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Increasing the Survival of Dendritic Cells In Vivo Does Not Replace the Requirement for CD4\(^+\) T Cell Help during Primary CD8\(^+\) T Cell Responses\(^1\)

Kate E. Matthews,* Jim S. Qin,* Jianping Yang,* Ian F. Hermans,*† Michael J. Palmowski,† Vincenzo Cerundolo,* and Franca Ronchese\(^2\)*

The survival of dendritic cells (DC) in vivo determines the duration of Ag presentation and is critical in determining the strength and magnitude of the resulting T cell response. We used a mouse model to show that Ag-loaded C57BL/6 DC (MHC class II\(^{+/+}\) (MHC II\(^{+/+}\))) that reach the lymph node survived longer than Ag-loaded MHC II\(^{-/-}\) DC, with the numbers of C57BL/6 DC being ~2.5-fold the number of the MHC II\(^{-/-}\) DC by day 4 and ~5-fold by day 7. The differential survival of DC in vivo was not affected by low doses of LPS, but in vitro pretreatment with CD40L or with high doses of LPS increased the numbers of MHC II\(^{-/-}\) DC to levels approaching those of C57BL/6 DC. Regardless of their numbers and relative survival in lymph nodes, MHC II\(^{-/-}\) DC were profoundly defective in their ability to induce CTL responses against the gp33 peptide epitope, and were unable to induce cells regulate DC survival, however, the results from these studies

Dendritic cells (DC)\(^3\) capture Ag in the periphery and transport it to lymph nodes to initiate T cell responses. DC are terminally differentiated cells and their survival in lymph nodes is limited, but is thought to be affected by signals derived from infectious agents as well as by signals from other immune cell populations (1). Effects on DC survival in turn affect both the number of cells that are presenting Ag at a given time, and the duration of Ag-specific T cell stimulation. Therefore, the survival of DC strongly influences the overall strength of signal that a T cell receives, and impacts upon the quality, magnitude, and duration of both CD4\(^+\) and CD8\(^+\) T cell responses (2–6). Experimental evidence indicates that the number of Ag-loaded DC in the lymph node correlates with the level of proliferation and cytokine secretion by T cells, and the generation of delayed-type hypersensitivity responses (7). Dysregulated DC survival has also been shown to result in chronic T cell stimulation, and the development of autoimmune symptoms (8).

The consequences of the interaction with CD4\(^+\) T cells on DC survival are complex. Several reports have suggested that CD4\(^+\) T cells regulate DC survival, however, the results from these studies are conflicting, with some suggesting that CD4\(^+\) T cells eliminate DC and others suggesting that they enhance DC survival in vivo (1, 9–11). The importance of prolonged presentation in CD8\(^+\) responses has clearly been demonstrated in vivo during HSV infection. Induction of primary CTL responses required Ag presentation for at least 4 days after infection, and when Ag presentation was abbreviated, the number of HSV-specific CD8\(^+\) T cells was reduced (12). Similarly, early elimination of injected DC loaded with an MHC class I (MHC I)-binding peptide decreased the magnitude of the induced CD8\(^+\) T cell response, without affecting development of effecter function or memory cells (4). How the effects of CD4\(^+\) T cells on DC survival contribute to CD8\(^+\) T cell proliferation, cytotoxicity, and cytokine production is unknown.

CD4\(^+\) T cell-dependent regulation of DC activation and survival have been proposed to be critical to the ability of DCs to initiate CD8\(^+\) T cell responses (13–15). DC that have been “licensed” to survive and up-regulate costimulatory signals through interaction with Ag-specific CD4\(^+\) T cells would significantly increase their opportunity to encounter rare Ag-specific CD8\(^+\) T cells, and induce their proliferation and activation. Increased DC survival would be critical to ensure that rare naive CD8\(^+\) T cells have sufficient opportunity to meet these stimulatory DC. Competing models have instead proposed that, upon interaction with CD4\(^+\) T cells, DC acquire the ability to secrete chemokines that attract naive CD8\(^+\) T cells (16) thereby facilitating contacts between multiple cell types (17). The opportunity of the DCs to form productive interactions with CD8\(^+\) T cells would therefore be maximized, without requiring increased survival.

To determine whether CD4\(^+\) T cells have a role in regulating DC survival in vivo, we compared MHC class II (MHC II\(^{-/-}\)) DC, which cannot form cognate interactions with CD4\(^+\) T cells, to MHC II\(^{+/+}\) DC. DC were labeled with fluorescent dyes and injected into mice, and their numbers in draining lymph nodes were monitored over several days. We found that prolonged DC survival in the lymph node required expression of MHC II on the DC, and that this requirement could be relieved by pretreating MHC II\(^{-/-}\) DC with CD40L or high doses of LPS.
before injection. However, improved survival did not seem to affect the activity of injected MHC II+ DC to induce CD8+ T cell responses in vivo. Therefore, CD4+ T cell help for CD8+ T cell responses does not appear to be mediated via increased survival of DC.

Materials and Methods

Mice

All mice were maintained at the Biomedical Research Unit (Wellington School of Medicine, Wellington, New Zealand) or at the Malaghan Experimental Research Facility (Victoria University, Wellington, New Zealand). Experimental procedures were approved by the relevant animal ethics committee and conducted in accordance with institutional guidelines. The C57BL/6 mice were from breeding pairs originally obtained from The Jackson Laboratory. TCR-transgenic OT-II (18) and line 318 (L318, 19) mice were provided by Prof. F. Carbone (Melbourne University, Melbourne, Australia) and Prof. H. Pircher (University of Freiburg, Freiburg, Germany), respectively. The MHC II+ B6A2F1/Ab1 (20) mice were provided by Dr. H. Bluthmann (Hoffman-La Roche, Basel, Switzerland).

In vitro culture medium, Abs, and reagents

All cultures were in complete IMDM, which consisted of IMDM supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (all obtained from Invitrogen Life Technologies) and 50 μM 2-ME (Sigma-Aldrich).

The gp33 (lymphocytic choriomeningitis virus glycoprotein fragment 33-41) KAYVYNFATM, OVA253-339 (ISQAVHAAHAEINEAGR), and UTY 246-254 (WMHHNMDL) peptides were purchased from Mimotopes.

The anti-CD40 (3/23), anti-CD44 (IM7), anti-Vα2 (B20.1), and anti-CD80 (16-10A1) mAbs were obtained from BD Pharmingen. The anti-FcγRI (2.4G2), anti-CD11c (N418), anti-MHC II (3IP), anti-CD86 (GL1), anti-Vβ8, and anti-CD43 (2.4D) Abs were purified from supernatants of B cell hybridoma lines using HyTrap protein G columns (Amersham Biosciences) and conjugated to FITC (Sigma-Aldrich) or to allophycocyanin (Prozyme).

Preparation of CD40L from the plasma membranes of NIH-3T3-transfected cells

CD40L was prepared by purifying the plasma membranes of CD40L-transfected NIH-3T3 cells (21) by mild osmotic lysis and differential centrifugation as described (22). The activity and optimal dose of CD40L were determined by titrating membranes into day 6 bone marrow DC culture wells. The plates were incubated for a further 48 h, and expression of MHC II, CD80, and CD86 on CD40L-treated cells was compared with untreated DC, and to DC that had been treated with 0.1 μg/ml LPS, by flow cytometry.

DC preparation and use for immunization

DC were prepared from the bone marrow of C57BL/6 or B6A2F1/Ab1 mice by culturing in GM-CSF and IL-4 as previously described (23). After 7 days of culture, loosely adherent cells were harvested. In some cases, DC were activated by adding 0,1 μg/ml LPS (Sigma-Aldrich) for the last 18–24 h of culture. Alternatively, DC were activated for the last 24 h of culture by treatment with CD40L, or by culture on a monolayer of CD40L-expressing NIH-3T3 cells. DC were routinely checked for purity and maturation status by assessing expression of CD11c, MHC II, CD40, CD80, and CD86 by flow cytometry.

In some experiments, DC were positively selected from the spleens of C57BL/6 or B6A2F1/Ab1 mice using anti-CD11c magnetic beads (Miltenyi Biotec) and magnetic selection. The purity of each population was over 90%.

In immunizations, DC were harvested and resuspended at 1 × 106 cells/ml. Peptide was added to the cells at a final concentration of 10 μM and the tubes were incubated at 37°C for 2 h. Cells were pelleted, washed three times in IMDM to remove excess peptide, and injected i.v. in the flank of mice in 100 μl.

Monitoring the migration and survival of DC in vivo

MHC II+ and C57BL/6 DC were labeled differentially with fluorescent dyes so that their survival in draining lymph nodes could be compared (24). One population of DC was labeled with the green fluorescent dye CFSE ( Molecular Probes) while the second population of DC was labeled with the orange dye Cell Tracker Orange (CTO; Molecular Probes). Each mouse received 1 × 106 CFSE-labeled DC and 1 × 106 CTO-labeled DC mixed in a volume of 50 μl, and injected intradermally in the volar aspect of the distal forelimb. At various times after injection, draining axillary and brachial lymph nodes were harvested and incubated for 1 h at 37°C in an enzyme mixture containing 2.4 mg/ml collagenase II (Invitrogen Life Technologies) and 1 mg/ml DNase I (Sigma-Aldrich). Cells were passed several times through an 18-g needle and syringe and then gauze before FACS staining. The number of labeled DC in lymph nodes was calculated by multiplying the percentage of labeled cells as determined by FACS by the number of cells in each sample. The relative survival of DC on day 4 was calculated using the following formula: (MHC II+/DC/C57BL/6 DC on day 4) × 100/(MHC II+/DC/C57BL/6 DC on day 1).

Immuno blotting

DC were washed, pelleted, and resuspended at 1 × 107 cells/ml in cell lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Nonidet P40, 0.9% Triton x-100, 1 mM PMSF, and 1 mM EDTA), and the equivalent of 30 μg of protein was applied to a 4–12% SDS-acrylamide gel (Invitrogen Life Technologies) for protein separation. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) and probed with anti-βc1-x1 (R&D Systems) or anti-actin 20-33 (Sigma-Aldrich) followed by goat anti-mouse Ig HRP (DakoCytomation/Med-Bio) or goat anti-rabbit IgG HRP (Santa Cruz Biotechnology), respectively. Bound Abs were revealed using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology) and quantified using Kodak Digital Science 1D software.

Adoptive transfer of naive CFSE-labeled TCR-transgenic T cells

Lymph nodes were harvested from L318 TCR-transgenic mice and made into single-cell suspensions. Cells were labeled by incubation with 5 μM CFSE in PBS for 8 min at room temperature. The dye reaction was quenched by adding excess FCS and then washing the cells three times in IMDM. Approximately 3 × 105 Vα2 Vβ8 cells were injected i.v. into the lateral tail vein of syngeneic recipient mice in a volume of 300 μl.

In vivo cytotoxicity assay

The in vivo cytotoxic activity of CD8+ T cells was assessed as previously described (25, 26). Syngeneic spleen cells were incubated at 37°C for 2 h either alone or with 10 or 100 nM gp33 peptide, and differentially labeled with CTO or 0.5 or 5 μM CFSE, respectively. The three populations of cells were combined in equal numbers and ~4 × 106 of each were injected i.v. into recipient mice. One to 3 days later, inguinal lymph nodes were harvested, digested in collagenase and DNase as described above, and analyzed by FACS. Percent-specific killing was calculated from the numbers of CFSE+ and CTO+ cells in experimental (exp) and control (nonimmunized) samples according to the following formula: 100 – (100 × [exp number CFSE+ cells/ep number CTO+ cells]/[control number CFSE+ cells/control number CTO+ cells]).

Male Ag-specific cytotoxic activity was determined by comparing a male spleen cell population, and/or a female spleen cell population loaded with 1 μM UTY peptide, to a control population of CTO-labeled female spleen cells.

Flow cytometry and tetramer staining

Cells were incubated with Abs diluted in PBS containing 2% FCS, 2 mM EDTA, and 0.01% sodium azide as previously described (27). Tetramers of H2-D6 bound to the UTY 246-254 peptide of the C57BL/6 H-Y protein were generated as previously described (28).

Peripheral blood was harvested from the tail veins of mice and the RBC were lysed with PhosphaLyse (BD Pharmingen). Cells were washed once in complete IMDM; 1 μl of tetramer diluted in 4 μl of PBS was added to each sample in a 96-well plate. Cells were incubated at 37°C for 20 min in the dark, washed, and incubated on ice for 10 min with anti-CD8-FITC and anti-CD44-allophycocyanin Abs diluted in PBS. Samples resuspended in FACS buffer were analyzed on a FACSsort using the CellQuest software (BD Biosciences). Live cells were identified by forward scatter/side scatter properties and exclusion of propidium iodide (PI; BD Pharmingen).

Results

Expression of MHC II+Ag complexes is necessary for the optimal survival of DC in the lymph node

We injected DC that had been loaded with OVA253-339 peptide or left untreated into syngenic C57BL/6 mice, or into OT-II TCR-transgenic mice where the majority of CD4+ T cells are specific for I-Aκ and OVA253-339. The two populations of DC were labeled...
were injected into C57BL/6 recipients. DC injection. Gates show C57BL/6 and MHC II and CFSE, respectively.

In contrast, there was a sharp decline in the numbers of DC only after injection. Bars show mean and SEM of six samples per group.

A further decrease was observed at day 7 (data not shown). These data suggest that the optimal survival of DC in vivo is dependent on the cognate interaction with CD4+ T cells, and that in C57BL/6 mice presentation of FCS-derived Ag, as in our cultured DC, is sufficient to support this interaction.

If interaction with FCS-specific CD4+ T cells is necessary for the optimal survival of DC in vivo, MHC II+/- DC, that cannot form cognate interactions with CD4+ T cells, should not survive as well in C57BL/6 hosts as C57BL/6 DC, which are MHC II+/. To test this hypothesis, we injected differentially labeled C57BL/6 and MHC II+/- DC into C57BL/6 hosts, and determined their numbers in draining lymph nodes at 1 and 4 days after injection. Representative FACS profiles from one such experiment are shown in Fig. 1B, while Fig. 1C shows average numbers and ratios. On day 1, the numbers of MHC II+/- and C57BL/6 DC recovered from lymph node were similar. By day 4, the numbers of both DC populations had decreased, but the number of MHC II+/- DC was more substantially decreased than the number of C57BL/6 DC. This differential decrease can be clearly appreciated by comparing the ratio of MHC II+/- to C57BL/6 DC at the two time points, as shown in the left panel of Fig. 1C. When recovery on day 4 was adjusted by the numbers of DC in lymph node at day 1, the number surviving MHC II+/- DC was 44% of the numbers of C57BL/6 DC. A further decrease was observed at day 7 (data not shown). These data suggest that C57BL/6 and MHC II+/- DC are equally able to reach the lymph node after injection, but the survival of MHC II+/- is impaired compared with the survival of C57BL/6 DC. Again, these data suggest that optimal DC survival in the lymph node requires cognate interaction with Ag-specific CD4+ T cells. In our experiments, FCS components taken up by DC during in vitro culture appear sufficient to elicit this interaction.

The survival of MHC II+/- DC is improved by pretreatment with CD40L or high doses of LPS

Within the lymph node, MHC II+/- DC may receive maturation signals through their interaction with CD4+ T cells; these signals would not be available to MHC II+/- DC, which are unable to form cognate interactions with CD4+ T cells. Activation of NF-kB

Table 1. Expression of MHC II-OVA complexes is required for the optimal survival of DC in OT-II mice

<table>
<thead>
<tr>
<th>DC Source</th>
<th>n</th>
<th>Adjusted Ratio ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen DC</td>
<td>6</td>
<td>19.1 ± 4.7</td>
</tr>
<tr>
<td>In vitro-generated DC</td>
<td>12</td>
<td>37.3 ± 14.8</td>
</tr>
</tbody>
</table>

* Number of samples in each group.

The ratios of OVA-loaded and untreated DC were calculated for each sample on days 1 and 4. In each experiment, day 4 ratios were adjusted to take into account differences in the ratio at which different DC populations were injected by using the following formula: (sample ratio on day 4) = 100/average ratio on day 1. Adjusted ratios from several experiments were combined and used for statistical calculations.

FIGURE 1. Expression of MHC II-Ag ligands is necessary for the optimal survival of injected DC in the lymph node. DC populations labeled with different fluorochromes were combined in equal numbers and injected s.c. into the forelimbs of mice. DC numbers in draining lymph nodes were determined by FACS at 1 and 4 days after injection. A, Labeled DC were loaded with OVA323-339 or left untreated, and injected into C57BL/6 or OT-II recipients. Average DC numbers and SEM for four samples/group are shown. B and C, Labeled C57BL/6 (MHC II+/-) and MHC II+/- DC were injected into C57BL/6 recipients. B, Representative FACS plots show each showing lymph node suspensions from individual mice 1 and 4 days after DC injection. Gates show C57BL/6 and MHC II+/- DC labeled with CTO and CFSE, respectively. C, Ratios (left panel) and numbers (right panel) of MHC II+/- and C57BL/6 DC in draining lymph nodes at different times after injection. Bars show mean and SEM of six samples per group.

with the fluorescent dyes CFSE and CTO, combined in equal numbers and injected intradermally into the forelimbs of C57BL/6 mice. Injected DC were identified in the draining axillary and brachial lymph nodes by FACS and quantified. Lymph nodes were harvested on day 1, to establish the rate of DC entry in the lymph node, and on day 4, when numbers of injected DC in lymph node are declining (29) but still high enough to be accurately determined. As shown in Fig. 1A, equal numbers of DC only and DC plus OVA323-339 could be recovered from the lymph nodes of C57BL/6 mice at days 1 and 4 after injection, suggesting that presentation of OVA323-339 did not improve the migration or the survival of DC in vivo. A similar trend was observed in OT-II mice injected with DC plus OVA323-339, although on day 1 the numbers of DC recovered from OT-II lymph nodes were somewhat lower than the numbers recovered from C57BL/6 mice, and a lesser decrease in the numbers of DC between days 1 and 4 was apparent. In contrast, there was a sharp decline in the numbers of DC only recovered from OT-II mice between days 1 and 4, with the number recovered at day 4 being only ~25–30% of the number recovered from other groups. Therefore, DC not presenting OVA323-339 do not survive well in OT-II mice where the repertoire of CD4+ T cells is limited by the presence of the transgenic receptor. Addition of OVA323-339 is sufficient to restore DC survival in OT-II mice.

To establish whether differential survival in vivo was due to the use of in vitro-generated DC, we set up a similar experiment using DC populations that were positively selected from mouse spleens using CD11c-magnetic beads. DC were prepared from C57BL/6 spleens and loaded with OVA323-339, or purified from MHC II+/- spleens and left untreated. Both populations were injected into OT-II mice. As shown in Table I, spleen DC that could not interact with OVA-specific CD4+ T cells in vivo did not survive well in the lymph node, and on day 4 their numbers were only ~20% of the numbers of C57BL/6 DC loaded with OVA323-339. This decline appeared even more marked than the decline observed using in vitro-generated DC (Fig. 1A and Table I). Together, these data suggest that the optimal survival of DC in vivo is dependent on the cognate interaction with CD4+ T cells, and that in C57BL/6 mice presentation of FCS-derived Ag, as in our cultured DC, is sufficient to support this interaction.
FIGURE 2. Phenotype of DC pretreated with CD40L or with high doses of LPS in vitro. C57BL/6 and MHC II \(^{-/-}\) DC were treated as indicated for 24 h, and then analyzed by flow cytometry. A, Expression of MHC II, CD86, CD80, and CD40 on CD11c \(^{+}\) live DC treated with CD40L membranes or LPS as indicated. Filled histograms, Unstained cells; gray dotted lines, untreated DC; thin black lines, C57BL/6 DC; thin dotted lines, MHC II \(^{-/-}\) DC. Data are representative of at least three similar experiments that gave comparable results. B, Expression of CD70 on CD11c \(^{+}\) live DC that had been cocultured with CD40L-expressing fibroblasts or treated with the indicated dose of LPS for 24 h as indicated. Filled histograms, Untreated or mock-treated DC (unstained DC gave indistinguishable results); thin black lines, C57BL/6 DC; thin dotted lines, MHC II \(^{-/-}\) DC.

FIGURE 3. Pretreatment with CD40L or with high doses of LPS increases the survival of MHC II \(^{-/-}\) DC in vivo. C57BL/6 and MHC II \(^{-/-}\) DC were treated with CD40L or LPS for 24 h, labeled with fluorochromes, and injected into C57BL/6 recipients. Bars show average \(\pm\) SEM of the number of C57BL/6 and MHC II \(^{-/-}\) DC in six samples per group. The relative survival of MHC II \(^{-/-}\) DC on day 4 is shown in each graph. Panels A–C refer to separate experiments.

and other signaling pathways in mature DC may contribute to the improved survival (30). To assess whether survival was related to maturation status, C57BL/6 and MHC II \(^{-/-}\) DC were treated with CD40L or with different doses of LPS for 24 h before injection into mice. Both 0.1 and 10 \(\mu\)g/ml LPS induced similar up-regulation of CD80, CD86, CD40 (Fig. 2A) and CD70 (Fig. 2B) expression in C57BL/6 and MHC II \(^{-/-}\) DC. Treatment with CD40L did not induce up-regulation of CD80 or CD86 on either DC population by 24 h (Fig. 2A), however, DC washed to remove CD40L, and cultured for a further 24 h showed an increase in the expression of each maturation marker that was similar to the increase induced by LPS treatment (data not shown). Notably increased CD70 expression on DC exposed to CD40L was already detectable by 24 h (Fig. 2B).

Treatment with CD40L substantially increased the numbers of C57BL/6 DC recovered from lymph nodes at both days 1 and 4. In addition, treatment with CD40L also increased the numbers of MHC II \(^{-/-}\) DC, and their relative survival compared with control C57BL/6 DC (Fig. 3A). When differences in the number of injected cells were taken into account, the number of recovered MHC II \(^{-/-}\) DC on day 4 was \(~77\%\) of the number of C57BL/6 DC in the experiment in Fig. 3A, and \(84\%\) when different experiments were combined (Table II); this increase was highly significant when compared over several experiments (Table II).

Pretreatment with LPS at 0.1 \(\mu\)g/ml also increased the numbers of both DC populations in lymph nodes at all time points, however, the relative recovery of MHC II \(^{-/-}\) DC was not significantly increased compared with C57BL/6 DC (Fig. 3B and Table I). Treatment with LPS at 1 or 10 \(\mu\)g/ml led to a further increase in the number of DC in lymph node (Fig. 3C). In addition, the numbers of LPS-treated MHC II \(^{-/-}\) DC in lymph node at day 4 were higher than the numbers of untreated C57BL/6 DC, and their relative survival was also significantly increased (Table I). Thus, the ability of MHC II \(^{-/-}\) DC to survive in vivo can be improved by treatment with CD40L or high doses of LPS.

To determine whether increased survival in vivo could be related to increased expression of prosurvival molecules that have been implicated in the regulation of DC survival, we used Western blotting to evaluate expression of the antiapoptotic molecule Bcl-x\(_L\), whose expression in DC has been reported to support survival in vitro (31). As shown in Fig. 4, expression of Bcl-x\(_L\) increased slightly during the spontaneous maturation of DC in vitro, while treatment with 0.1 \(\mu\)g/ml LPS led to a small and transient up-regulation of Bcl-x\(_L\). In contrast, treatment with LPS at 10 \(\mu\)g/ml
caused a rapid and sustained up-regulation of Bcl-xL in DC. Thus, at the time of in vivo transfer (18–24 h after the start of LPS treatment) DC treated with 10 μg/ml LPS expressed substantially higher levels of Bcl-xL compared with DC that were untreated, or had been treated with a lower dose of LPS. Expression of Bcl-xL after in vivo transfer was not investigated.

Reduced DC survival in the absence of CD4+ T cell help correlates with diminished ability to initiate CD8+ T cell responses

To determine whether differences in DC survival in vivo were related to differential ability of the DC to activate CD8+ T cells, the CD8+ T cell responses induced by MHC II−/− and C57BL/6 DC loaded with the MHC I-binding gp33 peptide were compared. To separate differences in costimulatory capacity from survival, both populations of DC were matured with 0.1 μg/ml LPS, CD40L 83.7 ± 19.2, C57BL/6 and MHC II−/− DC were treated with LPS and loaded with gp33 peptide as described. Therefore, subsequent experiments were conducted in mice that had not been adoptively transferred with TCR-transgenic T cells.

To ascertain whether DC survival affected induction of cytotoxic activity, C57BL/6 mice were immunized as above, and specific cytotoxicity was measured in vivo by examining the survival of differentially labeled spleen targets loaded with 10 or 100 nM gp33. As shown in Fig. 5B, 1 wk after immunization, mice injected with MHC II−/− or C57BL/6 DC showed similarly strong cytotoxic activities that eliminated 80% or more of

FIGURE 4. Up-regulation of Bcl-xL in LPS-treated DC. DC were treated with the indicated doses of LPS in culture, or left untreated, and harvested at the indicated time points. Thirty micrograms of each cell lysate was run on a SDS gel, transferred to a membrane, and probed with Bcl-xL or β-actin-specific Abs and goat anti-mouse IgHRP or goat anti-rabbit IgG HRP. Bars represent Bcl-xL expression after normalization for β-actin expression. Data are from one of two experiments that gave similar results.

FIGURE 5. The reduced survival of MHC II−/− DC in vivo is associated with defective induction of CTL responses. A. Division of TCR-transgenic L318 T cells after immunization with Ag-loaded C57BL/6 or MHC II−/− DC. The division of CFSE-labeled L318 TCR-transgenic T cells was examined in the lymph nodes of recipient C57BL/6 mice 3 days after immunization with 107 DC treated with 0.1 μg/ml LPS and loaded with 10 μM gp33 peptide. Mean and SEM of the number of Vα2+Vβ8+ cells at each division in a group of six mice are shown. B. C57BL/6 and MHC II−/− DC were matured with LPS and loaded with gp33 peptide as described in A, and used to immunize C57BL/6 recipients. At the indicated times, immunized mice were injected with differentially labeled spleen target cells that were loaded with 10 or 100 nM gp33 peptide, or no peptide. Percent killing was determined 24–48 h later in pooled lymph node suspensions. Average ± SEM of groups of five mice are shown. Results are from one of two similar experiments that gave the same result. *, p < 0.05 and **, p < 0.005 when compared with C57BL/6-immunized mice by a two-tailed unpaired Student’s t test.

Table II. The relative survival of MHC II−/− DC in vivo is increased by treatment with CD40L or high doses of LPS

<table>
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<tr>
<th>Treatments</th>
<th>Adjusted Ratio ± SD*</th>
<th>n°</th>
<th>p° (vs Untreated)</th>
<th>p° (vs LPS 0.1)</th>
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<td>Untreated</td>
<td>41.5 ± 14.2</td>
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<td>LPS, 0.1 μg/ml</td>
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<td>LPS, 10 μg/ml</td>
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<td>2.57 × 10^{-5}</td>
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<tr>
<td>CD40L</td>
<td>83.7 ± 19.2</td>
<td>12</td>
<td>1.2 × 10^{-9}</td>
<td>1.24 × 10^{-6}</td>
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</table>

* The ratios of MHC II−/− to C57BL/6 DC were calculated for each sample on days 1 and 4. In each experiment, day 4 ratios were adjusted to take into account differences in the ratio at which different DC populations were injected by using the following formula: (sample ratio on day 4) × 100/(average ratio on day 1). Adjusted ratios from several experiments were combined and used for statistical calculations.

° Number of samples in each group.

Value of p calculated using the two-tailed unpaired Student’s t test.
specific target cells. By 2 wk after immunization, in vivo cytotoxic activity had declined in all groups, but was still clearly measurable in mice immunized with C57BL/6 DC, while it had fallen to almost background levels in mice immunized with MHC II−/− DC (Fig. 5B). Therefore, the CTL response induced by activated C57BL/6 DC was stronger and/or more sustained than the response induced by activated MHC II−/− DC. A similar rapid decline of CTL activity has been described in situations where CD4+ help is not available at the time of CD8+ T cell priming (33, 34).

**Activated MHC II−/− male DC do not induce expansion of H2-Dα-UTY-specific CD8+ T cells**

To examine whether the effect of DC survival on CD8+ T cell responses was more pronounced when using DC that endogenously synthesize the relevant Ag, male DC were used to immunize syngeneic female recipients. This response is known to be strictly dependent on CD4+ help (35). To prevent complications arising from potential cross-presentation of Ag by host APC, male MHC II−/− or MHC II−/− DC were used to immunize MHC II+/− or MHC II−/− females, respectively. In preliminary experiments, the survival of MHC II−/− DC in MHC II−/− mice was examined, and found to be reduced compared with the survival of MHC II+/− DC in MHC II+/+ mice (data not shown).

The appearance of male-specific CD8+ T cells in the blood of immunized female C57BL/6 mice was detected using H-2Dα-UTY tetramers. As shown in Fig. 6A, the percentage of male-specific CD8+ T cells peaked at around day 14 and was declining by day 22. In mice immunized with 2 × 106 LPS-treated DC, UTY-specific cells comprised ~13% of the total CD8+ T cells in blood, while in mice immunized with 1 × 106 DC they were ~7%.

The effect of DC survival on the expansion of H2-Dα-UTY-specific CD8+ T cells in vivo was investigated under conditions where the number of DC was limiting. Female MHC II−/− and MHC II+/− mice were immunized with 1 × 106 LPS activated male DC from MHC II−/− or MHC II+/− mice, respectively. Representative dot plots of CD8+ T cells from naive, MHC II−/− or MHC II+/− immunized mice are shown in Fig. 6B. All UTY-specific CD8+ T cells expressed high levels of CD44 indicating that they were activated. However, there were notable differences in the percentages of UTY-specific T cells in the blood of immunized mice. Again, the percent UTY-specific CD8+ cells in MHC II−/− mice peaked at ~10% on day 14 and contracted by day 22 (Fig. 6C). In contrast, in mice immunized with MHC II−/− DC, the H2-Dα-UTY-specific CD8+ T cells failed to expand above 1% of CD8+ T cells.

CTL responses were also compared in these mice. Surprisingly, despite the low percentage of H2-Dα-UTY-specific CD8+ T cells in blood, mice immunized with MHC II−/− DC generated a detectable response that could lyse ~60% of male target cells at 14 days after immunization (Fig. 6D). In contrast, mice immunized with MHC II−/− DC lysed ~85% of male target cells. On day 22, when the percent of Ag-specific T cells in blood was declining, mice immunized with MHC II−/− DC still exhibited a high cytotoxic activity and killed >80% of the male target cells. In contrast, cytotoxic activity had sharply declined in mice immunized with MHC II−/− DC, and was <20%. We conclude that, even when expressing high levels of costimulatory molecules, MHC II−/− DC are unable to induce the expansion of UTY-specific T cells, and the development of sustained cytotoxic activity.

**FIGURE 6.** LPS-treated MHC II−/− DC fail to stimulate the expansion of H2-Dα-UTY-specific CD8+ T cells in vivo, and induce a reduced cytotoxic response. Male DC were treated with 0.1 μg/ml LPS for 24 h, and injected s.c. into the flank of syngeneic female mice. A, Percentages of male-specific CD8+ T cells in the blood of C57BL/6 female mice immunized with different numbers of C57BL/6 male DC. Male-specific CD8+ T cells were identified using H-2Dα-UTY tetramers. Each line shows the mean ± SEM for groups of four mice. B, Representative FACS plots showing the percent male-specific CD8+ T cells in the blood of MHC II+/− or MHC II−/− female mice immunized 14 days earlier with MHC II+/− or MHC II−/− DC, respectively. Only PI− CD8+ events are shown. C, Percent male-specific CD8+ T cells in the blood of MHC II+/− or MHC II−/− female mice immunized with MHC II+/− or MHC II−/− DC, respectively. Average ± SEM for groups of four mice are shown. Data are from one of two experiments that gave the same result. D, In vivo cytotoxic activity in female MHC II−/− and MHC II−/− mice immunized as in C. Cytotoxic activity was assessed at the indicated time points after DC immunization using MHC II−/− male and female spleen cells differentially labeled with fluorochromes and injected i.v. Target cells were harvested from pooled lymph nodes 48 h after injection. Bars show the mean ± SEM of the percent-specific lysis from groups of five mice. *, p < 0.05 and **, p < 0.005 when compared with mice immunized with MHC II+/− DC by a two-tailed unpaired Student t test. Data are from one of two experiments that gave the same results.

Treatment with high doses of LPS or with CD40L does not improve the ability of MHC II−/− DC to induce T cell expansion and cytotoxic activity in vivo

If impaired DC survival was the underlying cause of the inability of MHC II−/− to induce strong CD8+ T cell responses, treatments that improve DC survival should also improve their ability to induce CTL responses to levels that approximate or match those of C57BL/6 DC. To address this question, we used DC that had been treated with 10 μg LPS/ml, which substantially increased the numbers and relative survival of DC in vivo, or with 0.1 μg LPS/ml, which had no detectable effect on DC survival but induced a comparable up-regulation of costimulatory molecules on DC.

In a first experiment, we examined the expansion of UTY-specific T cells in the blood of mice immunized with male DC that had...
been treated with 0.1 or 10 µg LPS/ml. As shown in Fig. 7A, MHC II<sup>−/−</sup> female mice immunized with MHC<sup>+/−</sup> male DC showed a significant increase in the percentage of UTYSpecific T cells in peripheral blood; this increase was more marked in mice receiving DC treated with the higher dose of LPS. In contrast, no increase in the percentage of UTYSpecific T cells was observed in MHC II<sup>−/−</sup> mice immunized with MHC II<sup>−/−</sup> DC, regardless of the dose of LPS used to activate DC before injection. As a further readout of the response, we compared cytotoxic activity in vivo using spleen targets from male mice, or female targets loaded with UTYSpecific peptide. As shown in Fig. 7B, mice immunized with MHC<sup>+/−</sup> male DC generated strong cytotoxic responses regardless of whether the DC were activated with the low or high dose of LPS. In contrast, mice immunized with MHC<sup>−/−</sup> DC generated lower responses, and these were not improved by pretreatment with 10 µg LPS/ml.

The expansion of UTYSpecific T cells in blood was also examined after immunization with male DC that had been activated by simultaneous incubation with 0.1 µg/ml LPS and CD40L, a treatment that increased the survival of MHC II<sup>−/−</sup> DC in vivo. As shown in Fig. 7C, mice immunized with MHC II<sup>+/−</sup> DC pretreated with LPS had a higher percentage of UTYSpecific cells in blood than mice immunized with DC pretreated with LPS and CD40L. MHC II<sup>−/−</sup> DC pretreated with LPS only, or with LPS and antiCD40L, were both unable to induce expansion of UTYSpecific T cells to levels significantly above background. MHC II<sup>−/−</sup> DC pretreated with CD40L without LPS also failed to induce expansion of UTYSpecific T cells (data not shown).

In the experiments above, MHC II<sup>−/−</sup> DC were injected into MHC II<sup>−/−</sup> mice to avoid potential cross-presentation of male Ag on host DC. No CD4<sup>+</sup> T cells that might provide “bystander” help are available in this system. We wished to test a less stringent model where MHC II<sup>−/−</sup> DC are injected into C57BL/6 recipients, and to this purpose we used DC loaded with the gp33 epitope. DC were prepared from C57BL/6 mice and injected into C57BL/6 recipients, or prepared from MHC II<sup>−/−</sup> mice and injected into C57BL/6 or MHC II<sup>−/−</sup> recipients. DC were treated with 0.1 µg/ml or with 10 µg/ml LPS, and used in limiting numbers (5 × 10⁴ DC/mouse). As shown in Fig. 8, 1 wk after immunization mice injected with C57BL/6 DC exhibited strong CTL responses to the gp33 peptide, and killed ~70% of the injected target cells regardless of whether the immunizing DC were treated with 0.1 or 10 µg of LPS. In contrast, mice immunized with MHC II<sup>−/−</sup> DC generated lower CTL response in all cases, regardless of whether DC werepretreated with 0.1 or 10 µg/ml LPS, and of whether the recipients were C57BL/6 or MHC II<sup>−/−</sup>. When results from four separate experiments were combined, there was no statistical difference between cytotoxic activity in mice immunized with MHC II<sup>−/−</sup> DC treated with 0.1 or 10 µg/ml LPS (p = 0.337 in the C57BL/6 recipient group, and p = 0.732 in the MHC II<sup>−/−</sup> recipient group by a two-tailed unpaired Student t test) while the difference between C57BL/6 mice immunized with C57BL/6 or MHCII<sup>−/−</sup> DC treated with 10 µg/ml LPS was highly significant (p = 0.0032).

We conclude that treatments that extend DC survival in vivo have no detectable effect on the ability of the same DC to induce T cell expansion and cytotoxic responses in vivo.
Discussion

CD4⁺ T cells and high concentrations of LPS enhance DC survival

In this study, we examine the survival of C57BL/6 and MHC II⁻/⁻ DC in vivo. We used DC generated in vitro in medium containing FCS, which have taken up FCS proteins and thus model DC presenting Ag during an immune response. We show that MHC II⁻/⁻ DC were lost from the lymph node much faster than MHC II-expressing DC, suggesting that expression of MHC II allowed DC to receive signals that are necessary for their optimal survival in vivo. Similarly, DC not loaded with OVA were lost from the lymph nodes of OT-II mice much more rapidly than DC presenting OVA. Importantly, DC loss was also observed using DC freshly isolated from mouse spleen, indicating that it was not an artifact related to the use of in vitro-generated DC. Together, these results strongly suggest that the optimal survival of DC in vivo requires cognate signals mediated by CD4⁺ T cells.

Pretreatment with CD40L, or with high doses of LPS, improved the survival of MHC II⁻/⁻ DC to levels approaching those of C57BL/6 DC, indicating that signals from infectious agents and the innate and/or adaptive immune system are both able to regulate DC survival in vivo. Other studies have suggested that TNF-related activation-induced cytokine and other TLR ligands may also augment DC survival (10, 31), suggesting some redundancy among survival signals. Regardless of their ability to survive in vivo, MHC II⁻/⁻ DC were defective in their capacity to initiate CD8⁺ T cell immune responses. Thus, the mechanisms by which CD4⁺ T cells provide help to CD8⁺ T cells appear to be distinct or additional to improved DC survival.

The effects of LPS and CD40L on DC survival did not appear to directly correlate to the induction of an activated phenotype. DC treated with LPS at 0.1 or 10 μg/ml both rapidly increased their expression of maturation markers and, as shown by other authors, expression of the prosurvival molecule Bcl-xL was also increased (31). However, only the higher dose of LPS improved the survival of MHC II⁻/⁻ relative to C57BL/6 DC after in vitro transfer. The dose of CD40L used in our experiments did not induce up-regulation of maturation markers on DC at 24 h, although increased expression of costimulatory molecules was observed by 48 h. Nonetheless, CD40L was effective at improving the survival of MHC II⁻/⁻ DC in vivo. Thus, activation and survival did not appear to be coordinate events in our DC populations.

Several lines of evidence suggest that MHC II⁻/⁻ DC were not rejected by the host immune system, but were dying by neglect within the lymph node. The survival of MHC II⁻/⁻ DC could be enhanced by pretreatment with CD40L or high concentrations of LPS, which increase the stimulatory ability of DC, and could therefore be expected to accelerate rejection. In addition, MHC II⁻/⁻ DC showed reduced survival after injection into C57BL/6 mice, while MHC II⁻/⁻ DC did not. Both these DC populations express alleles from the mutant B6Aa²/Aa² strain, and should have been rejected to a similar extent. Lastly, OVA-loaded DC survived longer than non-OVA-loaded DC in OT-II TCR-transgenic mice. In those experiments, all DC were from C57BL/6 mice, and their differential survival cannot be explained by rejection. Thus, our results point to a mechanism of improved DC survival that is distinct from the protection from cell-mediated killing already reported by other authors (36, 37).

Although improved survival of DC through cognate interaction with CD4⁺ Th cells appears the most likely interpretation of the findings reported here, other scenarios are also possible. DC that are unable to establish productive interactions with CD4⁺ T cells may simply leave the lymph node and migrate to other sites. However, the correlation between DC numbers in lymph nodes and ability to initiate immune responses suggests that emigrated DC are unlikely to be functionally active. Survival signals might not be provided by CD4⁺ helpers but by CD4⁺ regulatory T cells, which are also able to form cognate interactions with MHC II⁻/⁻ DC. However, as regulatory T cells have not, to our knowledge, been reported to express CD40L, this possibility appears rather unlikely.

Survival of DC does not correlate with their ability to induce CD8⁺ T cell responses

Since it was first reported in 1982 (38), the requirement for CD4⁺ T cell help during primary and secondary CD8⁺ responses has been actively investigated (reviewed in Ref. 39). Experiments have shown that primary CD8⁺ immune responses to infectious agents could be generated regardless of the presence or absence of T cell help, while memory responses were impaired (33, 34). In contrast, primary responses to peptide Ag or to cross-presented minor histocompatibility Ag were mostly (35, 40, 41), but not always (42, 43), low or undetectable in the absence of T cell help. These differences may be due, at least in part, to differential activation of DC, and to inflammatory responses induced by the infectious agents.

In essentially all of our experiments, we examined primary CD8⁺ responses induced by LPS-treated DC that expressed high levels of costimulatory molecules. Using these conditions, we found that CD4⁺ help was clearly necessary for optimal CD8⁺ responses measured as T cell expansion and cytotoxic activity. Nonetheless, in vivo cytotoxic activity could be demonstrated after immunization with MHC II⁻/⁻ DC. This was mostly in experiments using high numbers of DC, and clear differences were observed as the response declined over time, or by using limiting numbers of DC. Interestingly, male-specific cytotoxic responses were observed in mice injected with MHC II⁻/⁻ male DC, in which circulating levels of male-specific CD8⁺ T cells were only marginally above background. This discrepancy between numbers of specific CD8⁺ T cells in blood and cytotoxic activity might be due to different thresholds of the two types of responses, or to a defect in the ability of male-specific T cells to leave the lymph node after activation. Defective emigration from the lymph node has been described during immune responses to tumor Ag (44), suggesting that high cytotoxic activity may not necessarily reflect the activation of a productive CTL response. It would have been interesting to determine whether a similar discrepancy occurred in the case of gp33-specific T cells; unfortunately, the percentages of gp33-specific T cells in the blood of our DC-immunized mice were too low to be accurately determined, preventing us from answering this question in a reliable manner.

Several models have been proposed to explain the mechanism by which CD4⁺ T cells “help” the development of CD8⁺ T cell responses. In one of these, “licensing” of DC by CD4⁺ T cells would make the DC better able to interact with CD8⁺ T cells and induce their productive activation, by either prolonging DC survival or by increasing their expression of costimulatory molecules (13–15, 45). Simultaneous interaction of APC with CD4⁺ and CD8⁺ T cells (17), induced expression of costimulatory molecules (46) or chemotactic chemokines by DC (16), effects of CD8⁺ precursor frequency (32), and induction of TRAIL expression (47) and cytokine secretion (48) on CD8⁺ T cells have all been invoked as additional or alternative possible mechanisms.

Our experiments examined the possibility that CD4⁺ help may be mediated via the increased survival of DC in vivo. We identified conditions in which the numbers and relative survival of MHC II⁻/⁻ DC in vivo were both increased, thus allowing us to test our hypothesis. Treatment with CD40L and with high doses of LPS...
increased the numbers of MHC II\(^+\) DC in lymph nodes at day 4 to well above the number of untreated C57BL/6 DC, and also increased their relative survival. We found that DC numbers and survival in vivo did not correlate with their ability to initiate CD8\(^+\) T cell responses, even when DC expressed very high levels of costimulatory molecules after LPS treatment. This was shown in MHC II\(^+\) female mice immunized with MHC II\(^+\) male DC, and in less stringent conditions where bystander help was present, in C57BL/6 mice immunized with MHC II\(^+\) DC loaded with gp33 peptide. Neither the expansion of specific CD8\(^+\) T cells nor the optimal induction of CTL responses were rescued by increasing the survival of MHC II\(^+\) DC. Thus, CD4-mediated "licensing" of DC must involve signals that are distinct or additional to the increased expression of costimulatory molecules and increased survival of stimulatory DC.

Pretreatment of MHC II\(^+\) DC with CD40L, with or without (data not shown) LPS, did not enable DC to induce CD8\(^+\) T cell responses. This was unexpected, given the reported ability of CD40/CD40L signaling to replace CD4\(^+\) help during the induction of CD8\(^+\) T cell responses (13–15, 45). Our CD40L treatment was effective, as treated DC were able to survive longer in vivo, but might not have been sufficient if higher amounts of CD40L were required to rescue induction of CD8\(^+\) responses as compared with DC survival. The fact that the survival of CD40L-treated MHC II\(^+\) DC was still lower than the survival of similarly treated C57BL/6 DC may suggest that the conditions of CD40L activation were indeed suboptimal, or may point to the existence of additional mechanisms by which DC survival is supported. Alternatively, the CD40 cross-linking conditions used in our experiments may not reproduce the conditions used by others. Studies using injection of anti-CD40 Abs in vivo found that this treatment was sufficient to replace help (13, 15, 45), however, anti-CD40 treatment may have additional in vivo effects besides inducing DC activation. Studies where the role of CD40/CD40L was examined by other means reported conflicting results, with some studies failing to demonstrate a sufficient role of CD40L in mediating help (49–51), and others successfully replacing CD4\(^+\) help by activating DC via CD40 (14, 52, 53). The conditions used in our experiments differ in at least some respects from the conditions used in those reports: we used only one DC injection into mice that had not been adoptively transferred with TCR-transgenic T cells, we always compared C57BL/6 and MHC II\(^+\) that had both been activated using similar conditions, and used as a model the UTY Ag which is known to be strictly dependent on the presence of T cell help. Any of these factors could explain the difference between the present findings and previous reports.

An alternative possibility is that the DC used in our experiments, and to which survival signals were applied, are not the same DC that present Ag and activate CD8\(^+\) T cells in vivo. Improved survival of those DC would then become irrelevant to the results of the experiment. We believe that this possibility is very unlikely; while Ag such as the male Ag might be cross-presented in vivo, minimal peptide epitopes such as the gp33 used in our experiments are known to be cross-presented very inefficiently by host APC (54, 55). In addition, we have shown that gp33-loaded DC induce comparable division of gp33-specific T cells regardless of the MHC of the host (56), again indicating that the observed responses are induced directly by the injected DC.

We show in this study that the survival of DC in vivo is increased during immune responses, and is determined by signals derived by infectious agents or by other immune cells. Although extended DC survival induced by high doses of LPS or by CD40 cross-linking may be critical for optimal immune responses, and may enhance the ability of DC to activate CD8\(^+\) T cells, it appears insufficient to replace the requirement for CD4\(^+\) T cell help. This view is consistent with reports where the duration of Ag presentation affected the magnitude but not the quality of the induced CTL response (4), and reports where the secretion of chemokines or cytokines by DC or T cells (16, 48) played a critical role in the delivery of CD4\(^+\) help to CD8\(^+\) cells.

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Disclosures

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