Priming in the Presence of IL-10 Results in Direct Enhancement of CD8+ T Cell Primary Responses and Inhibition of Secondary Responses

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Priming in the Presence of IL-10 Results in Direct Enhancement of CD8\(^+\) T Cell Primary Responses and Inhibition of Secondary Responses\(^1\)

Silvia S. Kang and Paul M. Allen\(^2\)

Although IL-10 acts as an inhibitory cytokine for APC and CD4\(^+\) T cell function, its effects on CD8\(^+\) T cells are unclear. Additionally, little is known about whether initial priming in the presence of IL-10 can have long-lasting effects and influence subsequent CD8\(^+\) T cell responses that occur in the absence of the cytokine. In the present study, we clarified the role of IL-10 during primary responses and examined whether exposure to IL-10 during initial priming of CD8\(^+\) T cells impacted secondary responses. To determine the effect of IL-10 on Ag-specific T cell responses, peptide-pulsed IL-10R2\(^{-/-}\) splenic dendritic cells were used to prime T cells from OT-I CD8\(^+\) TCR transgenic mice. During the primary response, the presence of IL-10 resulted in enhancement of CD8\(^+\) T cell numbers without detectable alterations in the kinetics or percentage of cells that underwent proliferation. A modest increase in survival, not attributable to Bcl-2 or Bcl-x\(_L\), was also observed with IL-10 treatment. Other parameters of CD8\(^+\) T cell function, including IL-2, IFN-\(\gamma\), TNF-\(\alpha\), and granzyme production, were unaltered. In contrast, initial exposure to IL-10 during the primary response resulted in decreased OT-I expansion during secondary stimulation. This was accompanied by lowered IL-2 levels and reduced percentages of proliferating BrdU\(^+\) cells and OT-I cells that were CD25\(^{hi}\), IFN-\(\gamma\), TNF-\(\alpha\), and granzyme production were unaltered. These data suggest that initial exposure of CD8\(^+\) T cells to IL-10 may be temporarily stimulatory; however, programming of the cells may be altered, resulting in diminished overall responses. The Journal of Immunology, 2005, 174: 5382–5389.

Interleukin-10 is a cytokine produced by various cell types, including T cells, B cells, thymocytes, keratinocytes, and macrophages (1). Its ability to modulate both macrophages and dendritic cells (DCs)\(^3\) in an inhibitory manner contributes to its anti-inflammatory properties. For example, decreases in the production of cytokines such as IL-12, IL-1\(\alpha\)\(\beta\), IL-6, IL-8, and TNF-\(\alpha\) (2–5) and chemokines including MIP-1\(\alpha\), MIP-3\(\alpha\)\(\beta\), MIP-2, and MCP-1 (6–8) by activated macrophages and monocytes have been demonstrated in the presence of IL-10. Additionally, IL-10 has been shown to diminish surface expression of MHC class II and costimulatory molecules such as CD80 and CD86 on monocytes and DCs, resulting in their decreased capacity to stimulate T cells (2, 9–15). In contrast, IL-10 has been shown to enhance B cell survival, proliferation, and differentiation (16–19), demonstrating its potential to also act in a stimulatory fashion.

The direct effects of IL-10 on T cells, in the absence of the indirect inhibitory effects mediated via the APC, are less clearly defined. In bulk T cell cultures, IL-10 has been shown to alter T cell fates in both a positive and negative fashion through its ability to increase survival of T cells (20–22) as well as to diminish proliferation and IFN-\(\gamma\) production (23–25). Studies using CD4\(^+\) T cell clones or T cells enriched from human or murine sources to examine the direct effects of IL-10 on T cells reveal a mainly inhibitory role for IL-10 on CD4\(^+\) T cells. For example, the presence of IL-10 has been demonstrated to decrease the ability of human CD4\(^+\) T cells and T cell clones to proliferate to various stimuli and to decrease production of cytokines such as IL-2 and IFN-\(\gamma\) by sorted CD4\(^+\) T cell murine cells as well as human and mouse T cell clones (23, 25–28). In contrast, the direct effects of IL-10 on purified murine and human CD8\(^+\) T cell populations are mixed with both enhancement and inhibition being reported. In the presence of IL-10, increases in CD8\(^+\) T cell proliferation have been observed in response to stimulation with the combination of IL-2 and either IL-4, allogeneic cells, CD3, or Con A (12, 29–32) in primary human and mouse CD8\(^+\) T cells or human CD8\(^+\) T cell clones. IL-10 treatment has also been associated with the enhancement of cytotoxicity and expansion of cytolytic cells generated from sorted mouse CD8\(^+\) T cells through Con A and IL-2 stimulation (30). However, decreases in IFN-\(\gamma\) production, cytotoxicity by a murine CD8\(^+\) T cell clone (28), and PHA-driven proliferation (23, 33) of human CD8\(^+\) T cells and T cell clones have also been shown in the presence of IL-10, suggesting an inhibitory role for this cytokine. In addition to the conflicting nature of the effects of IL-10 on CD8\(^+\) T cells, little is known about whether the presence of IL-10 during initial stimulation alters CD8\(^+\) T cell responses to secondary Ag stimulation.

In the present study, we sought to examine the role of the direct effects of IL-10 on CD8\(^+\) T cell function in response to Ag-specific stimulation, rather than polyclonal activation, to clarify the role of IL-10 on multiple aspects of CD8\(^+\) T cell function and to investigate whether initial priming in the presence of IL-10 would result in aberrant secondary responses. We used a unique approach to limit the effects of IL-10 to the CD8\(^+\) T cell population while priming with a physiologically relevant APC, by using peptide-pulsed IL-10R2\(^{-/-}\) splenic DCs to stimulate SIINFEKL (OVA (257–264))-specific OT-I CD8\(^+\) T cells. During initial stimulation,
IL-10 increased CD8+ T cell numbers with no major alterations seen in the kinetics of proliferation or in cytokine and granzyme production. In contrast, initial priming in the presence of IL-10 resulted in a decreased expansion of CD8+ T cells to secondary Ag exposure, even in the absence of IL-10, suggesting that IL-10 negatively influences the programming of CD8+ T cell effector function during priming.

Materials and Methods

Mice

Female or male OT-I CD8+ TCR transgenic mice (The Jackson Laboratory) and CRFB4-deficient mice (34) (Genentech) on the C57BL/6 background were used between 6 and 12 wk of age for this study. The CRFB4+/− mice lack the CRF2-4 (IL-10R2) receptor protein (referred to as IL-10R2−/−) in lymphoid tissues, and these animals do not display overt signs of illness during the time frame in which they were used. OT-I mice express a Vα2/Vβ8 transgenic TCR specific for the OVA (257–264)-derived SIINFEKL peptide in the context of H-2Kd (35). All mice were bred and housed in a specific pathogen-free barrier facility at Washington University.

Peptides

SIINFEKL peptide used in this study was generated by standard F-moc chemistry using a multiple peptide synthesizer (Symphony/Multiplex; Protein Technologies). Following synthesis, the peptide was HPLC purified and underwent mass spectrometry analysis (Washington University Mass Spectrometry Resource).

Purified OT-I cell preparation

Single cell suspensions from murine spleens were made using mechanical disruption with glass slides in HBSS and filtered using a 70-μm nylon cell strainer. Cell suspensions were counted, centrifuged, and resuspended in Dulbecco’s Solution A Ca2+/Mg2+-free PBS (Washington University Medical School Tissue Culture Support Center) supplemented with 2% bovine albumin (Sigma-Aldrich). Resuspended cells were incubated with CD8+ microbeads (Miltenyi Biotec) for 15 min at 4°C, washed, and passed over two LS+ columns (Miltenyi Biotec), according to manufacturer’s instructions. For each experiment, cells were split post positive selection, and a small aliquot of cells was taken for flow cytometric analysis to determine the purity of the cells. CD8+ purity generally ranged from 97 to 99% with the use of CD8+CD90 double-positive cells, falling in the range of 96–97% of the total cells. Cells were cultured in RPMI 1640 supplemented with 10% FCS (HyClone), 2 mM Glutamax (Invitrogen Life Technologies), 0.5 μM 2-ME (Sigma-Aldrich), and 50 μg/ml gentamicin (Invitrogen Life Technologies). Purified OT-I cells were used in all experiments.

DC preparation

For preparation of DCs, a syringe and 70-μm nylon cell strainer (Fisher Scientific) were used to mechanically disrupt the spleen into single cell suspensions. Cells were incubated with 10 μg/ml DNase I (Sigma-Aldrich) and 0.55 Wuensch units collagenase (Liberase Blendzyme 3; Roche) for 30 min at 37°C, with washing occurring every 10 min. An additional aliquot of Liberase was added, followed by another 30-min incubation. Cells were washed twice with medium containing 10% FCS (HyClone) and filtered before a 15-min room temperature (RT) incubation in selection buffer containing 10% FCS, 1 mM EDTA (Sigma-Aldrich), and Dulbecco’s Solution A Ca2+/Mg2+-free PBS (Washington University Medical School Tissue Culture Support Center). Splenocytes were centrifuged and resuspended in selection buffer containing EDTA. DCs were then purified by magnetic bead separation with CD11c microbeads (Miltenyi Biotec). Purity of CD11c+ cells generally ranged from 85 to 91%. DCs were pulsed with 1 μM SIINFEKL peptide for 1 h at 37°C. Cells were washed three times, counted, and normalized for CD11c+ cells before use. Purified 1 μM SIINFEKL-pulsed IL-10R2−/− splenic DCs were used in every experiment to stimulate OT-I cells. To ensure that the IL-10R2−/− splenic DCs used in our model were able to function similarly to wild-type splenic DCs, a proliferation assay using OT-I cells was conducted. DCs derived from IL-10R2−/− and wild-type C57BL/6 mice were comparable in their ability to stimulate OT-I cells as measured by [3H]Thymidine incorporation (data not shown). For every experiment in this study, we used purified OT-I cells stimulated with purified SIINFEKL-pulsed IL-10R2−/− splenic DCs at a DC:OT-I ratio of 1:25.

Flow cytometric analysis

Cells were surface stained at 4°C for 10–15 min with Abs against Vα2 FITC, CD8 CyChrome, CD69 FITC, CD25 PE, and CD62L FITC directly conjugated Abs (BD Pharmingen). Cells were analyzed by flow cytometry on a FACSscan (BD Biosciences) using CellQuest analysis software. A total of 5,000–10,000 lymphocyte gated events was collected. For annexin V-PE (BD Pharmingen) staining, cells were resuspended in binding buffer (10 nM HEPES, 140 mM NaCl, and 5 mM CaCl2, pH 7.4) with 7-amino-actinomycin D (7-AAD) (Sigma-Aldrich) and stained for 15 min at RT before immediate analysis.

Intracellular Bcl-2 staining

OT-I cells were harvested and surface stained with Abs to Vα2 FITC and CD8 CyChrome for 10 min at 4°C before fixation with 1% paraformaldehyde at RT for 10 min. Cells were washed with 0.03% saponin/2% FCS/PBS, followed by anti-Bcl-2 or an isotype control Ab (Ab (BD Pharmingen) in 0.3% saponin/20% FCS/PBS for 30 min at 4°C. Two washes in 0.03% saponin/2% FCS/PBS were conducted before resuspension in 2% BSA/PBS/azide. A total of 5,000 lymphocyte-gated events was collected and analyzed by flow cytometry.

Intracellular cytokine staining

Cells were harvested, centrifuged, and then resuspended in RPMI 1640 supplemented with 10% FCS (HyClone), 2 mM Glutamax (Invitrogen Life Technologies), 0.5 μM 2-ME (Sigma-Aldrich), and 50 μg/ml gentamicin (Invitrogen Life Technologies) containing 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) and incubated for 4 h at 37°C at the indicated time points. Brefeldin A (Sigma-Aldrich) was added during the last 2 h of culture at 10 μg/ml. Cells were stained with CD8 CyChrome for 10 min at 4°C. Stained cells were fixed in 2% paraformaldehyde for 20 min at RT before permeabilization with 0.05% saponin. Intracellular staining for the cells was conducted at RT for 30 min using anti-IFN-γ-FITC (2.5 μg/ml), anti-TNF/anti-PE (5 μg/ml) (BD Pharmingen), and anti-IL-2 PE (5 μg/ml) (Biolegend)-conjugated Abs. A total of 5,000 lymphocyte-gated cells was collected by flow cytometry. To determine background levels of cytokine staining, a set of cells was not restimulated, but was treated with brefeldin A and stained for intracellular cytokines, as was done for experimental cells.

T cell proliferation assays

Purified OT-I cells were centrifuged and resuspended at 106 cells/ml HBSS. Cells were incubated at 37°C for 5–10 min in 5 μM CFSE (Molecular Probes), washed, counted, and normalized for the percentage of OT-I CFSE-labeled cells. Cells were plated in a 48-well plate at a 1:25 DC:OT-I cell ratio, with a constant number of 1.5 × 105 OT-I cells/well. On the indicated day, cells were harvested, washed, counted, and analyzed by flow cytometry. To measure proliferation by BrdU incorporation, 10 μM BrdU (Sigma-Aldrich) was added to cell cultures during the final 30 min. Cells were harvested and fixed with 1% paraformaldehyde and 0.05% Tween 20 (Sigma-Aldrich) for 30 min at room temperature and then 30 min on ice. Afterward, cells were centrifuged and resuspended in a DNase solution (150 mM NaCl, 4.2 mM MgCl2, 10 μM HCl, and 100 U/ml DNase I (Sigma-Aldrich) for 30 min at 25°C. To stain for BrdU, anti-BrdU-FITC-conjugated Ab (eBioscience) in FACS buffer was added for 30 min at room temperature. Cells were washed twice and analyzed by flow cytometry. To test supernatants for the presence of IL-2, proliferation of an IL-2-dependent CTLL line was assessed. A total of 100 μl of supernatants was incubated with 5 × 105 CTLL cells/well in RPMI 1640 supplemented with 10% FCS (HyClone), 2 mM Glutamax (Invitrogen Life Technologies), and 50 μg/ml gentamicin (Invitrogen Life Technologies). Twenty-four hours later, cells were pulsed with 0.4 μCi/well [3H]thymidine and harvested 16–18 h later.

Restimulation of OT-I cells

OT-I cells were initially stimulated with 1 μM SIINFEKL-pulsed IL-10R2−/− DCs at a 1:25 DC:OT-I ratio in the presence or absence of 50 ng/ml murine rIL-10 (PeproTech). After 6 days of culture, the cells were harvested and washed extensively to remove any remaining IL-10. Cells were rested for 24 h in medium before secondary stimulation. At this point, both cultured cells were restimulated with freshly isolated 1 μM SIINFEKL-pulsed IL-10R2−/− DCs, again at a 1:25 DC:OT-I ratio. During restimulation, no IL-10 was added to either culture. For both the primary and secondary exposure to Ag, the number of OT-I cells added to each well was 1.5 × 105 cells.

Statistical analysis

To determine statistical significance, data were analyzed using paired Student’s t test using Microsoft Excel.
Results

**Increased CD8 T cell numbers in the presence of IL-10**

To examine the direct effects of IL-10 on CD8+ T cell responses, purified OT-I CD8+ TCR transgenic T cells, specific for SIINFEKL/H-2Kb, were stimulated in vitro using purified peptide-pulsed IL-10R2−/− splenic DCs. This provided a unique system that allowed for antigenic stimulation using a physiologically relevant APC, while restricting the effects of IL-10 to only the T cell population. Previous studies have demonstrated enhanced proliferation of CD8+ T cells to polyclonal stimuli (12, 29–32) in the presence of IL-10. To determine whether IL-10 had a direct effect on Ag-induced OT-I T cell expansion, the number of cells generated in the presence or absence of 50 ng/ml IL-10 was examined. As shown in Fig. 1A, OT-I cells cultured with IL-10 had a consistent increase in cell numbers over that seen in control cultures. Analysis of data from ≥9 separate experiments showed a mean increase in the number of IL-10-treated CD8+ T cells of 62% on day 2, 100% on day 3, 75% on day 4, and 21% on day 5 (Fig. 1A) over controls. A representative example of the difference in cell numbers seen on day 3 is shown in Fig. 1B. To ensure that the effects of IL-10 were mediated directly through the CD8+ T cell population and not through a small, yet detectable (0.85–1%) population of contaminating CD8−CD11c+ cells present in the OT-I T cell preparation, a pilot study using sorted CD8+ OT-I T cells was conducted. The OT-I cells were positively selected and then sorted to remove any CD4+, B220+, CD11b+, and CD11c+ cells. The sorted cells were 99.96% pure with only a 0.01% CD8−CD11c+ population remaining. Stimulation in the presence of IL-10 resulted in a 106% increase in cell counts on day 3, a 113% increase on day 4, and a 26% increase on day 5. These percentages are well within the typical range seen with our unsorted positively selected OT-I cells, indicating that the response is directly through the T cells and not via any contaminating cells. To assess whether the increased cell numbers were due to alterations in proliferation, CFSE-labeled OT-I cells were analyzed on days 2–4. In the presence of IL-10, the number of cell divisions remained the same as control cultures; however, higher numbers of cells were found in each division (Fig. 1C). This indicated that the increase in cell numbers with IL-10 was potentially due to a greater relative number of cells undergoing division and/or an increase in cell survival, rather than changes in the rapidity of proliferation. If there were an augmentation in the relative number of cells that underwent proliferation in the presence of IL-10, we would expect lower percentages of cells at earlier divisions and...
higher percentages at later divisions compared with untreated controls. In our system, similar percentages of cells in divisions 1–4 were seen in both conditions with slightly elevated percentages seen in IL-10-treated cultures for days 2–4 of culture. The levels of these antiapoptotic proteins did not differ between conditions (Fig. 2A and data not shown), indicating that this was not the underlying mechanism for the increased cell expansion. Annexin V staining was also conducted on day 3 of stimulation to measure the percentage of cells that were undergoing early apoptosis in each division at that time point. A significant decrease in the percentage of 7-AAD– annexin V+ cells was found in IL-10-treated cultures for cells found in the last few divisions. Additionally, a trend for decreased percentages of annexin V+ cells was found in the other divisions in the IL-10 condition (Fig. 2B). These data suggest that modest decreases in apoptosis over time may contribute to the overall enhancement of cell numbers during primary stimulation.

**Primary stimulation in the presence of IL-10 alters the activation phenotype, but not the intracellular levels of cytokines or granzymes**

To ascertain whether alterations in activation phenotype, cytokine, or granzyme expression accompanied the increase in cell numbers seen in the presence of IL-10, staining for surface activation markers and intracellular cytokine and granzyme levels was conducted. On days 2 and 3 (data not shown) and 3 (Fig. 3A), CD62L, CD69, and CD25 levels were similar in both conditions, while the percentage of cells expressing CD69 and CD25 was lower on days 4 (Fig. 3A) and 5 (data not shown) in IL-10-treated cultures. This suggested that the cultures exposed to IL-10 were less activated at later time points during primary stimulation. No differences in the patterns of additional markers including Fas, Fas ligand, CD44, CD62L, Vα2, and CD8 were seen on days 2–5 (data not shown). In the past, decreases in IL-2 production have been noted for CD4+ T cells in the presence of IL-10 (27, 38). Examination of intracellular staining of stimulated OT-I CD8+ T cells revealed no drastic alterations in the percentage of cells that were positive for IFN-γ, IL-2, or TNF-α in the presence of IL-10 on days 2 (Table I), 3 (Fig. 3B and Table I), and 4 (Table I). Using a separate CTL bioassay to assess the levels of IL-2 in the supernatants, we confirmed that cells in both conditions generated similar amounts of IL-2 (data not shown). Granzyme levels were also unaffected by IL-10 treatment (data not shown). These data suggest that IL-10 does not drastically change the ability of CD8+ T cells to commit to a normal cytokine and granzyme profile.

**Priming in the presence of IL-10 results in a decreased secondary expansion**

After investigating the role of IL-10 in the primary response, we next wanted to examine whether exposure to IL-10 during initial stimulation would affect CD8+ T cell responses to secondary antigenic stimulation. OT-I cells were primed in the presence or absence of IL-10

### Table 1. Priming in the presence of IL-10 has little effect on the percentage of cytokine-positive CD8+ T cells during the primary response

<table>
<thead>
<tr>
<th>Day</th>
<th>± 50 ng/ml IL-10</th>
<th>% IFN-γ Positive</th>
<th>% IL-2 Positive</th>
<th>% TNF-α Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>–</td>
<td>22.5 ± 4.6</td>
<td>69.1 ± 3.0</td>
<td>51.0 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>26.6 ± 5.6</td>
<td>64.2 ± 8.7</td>
<td>47.2 ± 2.6</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>33.5 ± 5.6</td>
<td>67.5 ± 2.6</td>
<td>63.5 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>41.8 ± 7.5</td>
<td>65.1 ± 3.7*</td>
<td>55.5 ± 4.5</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>52.6 ± 4.4</td>
<td>58.4 ± 7.1</td>
<td>38.9 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>53.3 ± 1.2</td>
<td>53.2 ± 6.5</td>
<td>34.1 ± 2.3</td>
</tr>
</tbody>
</table>

*The average percentage ± the SEM of cells staining positive for the presence of intracellular cytokines IFN-γ, IL-2, and TNF-α on days 2–4 is shown above. The numbers are averaged from three to five different experiments. Statistically significant differences (p ≤ 0.05), as determined by paired Student’s t test, are designated by *.

### Figures

**FIGURE 3.** Alteration of the activation phenotype, but not the percentages of IFN-γ, IL-2, or TNF-α-positive cells in the presence of IL-10. A, OT-I cells primed in the absence (filled histogram) or presence (open histogram) of 50 ng/ml IL-10 were stained with Abs to CD62L, CD69, and CD25 to examine their surface activation phenotype. Phenotypes on days 2 and 4 of stimulation are shown from one of four similar experiments. B, On days 2–4 (data for day 3 are shown), activated OT-I cells treated with line) or without (filled histogram) 50 ng/ml IL-10 were harvested and restimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 h. A subset of cells was not restimulated (dashed line; unstimulated control) as negative controls. Cells were intracellularly stained with Abs to IFN-γ, IL-2, or TNF-α, and lymphocyte-gated populations were examined by flow cytometry. Similar results were seen in all three experiments.
and were harvested on day 6. The IL-10 was removed and the cells were rested in medium for 24 h before restimulation. Analysis of the surface expression of CD69, CD25, and CD62L after the rest period revealed that the cells were CD69 negative, had lowered levels of CD25, but remained CD62L<sub>low</sub> (Fig. 4A). Although cells

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freshly isolated peptide-pulsed DCs, no IL-10 was added to the cultures. To ensure that initial priming in the presence of IL-10 did not induce endogenous IL-10 production by the OT-I T cells, preliminary experiments examining intracellular IL-10 levels were conducted during the primary response and during restimulation. Generally, <1% of the cells in IL-10-treated and untreated conditions were positive for intracellular IL-10, suggesting that there was no major generation of IL-10 by the CD8\(^{+}\) T cells during stimulation.

To investigate the effects of priming with IL-10 on the secondary response, the numbers of cells present in cultures that had initially been primed with or without IL-10 were examined at different time points on days 0–6 postrestimulation. Although both cultures demonstrated an ability to proliferate upon secondary Ag exposure, by 66 h of restimulation, CD8\(^{+}\) T cells that had been previously exposed to IL-10 did not expand to the same level as the untreated controls (Fig. 4B). This difference in cell numbers was most apparent between 90 and 114 h of culture. To determine whether the percentage of cells proliferating at each time point was altered as a result of the initial priming with IL-10, BrdU incorporation was measured. A statistically significant decrease in the percentage of BrdU\(^{+}\) cells at 48, 66, and 72 h of culture was observed in cells that were exposed to IL-10 during the primary response as compared with control cultures (Fig. 4C). This difference in the percentage of proliferating cells preceded the time points at which the greatest disparity in cell numbers occurred between control and IL-10-treated cultures.

**Initial stimulation in the presence of IL-10 decreases IL-2 accumulation and CD25 expression during secondary expansion**

To assess whether cytokine production or the activation phenotype of the cells in the secondary response was also affected by priming in the presence of IL-10, we examined IL-2 levels in the supernatants of restimulated cultures and conducted intracellular and surface staining for cytokines and activation markers. Proliferation of an IL-2-dependent CTLL cell line over an extensive time course (24, 42, 44, 66, 72, 90, 96, 114, and 120 h postrestimulation) revealed that the amount of IL-2 in cultures that had been initially primed in the presence of IL-10 was decreased in comparison with control cultures (Fig. 5A). Interestingly, although the levels of IL-2 present in the medium were altered with IL-10 treatment, the percentages of cells that stained positively for intracellular IL-2 (Table II) and the mean fluorescent intensities (data not shown) were the same. This was in contrast to the primary response in which both intracellular IL-2 staining (Table I) and CTLL supernatant analyses (data not shown) revealed similar levels of IL-2. However, it is important to note that the two assays address slightly different issues. Whereas intracellular staining examines the percentage of cells that have the ability to produce cytokines during a short period of restimulation, the CTLL assay focuses on the amount of IL-2 that has accumulated in the culture based on the production and usage of the cytokine over time. During restimulation, the proportion of cells that stained positive for IFN-\(\gamma\), TNF-\(\alpha\) (Table II), and granzyme (data not shown) was the same in both populations. Based on intracellular staining, the effects of initial stimulation in the presence of IL-10 appeared to have a minimal effect on the proportion of cells that had the capacity to generate IFN-\(\gamma\), TNF-\(\alpha\), and granzyme in the secondary response. Although the percentage of cytokine-positive cells was similar in both conditions, it is always possible that the actual amount of IFN-\(\gamma\) and TNF-\(\alpha\) released and accumulated in the supernatants may be different.

Because exposure to IL-2 up-regulates CD25, the high affinity chain of the IL-2R, resulting in increased sensitivity to IL-2, we wanted to determine whether cells exposed to IL-10 during priming also had impaired CD25 expression during secondary stimulation. Examination of CD25 levels on cells initially stimulated in the presence or absence of IL-10 at several time points during restimulation revealed a decrease in the percentage of cells expressing high levels of CD25 in IL-10-treated conditions (Fig. 5, B and C). Similar to the pattern seen with BrdU staining, the decrease in the percentages of the CD25\(^{hi}\) population primarily occurred at time points that preceded the 90- to 114-h time frame in which maximal discrepancies in cell counts were noted. Interestingly, other activation markers, including CD69 and CD44, were similar in both culture conditions (data not shown), demonstrating that other measures of activation were unaltered. Therefore, the presence of IL-10 in the primary culture led to a reduction of cell numbers during the secondary response, most likely due to impaired IL-2 production and decreased percentages of CD25\(^{hi}\) expressing cells in secondary cultures.

**Discussion**

IL-10 has been associated with decreases in resistance to infection and tumor rejection, control of organ-specific diseases such as colitis, and aspects of peripheral tolerance (1, 39–57). Its ability to negatively modulate various parameters of APC and CD4\(^{+}\) T cell function contributes to its ability to dampen the overall immune response. In contrast, studies examining the direct effect of IL-10 exposure on CD8\(^{+}\) T cells have shown either enhancement or inhibition of function (12, 23, 28–33). In the presence of IL-10, increases in proliferation, IFN-\(\gamma\) production, and cytotoxicity have been observed with stimuli such as anti-CD3 and Con A, whereas PHA-driven proliferation was decreased. Additionally, a study using tumor-specific CD8\(^{+}\) T cell clones demonstrated that cytotoxicity and IFN-\(\gamma\) production against tumor targets were decreased in a subset of clones due to their production of IL-10. Incubation of these clones with neutralizing Abs against IL-10 resulted in a regained ability to become cytolytic and lyse target cells (28). It is interesting to note that most studies demonstrating enhanced function with IL-10 treatment stimulated their cells in the presence of exogenously added IL-2, whereas studies demonstrating inhibition did not. In the present study, we assessed the direct effects of IL-10 exposure on CD8\(^{+}\) T cells during a primary response, using peptide-pulsed DCs that were unresponsive to IL-10 to provide antigenic stimulation. We also evaluated whether initial IL-10 treatment during primary stimulation affected the programming of effector functions by evaluating secondary responses generated by these cells in the absence of IL-10.

In our model system, the presence of IL-10 during Ag-driven stimulation in the primary response resulted in an increase in the number of cells that accumulated during the first 4 days of culture.
(Fig. 1). Although others have demonstrated enhancement of CD8⁺ T cell proliferation in response to various stimuli in the presence of IL-10, a caveat with these studies was that [³H]thyroidine incorporation rather than CFSE dilution was used as a read-out of proliferation (12, 29–31). This particular method does not differentiate between whether augmented proliferation resulted from increases in the number of cell divisions, the kinetics of proliferation, increases in the percentage of cells that actually divided, or, alternatively, changes in cell survival rather than proliferation. Our data illustrate that the overall increase in IL-10-treated cell numbers was not accompanied by alterations in the kinetics of proliferation or in the proportion of cells that underwent division (Fig. 1) as measured by CFSE proliferation. Additionally, a consistent trend for decreased annexin V⁺ staining of IL-10-treated cells (Fig. 2) suggested that the increased accumulation of cells seen during initial priming was partly due to an increase in viability. This would indicate that an increase in the number of cells that were alive to proliferate, combined with augmented survival, resulted in a greater number of CD8⁺ T cells observed in the presence of IL-10 during antigenic stimulation. In accordance with the altered CD8⁺ T cell survival we observed, previous work on bulk T cells or CD4⁺ T cells that were infected with para-influenza or EBV, or undergoing growth factor withdrawal (20–22, 36) demonstrated enhanced viability with IL-10 treatment. This outcome is not restricted to the T cell subset, as the presence of IL-10 has also been shown to increase the viability of activated B cells (16). In both B and T cells, the effect of IL-10 on survival has been attributed to increased levels of Bcl-2 (16, 37, 58). However, a previous study examining the effects of IL-10 on CD4⁺ T cell clones revealed a similar survival phenomenon with IL-10 treatment in the absence of any detectable variations in Bcl-2, Bcl-xL, or Fas levels (38), suggesting an alternative mechanism for survival. Our data are consistent with the latter finding in which the mechanism for increased viability did not depend upon alterations in either Bcl-2 or Bcl-xL levels (Fig. 2).

In contrast to the beneficial effects of IL-10 seen during the primary response, initial priming of CD8⁺ T cell populations in the presence of IL-10 actually results in diminished T cell expansion during secondary exposure to Ag. We observed decreased cell numbers and a lowered percentage of BrdU⁺ cells after restimulation of cultures initially exposed to IL-10 (Fig. 4). The levels of IL-2 found in cell culture supernatants were decreased in cultures initially primed with IL-10 (Fig. 5 and Table II), and this was accompanied by a decrease in the percentage of the CD25high population. Together, the diminished capacity to produce and respond to IL-10 most likely contributed to the muted expansion seen during restimulation. Therefore, while IL-10 appears to enhance the primary response by increasing cell numbers, its effects on CD8⁺ T cells are long lasting and result in a decrease in the magnitude of the secondary expansion. These data support a model in which initial exposure to IL-10 is enough to alter the programming of CD8⁺ T cells, producing aberrant secondary responses.

In recent years, several groups have examined the initial requirements needed for programming and fitness of CD8⁺ T cells (59–62). These studies culminated in the idea that initial priming of the CD8⁺ T cell activated a programmed pathway for the development of cytolytic capabilities and expansion. Once this pathway was initiated, it could be completed even upon removal of the original stimulus (59, 60, 63). Additionally, the idea of T cell fitness, defined by the ability of the cells to survive and to later proliferate to cytokines, was shown to be determined not only by the duration of stimulation, but also by the strength of signal received by the T cells during initial priming (61). Within this study, it was also shown that TGF-β was able to negatively influence the survival and fitness of T cells stimulated with anti-CD3. In contrast to TGF-β, IL-12 has been implicated as a third signal, apart from TCR engagement and costimulation, that is needed during priming of CD8⁺ T cells to achieve full effector functions (62, 64–66). The presence of IL-12 was able to augment proliferation of cultures stimulated with Ag and IL-2, and it was determined to be necessary for the development of cytolytic capabilities (66). Furthermore, to achieve full in vivo expansion of Ag-specific CD8⁺ T cells during a secondary challenge with peptide and LPS, the presence of IL-12 during the primary stimulation with Ag was required (62). Therefore, these results demonstrated that cytokine availability during early time points could influence subsequent T cell responses, adding new complexity to what dictates full differentiation programs of CD8⁺ T cells. Our report suggests that IL-10 should be included among those cytokines known to impact T cell programming during primary stimulation.

Although IL-10 is known for its ability to dampen APC function, our results illustrate that it has the potential to directly affect CD8⁺ T cell responses in both a stimulatory and inhibitory fashion. Although the presence of IL-10 results in a transient increase in cell numbers generated during primary stimulation, the same exposure to IL-10 results in a decreased secondary expansion. These data may help resolve some of the previous findings concerning the conflicting nature of IL-10 on CD8⁺ T cell responses by emphasizing the importance of the time frame in which responses to IL-10 are examined. Overall, our findings suggest a previously unappreciated mechanism by which IL-10 may inhibit CD8⁺ T cell responses. In addition to its well-characterized negative effects on APCs and CD4⁺ T cell function, IL-10 may further diminish a robust immune response through its long-lasting effects on CD8⁺ T cell function that result in decreased secondary expansions.

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Disclosures

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