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CD8$^+$ T Cell-Dependent Elimination of Dendritic Cells In Vivo Limits the Induction of Antitumor Immunity[1]

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The fate of dendritic cells (DC) after they have initiated a T cell immune response is still undefined. We have monitored the migration of DC labeled with a fluorescent tracer and injected s.c. into naive mice or into mice with an ongoing immune response. DC not loaded with Ag were detected in the draining lymph node in excess of 7 days after injection with maximum numbers detectable ~40 h after transfer. In contrast, DC that had been loaded with an MHC class I-binding peptide disappeared from the lymph node with kinetics that parallel the known kinetics of activation of CD8$^+$ T cells to effector function. In the presence of high numbers of specific CTL precursors, as in TCR transgenic mice, DC numbers were significantly decreased by 72 h after injection. The rate of DC disappearance was extremely rapid and efficient in recently immunized mice and was slower in "memory" mice in which memory CD8$^+$ cells needed to reacquire effector function before mediating DC elimination. We also show that CTL-mediated clearance of Ag-loaded DC has a notable effect on immune responses in vivo. Ag-specific CD8$^+$ T cells failed to divide in response to Ag presented on a DC if the DC were targets of a pre-existing CTL response. The induction of antitumor immunity by tumor Ag-loaded DC was also impaired. Therefore, CTL-mediated clearance of Ag-loaded DC may serve as a negative feedback mechanism to limit the activity of DC within the lymph node. *The Journal of Immunology,* **2000, 164: 3095–3101.**

Dendritic cells (DC)[3] are highly specialized APCs that serve a sentinel function in immunity by internalizing and processing peripheral Ags for presentation to naive T cells (1). DC residing in the peripheral tissues are initially of an "immature" phenotype characterized by a high propensity for Ag capture but relatively poor ability to stimulate naive T cells (2). However, upon exposure to cytokines associated with inflammation or tissue injury such as TNF-α or IL-1β, DC migrate from the peripheral tissues to regional lymph nodes (3, 4) and undergo a maturation process characterized by a reduction in Ag capture function and up-regulation of MHC and costimulatory molecules (5, 6). Final DC activation is induced by Ag-specific Th cells via the interaction of CD40-ligand on the Th cell with CD40 on the DC (7, 8). Activated DC can prime naive CD8$^+$ T cells to CTL (9–11), which have the capacity to recognize and kill cells that express specific Ag in the peripheral tissues. The fate of DC after they have reached the lymph node is unknown. Because no cells with the morphology of DC can be demonstrated in efferent lymph (12), it has been proposed that DC die in situ, although the mechanism remains unclear.

Previous studies have shown that DC injected s.c. or i.v. have the capacity to migrate to the regional lymph nodes or the spleen, respectively (13). Fluorescent-labeled DC injected s.c. have been shown to migrate to draining lymph nodes (DLN) where they interact with Ag-specific CD4$^+$ T cells to form clusters in the paracortex (14). DC that had not been loaded with specific Ag failed to form such clusters. Surprisingly, it was also observed that the numbers of Ag-loaded DC in the DLN had declined by 48 h, whereas the non-Ag-loaded DC persisted for longer periods of time. This suggested that the Ag-loaded DC may have been eliminated by an immune-mediated mechanism.

We wished to examine in more detail the fate of peripherally administered DC in a model of CTL-mediated immunity and to establish how the immune response affected the persistence of DC in the lymph node. Our results suggest that DC clearance occurs in the presence of previously activated CTL and also during the activation of naive Ag-specific CTL precursors. Furthermore, the rapid clearance of tumor Ag-loaded DC was associated with impaired capacity to induce tumor immunity. Therefore, CTL-mediated clearance of Ag-loaded DC may serve as a negative feedback mechanism to limit the activity of DC within the lymph node.

Materials and Methods

Mice

C57BL/6 mice were from breeding pairs originally obtained from The Jackson Laboratory (Bar Harbor, ME). Strain 318 mice (15), transgenic for a TCR specific for H-2 D^b plus fragment 33–41 of the lymphocytic choriomeningitis virus glycoprotein (LCMV$_{33-41}$),[3] were kindly provided by Dr. H. Pircher (Institute of Medical Microbiology and Hygiene, University of Freiburg, Freiburg, Germany). The B6Aa^0/Aa^0 MHC class II$^{-/-}$ mice (16) were supplied by Dr. H. Bluethmann (Hoffmann-LaRoche, Basel, Switzerland). All mice were maintained at the Biomedical Research Unit of the Wellington School of Medicine by brother × sister mating; in vivo experimental protocols were approved by the Wellington School of Medicine Animal Ethics Committee and were performed according to institutional guidelines.

Tumor cell line

LL-LCMV is a derivative of the Lewis lung carcinoma LLTC (C57BL/6, H-2^b) that has been modified to express a minigene encoding LCMV$_{33-41}$ under the control of a CMV promoter (17).

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[3] Abbreviations used in this paper: DC, dendritic cell; DLN, draining lymph node; LCMV, lymphocytic choriomeningitis virus; CM, complete medium; CFSE, carboxyfluorescein succinimidyl ester.

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In vitro culture media and reagents

Unless otherwise stated, all cultures were maintained in complete medium (CM) comprising of IMDM with 2 mM glutamine, 1% penicillin-streptomycin, 5×10^{-5} M 2-ME, and 5% FBS (all from Life Technologies, Auckland, New Zealand). The synthetic peptides $LCMV_{33-41}$ (KAVYN FATM) and $OVA_{257-264}$ (SIINFEKL) were from Chiron Mimotopes (Clayton, Australia).

Culture of bone marrow-derived DC

Bone marrow cells from C57BL/6 mice or $B6Aa^0/Aa^0$ mice were cultured at 4×10^5 cells/ml in CM containing 20 ng/ml IL-4 and 20 ng/ml GM-CSF as described previously (18). Cultures were provided fresh CM and cytokines every 3 days and incubated at 37°C until the time of assay (6–8 days). Cultures typically contained 70–100% DC as determined by fluorescent staining with the anti-CD11c Ab N418. DC (1×10^6 cells/ml) were loaded with peptide Ag by incubation at 37°C in CM containing 10 μM synthetic peptide for 2 h and then were washed three times with IMDM to remove excess peptide.

DC migration assay

DC were labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) by incubation in PBS containing 1 μM CFSE for 10 min at 37°C before one wash in five volumes of ice-cold PBS and two washes with IMDM. Unless otherwise stated, mice were injected with 1×10^6 $CFSE^+$ DC by s.c. injection in the distal forelimb (volar aspect). At the indicated times, both axillary and brachial lymph nodes were removed from immunized mice and incubated in 2.4 mg/ml collagenase type II (Life Technologies) and 1 mg/ml DNase I (Sigma, St. Louis, MO) for 90 min at 37°C. The tissue was then disrupted by aspiration through an 18-gauge needle. The cell suspension was sieved through gauze, washed in PBS, and resuspended for flow cytometric analysis in PBS containing 2% FBS and 0.01% sodium azide.

Lymph node suspensions were analyzed using a FACSort and CellQuest software (both from Becton Dickinson, Mountain View, CA). The region containing DC was identified on the basis of forward/side light scatter profile. No $CFSE^+$ cells were found outside this region. In initial experiments, the total number of DC within the DLN was calculated as follows: % $CFSE^+$ cells in the DC region × % of DLN cells in the DC region × number cells in DLN; data are expressed as average number of DC within DLN for each experimental group. Because it was observed over repeated experiments that the percentage of $CFSE^+$ cells in the DC region was always proportional to the absolute number of $CFSE^+$ cells across the experimental groups, data were expressed as average percentage of $CFSE^+$ cells thereafter. Only events falling within the gated DC region were collected and stored (>250,000 events for each DLN suspension).

Frozen sections (8 μm) were prepared from the axillary lymph nodes of animals injected with $CFSE^+$ DC 20 h earlier. The sections were stained with hematoxylin and eosin and analyzed by standard and fluorescence microscopy. $CD8^+$ T cells are depleted by one i.v. injection of 500 μg purified 2.43 mAb 24 h before DC immunization.

Ab staining

Ab staining was in PBS containing 2% FBS and 0.01% sodium azide. The anti-FcγRII mAb 2.4G2 was used at 10 μg/ml to inhibit nonspecific staining. Anti-MHC class II (3JP) and anti-CD11c (N418) were affinity purified from culture supernatants using protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden), conjugated to biotin as described (19), and revealed using streptavidin-PE (PharMingen, San Diego, CA). Instrument compensation was set in each experiment using single color-stained samples.

Adoptive transfer and DC immunization

Pooled lymph node cell suspensions were prepared from strain 318 mice, and the percentage of T cells expressing the transgenic TCR was determined on one sample of cells by staining with anti-Vα2 and anti-Vβ8.1, 8.2 Abs and by FACS analysis. The cells were then labeled by incubation in 1.25 μM CFSE (Molecular Probes) in PBS for 10 min at room temperature at a cell concentration of 2×10^7 cells/ml. The cells were pelleted by centrifugation in the presence of 50% FCS, washed two times in CM, and then washed again in IMDM. Suspensions containing 5×10^6 $V\alpha2^+V\beta8^+$ cells were transferred into C57BL/6 recipients by i.v. injection into the tail vein. One day after adoptive transfer, the mice were immunized s.c. with 10^5 DC. After 66 h, the draining axillary and brachial lymph nodes were removed, teased through gauze to prepare single-cell suspensions, and analyzed by FACS.

Tumor immunity assay

Groups of C57BL/6 mice ($n = 5$) were immunized by s.c. injection into the left flank with 10^5 DC. Secondary immunizations were by s.c. injection into the contralateral flank 7 days later. One week after the last immunization, all animals were challenged with 10^6 LL-LCMV tumor cells injected s.c. into the left flank. Mice were monitored for tumor growth every 3–4 days, and tumor size for each group was calculated as the mean of the products of bisecting diameters (±SE). Measurements were terminated for each group when the first animal developed a tumor in excess of 200 mm^2.

Results

$CFSE^+$ DC injected s.c. are detected in the DLN

We examined the migration of injected DC to the secondary lymphoid organs of mice. DC were cultured from bone marrow precursors in the presence of GM-CSF and IL-4 for 7 days and labeled with CFSE, an amine-reactive dye that can be retained in live cells for many generations. DC were administered s.c. into the distal forelimb of mice without any deliberate preincubation with Ag. As can be seen in Fig. 1, 20 h after injection $CFSE^+$ cells could be detected in the DLN by fluorescence microscopy on frozen tissue sections and were located in the paracortical region of the lymph node. Analysis of DLN cell preparations by flow cytometry also revealed the presence of $CFSE^+$ cells in the DLN (Fig. 2). No $CFSE^+$ cells could be detected in the non-DLN. In addition, $CFSE^+$ cells could not be detected in the DLN if the DC were heat killed before injection. These results imply that the $CFSE^+$ cells had reached the DLN via an active migratory process.

The possibility that the $CFSE^+$ cells found in the DLN were in fact endogenous cells that had acquired CFSE from the injected cells was examined by injecting MHC class $II^{-/-}$ DC and then using MHC class II expression to distinguish the endogenous from the injected DC. C57BL/6 mice were injected with $CFSE^+$ DC cultured from either C57BL/6 mice or MHC class $II^{-/-}$ $B6Aa^0/Aa^0$ mice. MHC class II expression was then examined on $CFSE^+$ cells in the DLN. As shown in Fig. 2*B*, MHC class II expression could be demonstrated on $CFSE^+$ cells obtained from mice that had received C57BL/6 DC. In contrast, no expression of MHC class II was found on $CFSE^+$ cells from mice that received MHC class $II^{-/-}$ DC. We conclude that the $CFSE^+$ cells detected in the DLN were the same DC that had been injected peripherally and that these cells had actively migrated from the site of injection to the DLN.

The proportion of $CFSE^+$ DC that reached the DLN and their kinetics of appearance were also examined. As shown in Fig. 3*A*, the absolute number of $CFSE^+$ DC found within the DLN represented only a small proportion (~0.1%) of the original number of DC injected. The injected DC appeared in the DLN within 12 h of injection, reaching maximum numbers before 48 h. Considerable numbers of $CFSE^+$ DC could still be demonstrated in the DLN at 172 h postinjection (Fig. 3*B*), the latest time point examined.

In summary, $CFSE^+$ DC can be demonstrated to actively migrate from the site of s.c. injection to the DLN and to persist in vivo in excess of 7 days from the time of injection.

DC loaded with MHC class I-binding peptide Ag are cleared from the DLN

Next, we sought to determine the fate of Ag-loaded DC after s.c. injection into mice. $CFSE^+$ DC were loaded with $LCMV_{33-41}$, an MHC class I-binding peptide from the LCMV glycoprotein, and injected into the forelimbs of C57BL/6 recipients. The numbers of Ag-loaded DC in the DLN were compared with the numbers in the DLN of mice that received DC not loaded with Ag. As can be seen in Fig. 4*A*, loading with Ag had no effect on the number of $CFSE^+$ DC recovered from the DLN at 16 h after injection. By 72 h, a small reduction in the number of DC in the DLN was observed in

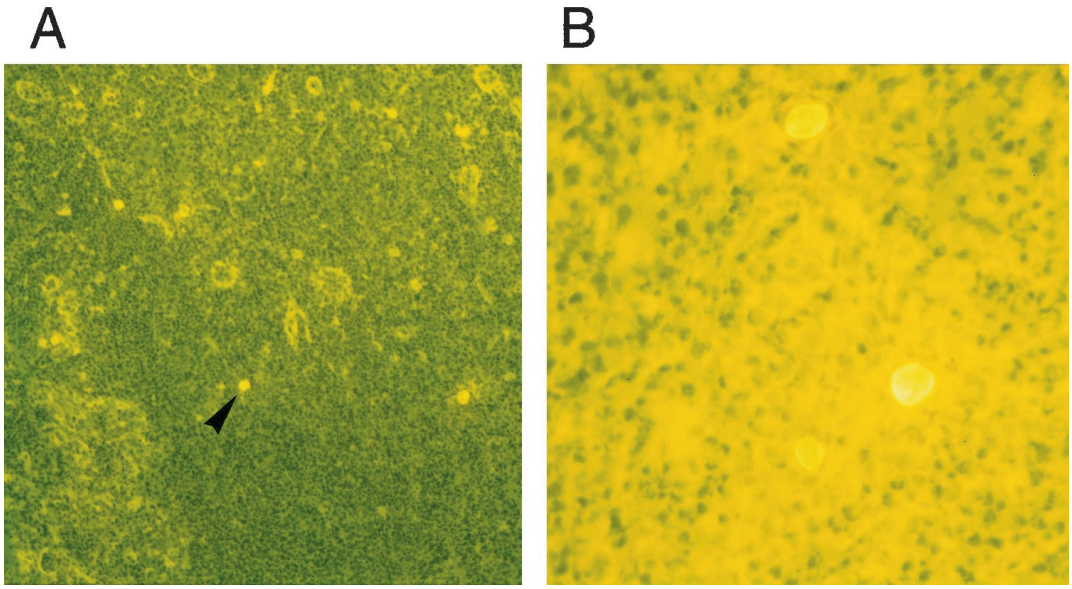

FIGURE 1. DC injected s.c. migrate to the paracortex of the DLN. Mice were injected s.c. with 5×10^5 CFSE$^+$ DC in the distal forelimb, and axillary and brachial lymph nodes were removed for histological analysis 20 h after injection. Frozen tissue sections of the DLN were prepared and stained with hematoxylin and eosin. CFSE$^+$ cells were detected by fluorescence microscopy. *A*, Low-power magnification with a representative CFSE$^+$ cell indicated (arrowhead). *B*, High-power magnification showing three CFSE$^+$ cells.

the experiment shown, but this reduction was not always observed in repeated experiments. In contrast, when the number of CD8$^+$ T cells specific for the Ag presented on DC was increased as in TCR transgenic recipients, a significant and highly reproducible reduction in the number of Ag-loaded DC in the DLN was observed 72 h after injection. No reduction was apparent at 16 h. Depletion of CD8$^+$ T cells from TCR transgenic recipients before DC injection prevented the reduction in number of Ag-loaded DC in the DLN

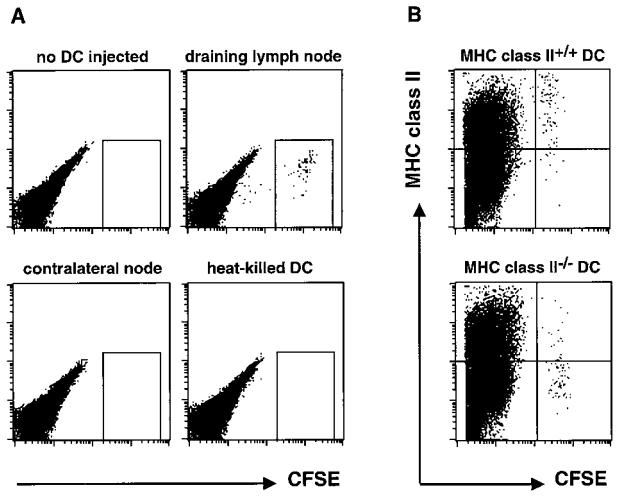

FIGURE 2. DC that have migrated to the DLN can be detected by FACS analysis. Mice were injected s.c. with 5×10^5 CFSE$^+$ DC in the distal forelimb, and axillary and brachial lymph nodes were removed for FACS analysis 20 h after injection. Data were collected in a gate encompassing only large cells as based on the known size of cultured DC. No CFSE$^+$ cells were found outside this gate. *A*, Results are shown for a noninjected animal, an animal injected with CFSE$^+$ DC (DLN and contralateral nodes), and an animal that was injected with CFSE$^+$ DC that had previously been heated to 80°C for 10 min. *B*, DLN from C57BL/6 mice injected with either C57BL/6 DC (MHC class II$^{+/+}$ DC) or B6Aa^0/Aa^0 DC (MHC class II$^{-/-}$ DC). DLN cell suspensions were stained with anti-MHC class II to determine MHC class II expression on CFSE$^+$ cells.

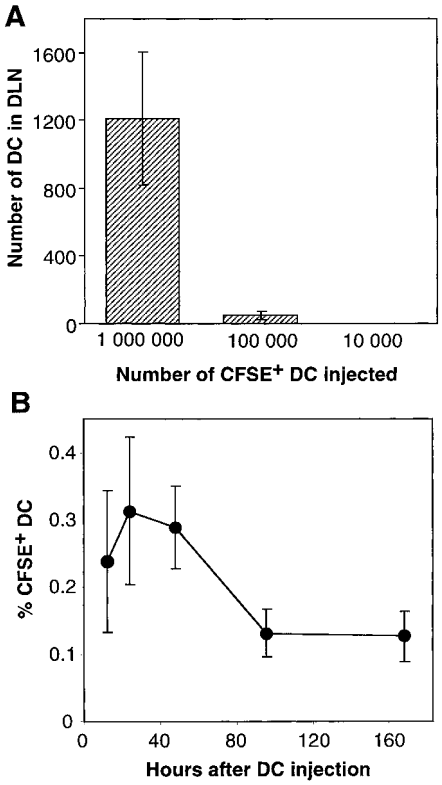

FIGURE 3. Quantitation and kinetics of DC migration to the DLN. *A*, Groups of mice were given s.c. injections in both distal forelimbs with the indicated numbers of CFSE$^+$ DC; DLN were removed for FACS analysis 20 h later. Each group contained three animals with each injected forelimb treated as an independent event. *B*, Mice were injected with 10^6 CFSE$^+$ DC, and the DLN were removed for FACS analysis at the indicated time points. Three animals were analyzed at each time point with each injected forelimb treated independently ($n = 6$). Data are presented as the mean percentage of DC collected in a gate encompassing only large cells (±SE), as outlined in *Materials and Methods*.

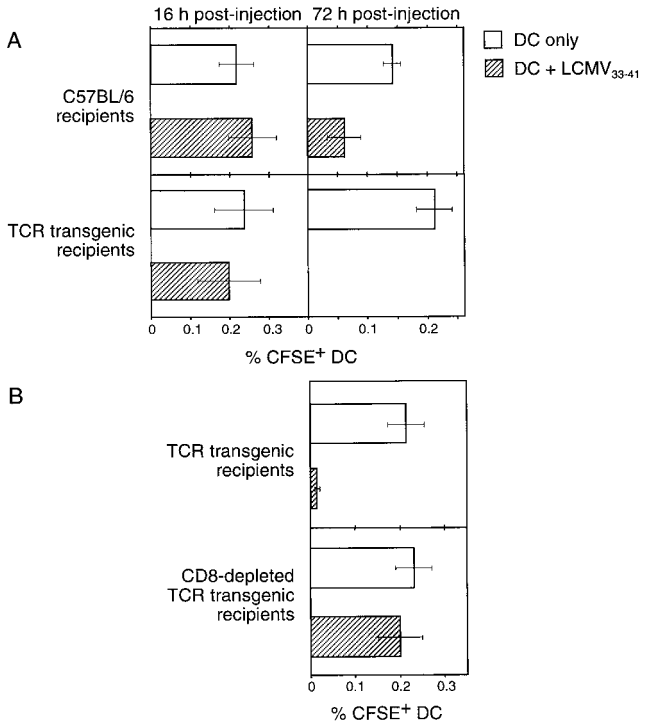

FIGURE 4. DC loaded with MHC class I-binding peptide Ag are cleared from the DLN. Groups of C57BL/6 or TCR transgenic mice were injected s.c. in the forelimb with either 10^6 $CFSE^+$ DC or 10^6 $CFSE^+$ DC that had been loaded with the $LCMV_{33–41}$ peptide recognized by the transgenic TCR. *A*, DLN were removed for FACS analysis 16 or 72 h after $CFSE^+$ DC injection. *B*, TCR transgenic recipients were depleted of $CD8^+$ T cells or were left untreated before injection with $CFSE^+$ DC. DLN were removed for FACS analysis 72 h later. All data are presented as in Fig. 3*B*.

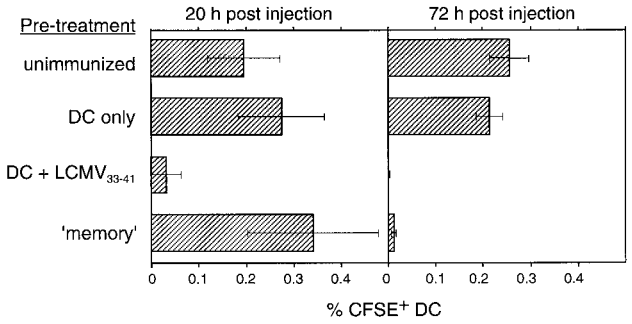

FIGURE 5. Clearance of Ag-loaded DC is accelerated in the presence of an Ag-specific immune response. The presence of $CFSE^+$, $LCMV_{33–41}$ peptide-loaded DC was monitored in the DLN of mice that had been immunized 1 wk before with 10^5 $LCMV_{33–41}$-loaded DC injected s.c. in the flank or with 10^5 DC not loaded with Ag or in mice that had received no previous treatment. A fourth group, termed the "memory" group, was also assessed. This group had been immunized with 10^5 $LCMV_{33–41}$ peptide-loaded DC 6 mo before and had also successfully rejected a challenge with 10^6 LL-LCMV tumor cells 7 days after immunization. All groups were injected s.c. with 10^6 $CFSE^+$, $LCMV_{33–41}$ peptide-loaded DC in each forelimb. Three animals from each group were analyzed at the indicated time points after $CFSE^+$ DC administration. Data are presented as in Fig. 3*B*.

(Fig. 4*B*). These results suggest that activation of TCR transgenic $CD8^+$ T cells to cytotoxic effector cells is associated with the disappearance of DC expressing specific Ag from the DLN. DC clearance occurs between 16 and 72 h after injection, a period consistent with the acquisition of effector function by $CD8^+$ T cells as reported by other investigators (20, 21). The minimal clearance of Ag-loaded DC demonstrated in the DLN of naive, non-TCR transgenic mice probably reflects the smaller number of Ag-specific T cells that become activated in these mice and hence the lower number of interactions that result in DC clearance.

Clearance of Ag-loaded DC is accelerated in the presence of an Ag-specific immune response

Next, we next investigated the fate of Ag-loaded DC in the context of an ongoing immune response. To this end, $CFSE^+$ DC loaded with the MHC class I-binding peptide $LCMV_{33–41}$ were administered to mice that had been injected s.c. in the flank 7 days before with 10^5 DC alone or with 10^5 DC loaded with the same $LCMV_{33–41}$ peptide. The 7-day interval was chosen because our previous studies have shown that Ag-specific T cell expansion is maximal at this time (22); also, resistance to challenge with $LCMV_{33–41}$-expressing tumors can be demonstrated (17). In addition, "memory" recipients were also used. These animals had received $LCMV_{33–41}$-loaded DC 6 mo before and had subsequently rejected a challenge with tumor cells expressing the $LCMV_{33–41}$ epitope. All mice received the $LCMV_{33–41}$-loaded, $CFSE^+$ DC by s.c. injection into the anterior forelimb. As can be seen in Fig. 5, similar numbers of $CFSE^+$ DC were observed in the DLN of mice that were either previously immunized with DC without Ag or in those of mice that had received no previous treatment. In contrast, a large reduction in the number of $CFSE^+$ DC was observed in the DLN of animals immunized with $LCMV_{33–41}$ peptide-loaded DC 7 days earlier. The reduction was clearly detectable as early as 20 h after injection, suggesting that it was related to the presence of peptide-specific effector cells in the recipient mice. Similar results were also obtained with MHC class $II^{-/-}$ DC (data not shown), indicating that $CD4^+$ T cells were not involved in DC elimination. A decrease in the number of $CFSE^+$ DC was also observed in the DLN of mice from the "memory" group. However, in this latter case the decrease in $CFSE^+$ DC was observed only at 72 h and not at 20 h after in vivo injection. This result suggests that the reduction in the number of $CFSE^+$ DC was related to the acquisition of effector activity by pre-existing memory $CD8^+$ T cells in the recipient mice.

Taken together, the data presented above indicate that the disappearance of $CFSE^+$ DC from the DLN of recipient mice is associated with the presence or the development of effector CTL. We suggest that in the course of an immune response, Ag-loaded DC become targets for CTL activity and are eliminated from the lymph node.

CTL-mediated clearance of DC from the lymph node impairs induction of further immune responses

We wished to establish whether elimination of Ag-loaded DC during an immune response impacts upon T cell activation initiated by the same DC. As an in vivo readout of T cell activation, we examined the proliferation of CFSE-labeled, $LCMV_{33–41}$-specific $CD8^+$ T cells adoptively transferred into syngeneic recipients. Proliferation of $CFSE^+$ T cells can be monitored in the DLN of immunized mice by flow cytometry and is detected as progressive halving of cellular fluorescence with every cell division completed (23). Thus, we examined the specific proliferation induced by DC that were or were not the targets of a pre-existing immune response. Mice were preimmunized s.c. in the flank with DC + $OVA_{257–264}$ peptide to elicit an OVA-specific CTL immune response or with DC only as a control. One week later, both groups of mice received an adoptive transfer of $CFSE^+$ $LCMV_{33–41}$-specific T cells and were immunized s.c. in the forelimb with DC loaded with both the $OVA_{257–264}$ and $LCMV_{33–41}$ peptides. These

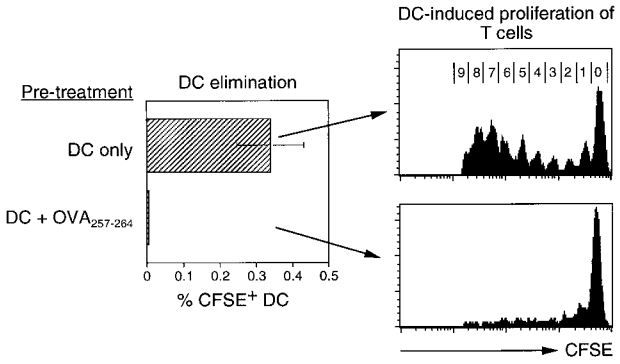

FIGURE 6. CTL-mediated clearance of Ag-loaded DC from the DLN limits the induction of further immune responses. Groups of mice were immunized s.c. in the flank with either 10^5 $OVA_{257–264}$-loaded DC or with 10^5 DC alone. All mice received a second injection of DC loaded with $OVA_{257–264}$ and $LCMV_{33–41}$ s.c. in the forelimb. Survival of the DC administered with the latter injection and their induction of a T cell response were examined. *Left panel*, $CFSE^+$ DC simultaneously loaded with $OVA_{257–264}$ and $LCMV_{33–41}$ are eliminated in OVA-immunized mice. Data were collected 66 h after injection of $CFSE^+$ DC and are presented as in Fig. 3*B*. *Right panel*, Adoptively transferred $CFSE^+$ TCR transgenic cells specific for $LCMV_{33–41}$ fail to divide in response to specific peptide presented on DC if the DC are eliminated by an existing CTL response. Data were collected 66 h after DC immunization; only $CFSE^+$, $V\alpha2^+$ $V\beta8.1,8.2^+$ events are shown. FACS profiles shown are of one representative mouse of the six in each group.

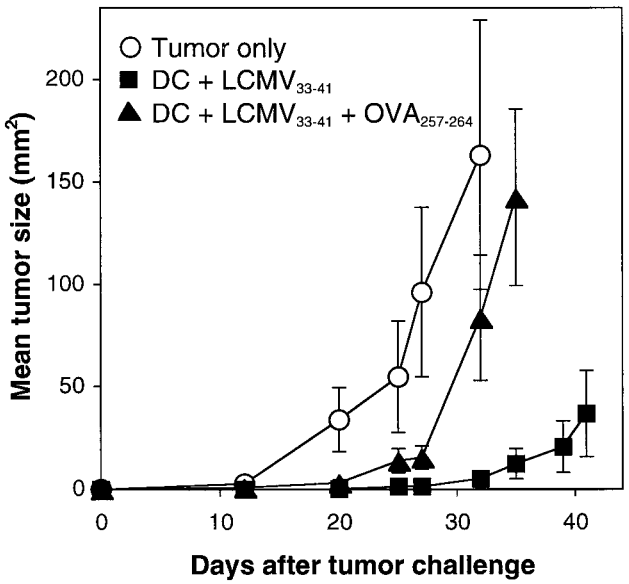

FIGURE 7. CTL-mediated clearance of tumor Ag-loaded DC from the DLN impairs the generation of antitumor immune responses. Two groups of mice ($n = 5$) were immunized with 10^5 $OVA_{257–264}$ peptide-loaded DC by s.c. injection in the flank, and then 1 wk later, one group was immunized with $LCMV_{33–41}$ peptide-loaded MHC class $II^{-/-}$ DC in the contralateral flank, and the second group was immunized with MHC class $II^{-/-}$ DC loaded with both $LCMV_{33–41}$ and $OVA_{257–264}$ peptides. After 7 days, both of these groups and an additional control group were challenged with 10^6 LL-LCMV tumor cells presenting the $LCMV_{33–41}$ epitope. Mice were monitored for tumor growth every 3–4 days, and tumor size for each group was calculated as the mean of the products of bisecting diameters (±SE). Measurements were terminated for each group when the first animal developed a tumor in excess of 200 mm^2.

mice were used to examine the proliferation of $LCMV_{33–41}$-specific T cells. In addition, separate mice that had also been immunized in the flank with DC + $OVA_{257–264}$ or with DC only received $CFSE^+$ DC that had been loaded with both the $OVA_{257–264}$ and $LCMV_{33–41}$ peptides by s.c. injection in the forelimb. These latter mice were used to evaluate the numbers of $CFSE^+$ DC in the DLN. As shown in Fig. 6, $OVA_{257–264}$ + $LCMV_{33–41}$-loaded DC were rapidly eliminated in mice that had previously been immunized with DC + $OVA_{257–264}$, most likely due to the induced OVA-specific CTL response. In contrast, a considerable proportion of $OVA_{257–264}$ + $LCMV_{33–41}$-loaded DC could be demonstrated in the DLN of mice previously immunized with DC only. Fig. 6 also shows the proliferative response of $LCMV_{33–41}$-specific T cells in mice that had received the same DC immunizations. In mice in which DC could be demonstrated in the DLN, $LCMV_{33–41}$-specific T cells underwent several cycles of cell division, indicating the onset of an active immune response. In contrast, in mice in which DC had disappeared from the DLN, only limited proliferation of $LCMV_{33–41}$-specific T cells could be demonstrated. We conclude from these results that CTL-mediated elimination of DC may have a profound impact on T cell immune responses and may prevent the initiation of immune responses to other Ags that are simultaneously presented on the same DC.

CTL-mediated clearance of tumor Ag-loaded DC from the lymph node impairs antitumor immune responses

We wished to extend our findings by examining whether elimination of Ag-loaded DC during the induction of an antitumor immune response has a significant negative impact on antitumor immunity. For these studies we used the tumor cell line LL-LCMV, a Lewis lung carcinoma expressing the $LCMV_{33–41}$ epitope (17). All mice were preimmunized s.c. in the flank with DC loaded with $OVA_{257–264}$ to induce $OVA_{257–264}$-specific CTL responses. One week later, mice received a second s.c. immunization in the opposite flank with MHC class $II^{-/-}$ DC that were loaded with both $OVA_{257–264}$ and the "tumor Ag" $LCMV_{33–41}$ or with MHC class $II^{-/-}$ DC loaded with the "tumor Ag" $LCMV_{33–41}$ only. MHC class $II^{-/-}$ DC were used in this experiment to rule out any effects mediated by $CD4^+$ T cells. Similar results were obtained when MHC class $II^{+/+}$ DC were used (data not shown), because DC are eliminated by pre-existing CTL responses regardless of MHC class II expression. DC presenting $OVA_{257–264}$ and tumor Ag became targets of the anti-$OVA_{257–264}$ CTL response and were eliminated (data not shown). In contrast, DC presenting tumor Ag alone remained unaffected. One week after the second DC immunization, mice were challenged with LL-LCMV, and tumor size was measured. In Fig. 7, we show that DC presenting both $OVA_{257–264}$ and $LCMV_{33–41}$ were less effective at inducing antitumor immunity than DC presenting the $LCMV_{33–41}$ Ag alone were. This reduction in efficacy was most likely due to the rapid clearance of $OVA_{257–264}$-loaded DC by $OVA_{257–264}$-specific CTL, which prevented the productive initiation of an $LCMV_{33–41}$-specific immune response. However, a limited degree of antitumor immunity was reproducibly induced by $OVA_{257–264}$-loaded DC in these experiments, suggesting that the DC were able to induce some degree of T cell activation before being eliminated. Alternatively, DC-associated Ag had been released and taken up by other APC, allowing a specific immune response to be initiated.

Discussion

The capacity of DC to migrate from peripheral tissues to lymphoid organs and to initiate immune responses has been the subject of a number of investigations (reviewed in Ref. 1). In contrast, the subsequent fate of DC has received little attention. DC are not observed in the efferent lymph (12), suggesting that they may not be

capable of leaving the lymph node and may die in situ. It has been speculated that DC may be eliminated by the very immune responses they elicit. This would allow the activated T cells to proliferate freely and to migrate away from the lymph node to peripheral tissues or, in the case of $CD4^+$ T cells, it would allow the T cells to migrate to the B cell areas of the lymph node to deliver help for Ab production (14, 24). Furthermore, elimination of DC could function as a negative feedback mechanism to limit T cell responses and also could prevent excessive accumulation in the lymph node of DC that carry Ags to which the immune system has already been sensitized.

In this paper we have monitored the migration of $CFSE^+$ DC injected s.c. into mice. The effect of Ag presented on MHC class I on DC survival in the DLN was also examined. We show that Ag-loaded DC disappear from the lymph node during the course of a $CD8^+$ T cell response, with kinetics that parallel the known kinetics of activation of $CD8^+$ T cells to effector function (20, 25). Ag-loaded DC were immediately cleared in recently immunized mice with a reduction in DC numbers observed in the DLN within 20 h of injection, suggesting that some DC may have been eliminated before reaching the lymph node. The kinetics of clearance were somewhat slower in "memory" mice, in which labeled DC were clearly demonstrated in the DLN at 20 h after injection but not at 72 h after injection. Memory $CD8^+$ cells presumably would need to reacquire effector function before mediating elimination of DC in these mice (21). Lastly, Ag-loaded DC were cleared from the DLN between 16 h and 72 h after s.c. injection into naive, TCR transgenic mice. This indicates that Ag-loaded DC may be eliminated as a consequence of a primary $CD8^+$ T cell immune response, perhaps as a mechanism to allow turnover of DC within the lymph node. This process may contribute to the restriction of the number of T cell specificities generated in a given immune response, a phenomenon that has been reported for viral infections (26–28). High-avidity T cells that become successfully activated in response to Ag on a given DC may eliminate that DC, thereby preventing the full activation of lower-avidity T cells that recognize different antigenic epitopes presented on the same DC.

The close parallel of the kinetics of DC clearance with the development of a CTL response suggests that the failure to demonstrate $CFSE^+$ DC in the DLN is most likely due to active elimination of labeled DC rather than to their migration to another site or to selective loss of the CFSE label from otherwise viable DC. This hypothesis is also supported by the observation that DC elimination was inhibited by anti-CD8 Ab treatment and did not require MHC class II expression by the DC. Elimination of DC was clearly a systemic event in that it was observed at sites distant from the site of original immunization, as would be expected on the basis of the CTL's ability to recirculate to different sites. However, the mechanism of DC clearance, although clearly Ag-specific, is as yet undefined. We cannot exclude the possibility that there was a constant rapid turnover of DC in the DLN and that the clearance observed represented a failure of DC to migrate to the DLN in the presence of activated CTL. However, the fact that Ag-loaded DC were present in the DLN of naive TCR transgenic mice at 16 h and then were lost by 72 h is highly suggestive of a cytolytic mechanism operating within the node. Furthermore, the kinetics of DC clearance in naive animals are consistent with the reported time required for CTL to gain cytolytic function upon activation (20, 25). Preliminary experiments indicated that DC clearance is not impaired in IFN-γ-deficient mice (data not shown), suggesting that this cytokine is not critical to the clearance process and that other effector mechanisms, presumably cytotoxicity, have a greater role. Other authors have also reported a decrease in the number of Ag-loaded DC in the lymph node during a $CD4^+$ T cell immune response (14). That decrease appeared less profound than the one we describe here, probably reflecting different mechanisms of DC elimination during $CD4^+$ T cell responses compared with $CD8^+$ T cell responses. DC elimination has also been demonstrated by histological means in mice exposed to viruses that induce strong CTL immune responses (29).

We also show here that CTL-mediated clearance of Ag-loaded DC can have significant consequences on immune responses. Ag-specific T cell activation and proliferation, and induction of antitumor immunity were severely impaired when the DC were cleared by an existing CTL response. However, it should be noted that a limited degree of T cell proliferation and antitumor immunity was reproducibly demonstrated in these experiments, suggesting that some T cell activation was occurring despite the rapid clearance of DC. This weakened T cell response may have resulted from suboptimal stimulation by DC in the process of being eliminated or may have been induced by a low number of healthy DC that had escaped elimination.

It has been reported that Ag from short-lived migratory DC can be processed and presented by recipient DC within the lymph node (30). If DC are cleared from the DLN by a cytolytic mechanism, these dying cells may provide a ready source of Ag for lymph node-resident DC. The recipient DC may be of the "lymphoid" subclass that has been proposed to serve a regulatory, tolerogenic function (31, 32). This process could account for the reduced antitumor responses observed when the DC were "cleared" by an existing CTL response. Alternatively, it is possible that transfer of Ag from migratory DC to lymph node-resident DC, in fact, may be stimulatory but that this process was inefficient in our experimental system.

One surprising finding in our study is that a proportion of the injected DC appeared to persist in the DLN for a long time (in excess of 7 days from the in vivo transfer). This suggests that DC may have the ability to "wait" for T cells of the appropriate specificity to migrate through the lymph node and to recognize Ag on their surface. The prolonged survival of some DC may be simply a stochastic process or alternatively may reflect heterogeneity in the degree of activation of our DC before injection in vivo. It is also possible that the DC become activated in vivo by host $CD4^+$ T cells to become the "temporal bridge" hypothesized by Ridge et al. (10), thereby becoming "conditioned" to stimulate further immune responses. Our experiments do not distinguish among these possibilities. In addition, we cannot exclude the possibility that this apparent prolonged persistence of DC in the DLN may in fact represent a slow turnover of DC that are continuously replaced by fresh DC migrating from the site of injection. We find this possibility less attractive in that it would appear that the lymph node should offer a more favorable environment for DC survival compared with that of the periphery. However, additional experiments are required to clarify this issue.

The process of DC clearance has implications for the design of DC-based immunotherapy regimes. From our results it can be concluded that repeated immunizations at short intervals with DC loaded with a given MHC class I-binding peptide may not be effective at enhancing responses to this Ag because the DC used in the secondary injections would be rapidly cleared by existing CTL. Indeed, our own experiments have suggested that repeated immunization with $LCMV_{33-41}$-loaded DC does not lead to enhanced CTL responses against LL-LCMV challenge (data not shown). However, repeated DC injections may be effective in maintaining effector function in "memory" $CD8^+$ T cells that may have lost activity due to suboptimal presentation of Ag in the context of tumor tissue.

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