present in such a system. For this purpose, we used L-DR53 transfected with CD80 or CD86 as APC. Note that our flow cytometric results confirmed that PBMC expressed HLA-DR, CD80, and CD86; L-DR53 expressed only HLA-DR53; CD80-L-DR53 expressed HLA-DR53 and CD80 but not CD86; and CD86-L-DR53 expressed HLA-DR53 and CD86 but not CD80 (data not shown).

Proliferation assays of TCC using L-DR53, CD80-L-DR53, CD86-L-DR53, or PBMC as APC

Our previous experiments indicated that, under normal circumstances, anergy to human PDC-E2 or E. coli OGDC-E2 or both could only be induced in TCC with a costimulation-dependent response to the respective Ag, i.e., TCC-3–8. We continued this analysis by first determining the proliferative response of these TCC to their relevant Ags using CD80-L-DR53 or CD86-L-DR53 as APC. As in our previous experiments (Fig. 1), TCC-3, -4, and -5 did not proliferate in the presence of L-DR53 pulsed with E. coli OGDC-E2, but did proliferate when pulsed PBMC were used as APC. These clones also showed a proliferative response to E. coli OGDC-E2 when presented by CD80-L-DR53 or CD86-L-DR53, but this response was clearly reduced compared with that obtained with PBMC (Fig. 7A for representative results). In the case of TCC-6, which did not proliferate in response to L-DR53 pulsed with human PDC-E2 or E. coli OGDC-E2 but did respond to PBMC pulsed with these Ags, there was also a reduced proliferation when CD80-L-DR53 or CD86-L-DR53 were used as APC compared with PBMC (Fig. 7B). In the case of TCC-7 and TCC-8, which did not proliferate in the presence of PDC-E2-pulsed L-DR53 but did proliferate with Ag-pulsed PBMC, the proliferation was again reduced for CD80-L-DR53 or CD86-L-DR53 as APC compared with PBMC (Fig. 7C). Thus, TCC with a costimulation-dependent response to a specific Ag proliferate in the presence of the costimulation molecules CD80 or CD86, although to a lesser extent than seen in the presence of a complete costimulation system as provided by PBMC.

Anergy assay of TCC after coculture with Ag-pulsed CD80-L-DR53 or CD86-L-DR53 cells

Next, we determined whether preincubation with Ag-pulsed CD80- or CD86-transfected L-DR53, like untransfected L-DR53, could induce anergy in costimulation-dependent TCC. TCC-3, -4, and -5 were preincubated with CD80-L-DR53 or CD86-L-DR53.
pulsed with *E. coli* OGDC-E2, followed by a proliferation assay with human PDC-E2-pulsed PBMC. In this case, anergy was not observed (representative results are shown in Fig. 8A). Similarly, when using the same Ag presentation system for stimulating TCC-6 with human PDC-E2 or *E. coli* OGDC-E2, no induction of anergy occurred (Fig. 8B). Finally, preincubation with CD80-L-DR53 or CD86-L-DR53 pulsed with human PDC-E2 failed to induce anergy to human PDC-E2 in TCC-7 and TCC-8 (Fig. 8C). These results indicate that peripheral regulation is easily broken with the costimulation molecules CD80 and CD86.

**Discussion**

Previously we demonstrated that some PBC patients have autoreactive effector T cells that are completely independent of costimulation (27). In the current study, we evaluated these TCC in greater detail by using both the important autoantigen PDC-E2163–176 and the putative mimic *E. coli* OGDC-E234–47 (12, 13) and by using not only PBMC and L-DR53 as APC, but also CD80-L-DR53 and CD86-L-DR53.

Based on their ability to recognize one or both of these peptides when presented by costimulation-deficient L-DR53 or costimulation-competent PBMC, the autoreactive TCC analyzed in the present study could be classified into four groups. The first group (group A) is costimulation independent in its recognition of both human PDC-E2163–176 and *E. coli* OGDC-E234–47. Group B shows a costimulation-independent response to human PDC-E2163–176 but requires costimulation for recognition of *E. coli* OGDC-E234–47. Group C depends on costimulation for recognition of both human PDC-E2163–176 and *E. coli* OGDC-E234–47. Group D requires costimulation to respond to human PDC-E2163–176, and does not recognize *E. coli* OGDC-E2. Costimulation-dependent, but not costimulation-independent clones could be readily induced to become anergic.

Herein, we demonstrate that TCC belonging to Group B can be induced to become anergic with the mimic *E. coli* OGDC-E234–47, even though they are costimulation independent in their recognition of human PDC-E2163–176. Generally, molecular mimicry is considered as a potential initiator of autoimmune responses. However, our results suggest that, in the case of costimulation-deficient Ag presentation systems, molecular mimicry also has the possibility to maintain peripheral tolerance (Fig. 2, group B). Furthermore, TCC that could be induced to become anergic produced IL-10 (Fig. 3, group B) and exhibited regulatory functions (Fig. 4B).

Altered peptide ligands with single residue substitutions in the antigenic peptide can induce agonism, antagonism, and antagonism with partial activation (26). The frequencies of analog peptides exhibiting these three different effects on TCC differ depending on the residue of the peptide substituted (28). Furthermore, peptide ligands with substitutions at TCR contact residues, by acting as specific TCR antagonists or partial agonists, can play a role in down-modulating autoreactive T cell responses (29–32). Previously, we reported that position 168E of human PDC-E2163–176 is the first TCR recognition site (25). In the present work, we used peptide analogs of human PDC-E2163–176 with single amino acid substitutions at this position. TCC-1 proliferated in response to all of the substituted peptide analogs when presented by professional APC (PBMC) (Fig. 5). In contrast, TCC-1 did not respond to the substituted peptides 168D, 168K, 168R, or 168W when presented by costimulation-deficient L-DR53. Our results obtained with 168K and 168W (Fig. 6) indicate that certain specific substituted peptide analogs of position 168E of human PDC-E2163–176 can induce anergy. The resulting anergized clones were found to produce IL-10, but not IFN-γ, and to function as regulators of the proliferative response of their corresponding nonanergized clones.

It is now well established that T cells capable of suppressing the effector functions of other T cells play an important role in the control of immune responses, particularly to self-Ags. Several subsets of such suppressor cells have been identified, including thymus-derived regulatory T cells (Tregs) expressing CD4, CD25, and the transcription factor Foxp3, Th3 cells that produce high levels of TGFβ, and Tr1 cells that exert their regulatory function by secreting mostly IL-10. Of note, adaptive Tregs that are phenotypically and functionally indistinguishable from Foxp3+ Tregs can arise from the conversion of peripheral CD25+ T cells. Previous work from our laboratory showed that the anergic suppressor T cells generated by stimulation with PDC-E2163–176, in a costimulation-incompetent system did not exhibit some of the characteristics typical of Tregs, such as high expression of CD25 and contact-dependent suppressor activity (15). This suggests that the anergic suppressor TCC generated in our system belong to a different subset of regulatory cells. In our previous work, we also demonstrated that these TCC produced IFN-γ, IL-10, and low amounts of IL-4 in response to PDC-E2163–176 before the induction of anergy (15). Once anergic, they synthesized only IL-10, and their regulatory activity could be partially abrogated by blocking IL-10. Some of the TCC underwent an intermediate stage during which they no longer proliferated in response to PDC-E2163–176 but still secreted both IL-10 and IFN-γ. Since we did not analyze IL-4 production in partially and completely anergized TCC, we cannot determine whether the suppressor TCC exhibited a Th2 cytokine profile or had become Tr1 cells producing only IL-10.

If T cells can easily be induced to become not only anergic but also regulatory as described above, peptide analog vaccine therapy would be expected to be successful in autoimmune diseases. Actually, even though this approach shows some promise in the induction of strong immune responses in some types of infection or cancer (33), it did not work well in autoimmune diseases. To clarify why anergy is not induced in vivo, we focused on the function of the costimulation molecules CD80 and CD86, which play a crucial role in the induction of autoimmune diseases (21). Note, however, that we used TCC obtained after several rounds of Ag stimulation, i.e., activated memory T cells, whereas the breaking of tolerance that eventually results in the development of autoimmune disease involves naïve T cells.

In our experimental systems, proliferation of some autoreactive TCC in response to PDC-E2 or OGDC-E2 was supported by professional APC (PBMC), but not by costimulation-deficient APC (L-DR53), indicating that CD80 or CD86 are central to this process. This is further supported by the finding that the proliferative response was diminished, but complete anergy was not achieved when these peptides were presented by L-DR53 transfected with CD80 or CD86 (Fig. 7). This suggests that peripheral tolerance cannot be maintained in the presence of costimulation (Fig. 8). Note that the major autoantigen in PBC, PDC-E2, is a ubiquitous protein, yet, the specific target of the autoimmune response in PBC are biliary epithelial cells. It has been hypothesized that biliary epithelial cells act as APC, based on the observation that these cells express MHC class II. However, since they lack costimulation molecules, they would be expected to silence rather than activate autoreactive T cells. Indeed, we found that biliary epithelial cells can induce T cell anergy (15, 34). Therefore, we submit that biliary epithelial cells themselves may be a candidate for induction of peripheral tolerance. However, if costimulation molecules such as CD80 or CD86 exist around biliary epithelial cells, as has been suggested in several studies (35–37), peripheral tolerance may be broken. This is important as biliary cells are surrounded by dendritic cells, macrophages (38), and activated B cells (39).
These data are of great interest since, if autoreactive T cells can be induced to become anergic, this would represent a strong tool to control autoimmune diseases such as PBC. However, as costimulatory molecules are expressed in the inflammatory area where regulatory function must work, T cell anergy may not be induced. In that case, a better therapeutic approach would be to transfer anergic T cells (adoptive cellular immunotherapy) for control of the autoimmune process.

Acknowledgment
We appreciate the outstanding work of Dr. Andrea T. Borchers.

Disclosures
The authors have no financial conflict of interest.

References
1. Kaplan, M. M., and M. E. Gershwin. 2005. Primary biliary cirrhosis. N. Engl. J. Med. 353: 353–360.
2. Aoki, C. A., C. M. Roifman, Z. X. Lian, C. L. Bowlus, G. L. Norman, T. Spengler, U., L. Leifeld, I. Braunschweiger, F. L. Dumoulin, M. Lechmann, and Y. Nakanuma. 2000. Fas ligand expressing mononuclear cells around intrahepatic bile ducts in primary biliary cirrhosis and primary sclerosing cholangitis: an immunohistochemical study. J. Pathol. 195: 225–234.
3. Kamihira, T., S. Shimoda, M. Nakamura, T. Yokoyama, Y. Takii, A. Kawano, M. Handa, H. Ishibashi, M. E. Gershwin, and M. Harada. 2003. Distinct costimulation dependent and independent autoreactive T cell clones in primary biliary cirrhosis. Gastroenterology 125: 1379–1387.
4. Tsuneyama, K., M. E. Gershwin, and Y. Nakanuma. 1996. Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide: characterization and frequencies of TCR agonism and TCR antagonism with or without partial activation. J. Immunol. 157: 3783–3790.
5. Allen, P. M. 1994. Peptides in positive and negative selection: a delicate balance. Cell 76: 593–596.
6. Goldrath, A. W., and M. J. Bevan. 1999. Selecting and maintaining a diverse T-cell repertoire. Nature 402: 255–260.
7. Kamihira, T., S. Shimoda, K. Harada, M. Handa, E. Baba, K. Tsuneyama, M. Nakamura, H. Ishibashi, Y. Nakanuma, M. E. Gershwin, and M. Harada. 2003. Distinct costimulation dependent and independent autoreactive T cell clones in primary biliary cirrhosis. Gastroenterology 125: 1379–1387.
8. Tsuneyama, K., M. E. Gershwin, and Y. Nakanuma. 1996. Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide: characterization and frequencies of TCR agonism and TCR antagonism with or without partial activation. J. Immunol. 157: 3783–3790.
9. Allen, P. M. 1994. Peptides in positive and negative selection: a delicate balance. Cell 76: 593–596.
10. Goldrath, A. W., and M. J. Bevan. 1999. Selecting and maintaining a diverse T-cell repertoire. Nature 402: 255–260.
11. Kamihira, T., S. Shimoda, M. Nakamura, T. Yokoyama, Y. Takii, A. Kawano, M. Handa, H. Ishibashi, M. E. Gershwin, and M. Harada. 2003. Biliary epithelial cells regulate autoreactive T cells: implications for biliary-specific diseases. Hepatology 38: 151–159.
12. Kaji, K., S. Tsuneyama, Y. Nakanuma, K. Harada, M. Sasaki, S. Kaneko, and K. Kobayashi. 1997. B7-2 positive cells around interlobular bile ducts in primary biliary cirrhosis and chronic hepatitis C. J. Gastroenterol. Hepatol. 12: 413–419.
13. Tsuneyama, K., M. E. Gershwin, and Y. Nakanuma. 1998. Expression of co-stimulatory factor B7-2 on the intrahepatic bile ducts in primary biliary cirrhosis and primary sclerosing cholangitis: an immunohistochemical study. J. Pathol. 186: 126–130.
14. Iwata, M., K. Harada, K. Hiramatsu, K. Tsuneyama, S. Kaneko, K. Kobayashi, and Y. Nakanuma. 2000. Fas ligand expressing mononuclear cells around intrahepatic bile ducts express co-stimulatory molecule CD68 in primary biliary cirrhosis. Liver 20: 129–135.
15. Bjorkland, A., K. Harada, K. Hiramatsu, K. Tsuneyama, S. Kaneko, K. Kobayashi, and Y. Nakanuma. 2000. Fas ligand expressing mononuclear cells around intrahepatic bile ducts express co-stimulatory molecule CD68 in primary biliary cirrhosis. Liver 20: 129–135.
16. Borchers, A. T., L. Loof, I. Mendel-Hartvig, and T. H. Totterman. 1994. Primary biliary cirrhosis. High proportions of B cells in blood and liver tissue produce anti-mitochondrial antibodies of several Ig classes. J. Immunol. 153: 2750–2757.
17. Azuma, M., M. Cayabyab, B. Duck, J. H. Phillips, and L. L. Lanier. 1992. CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. J. Exp. Med. 175: 353–360.