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Acquisition of CD80 (B7-1) by T Cells

Helen Sabzevari,* Judy Kantor,* Adnan Jaigirdar,* Yutaka Tagaya,† Mayumi Naramura,‡ James W. Hodge,* John Bernon,* and Jeffrey Schlom†*

Activation of T cells usually requires two signals. Signal 1 is mediated via a peptide-MHC on the APC; signal 2 is mediated via a costimulatory molecule on the APC surface. We demonstrate here that naive CD4+ T cells actually acquire the costimulatory molecule CD80 (B7-1) from syngeneic APCs after activation. This phenomenon was demonstrated showing acquisition of CD80 by T cells from CD80/CD86 (B7-2) knockout mice, and by treating T cells with cyclohexamide to further rule out endogenous expression of CD80 by T cells. Moreover, no CD80 mRNA could be detected in T cells that had acquired CD80. The amount of acquisition of CD80 by T cells was shown to be directly related to both the strength of signal 1 and the amount of CD80 on the APC. Specificity of this acquisition was also shown by the lack of acquisition by T cells from CD28 knockout mice (implicating CD28 in this process), the lack of acquisition of CD40 (another molecule on the APC surface) by T cells, and confocal microscopy studies. We demonstrate for the first time that 1) naive T cells, following acquisition of CD80 from APCs, were themselves shown to be capable of acting as APCs; and 2) memory T cells that have acquired CD80 from APCs undergo apoptosis in the presence of increased levels of signal 1. Thus we demonstrate both immunostimulatory and immunoregulatory functions as a result of CD80 acquisition by different T cell populations. The Journal of Immunology, 2001, 166: 2505–2513.

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‡ Abbreviations used in this paper: DC, dendritic cell; KO, knockout; B7/4KO, CD80/CD86 double-knockout; PCC/TCR-Tg, TCR transgenic for pigeon cytochrome C6a,105; rF-CD80, recombinant fowlpox virus expressing CD80; FP-WT, wild-type fowlpox; GFP, green fluorescence protein; CHX, cyclohexamide.

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Drosophila cells as APCs may result in the rapid transfer of MHC molecules and other costimulatory molecules, such as CD80, from APCs to T cells (21).

Although the concept of molecule acquisition by T cells originated 20 years ago, the actual interactions of receptor ligands and the factors affecting ligand acquisition still remain unclear. In this regard, we analyzed CD80 levels on murine T cells upon stimulation with various APCs expressing different levels of CD80 on their surface. Although CD80 was previously thought to be endogenously up-regulated, results of studies of CD80/CD86 double-knockout (B7dKO) mice reported here demonstrate that CD80 is physically acquired by T cells from APCs shortly after T cell activation. We also demonstrate here that CD80 acquisition by T cells is mediated by the TCR and its CD28 ligand (confirming the recent data by Hwang et al. (21)) and that the level of CD80 acquisition by T cells is related to both the level of CD80 expression on APCs and the strength of signal 1. Moreover, our data for the first time suggest that acquisition of CD80 by T cells might be of importance for the Ag-presenting capacity of T cells. Thus, these findings might have important implications for a further understanding of immune regulation and the pathogenesis of immunopathological diseases.

Materials and Methods

Animals

BALB/c, C57BL/6, B10.A, and CD28KO mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. B7dKO mice were provided by R. Hodes (National Institute on Aging, National Institutes of Health, Bethesda, MD) and were originally obtained from A. Sharpe (Harvard University, Boston, MA). Pigeon cytochrome c-53-transgenic (PCC/TCR-Tg) (5C7/TCR-Tg) mice, which are TCR transgenic for PCC, were obtained from Taconic Farms (Germantown, NY) and bred in the National Cancer Institute animal facility under pathogen-free conditions. PCC/TCR-Tg IL-2 green fluorescence protein (GFP)30 (PCC/I-Ek-specific) mice were provided by M. Narumura (National Institute of Allergy and Infectious Diseases, National Institutes of Health).

mAbs and flow cytometry

To evaluate CD80 expression on T cells, cell suspensions were stained with directly conjugated mAbs (anti-CD4PE, anti-CD8 FITC). The proportion of CD4+ cells expressing CD80 was determined by gating on CD4+ T cells, excluding the dead cells. Phytochrome mAb Abs to CD4, CD8, MHC class II I-Ek, CD28, and purified anti-CD28 Abs were purchased from PharMingen (San Diego, CA), and anti-CD28 Abs were purchased from BioXtack (Walkersville, MD).

APCs

DCs were prepared as described previously (22). Briefly, bone marrow cells from 6- to 8-wk-old mice were depleted of lymphocytes using a mixture of magnetic beads specific for CD4, CD8, MHC class I, and MHC class II (MiniMACS, Miltenyi Biotec, Auburn, CA). Cells were incubated in six-well plates (5 x 10^5 cells/well) with medium supplemented with 10 ng/ml GM-CSF and 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN). Cells were depleted in fresh cytokine-supplemented medium on days 2 and 4. On day 6, cells were harvested for infection and analysis. The following cell lines were provided by R. Germain (National Institute of Allergy and Infectious Diseases, National Institutes of Health): fibroblast cell line DCER, which expressed a low level of CD80; MHC class II I-Ek and 13.9 fibroblast cell line, which expressed a high level of CD80; and COS (monkey kidney) cells, which expressed MHC class II I-Ek. Murine colonic adenocarcinoma cells (MC38) and the retrovirally transduced MC38/CD80 cell line have been described previously (23). A20 cells are murine B cell lymphoma cells expressing 37% CD80 on their surface.

Separation of APCs by beads

Fibroblast cells (2 x 10^5) were cultured with 250 μl of goat anti-mouse Dynabeads (Dynal, Lake Success, NY) in the presence of various concentrations of peptide. After 2 days, the fibroblasts that had absorbed the beads were separated by a magnet. These cells were then used as APCs (2 x 10^5 cells) and cultured with 1 x 10^6 effector/memory T cells. The APCs were separated from T cells 24 h later by a magnet. This procedure yielded a preparation of T cells devoid of any APCs.

Recombinant fowlpox viruses and infection of DCs

Recombinant fowlpox virus expressing CD80 (rF-CD80) and wild-type fowlpox (FP-WT) have been described previously (24). DCs were harvested on day 6 of culture and washed with OptiMem (Life Technologies, Gaithersburg, MD). The cells (1.2 x 10^7/ml) were infected with FP-WT or rF-CD80 at 50 MOI (multiplicity of infection; PFU/cells) for 2 h at 37°C. Cells were then washed and incubated in condition medium at 37°C overnight. After 18 h, DCs were harvested for flow assay.

Peptide

The I-E-restricted PCC peptide was synthesized and HPLC-purified by American Peptide (Sunnyvale, CA).

Cell preparation and culture

CD4+ cells were purified using microbeads conjugated to anti-CD4 mAb, according to the manufacturer’s instructions (MiniMACS, Miltenyi Biotech), on MACS columns. Isolated CD4+ cells (5 x 10^5 cells/ml) were cultured with APCs (1 x 10^5 cells/ml) with or without signal 1, with either anti-CD3 Ab or PCC peptide (at concentrations indicated in each figure legend), in RPMI 1640 supplemented with the following: 10% heat-inactivated FBS, 5 x 10^-3 M 2-ME, 2 mM l-glutamine, 100 μM/ml penicillin, 100 μM/ml streptomycin, and 10 mM HEPEs (all obtained from Life Technologies, Gaithersburg, MD), and 15 μg/ml gentamicin (BioWhittaker, Walkersville, MD).

Effector/memory CD4+ T cells

Effector/memory CD4+ T cells were generated by activating naive splenic CD4+ T cells from PCC/TCR-Tg mice in culture with 10 μg/ml PCC peptide for 3 days. Following centrifugation with ficoll gradient, the live cells were washed and then rested for 4–6 days in six-well plates with 5 IU/ml of murine IL-2 medium. The cells were washed and given IL-2 every 3 days following the 3-day PCC activation. The effector/memory CD4+ T cells were analyzed for markers as follows: FITC-conjugated CD62 ligand, PE-conjugated CD44, FITC-conjugated CD45RB, and FITC-conjugated CD90. Appropriate isotype control was used for all of the markers.

Aptosis assay

Effector/memory CD4+ cells were cultured for 24 h in the presence of various concentrations of peptide as described in Results. Apoptosis was assessed using the TUNEL kit (PharMingen) according to manufacturer’s instructions.

RNA extraction and PCR

RNA was purified using an RNA STAT-60 kit (Tel-Test, Friendswood, TX), and 2 μg of total RNA was used to synthesize cDNA with the SuperScript Preamplification System (Life Technologies, Gaithersburg, MD), using oligo(dT) according to the manufacturer’s instructions. Oligonucleotides specific for CD80 and β-actin were prepared by cruelchem (Dulles, VA) and Invitrogen (Carlsbad, CA). Primers for CD80 (517-bp fragment) were 5′-TCTCCAGATACACCTCCTC-3′ (sense) and 5′-TCCAAACCA GAGAAGCGGAG-3′ (antisense). Primers for β-actin (1-kb fragment) were 5′-GCTCACTCAGTGATGATATGC-3′ (sense) and 5′-GGA GGCGAATGTCTATGCTTC-3′ (antisense). cDNA samples were subjected to amplification using PCR (model 9600) (Perkin-Elmer Cetus, Norwalk, CT). Samples were initially denatured for 2 min at 94°C. CD80 and β-actin-specific amplification was conducted for 25 cycles, where each cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and polymerization at 72° for 30 s, followed by a finishing step at 72° for 10 min. Samples (20 μl) were resolved on 1% agarose gels in Tris-borate-EDTA (TBE), electrophoresed at 100 V, and stained with ethidium bromide; the cDNA fragments were visualized directly by UV light.

GFP-CD80 fusion protein

A cDNA clone of murine CD80 was cloned and sequenced as described previously (25). Restriction endonuclease digestion of this clone with KpnI and ApaiI released a 1-kb fragment that was gel purified and ligated into the KpnI/ApaiI sites of pEGFP-C1, the C-terminal protein-fusion expression vector (Clontech Laboratories, Palo Alto, CA). DH5α Escherichia coli cells were transformed with this construct (Life Technologies, Rockville, MD), and kanamycin-resistant colonies were selected and sequenced for
CD80 expression. pEGFP-CD80 DNA was CsCl-banded and subsequently used to transfect COS cells for confocal studies.

**Results**

**CD80 acquisition by T cells in B7dKO mice**

To establish the innate expression of CD80 on naive T cells, CD4+ cells from four strains of mice were analyzed by two-color FACS analysis. T cell expression of CD80 is a relatively rare occurrence in these strains, as determined by immunofluorescence and flow cytometry. CD80 expression levels on T cells varied among the different strains of mice, ranging from 2 to 9% in C57BL/6 mice, to very small (1–2%) or undetectable levels in B7dKO, CD28KO, PCC/TCR-Tg mice.

Previous studies have reported that activated T cells express CD80 molecules on their surface 3–7 days after activation (15). However, none of these studies used B7dKO mice as the source of T cells. CD4+ naive T cells from B7dKO mice were used to investigate whether CD80 expression on T cells shortly after activation is induced endogenously or acquired from APCs. Splenocytes from B7dKO mice lack functional genes for both CD80 and CD86; upon activation, these cells are unable to express CD80 or CD86 on their surface (26). CD4+ cells from B7dKO mice were incubated with 10 μg/ml of anti-CD3 as signal 1 and A20 cells (a B cell line expressing 37% CD80) as APCs. The T cells were monitored for 24 h. As shown in Fig. 1A, CD4+ cells from B7dKO mice did not express CD80; levels were similar to isotype control. Activation of B7dKO CD4+ cells with 10 μg of anti-CD3, in the absence of APCs (A20 cells), did not change CD80 expression (Fig. 1B). As a result of incubating B7dKO CD4+ cells with A20 cells in the absence of signal 1, 4% of CD4+ cells acquired CD80 (Fig. 1C). However, when CD4+ cells were incubated with A20 cells as APCs in the presence of anti-CD3 (10 μg/ml) as signal 1, 30% of the CD4+ cells from the B7dKO mice acquired CD80 (Fig. 1D).

To further address the hypothesis that CD80 is acquired by T cells upon activation, CD4+ cells from B7dKO mice were then incubated for 24 h with either normal DCs or DCs that were infected with WT-FP virus or rF-CD80. Normal DCs expressed high levels of CD80 (70%), and infection with WT virus did not affect CD80 expression. However, infection of DCs with rF-CD80 increased expression of CD80 on 90% of cells. In the absence of signal 1, incubation of T cells from B7dKO mice with both regular DCs and those infected with WT-FP led to the acquisition of relatively low levels of CD80 (7–15%) (Table I). However, in the presence of signal 1 (10 μg/ml anti-CD3), 55–57% of CD4+ cells acquired CD80 on their surface (Table I). When B7dKO CD4+ cells were incubated with rF-CD80-infected DCs, the CD4+ cells acquired more CD80 in both the absence (53%) and presence (87%) of signal 1 (Table I). These results using T cells from B7dKO mice further demonstrate that CD80 levels on T cells after a short period of activation are due to the acquisition of CD80 from APCs, and not the constitutive expression and/or up-regulation of endogenous CD80. Moreover, these results demonstrate that both the presence of signal 1 and the level of CD80 expression on APCs influence this phenomenon.

**CD80 is acquired by T cells through CD28 interaction**

CD80 acquisition by T cells raises the question of the role of CD28 in this process. To examine the role of the CD28 molecule in acquiring CD80, CD4+ cells from CD28KO (CD28−/−) mice and normal C57BL/6 mice were incubated for 24 h with MC38/CD80 cells; MC38/CD80 are retrovirally transduced murine carcinoma cells expressing CD80. These experiments were conducted either in the presence or absence of anti-CD3 (10 μg/ml) as signal 1. As shown in Table II, T cells from CD28KO mice did not acquire CD80 upon incubation with MC38/CD80 in the absence or presence of signal 1 (1–2% CD80 acquisition). However, 19% of CD4+ cells from C57BL/6 mice (expressing constitutively high levels of CD28) did acquire CD80 upon stimulation with MC38/CD80 in the presence of signal 1. These studies demonstrate that the acquisition of CD80 by T cells is mediated through the CD28 ligand and not by way of a random process. Experiments were also conducted to show that the ligand on resting T cells for CD80 acquisition is not CTLA-4. T cells from PCC/TCR-Tg mice were incubated with fibroblasts expressing CD80 in the presence and absence of anti-CTLA-4 Ab. In both cases, there was no reduction in the acquisition of CD80 by T cells (data not shown).

**Physical nature of CD80 transfer to T cells**

To investigate the physical nature of the CD80 binding to the surface of T cells, COS (monkey-kidney) cells expressing I-Ek MHC class II were transfected with a construct containing GFP-CD80. As determined by FACS analysis, 30% of COS cells expressed membrane-bound GFP-CD80 fusion protein (data not shown). Confocal microscopy studies of COS cells demonstrated that when these cells were transfected with GFP-CD80 construct, CD80 was acquired on their surface (Table I). However, in the presence of signal 1 (10 μg/ml anti-CD3), 55–57% of CD4+ cells acquired CD80 on their surface (Table I). When B7dKO CD4+ cells were incubated with rF-CD80-infected DCs, the CD4+ cells acquired more CD80 in both the absence (53%) and presence (87%) of signal 1 (Table I). These results using T cells from B7dKO mice further demonstrate that CD80 levels on T cells after a short period of activation are due to the acquisition of CD80 from APCs, and not the constitutive expression and/or up-regulation of endogenous CD80. Moreover, these results demonstrate that both the presence of signal 1 and the level of CD80 expression on APCs influence this phenomenon.

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Acquisition of CD80 from the A20 cell line by B7dKO T cells. Two-color FACS analysis was performed on T cells for CD4 and CD80. A. Expression of CD80 on CD4+ T cells from B7dKO mouse; B. CD80 expression on CD4+ cells upon stimulation with 10 μg/ml of anti-CD3 Ab for 24 h; C. acquisition of CD80 by B7dKO CD4+ cells in the presence of the A20 cell line as APCs, but in the absence of signal 1; or D. acquisition of CD80 by B7dKO CD4+ cells in the presence of A20 cells and anti-CD3 as signal 1. The data are representative of two independent experiments. All gates were set based on negative controls.

**Table I.** CD80 acquisition by CD4+ T cells from double knockout mice

<table>
<thead>
<tr>
<th>APC</th>
<th>Signal 1 (anti-CD3 μg/ml)</th>
<th>% CD80</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>1.1</td>
</tr>
<tr>
<td>DC</td>
<td>0</td>
<td>7.5</td>
</tr>
<tr>
<td>DC (FP-WT)</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td>DC (rF-CD80)</td>
<td>0</td>
<td>53</td>
</tr>
<tr>
<td>DC (rF-CD80)</td>
<td>10</td>
<td>87</td>
</tr>
</tbody>
</table>

*CD4+ T cells from B7dKO mice were purified using magnetic beads. CD4+ cells (5 × 10^7/ml) were cultured with DCs (1 × 10^7/ml) or DCs infected with either FP-WT or rF-CD80 (as described in Materials and Methods) in the presence or absence of anti-CD3 as signal 1. Two-color FACS analysis for CD80 and CD4+ cells was performed 24 h later. The data are representative of two independent experiments.*
Table II.  Analysis of CD80 acquisition by T cells from CD28+ vs CD28 knockout mice

<table>
<thead>
<tr>
<th>APC</th>
<th>Signal 1 (anti-CD3 μg/ml)</th>
<th>Source of CD4+ T Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>C57BL/6 8.0 CD28KO 1.8</td>
</tr>
<tr>
<td>MC38/CD80</td>
<td>10.0</td>
<td>C57BL/6 8.0 CD28KO 1.4</td>
</tr>
</tbody>
</table>

* CD4+ T cells were prepared from C57BL/6 mice or CD28KO mice using magnetic beads. CD4+ cells (5 × 10^⁶/ml) were cultured with 1 × 10^⁷/ml MC38 carcinoma cells that had been transduced with CD80 (MC38/CD80) in the absence or presence of anti-CD3 for 24 h and analyzed with two-color FACS analysis for CD4 and CD80 expression. The data are representative of two independent experiments.

expressed on cell surfaces (Fig. 2A). To further demonstrate cell surface expression of CD80, these cells were double-stained with PE-anti-CD80. Superimposition of red PE on GFP led to the appearance of a yellow/orange color on cell membranes (Fig. 2, B–D). When PCC/TCR-Tg CD4+ cells were incubated with transfected COS cells in the presence of PCC peptide (10 μg/ml) as signal 1, T cells were shown to acquire CD80 molecules (Fig. 2, E–H). These results further demonstrate the physical acquisition of CD80 by T cells.

**Effect of cyclohexamide (CHX) on CD80 acquisition**

Recent studies have demonstrated the existence of a new homolog of the B7 family of molecules (27). This observation raises the possibility that there might be a CD80 homolog that can be endogenously up-regulated rapidly on T cells upon stimulation, and that the molecule might interact with the anti-CD80 Ab. To exclude this scenario and further rule out the de novo generation of CD80 in short-term activated T cells, CD4+ cells from PCC/TCR-Tg mice were treated with the protein synthesis inhibitor CHX (20 μg/ml) for 2 h. After CHX treatment, CD4+ cells were incubated with irradiated fibroblast cells expressing high levels of CD80 and L-Eα MHC class II molecules for 4 h and for 24 h. There were no significant differences in CD80 acquisition between the CHX-treated and the untreated CD4+ cells at either 4 h (22% for CHX-treated and 30% for untreated cells) or 24 h (99% acquisition of CD80 for both treated and untreated cells). These results further indicate that CD80 expression on T cells upon activation is indeed due to acquisition, rather than up-regulation, of CD80 or any other homolog of the B7 family.

**Analysis of naive and activated CD4+ T cells for CD80 mRNA by PCR**

To further demonstrate that CD80 is acquired by T cells upon activation, and not by the endogenous up-regulation of CD80 mRNA, PCR studies were performed (Fig. 3). Purified CD4+ cells of PCC/TCR-Tg mice were incubated with fibroblast cells expressing high levels of CD80 (Fig. 4C) and 10 μg/ml of PCC peptide as signal 1. After 24 h of incubation, CD4+ cells were carefully removed from supernatant fluid and cultured two additional times (each time recovering cells in the supernatant) to ensure that no fibroblast (an APC that expresses CD80) was contaminating the T cell population. Purity of CD4+ cells, as well as the expression of CD80, was checked by FACS analysis. The cells were 100% CD4+, and 79–80% of these cells had acquired CD80 on their surface (Fig. 4D). Simultaneously, as controls, naive PCC/TCR-Tg

**FIGURE 2.** Physical transfer of CD80 from COS cells (transfected with GFP-CD80 fusion protein) to T cells. Binding and transfer of CD80 from COS cells to CD4+ cells were visualized by fluorescence and confocal microscopy. Transfection of COS cells with GFP-CD80 vector led to cell surface expression (green) of the CD80 molecule (A) and double-staining for CD80 (using PE CD80 Ab) (B–D), demonstrating that CD80 is membrane-bound on the COS cell surface. Incubation of GFP-CD80 COS cells with PCC/TCR-Tg CD4+ cells in the presence of PCC peptide (10 μg/ml) led to physical acquisition of CD80 from COS cells (E and F). G and H, Higher magnifications of E and F, respectively.

**FIGURE 3.** Analysis of CD4+ cells for CD80 mRNA. A 517-bp fragment of CD80 cDNA was amplified by RT-PCR, as described in Materials and Methods. Lane A, DNA-molecular size standard; lane B, fibroblast transfected with plasmid expressing CD80 (positive control); lanes C and D, two different preparations from naive (unstimulated) PCC/TCR-Tg CD4+ cells; lane E, PCC/TCR-Tg CD4+ cells stimulated for 24 h in the presence of APCs (fibroblasts expressing CD80) and 10 μg/ml of PCC peptide that had acquired CD80 (see Fig. 4D). These cells were depleted of APCs as described in Materials and Methods.
CD4+ cells were also purified from PCC/TCR-Tg animals and were checked for CD80 expression by FACS. These cells had very little or no CD80 on their surface. Using RT-PCR, we then analyzed the expression of CD80 mRNA on unstimulated PCC/TCR-Tg CD4+ cells or on CD4+ cells that were stimulated for 24 h. As shown in Fig. 3, a 517-bp CD80 fragment could readily be amplified from the APC fibroblast cell line that expresses CD80 (positive control, lane B). However, CD80 could not be amplified from two different preparations of unstimulated CD4+ cells (Fig. 3, lanes C and D) or CD4+ cells that were stimulated for 24 h (Fig. 3, lane E). β-actin expression demonstrated that equal amounts of RNA were added in each PCR. To establish the sensitivity of detection of CD80 in PCR, a template dilution experiment was performed on a CD80-transfected fibroblast cell line. cDNA encoding CD80 was synthesized using 2 μg of RNA from transfected fibroblasts; serial dilutions of cDNA (1:10 to 1:320-fold dilutions) were used as a template for PCR amplification. Analysis of the product on an agarose gel revealed that the DNA bands could not be detected when the template was diluted >1:160-fold, which is ≤1 ng of RNA. Therefore, we can conclude that the T cells that have been activated and acquired CD80 express ≤1 ng of CD80 RNA. These results illustrate that upon activation of CD4+ cells, there is no up-regulation of detectable levels of mRNA for CD80 at the 24-h time point; a 24-h time point was used in the above studies to detect the acquisition of CD80.

**Acquisition of CD80 by T cells is proportional to CD80 expression on APCs**

Studies were undertaken to investigate whether increased density of CD80 on APCs might further stabilize the interaction and, therefore, lead to more acquisition of CD80 by CD28 on T cells. To address this question, purified CD4+ cells of PCC/TCR-Tg mice were incubated for 24 h with fibroblast cells expressing either relatively low levels of CD80 (Fig. 4A) or high levels of CD80 (Fig. 4C) and PCC peptide (10 μg/ml) as signal 1. CD80 acquisition on T cells was proportional to CD80 expression by APCs. Although CD4+ cells stimulated with fibroblast cells expressing a low level of CD80 acquired much less CD80 on their surface (26%, Fig. 4B); 79% of CD4+ cells that were incubated with fibroblast cells expressing a high level of CD80 acquired CD80 (Fig. 4D).

To further determine whether this level of CD80 on APCs influenced CD80 acquisition by T cells, purified CD4+ cells from PCC/TCR-Tg mice were incubated in the presence of PCC peptide (10 μg/ml) with either uninfected or rF-CD80-infected DCs. Normal DCs express very high levels of CD80 (75%); DCs infected with rF-CD80 showed 90% CD80 expression. Incubation of CD4+ cells with uninfected DCs led to 35% of CD4+ cells acquiring CD80 on their surface. Moreover, CD4+ cells incubated with rF-CD80 DCs obtained markedly higher levels of CD80 (81%) on their surface.

Studies were then conducted to investigate T cell selectivity in the acquisition of CD80 molecules; toward this goal, the fate of another surface marker, CD40, on APCs was investigated in terms of acquisition by T cells. CD4+ cells were incubated with either normal DCs or DCs infected with FP-WT or rF-CD80 in the presence or absence of various concentrations of PCC peptide as signal 1. FACS analysis was performed on gated T cells, based on size and cell surface markers for T cells. An average of 30–40% of DCs express CD40 on their surface, whereas only 3–4% of T cells express CD40. Incubation of CD4+ cells with APCs in the presence of different concentrations of signal 1 PCC peptide led to little or no increase in the acquisition of CD40 by CD4+ cells, which remained 3–4% positive for CD40. These results further demonstrate that CD80 expression on T cells is not due to contaminating APCs, and that CD4+ cells show selectivity in the acquisition of molecules from APCs.

To further investigate the selectivity in CD80 acquisition, naive CD4+ cells from PCC/TCR-Tg mice were incubated with a fibroblast cell line expressing high levels of CD80 and PCC peptide (10 μg/ml) as signal 1 for 24 h (as described previously) in the presence of CD80+ cells from non-Tg mice. As shown in Fig. 4E, non-Tg CD80+ cells acquired relatively little CD80 (18%) above their 6–7% normal level of expression, whereas PCC/TCR-Tg CD4+ cells acquired high levels of CD80 (77%, Fig. 4F). These results further demonstrate that CD80 acquisition by T cells is specific and not due to random acquisition or incorporation.

**CD80 acquisition by T cells is directly related to the strength of signal 1**

Naive CD4+ cells from PCC/TCR-Tg mice were incubated with fibroblast cells expressing low and high levels of CD80 in the presence of various concentrations of PCC peptide to investigate the effect of the concentration of signal 1 on T cell acquisition of CD80. Acquisition of CD80 by CD4+ cells upon stimulation with fibroblast cells expressing a low level of CD80 was shown at the high level of signal 1 (Fig. 5A). When PCC/TCR-Tg CD4+ cells were stimulated with fibroblast cells expressing high levels of CD80, a direct relationship between the strength of signal 1 and the acquisition of CD80 by T cells could be more clearly defined (Fig. 5B).
5B). These results further demonstrate that the acquisition of CD80 by T cells can be influenced by the level of signal 1.

Differential acquisition of CD80 by naive and effector/memory CD4\(^+\) T cells

To investigate the biological consequence of CD80 acquisition by effector/memory T cells, effector/memory CD4\(^+\) cells were generated from naive CD4\(^+\) cells, as described above, and then cultured with fibroblasts expressing high levels of CD80 as APCs in the presence of various concentrations of PCC peptide for 24 h. Before incubation with APCs, 8–9% of effector T cells were positive for CD80 expression, as compared with 1–2% of naive T cells (data not shown). When effector/memory CD4\(^+\) cells were cultured with fibroblasts expressing high levels of CD80 as APCs, ~40% of the cells acquired CD80. This is in contrast to the relatively low percent CD80 acquisition by naive CD4\(^+\) cells when cultured with the same APCs expressing high levels of CD80 (Fig. 6). Based on our observations, the probable reason for this difference between effector/memory and naive T cell acquisition is that effector/memory T cells express higher levels of CD80 ligand, than naive T cells. Indeed, the effector/memory CD4\(^+\) cells from PCC/TCR-Tg mice used here expressed much higher levels of CD80 than naive T cells from these mice (70% for memory vs 32% for naive). When both naive and effector/memory CD4\(^+\) cells were cultured with APCs (expressing high amounts of CD80) and various concentrations of peptide, the effector/memory cells always acquired more CD80 at a given peptide concentration (Fig. 6). At a peptide concentration of 0.1 \(\mu\)g/ml, ~90% of naive T cells acquired CD80; no cell death was observed in these T cells.

Acquisition of CD80 by memory cells results in apoptosis

To determine whether the cell death in effector/memory T cell populations (as shown in Fig. 6) was due to apoptosis, effector/memory CD4\(^+\) T cells were generated by activating naive cells with PCC peptide for 3 days and then restimulating them with IL-2 for 4 days. These effector/memory cells were then divided into two groups. Cells in the first group were cultured alone with various concentrations of PCC peptide for 24 h. As shown in Fig. 7A–C, these cells demonstrated minimal apoptosis when cultured with or without peptide. The second group consisted of effector/memory T cells that were cultured with APCs (fibroblasts expressing high amounts of CD80) in the presence of 0.001 \(\mu\)g/ml of PCC peptide for 24 h. Approximately 80% of these T cells acquired CD80 (as shown in Fig. 6), and the T cells were then separated from the fibroblast APCs by magnetic beads and cultured further in the presence of various concentrations of PCC peptide (i.e., 0, 0.001, or 0.1 \(\mu\)g/ml) for 24 h. Upon acquisition of CD80, 34% of these effector/memory T cells underwent spontaneous apoptosis (Fig. 7D) in the absence of exogenous peptide. Although the addition of...
CD4+ T cells treated with anti-CD80 Ab demonstrated a meaningful decrease in their apoptosis (46%, Fig. 8C). Based on these results, we propose that CD80 acquisition can in part lead to apoptosis of effector/memory cells and the level of apoptosis is enhanced with increased levels of signal 1.

**CD80 acquisition can lead to Ag presentation by T cells**

Studies were then conducted to determine the biologic consequence that results when naive T cells acquire CD80. We asked specifically whether T cells that have acquired CD80 can now function as APCs to naive CD4+ T cells. To this end, IL-2 production was measured as an indicator of T cell activation. However, one cannot simply measure IL-2 levels in the supernatant, because IL-2 can be produced not only from T cells functioning as APCs but also from T cells receiving the costimulatory signal, if any. We took advantage of an IL-2-GFPki mouse (28) in which the endogenous IL-2 promoter (of the producer cell) fluorescence from GFP expression driven by the endogenous IL-2 promoter.

**PCC/TCR-Tg CD4+ cells that have acquired CD80 were prepared in the manner described above. Fibroblasts were used as APCs of choice to minimize the possibility of contamination of CD4+ cells that had acquired CD80 from professional APCs because they can easily be separated from T cells by magnetic bead separation. Isolated CD4+ cells (2–3% expressing CD80 spontaneously) were irradiated and used as control APCs. In the absence of PCC peptide, these control APCs were unable to activate the responding T cells (Fig. 9A); in the presence of PCC peptide (10 μg/ml), they could activate 16% of PCC/TCR-Tg/IL-2-GFPki APCs.**

![FIGURE 7](Image 51x556 to 278x734)

**FIGURE 7.** Analysis of apoptosis in effector/memory CD4+ T cells before and after acquisition of CD80. Naive CD4+ cells from PCC/TCR-Tg mice were cultured with 1 μg/ml of PCC peptide for 3 days and then rested with IL-2 (5 μg/ml) for 4 days to generate the effector/memory T cells. These cells were divided into two groups. Cells in the first group were cultured overnight with various concentrations of peptide (i.e., 0, 0.001, or 0.1 μg/ml) (A–C, respectively). Cells in the second group were first cultured with fibroblasts (which express high levels of CD80) for 24 h to acquire CD80 and were then separated from APCs as described in Materials and Methods. The T cells were then cultured alone in the presence of various concentrations of peptide for 24 h (0, 0.001, or 0.1 μg/ml) (D–F, respectively). All of the above cells (in groups I and II) were analyzed for apoptosis using the TUNEL assay. Percent apoptosis is given in each panel.

![FIGURE 8](Image 307x197 to 537x384)

**FIGURE 8.** CD80 acquisition can lead to apoptosis of effector/memory CD4+ T cells. Cells were cultured with fibroblasts for 24 h to acquire CD80 and were then separated from APCs as described in Materials and Methods. The CD4+ cells were then treated with either no Ab (A), isotype control Ab (hamster IgG) (B), or anti-CD80 Ab (10 μg/ml) (C) for 30 min and replated with 1 μg/ml of PCC peptide for 24 h. Cells were analyzed for apoptosis using the TUNEL assay. Percent apoptosis is given in each panel.

0.001 μg/ml PCC peptide had minimal effect (Fig. 7E), the addition of 0.1 μg/ml of PCC clearly augmented the occurrence of apoptosis of CD4+ T cells (Fig. 7F). To further ascertain that the acquisition of CD80 caused the effector/memory cells apoptotic death, CD80 Ab was included in the assay, with an even higher concentration (1 μg/ml) of peptide as signal 1. As seen in Fig. 8A and B, isotype control Ab showed no inhibitory effects on the apoptotic death of these CD4+ T cells, whereas effector/memory

**FIGURE 9.** T cells that have acquired CD80 can themselves become APCs. Naive CD4+ cells from PCC/TCR-Tg mice were purified, irradiated (as APCs), and cultured with CD4+ cells from PCC/TCR-Tg/IL-2-GFPki mice as effector cells for 24 h in the absence of PCC peptide (A) or presence of 10 μg/ml of PCC peptide (B). Furthermore, PCC/TCR-Tg CD4+ cells that had acquired CD80 from a fibroblast cell line expressing high levels of CD80 were purified, irradiated, and used as APCs in culture with PCC/TCR-Tg/IL-2-GFPki CD4+ cells as effectors. C and D, Activation of the effector cells (PCC/TCR-Tg/IL-2-GFPki) in the absence of peptide (C) and in the presence of 10 μg/ml of PCC peptide (D). Moreover, PCC/TCR-Tg CD4+ cells that acquired CD80 were treated with either 10 μg/ml of isotype-control Ab (E) or purified anti-CD80 Ab (F) for 30 min on ice, irradiated, and then incubated with PCC/TCR-Tg/IL-2-GFPki CD4+ cells in the presence of peptide (10 μg/ml) for 24 h. Two-color FACS analysis was performed to study the presence of activated PCC/TCR-Tg/IL-2-GFPki CD4+ cells. The data represent two different experiments.
CD4+ cells, possibly due to the spontaneous expression of CD80 (Fig. 9B). In contrast, CD4+ cells that had acquired CD80 from fibroblasts were much more potent as stimulators of PCC/TCR-Tg/IL-2-GFPAb cells to produce IL-2 at 24 h in the absence (Fig. 9C) or presence (Fig. 9D) of peptide. The possible reason for this will be discussed below. To further demonstrate that CD80 acquisition allows naive CD4+ cells to become APCs, anti-CD80 Ab was included in the assay. As seen in Fig. 9F, APCs treated with anti-CD80 Ab led to a marked decrease of GFP-IL-2 CD4+ cells becoming activated (Fig. 9F), when compared with APCs treated with control Ab (Fig. 9E).

Discussion

In this report, we demonstrate for the first time the acquisition of the CD80 costimulatory molecule by naive CD4+ T cells using syngeneic APCs. Several studies were conducted to rule out the endogenous expression of CD80 by T cells. When T cells from B7dKO mice were incubated with either a B cell line or DCs in the presence of signal 1, they acquired CD80 on their cell surface (Fig. 1; Table I). Because the CD80 and CD86 genes are deleted in B7dKO mice, neither one of these molecules can be expressed (26). In these studies, we also used normal DCs and a B cell line as APCs to eliminate the possibility that CD80 acquisition might be due to some abnormal phenomenon on vector-transfected APC membranes. The PCR studies (Fig. 2) and the studies with CHX-treated T cells further demonstrated that the expression of CD80 on T cells is not de novo. In view of the recent discovery of B7h (a new homolog of CD80 and CD86), these results also ruled out the possibility of interaction between anti-CD80 Ab and this homolog. The confocal microscopy studies using GFP/CD80 fusion protein further strengthened the notion that CD80 molecules are actually acquired upon T cell/APC interaction (Fig. 2). Moreover, we demonstrated here that the level of acquisition by T cells is related to the strength of signal 1 (Fig. 5).

It has been demonstrated that shortly after T cell/APC interaction, immunological synapses or supramolecular activation clusters form at the contact site (29). Formation of these immunological synapses is associated not only with TCRs and MHC molecules, but also with various costimulatory molecules such as LFA/ICAM, CD2/CD48, and CD28/B7 receptor ligands (30–32). Wade et al. (33) have demonstrated that truncation of the intracytoplasmic tail of MHC molecules enhances lateral movement of these complexes but decreases immunogenicity. Presentation of MHC/peptide complexes in a cross-linked form can lead to overt T cell activation (33). It has been suggested that the accumulation and interaction of costimulatory molecules with their receptors and, perhaps, cytoskeletal proteins may promote the stabilization of TCR/MHC interactions. In addition, costimulatory molecules at the early stages of T cell activation may function by enhancing TCR cross-linking, therefore intensifying signal 1. In light of the above-mentioned studies, it is possible that increased CD80 expression on APCs, along with increased signal 1 in our models, may stabilize TCR/MHC interactions, contribute to the overall avidity of T cell/APC interactions, and thus lead to the increased transfer of CD80 to T cells. The data reported here on CD80 acquisition by CD28−/− cells support the hypothesis by Hwang et al. (21) that a high enough affinity for adhesion between CD28 and CD80 may actually result in the transfer of CD80 molecules from APCs to T cells.

Previous studies have shown that varying the strength of costimulation dramatically reduces the Ag dose required for T cell activation (34). Our observations indicate that increasing the density of CD80 on APCs may facilitate a strong interaction with CD28, which would lead to stabilization of the T cell/APC interaction and, therefore, acquisition of higher levels of costimulatory molecules in the presence of less signal 1.

Several groups have previously reported the expression of CD80 on T cell clones as well as on T cells from patients with autoimmune diseases or HIV infection (6–13). Interestingly, there has been no study to address the up-regulation of CD80 message in T cells. T cells in these studies have always been kept in the presence of APCs either in vitro or in vivo; therefore, it is reasonable to infer that reported CD80 expression on T cells in these studies might have actually been due to acquisition, rather than up-regulation of expression, of CD80. The studies reported here demonstrate that at the time of acquisition of CD80 by CD4+ cells (24 h), no CD80 mRNA could be detected by PCR (Fig. 3).

Although two different biological consequences of CD80 acquisition by T cells are demonstrated here, this phenomenon will most likely be the subject of numerous investigations in the future. During an initial encounter with Ag, naive Ag-specific lymphocytes proliferate and differentiate to become activated effector/memory T cells. The current dogma states that most of these activated effector T cells die after a brief life span (35–37). Mechanisms underlying the regulated expansion of effector/memory T cells are not well understood. However, two of the findings reported here may indicate negative regulatory consequences upon acquisition of CD80 by memory cells: 1) effector/memory T cells can acquire significantly higher levels of CD80 in the presence of low levels of signal 1; and 2) these cells undergo apoptosis upon acquisition of CD80 and increased levels of signal 1. The apoptosis observed in the results of Figs. 7 and 8 is most likely not due to a consequence of the way cells were handled because the apoptosis was dependent on the amount of peptide present (including no apoptosis with no peptide), and all cells were handled in the same way. Also, it should be mentioned that our data do not exclude the possibility that effector/memory CD4+ cells could also acquire the MHC/peptide complex; therefore, these MHC complexes might play a role in the apoptosis of these cells. It has been demonstrated that activated T cells can process and present Ags to other T cells in vitro (38). The studies reported here using CD4+ cells from PCC/TCR-Tg mice that have acquired CD80 show not only that these cells expressed MHC class II molecules, but also that they were able to present soluble PCC peptide to T cells from PCC/TCR-Tg/IL-2-GFPAb mice more efficiently than CD4+ cells from PCC/TCR-Tg mice that had not acquired CD80; this led to activation and production of IL-2. Interestingly, the CD4+ cells that had acquired CD80 were able to activate the PCC/TCR-Tg/IL-2-GFPAb CD4+ cells without adding additional peptide. One possible explanation of this is that, because these CD4+ cells had been incubated with fibroblast cells in the presence of peptide and had up-regulated their MHC class II molecules (data not shown), these T cells actually also acquired peptide-MHC and were able to activate PCC/TCR-Tg/IL-2-GFPAb CD4+ cells in the absence of the addition of exogenous PCC peptide. These results suggest that activated T cells that have acquired CD80 molecules have all the characteristics of professional APCs. However, these functions are not expressed constitutively; instead, they occur only after T cell activation, when class II synthesis is up-regulated and T cells have acquired the CD80 ligand. Studies are now in progress investigating whether the APC function of CD4+ cells that had acquired CD80 as reported here is dependent on other acquired molecules (data not shown). There are at least two possible reasons why T cells become APCs in vivo. One potential advantage is that activated T cells...
would deliver both signals 1 and 2 to other T cells, thus amplifying an immune response; the other is that T cells could deliver only signal 2 to T cells that have received signal 1 from nonprofessional APCs (38). Consequently, although the initiation of the T cell response is dependent on professional APCs, it is possible that T cells may costimulate each other by acquiring CD80 under certain conditions (e.g., crowding of many T cells around the same DC). This would allow the APCs to interact further with other T cells. This mechanism of “T cell to T cell” costimulation could facilitate the regulation of the immune response.

In conclusion, the studies reported here demonstrate for the first time that naive CD4+ cells acquire CD80 upon activation by syngeneic APCs. This acquisition was shown to be directly related to both the strength of signal 1 and the level of signal 2 on APCs. Moreover, we demonstrate that CD80 acquisition is CD28 mediated. The specificity of the process of CD80 acquisition was demonstrated using both CD80 and CD28KO mice. Moreover, the acquisition of high levels of CD80 by effector/memory CD4+ cells and the resulting increased apoptosis of these cells together indicate a negative regulatory function. In contrast, when naive T cells acquire CD80, these cells themselves can act as more efficient APCs. Therefore, CD80 acquisition by T cells might play an important role in activating and/or regulating the immune response. The specific consequences of CD80 acquisition by T cells in the regulation of the immune response to pathogens, in anti-tumor responses, and in diseases of the immune system will require further investigation.

Acknowledgments

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References