Direct CD4 Help Provision following Interaction of Memory CD4 and CD8 T Cells with Distinct Antigen-Presenting Dendritic Cells

Marie-Ghislaine de Goër de Herve, Bamory Dembele, Mélissa Vallée, Florence Herr, Anne Cariou and Yassine Taoufik

*J Immunol* 2010; 185:1028-1036; Prepublished online 18 June 2010; doi: 10.4049/jimmunol.0904209

http://www.jimmunol.org/content/185/2/1028

References

This article cites 44 articles, 24 of which you can access for free at:

http://www.jimmunol.org/content/185/2/1028.full#ref-list-1

**Why The JI?**

Submit online.

• **Rapid Reviews! 30 days** from submission to initial decision

• **No Triage!** Every submission reviewed by practicing scientists

• **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Direct CD4 Help Provision following Interaction of Memory CD4 and CD8 T Cells with Distinct Antigen-Presenting Dendritic Cells

Marie-Ghislaine de Goër de Herve,*†‡,1 Bamory Dembele,*† Mélissa Vallée,* Florence Herr,* Anne Cariou,* and Yassine Taoufik*†‡‡

Accumulating evidence suggests that CD4 help is needed at the memory stage to mount effective secondary CD8 T cell responses. In this paper, we report that memory CD4 T cells can provide efficient help to memory CD8 T cells after interaction of the two lymphocytes with distinct dendritic cells. Provision of help to CD8 T cells required direct cell–cell contact and involved both IL-2 and CD40 ligation, within a CD4–CD8 T cell synapse. Thus, following antigenic interaction with APCs, activated memory CD4 and CD8 T cells appear to separate from their respective APCs before meeting each other for help provision, regardless of their Ag specificity. CD4 help for memory CD8 T cells therefore appears to be conditioned primarily not by Ag specificity but by activation status. The Journal of Immunology, 2010, 185: 1028–1036.

Although signals from CD4 T cells may not always be required for primary CD8 T cell responses, particularly in inflammatory environments, their absence undermines CD8 T cell memory responses (1). Help from CD4 T cells appears to be necessary at the time of initial antigenic activation of naive CD8 T cells (2, 3), and CD4 T cells may also be required for long-term maintenance of memory CD8 T cells after Ag eradication (2–4). Deficient secondary CD8 T cell responses have also been reported when CD4 T cells are lacking at the time of Ag reactivation of memory CD8 T cells (5–10), even if the CD8 T cells are generated in normal help conditions (5–10). The nature of the help provided by CD4 T cells during priming of naive CD8 T cells and during maintenance or reactivation of memory cells is largely unknown and may differ in these three situations. Yet the mechanisms of CD4 help during memory CD8 T cell reactivation are of major fundamental and therapeutic importance. Indeed, whereas Ag-specific help during CD8 T cell priming is provided by naive CD4 T cells, help during secondary activation is likely provided by memory CD4 T cells, which could be subject to complex tolerance phenomena. It has been reported that Ag-specific CD4 T cell memory responses are selectively impaired in several human and mouse chronic viral infections and malignancies, and that this undermines CD8 T cell responses (6, 11–22). A key player in CD4 help is the Ag-presenting dendritic cell (DC), which allows CD4 and CD8 T cells to meet and become activated. A longstanding concept is that CD4 and CD8 T cells must recognize their cognate Ag on the same DC for help to be provided (23, 24). However, these experiments involved CD8 T cells in the early stages of differentiation. Moreover, accumulating evidence, including some obtained in vivo, suggests that CD8α and non-CD8α DC subsets are, respectively, preferentially able to cross-present Ags and to activate CD8 and CD4 T cells (25–32). Finally, if helper CD4 T cells must indeed interact with the same Ag-presenting DC as CD8 T cells, this would entail markedly unbalanced competition between CD4 and CD8 T cells for physical access to the DC, owing to differential expression of MHC class I and class II peptide complexes on different DC subsets (CD8α and non-CD8α), particularly at the memory stage, in which Ag-specific cells are present at high frequencies.

In this paper, we examined whether memory CD4 T cells must physically interact with the same DC as memory CD8 T cells to provide help for optimal secondary CD8 T cell responses. We found that help provision does not necessarily require Ag activation of CD4 and CD8 T cells by the same DC. Following release of activated memory CD4 and CD8 T cells from distinct Ag-presenting DCs, help is provided via direct CD4 T cell–CD8 T cell contact and involves both IL-2 and CD40 ligation.

Materials and Methods

Mice

Mice with a C57BL/6 background were used for all experiments. Wild-type (WT) C57BL/6 mice were purchased from Janvier Laboratories (Le Genest-St-Ise, France). MHC class IIα−/− mice were kindly provided by Dr. B. Lucas (Centre National de la Recherche Scientifique Unité Mixte de Recherche 8104, Paris, France) (33). MHC class I−−/− mice (H-2Kb/H-2Db/β-2m triple knockouts [KO]) were kindly provided by Dr. F. Lemonnier (Institut Pasteur, Paris, France). H-2Kb/H-2Db/β-2m triple KO mice were generated by crossing H-2Kb/H-2Db/β-2m triple KO mice with β-2m KO mice (from The Jackson Laboratory, Bar Harbor, ME) (34). CD11c DTR mice were purchased from The Jackson Laboratory. These latter mice express the diphtheria toxin receptor (DTR) fused to enhanced GFP under the control of the CD11c promoter (35). Their DCs therefore selectively express DTR, allowing their depletion by DT exposure (35). All the protocols were approved by the local ethics committee (Villejuif, France).
**Immunization protocols**

We used two different model Ags, namely, the male Ag (H-Y) and OVA. Virgin female WT C57BL/6 mice aged 6–12 wk received either 1) i.p. injection of 150 μg purified OVA emulsified in CFA (Sigma-Aldrich, St. Louis, MO) and, 2 wk later, 150 μg purified OVA emulsified in IFA; or 2) two i.p. injections, 2 wk apart, of 2 × 10⁶ male splenocytes in 100 μl HBSS. Two weeks later, the mice were killed and their spleen cells were pooled.

**Cells and culture**

Lewis lung carcinoma (LLC) cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and were cultured as previously described (10). LLC tumor lysates were prepared as outlined before (10). Male apoptotic splenocytes, obtained by osmotic shock, were used as a source of male Ag. DCs were isolated from the spleen of untreated mice, using anti-CD11c microbeads (Miltenyi Biotec, Auburn, CA). Bone marrow-derived DCs (BMDCs) were differentiated from bone marrow progenitors, as previously described (36). CD8α and CD4+CD8α−CD11c+DCs were isolated from splenic DCs by cell sorting after CD3/CD11c/CD8/CD4 staining (FACSVantage Cell Sorter; BD Biosciences, San Jose, CA).

For T cell/DC coculture, CD4 and CD8 T cells were purified from the spleens of H-Y- or OVA-immunized animals, using anti-CD4 and anti-CD8 microbeads (Miltenyi Biotec). Spleenic DCs were loaded with male apoptotic cells (2 × 10⁶ apoptotic cells/1 ml CFA) and matured with 0.5 μg/ml LPS (Sigma-Aldrich) for 24 h. DCs were then washed extensively before use. A total of 100,000 DCs were cocultured with 10⁶ CD8 T cells in the presence or absence of 10⁶ CD4 T cells in 1 ml complete medium for 5 d. In some experiments, CD8 and CD4 T cells were separated by a semipermeable membrane (0.4-μm pore size; Costar, Cambridge, MA).

In some experiments, we used CD11c-DTR DCs isolated from spleens. CD8 T cells (labeled or unlabeled with CFSE) and CD4 T cells were isolated from spleens of H-Y-immunized animals and cocultured in distinct wells with HY-loaded mature CD11c-DTR Dcs for 2 d. DT (Sigma-Aldrich) was added at 0.5 μg/ml for 15 h. DC killing was checked by flow cytometry. CD4 and CD8 T cells were washed twice and cultured together for a further 2 d before proliferation and Granzyme B assays.

**Tumor experiments**

WT mice 6–12 wk of age were vaccinated with 150 μg purified OVA emulsified in 100 μl CFA. Two weeks later, 10⁶ LLC cells were injected s.c. in the left flank. Then, 10–12 d later, when the tumor was palpable, the mice received an s.c. injection in the right flank of 5 × 10⁶ Ag-loaded mature BMDCs prepared as follows: Immature BMDCs were loaded for 3 h with 10 μM OVA₂₃₃–₃₅₀ and/or 100 μg/ml LLC tumor lysate, then washed and matured for 24 h with LPS (0.5 μg/ml). Two weeks after the s.c. injection, the mice received a second injection of Ag-loaded mature BMDCs. The tumor was measured three times per week with a digital caliper. Tumor volume (mm³) was estimated as width × width × length × π/6.

**Proliferation assays**

CD8 T cells were stained with 0.5 μM CFSE and cocultured for 5 d with CD4 T cells and Ag-loaded mature DCs, as described above. In some experiments, the following neutralizing Abs were used at 10 μg/ml: anti–IL-7, anti–IL-15, anti–CD40L (all from ebioscience, San Diego, CA), anti–IL-2 and anti–IL-21 (from R&D Systems, Minneapolis, MN). On day 5, the cells were stained with anti–CD8-PE-Cy5, anti–CD4-PE-Cy7, anti–CD3-APC-Alexa750, and APC-labeled Ag-specific dextramer. CFSE dilution was analyzed in the CD₈⁺ gate. Data were analyzed with FlowJo software (Tree Star, Ashland, OR), and results were expressed as the percent of divided cells among initial CD₈⁺ T cells.

**Flow cytometry and Elispot**

For CD107/dextramer staining, 10⁵ male target cells per well were added to DC/T cell cocultures (see above), together with 1 μl FITC-labeled anti–CD107a and anti–CD107b (BD Biosciences) and 10 μM monensin (Sigma-Aldrich) for 6 h. The cells were then stained with APC-labeled Uty-specific or OVA₃₂₃–₃₅₀–specific dextramer (Immudex, Copenhagen, Denmark), anti–CD8-PE-Cy5, anti–CD4-PE-Cy7, and anti–CD3-APC-Alexa750 (ebioscience) prior to flow cytometry (FACSCanto; BD Biosciences). CD107 expression at the cell surface was analyzed in the CD₈⁺ T cell population.

For Granzyme B staining, 10⁵ male target cells per well were added to DC/T cell cocultures for 6 h. The cells were then stained with APC-labeled Uty-specific dextramer and anti–CD8/CD4/CD3, as described above. The cells were next permeabilized and stained with PE-conjugated anti–Granzyme B (ebioscience) before analysis. Granzyme B expression was analyzed on CD₈⁺ cells. In tumor experiments, Granzyme B⁺ tumor-infiltrating CD8 T cells were evaluated as previously described (10). In some experiments, the following neutralizing Abs were used at 10 μg/ml: anti–IL-7, anti–IL-15, anti–CD40L, and anti–LFA-1 (all from ebioscience), anti–IL-2, and anti–IL-21 (from R&D Systems).

For Granzyme B Elispot experiments, serial dilutions of cocultured DC/T cells were incubated with target cells at a 10:1 E:T ratio for an additional

---

**FIGURE 1.** CD4 help provision in vivo following Ag activation of CD4 and CD8 T cells with distinct DCs. A, Mice were vaccinated with OVA emulsified in CFA, and an LLC tumor was induced 2 wk later by s.c. injection in the left flank. When the tumor was palpable (10–12 d), mice were injected contralaterally with 10⁶ mature BMDCs loaded with tumor lysate alone (DC₈₊₄ Tum) or with tumor lysate + OVA₃₂₃–₃₅₀ (DC₈₊₄ + OVA₃₂₃–₃₅₀ Tum), or with a mixture of singly loaded DC₈₊₄ Tum and DC₈₊₄ + OVA₃₂₃–₃₅₀. Two weeks later, the mice received a second injection of Ag-loaded mature BMDCs, as above (injections are indicated by arrows). Tumor size was recorded three times per week. The data correspond to one experiment representative of a total of four independent experiments, with four to five mice per condition per independent experiment. B and C, Three weeks following BMDC injection, tumors were analyzed for CD8 T cell infiltration. B, Absolute number of CD8 T cells/10⁶ mm³ of tumor. C, Percentage of Granzyme B⁺ cells among infiltrating CD8 T cells. Results correspond to the mean ± SEM of three independent experiments, six mice per group per experiment.
24 h on an anti-Granzyme B–coated plate (R&D Systems). Spots were counted with a Zeiss Elispot counter. The results are expressed as the number of Granzyme B–producing cells per 10^6 CD8 T cells plated at the beginning of coculture.

In cytotoxicity experiments, the target cells consisted of a T cell line expressing OVA peptides (E.G7-OVA derivative of the EL-4 cell line, ATCC). The EL-4 cell line (ATCC) was used as a control. CFSE^hi-labeled E.G7-OVA target cells (0.3 μM) and CFSE^lo-labeled EL-4 control cells (0.075 μM) were mixed at a 1:1 ratio, and 2 × 10^5 total cells were added to DC/T cell cocultures. After overnight incubation, the CFSE^hi/CFSE^lo cell ratio in the live cell gate was analyzed and compared with control values (CFSE^hi/ CFSE^lo cell mix incubated alone). Results were expressed as percentages of specific cytotoxicity.

**IL-2 catch assay**

CD4 and CD8 T cells were activated separately with CD11c-DTR-DC. After DC killing, CD8 T cells were labeled with an IL-2 catch reagent (rat IgG2a anti–IL-2 conjugated with a rat IgG2b anti-CD45R; Miltenyi Biotec) and cocultured for 18 h with CD4 T cells, either in direct contact or separated by a semipermeable membrane. Cells were then stained with anti-CD3/CD4/CD8 Abs, Uty dextramer, and anti–IL-2-PE. IL-2 staining was analyzed in the CD3^+CD4^-/CD8^+Uty^+ cell population.

**Statistical analysis**

The Wilcoxon test was used.

**Results**

*In vivo, effective CD4 help can be provided to antitumoral CD8 T cells by heterospecific CD4 T cells interacting with distinct DCs*

We examined whether memory CD4 T cell help required interaction with the same DC as memory CD8 T cells. We have previously shown in the LLC model that functional anti-tumoral CD4 T cells are lacking and that functional heterospecific helpers directed against OVA^323–339 peptide are capable of providing effective help to anti-tumoral CD8 T cells interacting with the same
APC in vivo, thereby improving their function and survival and leading to significant tumor growth reduction (10). Following LLC tumor induction, we injected s.c. a mixture of mature DCs singly loaded with either the MHC II-restricted OVA\textsuperscript{323–339} peptide or an LLC tumor lysate, in the flank opposite the tumor. This had an antitumoral effect similar to that obtained by providing heterospecific help via interaction of OVA-specific memory CD4 T cells with the same mature DCs as anti-tumoral CD8 T cells (Fig. 1A). Tumor growth inhibition was associated with increased tumor infiltration by CD8 T cells (Fig. 1B). Tumor-infiltrating CD8 T cells expressed higher levels of Granzyme B (Fig. 1C). This finding showed that help could be provided at the time of Ag reactivation of memory CD8 T cells through antigenic interaction of CD4 and CD8 T cells with distinct mature DCs.

Effective help can be provided following interaction of CD4 and CD8 T cells with distinct DCs, including non-CD8\textsuperscript{a} and CD8\textsuperscript{a} DCs

We then addressed this issue in vitro. Following antigenic reactivation, memory CD8 T cells undergo secondary expansion and become secondary cytotoxic effectors. CD8 T cells isolated from female mice immunized with male splenocytes were cocultured with mature DCs loaded with both H-Y (lysate of male splenocytes) and OVA or with a mix of DCs singly loaded with either H-Y or OVA. Helpers were CD4 T cells isolated from OVA-immunized mice. As shown in Fig. 2A and 2B, effective help for secondary expansion of memory CD8 T cells was provided whether CD4 and CD8 T cells recognized their Ags on the same or distinct mature DCs. These results were confirmed using a mixture of HMC class I\textsuperscript{−/−} and MHC class II\textsuperscript{−/−} mature DCs interacting antigenically with memory CD4 and CD8 T cells, respectively (Fig. 2C). That help could be provided following interaction of CD4 and CD8 T cells with distinct DCs raised the possibility that help might be mediated by soluble factors. However, following physical separation of activated CD4 and CD8 T cells by a semipermeable membrane, CD8 T cells expanded less efficiently, whether the DCs used to activate T cells were MHC class I\textsuperscript{−/−} and class II\textsuperscript{−/−} (Fig. 2C) or WT (Fig. 2D). Effective help for CD107\textsuperscript{a} expression and Granzyme B secretion was also provided following interaction of CD4 T cells and memory CD8 T cells with distinct mature DCs (Figs. 3A, 3B, 4A, 4B). Experiments also included analysis of CD8 T cell cytotoxicity against an OVA-expressing cell line. In this case, both CD4 and CD8 T cells were isolated from OVA-immunized mice. These experiments also showed that help provision does not require

**FIGURE 3.** Effective degranulation of CD8 effectors following Ag activation of memory CD4 and CD8 T cells by distinct DCs. CD8 T cells isolated from H-Y–vaccinated mice were cocultured with CD4 T cells isolated from OVA- (A) or H-Y– (A, B) vaccinated mice and mature WT DCs (A, B) or a mixture of class I\textsuperscript{−/−} and class II\textsuperscript{−/−} DCs (B) presenting H-Y and/or OVA Ags for 5 d. CD107\textsuperscript{a} expression on the surface of CD8 T cells was measured by flow cytometry after challenge with male target splenocytes. In some conditions, CD4 and CD8 T cells with the corresponding Ag-loaded DCs were separated by a semipermeable membrane, symbolized in the graph by a horizontal line. Results are the mean ± SEM of two or three independent experiments. In A, representative plots of CD107 labeling among CD8 T cells are also shown.
interaction of the CD4 cell and the CD8 T cell with the same DC (Fig. 5A). Physical separation of activated CD4 and CD8 T cells by a semipermeable membrane reduced Granzyme B secretion and cytotoxicity (Figs. 4A, 4B, 5A) but had no impact on degranulation, based on CD107a cell surface expression (Fig. 3B). This observation suggested that distinct soluble and cognate help signals are required for secondary expansion, Granzyme B production, and cytotoxic granule release.

Accumulating evidence suggests that CD8α+ DCs are important for CD8 T cell activation, through their ability to cross-present Ags (25–32). As shown in Fig. 5B, we found that memory CD4 T cells interacting with MHC class I−/− CD8−/− DCs could provide effective help.
to cytotoxic memory CD8 T cells interacting with MHC class II−
CD8α+ DCs, leading to increased cytotoxicity (Fig. 5B).

Direct interaction between activated CD4 and CD8 T cells
during help provision does not require the presence of DCs

The above results suggested that CD4 and CD8 T cells can be
activated by distinct DCs but that they then have to meet for
optimal differentiation into secondary effectors. We therefore
examined whether DCs were necessary during this encounter; in
other words, whether the CD4 cell or the CD8 T cell must still be
interacting with its APC when the helper and the killer meet. For
this purpose, we used splenic DCs from CD11c DTR mice, which
express a receptor for DT, enabling their rapid depletion on DT
exposure (Fig. 6B). CD4 and CD8 T cells were activated sepa-
ately with DTR DCs; then the DCs were killed, and CD4 and
CD8 T cells were placed in contact with each other (Fig. 6A, 6B).
The results showed that direct interaction between activated CD8
T cells and homospecific or heterospecific CD4 T cells, which is
required for optimal differentiation of memory CD8 T cells into
secondary cytotoxic effectors, did not require the presence of DCs (Fig. 6C).

CD4 help involves IL-2 and CD40 ligation provided through
adhesion molecule-stabilized contact between memory CD4
and CD8 T cells

To examine the signals emitted by CD4 T cells during direct
interaction with CD8 T cells, we used neutralizing Abs to cyto-
kines that are known to influence the functions of memory CD8
T cells, namely, IL-2, IL-7, IL-15, and IL-21. IL-7 and IL-15 were
used as controls, as they are not secreted by CD4 T cells. We also
used a neutralizing Ab to CD40L, as CD40 is expressed at the
surface of activated human and mouse CD8 T cells (24, 37). CD4
and CD8 T cells were again activated separately with DTR DCs; then
DTR DCs were depleted, and CD4 and CD8 T cells were placed
in contact. IL-2 neutralization led to less efficient secondary ex-
pansion of memory CD8 T cells, whereas the other neutralizing
Abs had no clear effect (Fig. 7A). As shown in Fig. 7B, CD4 help
for Granzyme B production by activated memory CD8 T cells
required CD40–CD40L interaction and IL-2. As reported above,
CD4 help was abrogated by physical separation with a semiper-
meable membrane, suggesting that close physical proximity is
required for CD4 T cell-secreted IL-2 to efficiently enhance
secondary expansion and cytotoxic functions of memory CD8

**FIGURE 6.** Provision of help by activated memory CD4 T cells to activated memory CD8 T cells no longer requires the presence of DCs. A–C, CD8 T cells isolated from H-Y–vaccinated mice and CD4 T cells isolated from H-Y– or OVA-vaccinated mice were activated in distinct wells with H-Y– or OVA-loaded mature CD11c DTR DCs for 2 d. DT was then added or not to the cultures to deplete DTR DCs. Activated CD4 and CD8 T cells were next cocultured for 2 d, then challenged with male target splenocytes, and intracellular Granzyme B was assayed in Uty+ CD8 T cells. A positive control with CD4 T cells, CD8 T cells, and DTR DCs cocultured in the same well without DT was used. A control without CD4 T cells was also used. The experimental scheme is depicted in A. CD11c DTR DC depletion following DT treatment is shown in B. Results are the mean ± SEM of three independent experiments.
T cells, possibly owing to the need for a high local IL-2 concentration. We used an IL-2 detection assay to evaluate IL-2 levels at the surface of CD8 T cells (see Materials and Methods). As shown in Fig. 7C, IL-2 was undetectable at the surface of most CD8 T cells when the latter were separated from CD4 T cells by a semipermeable membrane, whereas IL-2 was detected at the surface of most CD8 T cells placed in direct contact with CD4 T cells. Together, these results suggest that optimal differentiation into secondary CD8 T effectors requires direct contact with activated CD4 T cells. This contact involves CD40 ligation and allows optimal capture of IL-2. As shown in Fig. 7B, CD4 help provision to CD8 T cells also required the adhesion molecule CD40.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** IL-2 and CD40 signaling, two help signals emitted by activated memory CD4 to CD8 T cells within an immunological synapse. A, CD8 and CD4 T cells were isolated from H-Y–vaccinated mice and labeled with CFSE. CD4 and CD8 T cells were activated in distinct wells with H-Y–loaded mature CD11c DTR DCs for 2 d. DT was then added to cultures to deplete DCs. Activated CD4 and CD8 T cells were next cocultured for another 2 d in the presence of the indicated neutralizing Abs. CFSE dilution was assayed in CD8^{H-Y+} T cells. Results are expressed as the percent of divided cells among initial CD8^{H-Y+} T cells. B, Cells were obtained and cultured as in A (but without CFSE labeling); then CD8^{H-Y+} T cells were tested for intracellular Granzyme B following challenge with male target splenocytes. Results are the mean ± SEM of three to four independent experiments. C, CD8 and CD4 T cells were isolated from H-Y–vaccinated mice, then activated in distinct wells by H-Y–loaded mature CD11c DTR DCs for 2 d. DT was then added to the cultures to deplete DCs. CD8 T cells were next labeled with IL-2 catch reagent and cocultured overnight with CD4 T cells, with or without a semipermeable separating membrane, symbolized in the graph by a horizontal line. Captured IL-2 was revealed with PE-labeled anti–IL-2.

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Injection of DCs singly loaded with a recall Ag reduced tumor growth in vivo. A, Mice were vaccinated with OVA emulsified in CFA, and an LLC tumor was induced 2 wk later by s.c. injection in the left flank. When the tumor was palpable (10–12 d), mice were injected contralaterally with 10^5 singly loaded mature DC^{OVA}. Two weeks later, the mice received a second injection of Ag-loaded mature BMDCs, as above (injections are indicated by arrows). Tumor size was recorded three times per week. The data correspond to the mean ± SEM of four independent experiments, with three mice per condition per independent experiment. B and C, Three weeks following DC injection, tumors were analyzed for CD8 T cell infiltration. B, Absolute number of CD8 T cells/100 mm^3 of tumor. C, Percentage of Granzyme B+ cells among infiltrating CD8 T cells. The data correspond to the mean ± SEM of four independent experiments, with three mice per condition per independent experiment. DC^{OVA}, BMDC^{OVA}_323–339 alone.
LFA-1, suggesting the formation of an adhesion molecule-stabilized immunological synapse between memory CD4 and CD8 T cells through which help signals are conveyed.

**In vivo, DCs singly loaded with the OVA\textsubscript{323–339} peptide reduced tumor growth**

The above results raised the possibility that injection of DCs singly loaded with a recall Ag might activate functional heterospecific memory CD4 T cells that could therefore provide direct help to anti-tumoral CD8 T cells activated by endogenous DCs. Following LLC tumor induction in mice previously immunized against OVA, we injected s.c. mature DCs singly loaded with the HII-restricted OVA\textsubscript{323–339} peptide, in the flank opposite the tumor. This led to significant inhibition of tumor growth (Fig. 8A) and increased tumor infiltration by CD8 T cells expressing higher levels of Granzyme B (Fig. 8B, 8C).

**Discussion**

A better understanding of how memory CD4 T cells, CD8 T cells, and DCs interact during CD4 help provision is needed to design effective immunotherapies for chronic diseases associated with defective CD4 help, such as cancer and chronic infections by viruses such as hepatitis C virus and HIV (6, 11–22). It is conceivable that CD4 T cells provide help in different ways for the generation, long-term maintenance, and reactivation of memory CD8 T cells.

The results presented in this paper suggest that, during Ag reactivation of memory CD8 T cells, CD4 T cells and CD8 T cells can interact antigenically with distinct DCs, and that the help thus provided is as effective as when the two lymphocytes recognize their cognate Ags on the same DC. We found that the help provided to memory CD8 T cells involved both soluble and cognate components, including IL-2 and CD40 ligation. In addition, IL-2 secreted by CD4 T cells was effective only when CD4 and CD8 T cells were in proximity. This is in line with previous results suggesting a synaptic basis for paracrine IL-2 signaling during T–T cell interactions in vivo (38). Multifocal synapses between T cells may polarize both IL-2 secretion and IL-2 signaling subunits (38). Physical contacts between activated CD4 and CD8 T cells may allow efficient IL-2 capture and CD40 signaling, promoting optimal clonal expansion and acquisition of effector functions. The key role of IL-2 in secondary expansion of memory CD8 T cells has previously been reported (39, 40), but it was unclear at which step—priming and/or reactivation—this signal was required (40). The role of IL-2 during priming was not investigated in this study, because CD8 T cells were primed in nonlimiting CD4 help conditions, but IL-2 was clearly required at the time of Ag reactivation. Both human and mouse CD8 T cells express CD40 following Ag activation (24, 37). Evidence pointing to the involvement of direct CD40–CD40L interaction between CD8 and CD8 T cells in CD4 help during priming has been obtained, mainly in terms of help for CD8 T cell proliferation (24), but was not subsequently confirmed in other experimental models (41). In this study, CD40L blockade had no effect on secondary expansion of activated memory CD8 T cells; however, like IL-2, and in a nonredundant manner, it prevented Granzyme B production, pointing to the involvement of this interaction in CD4 help at the memory stage.

These results are in line with the emerging concept of “division of labor” among DC subsets with respect to CD4 and CD8 T cell activation. Among lymphoid tissue-resident DCs, CD8\textsuperscript{+} cells specialize in Ag cross-presentation, whereas non-CD8\textsuperscript{α} DCs preferentially activate CD4 T cells (26, 32).

On the basis of these results, we propose the following scenario. In lymphoid tissues, CD4 T cells interact antigenically with DCs migrating from the periphery or with resident non-CD8\textsuperscript{α} DCs, whereas CD8 T cells are activated by CD8\textsuperscript{α} DCs. Activated CD4 and CD8 cells then need to interact physically for efficient help provision. Physical contact between CD4 and CD8 T cells requires adhesion molecules, implying the formation of an immunological synapse that could provide an appropriate microenvironment for help signal transmission. Importantly, DCs are no longer required at the stage when CD4 and CD8 T cells meet. Activation of CD4 and CD8 T cells by distinct DCs may avoid competition for the same DC, particularly at the memory stage, when specific T cells are present at high frequencies. Crucially, our results also suggest that a CD4 T cell may provide help to a CD8 T cell, whatever the respective Ag specificities of the two cells, provided that both have been antigenically activated. However, it must be pointed out that constraints existing in vivo may limit Ag-unmatched CD4–CD8 T cell collaboration. These include constraints in space and time. The number of foreign Ags presented in a given local lymphoid microenvironment may be limited. In addition, the temporal window of CD40L expression by CD4 T cells is narrow (42). Other mechanisms are known to limit autoimmunity and excessive activation, including regulatory T cells (Tregs), which are activated in an Ag-specific manner. However, Tregs inhibit conventional T cells in an Ag-nonspecific manner, possibly via direct T–T cell contact (43), mirroring our present findings. Tregs may also modulate the maturation and/or function of DCs, thereby downregulating T cell activation (43).

We have shown in an experimental cancer model, in which anti-tumoral CD4 help is defective (10), that injection of OVA-immunized mice with a mixture of mature DCs preloaded with either a single class II-restricted OVA peptide or a tumor lysate reduces tumor growth as efficiently as injection of mature DCs loaded with both the OVA peptide and the tumor lysate. The use of DCs singly loaded with a helper peptide may prevent the re-creation of Tregs with antitumoral specificity to the same DC as heterospecific helper CD4 T cells. This could limit inhibitory interference mediated by inhibitory T cells with tumor Ag specificity. We have also shown that injection of only DCs loaded with a class II-restricted OVA peptide can provide help to anti-tumoral CD8 T cells activated by endogenous tumor Ag-presenting DCs, leading to tumor reduction. This could prevent the need for invasive procedures to obtain tumor Ags. However, one potential limitation of such an approach is that endogenous DCs in tumor-infiltrated lymph nodes could be functionally impaired (44).

In conclusion, we show that memory CD4 and CD8 T cells can be activated by distinct DCs, such as non-CD8\textsuperscript{α} and CD8\textsuperscript{α} DCs. CD4 and CD8 T cells then appear to dissociate from their respective DCs and enter into physical contact for full help provision. This finding points to a new model of CD4–CD8 T cell interaction at the memory stage and may have important implications for the development of immunotherapies for diseases associated with defective help.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


