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Synergistic CD40 signaling on APCs and CD8 T cells drives efficient CD8 response and memory differentiation

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ABSTRACT

The role of CD4 help during CD8 response and memory differentiation has been clearly demonstrated in different experimental models. However, the exact mechanisms of CD4 help remain largely unknown and preclude replacement therapy to develop. Interestingly, studies have shown that administration of an agonist aCD40ab can substitute CD4 help in vitro and in vivo, whereas the targets of this antibody remain elusive. In this study, we address the exact role of CD40 expression on APCs and CD8 T cells using aCD40ab treatment in mice. We demonstrate that aCD40 antibodies have synergetic effects on APCs and CD8 T cells. Full efficiency of aCD40 treatment requires CD40 expression on both populations: if one of these cell populations is CD40-deficient, the CD8 T cell response is impaired. Most importantly, direct CD40 signaling on APCs and CD8 T cells affects CD8 T cell differentiation differently. In our model, CD40 expression on APCs plays an important but dispensable role on CD8 T cell expansion and effector functions during the early phase of the immune response. Conversely, CD40 on CD8 T cells is crucial and nonredundant for their progressive differentiation into memory cells. Altogether, these results highlight that CD40–CD40L-dependent and independent effects of CD4 help to drive a complete CD8 T cell differentiation. *J. Leukoc. Biol.* 91: 859–869; 2012.

INTRODUCTION

The generation of memory CD8 T cells is a key factor for clinical immunotherapy. However, the mechanisms underlying effector and memory CD8 T cell differentiation are far from being established entirely [1–3]. Elucidation of these mechanisms, leading to the generation of long-lasting and highly efficient cells, may pave new pathways toward improved clinical therapy and vaccination.

A few years ago, we and others [4–8] demonstrated that the generation of efficient memory CD8 T cells requires the presence of CD4 T cells. Vaccinations and treatments relying on CD8 response should consequently target CD4 and CD8 T cell populations. An alternative is to bypass the requirement for CD4 T cells. This implies that the underlying mechanisms of CD4 help, which are still under extensive debates, should be dissected further [9–11].

CD4 help on CD8 T cell responses was described initially during CD8 T cell activation and involved CD40–CD40L interactions, expressed, respectively, on APCs and CD4 T cells [12]. Based on these observations, a sequential model has been proposed. This model supports the hypothesis that CD4 T cells express CD40L upon activation and activate the APCs, which express CD40. The licensed APCs would then drive CD8 responses [12]. Additionally, stimulations by an agonist aCD40ab have been proven to be sufficient to induce efficient CD8 responses in the absence of CD4 T cell help in vitro and in vivo [13–16]. Such aCD40ab treatments are used in tumor [17, 18] as well as in viral models [19] to increase CD8 T cell responsiveness. However, injection of aCD40ab may also induce side-effects. The administration of aCD40ab in certain tumor models reduced CD8 T cell response [20] and provoked the expression of several angiogenic factors enhancing tumor growth [21–24]. Other studies indicated that aCD40ab could profoundly suppress CD8 response to LCMV infection [25] and failed to induce effector functions in an influenza model [26]. Breakdowns of peripheral tolerance, inducing autoimmune diseases, have also been reported [27, 28].

Additionally to the APCs, CD8 T cells can express CD40 as well [5, 12]. Therefore, we hypothesize that such diversity of

Abbreviations: aCD40ab=anti-CD40 antibody, ARC=Association de la recherche contre le cancer, BM=bone marrow, CD3-$\varepsilon^{-/-}$=CD3-ε-deficient, Ct=threshold cycle, d=dilution, *hprt*=hypoxanthine guanine phosphoribosyl transferase, L=ligand, LCMV=lymphocytic choriomeningitis virus, LIP=lymphopenia-induced proliferation, T_{CM}=central memory T cells, Tg=transgenic, *Zfy-1*=zinc finger protein Y-linked

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effects is related to the diversity of targets. Indeed, we have demonstrated, in our experimental model, that CD40-deficient CD8 T cells were not able to receive CD4 T cell help and were incapable of differentiating into memory cells [5]. CD8CD40$^{-/-}$ T cells exhibited major defects in secondary response and on expansion, antigenic load control, effector functions, cytokine receptor expression patterns, as well as higher sensitivity to inhibitory cytokines [29]. These and other studies highlighted the significant importance of CD40–CD40L interactions in CD8 responses [30] and led to a reconsideration of aCD40 adjuvant treatment.

In this study, we investigated the effect of aCD40ab treatment on CD8 immune responses compared with the provision of CD4 help and determine the relative importance of CD40 expression on APCs and CD8 T cells. For this purpose, we used an experimental procedure permitting the restriction of CD40 expression on none, only one, or both of these populations.

MATERIALS AND METHODS

Mice

CD3-$\varepsilon^{-/-}$ mice; CD3-$\varepsilon^{-/-}$CD40$^{-/-}$ mice; Rag2$^{-/-}$ mice expressing a TCR-$\alpha\beta$ Tg specific for the HY male antigens, restricted to MHC class II IA^b [31] or restricted to MHC class I D^b [4]; and Rag2$^{-/-}$ CD40$^{-/-}$ HY Tg mice [5] were bred at the Center for the Development of Advanced Experimental Techniques (Orleans, France). Experimental procedures were approved by the French University Animals Ethics Committee and conducted according to the institutional guidelines of the European Community.

Immunization protocol

Sublethally irradiated (400 Rad) CD3-$\varepsilon^{-/-}$ and CD3-$\varepsilon^{-/-}$CD40$^{-/-}$ female mice were injected with 0.5×10^6 male + 4.5×10^6 female BM cells from CD3-$\varepsilon^{-/-}$ or CD3-$\varepsilon^{-/-}$CD40$^{-/-}$ mice, respectively. BM cells have a high capacity for cell divisions, allowing male cells to grow in host mice at early time-points after immunization, thus mimicking infectious antigenic spread or tumoral antigenic proliferation. Three days later, 0.5×10^6 LN CD8 HY Tg T cells (from Rag2$^{-/-}$ HY Tg CD40$^{+/+}$ or CD40$^{-/-}$ female mice) were injected alone, with an equal number of LN CD4 HY Tg T cells or with an agonist aCD40ab (FGK45; **Fig. 1A**). These HY Tg T cells are specific for male cells (expressing the HY antigen). The aCD40ab was injected at 50 μg/mouse at Days 0, 2, and 4. This administration protocol insures the presence of aCD40ab when APCs and CD8 T cells express the CD40 molecule. The activation of APCs results in a rapid up-regulation of CD40, whereas CD8 T cells in our in vivo experimental system express CD40 transitory, with an expression peak reached 4 days postimmunization [5]. At different time-points after the immunization, the number of CD8 T cells recovered from the spleen, a pool of LN, and liver was determined and referred to as number of CD8 T cells/mouse.

To perform in vivo secondary response, CD8 T cells were isolated and purified from the spleen of the different chimeras at Day 60 of the primary immune response. Then, 0.5×10^6 CD8 T cells were injected with 0.5×10^6 naive CD4 T cells into new chimeras immunized with the male antigen. As control, lethargic CD8 T cells (injected without CD4 help) were also isolated at Day 60 and injected alone into new chimeras immunized with the male antigen.

Immunofluorescence analysis

The following mAb were used: biotin-labeled anti-CD127 (IL-7Rα) and anti-CD62L revealed by streptavidin-allophycocyanin; PerCP-labeled anti-CD4 and anti-CD8; PE-labeled anti-IFN-γ; and FITC-labeled anti-T3.70 (anti-

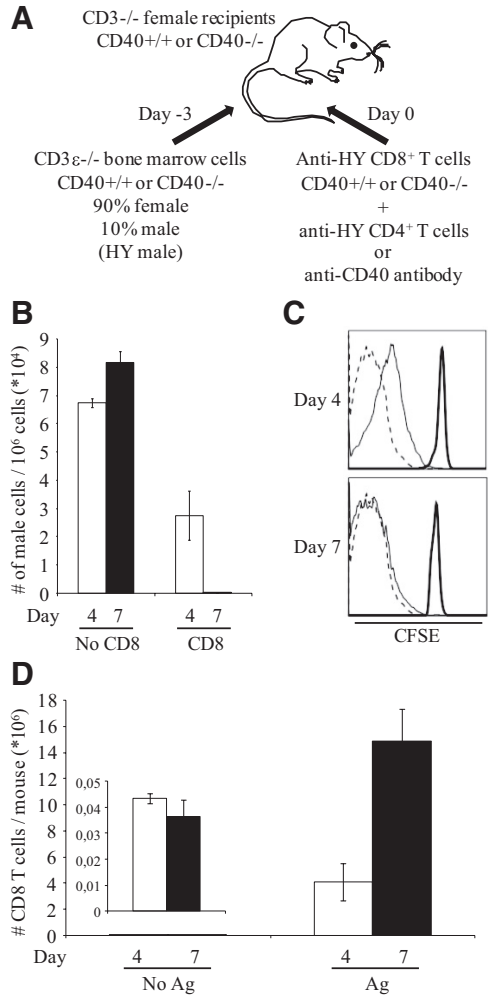

Figure 1. Description and characteristic of the experimental model. (A) Schema of the experimental strategy as described in Materials and Methods. (B) CD3-$\varepsilon^{-/-}$ female mice were injected with 0.5×10^6 male + 4.5×10^6 female BM cells from CD3-$\varepsilon^{-/-}$ mice. Three days later, one-half of them was injected with 0.5×10^6 CD8 + 0.5×10^6 CD4 T cells, and one-half of them was not injected with T cells. Results show the number of male cells detected/million splenic cells recovered at Day 4 (white histograms) and Day 7 (black histograms) from hosts injected (CD8) or not (No CD8) with T cells. Each sample was performed in triplicate. Data show the average ± SD of two mice/group and are representative of one experiment. (C and D) CD3-$\varepsilon^{-/-}$ female mice were injected with 5×10^6 female BM cells from CD3-$\varepsilon^{-/-}$ mice. Three days later, they were injected with 0.5×10^6 CD8 T cells. In parallel, CD3-$\varepsilon^{-/-}$ female mice were injected with 0.5×10^6 male + 4.5×10^6 female BM cells from CD3-$\varepsilon^{-/-}$ mice. Three days later, they were injected with 0.5×10^6 CD8 + 0.5×10^6 CD4 T cells. CD8 T cells were stained with CFSE before injection. (C) Results show CFSE staining of CD8 T cells isolated from female hosts injected (thin lines) or not (bold lines) with male BM cells at Days 4 and 7, respectively. Dotted lines represents the isotype control. (D) Results show the absolute number of CD8 T cells recovered at Day 4 (white histograms) and Day 7 (black histograms) from female hosts injected (Ag) or not (No Ag) with male BM cells. Data show the average ± SD of two mice/group and are representative of one experiment.

TCR-α Tg; PharMingen, San Diego, CA, USA). For some experiments, CD8 T cells were stained with CFSE before injection, as described previously [5]. Flow cytometry was performed using a FACSCalibur cytometer and data analysis via Flow Jo software (Becton Dickinson, San Jose, CA, USA).

Antigen load

The antigen load was determined directly by quantifying the number of male cells remaining in the spleen during the primary response, as described previously [29]. Briefly, we quantified the genomic DNA-encoded *Zfy-1* gene (present at one copy/antigenic male cells only) and *hprt* gene (present at two copies on male and female cells) using real-time PCR (7900 HT, Applied Biosystems, Warrington, UK) with SYBR Green dye (Applied Biosystems).

The number of male cells/million of total cells was calculated as follows: $2 \times 2^{(Ct^{hprt} - Ct^{Zfy\text{-}1})} \times (d^{hprt}/d^{Zfy\text{-}1}) \times 10^6$.

Cytokine secretion

Splenic CD8 T cells recovered from the chimeras were purified further by negative selection using a cocktail of mAb coated with Dynabeads (Dynal, A.S., Oslo, Norway) recognizing B cells, macrophages, and CD4 T cells (purity >99%). Purified CD8 T cells (0.5×10^6) were then incubated at 37°C with 1×10^6 spleen APCs (from CD3-$\varepsilon^{-/-}$ female mice) and 2.5 μg anti-CD3 mAb (clone 2C11, PharMingen)/well. After 2 h of incubation in the presence of Brefeldin A (10 μg/mL, Becton Dickinson), intracellular staining was performed as described previously [5]. IFN-γ and IL-2 secretions were determined in culture medium supernatants by ELISA (R&D Systems, Minneapolis, MN, USA) after 24 h of incubation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and the two-tailed Mann-Whitney test. All mentioned differences are statistically significant: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

RESULTS

Experimental strategy

To characterize CD8 response and memory differentiation, we used a well-described, noninfectious immunization system directed against the HY male antigen. Figure 1A recapitulates the experimental strategy. CD3-$\varepsilon^{-/-}$ female mice were injected with male BM cells. As BM cells have a high capacity for cell divisions, it allows male cells to grow in host mice. Indeed, the number of male cells increased constantly into host mice if the naïve HY CD8 Tg T cell were not injected (Fig. 1B). On the contrary, when CD8 T cells were injected, they acquired cytotoxic function and eliminated male cells (Fig. 1B). At Day 7, the elimination was almost completed, and by Day 14, no male cells were detected in the hosts. This experimental system relies on adoptive transfer of naïve T cells into an empty host, which could introduce a bias as a result of LIP. However, this particular naïve HY CD8 Tg T cell population did not suffer from LIP, as we and others have already shown [5, 32, 33]. We confirmed this in the present study. CD8 T cells injected into CD3-$\varepsilon^{-/-}$ female mice, not reconstituted with male BM cells, did not divide (Fig. 1C). Accordingly, the number of CD8 T cells recovered at Days 4 and 7 was similar and corresponded to the homing of CD8 T cells in the spleen (10% of the injected population; Fig. 1D). Contrary, in the presence of male cells, CD8 T cells have already strongly divided at Day 4, as no CFSE staining was detected at Day 7 (Fig. 1C). This correlated to a strong increase in absolute number of CD8 T cells (Fig. 1D). Thus, in this system, stimulation with the male antigen is strictly required for in vivo proliferation and differentiation of naïve CD8 T cells.

To evaluate the relative implication of CD40 expression on APCs and CD8 T cells, different chimeras immunized with the male antigen have been generated. Four groups of chimeras have been designed: CD40+ chimeras, APCs and CD8 T cells were $CD40^{+/+}$; CD40− chimeras, APCs and CD8 T cells were $CD40^{-/-}$; CD8 CD40− chimeras, APCs were $CD40^{+/+}$, and CD8 T cells were $CD40^{-/-}$; APC CD40− chimeras, APCs were $CD40^{-/-}$, and CD8 T cells were $CD40^{+/+}$. These different settings allow restricting CD40 expression on APCs and/or CD8 T cells or neither of them. Within each group, CD8 T cells were injected alone, with CD4 T cells or with aCD40ab (**Table 1**).

CD40 signaling on APCs is important for CD8 T cell expansion

We first determined the absolute number of CD8 T cells recovered from lymphoid organs in the different chimeras (**Fig. 2**).

TABLE 1. Description of the different groups of immunization

		CD8 T cells		
		$CD40^{+/+}$	$CD40^{-/-}$	
APCs	$CD40^{+/+}$	CD40+ chimeras	CD8 CD40− chimeras	Alone CD4 T cells aCD40ab
	$CD40^{-/-}$	APC CD40− chimeras	CD40− chimeras	Alone CD4 T cells aCD40ab

Different chimeras immunized with the male antigen have been generated, allowing restricted CD40 expression on different target cells. Four different groups can thus be considered: 1) CD40+ chimeras, APCs and CD8 T cells were $CD40^{+/+}$; 2) CD40− chimeras, APCs and CD8 T cells were $CD40^{-/-}$; 3) CD8 CD40− chimeras, APCs were $CD40^{+/+}$, and CD8 T cells were $CD40^{-/-}$; 4) APC CD40− chimeras, APCs were $CD40^{-/-}$, and CD8 T cells were $CD40^{+/+}$. In each group, CD8 T cells have been injected alone, with CD4 T cells or with aCD40ab. For all chimeras injected with CD8 T cells alone (groups 1–4), similar results were obtained. Therefore, only the chimeras, where both APCs and CD8 T cells were competent for CD40 (CD40+ chimeras), are shown in Results.

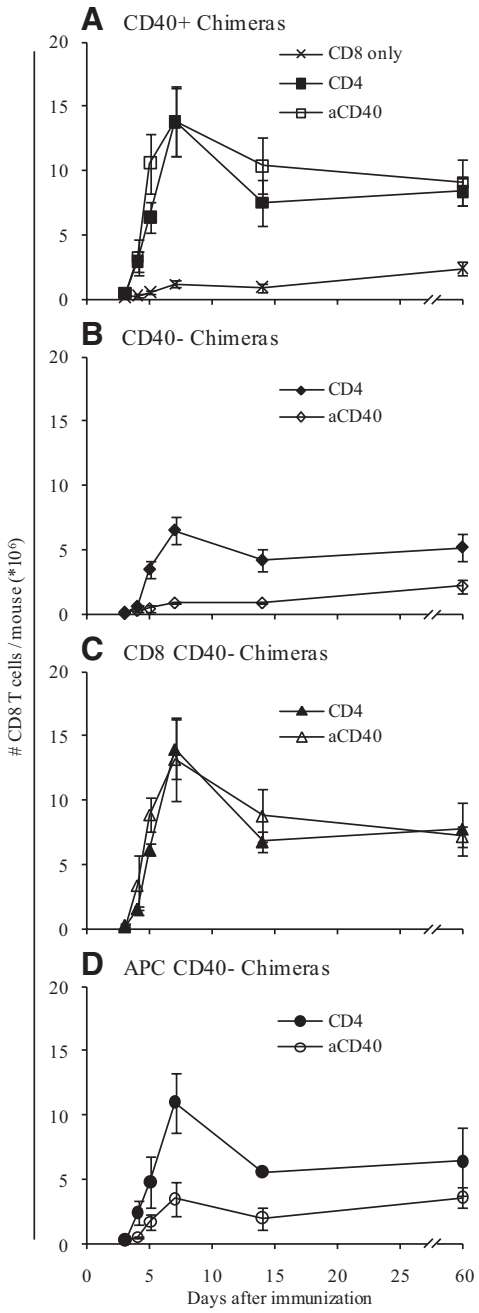

Figure 2. Synergic effect of CD40 pathways on CD8 T cell expansion in lymphoid organs. Total number of CD8 T cells recovered from lymphoid organs (spleen+LN) of individual mice at different time-points after immunization. (A) CD8 T cells recovered from CD40+ chimeras. CD8 T cells were injected alone (×) or coinjected with CD4 T cells (■) or aCD40ab (□). (B) CD8 T cells recovered from CD40− chimeras. CD8 T cells were coinjected with CD4 T cells (♦) or aCD40ab (◇). (C) CD8 T cells recovered from CD8 CD40− chimeras. CD8 T cells were coinjected with CD4 T cells (▲) or aCD40ab (△). (D) CD8 T cells recovered from APC CD40− chimeras. CD8 T cells were coinjected with CD4 T cells (●) or aCD40ab (○). Data show the average ± SEM of three mice/group and are representative of six independent experiments.

In CD40+ chimeras, the expansion of CD8 T cells, injected alone, was extremely low (Fig. 2A), as previously described for lethargic cells [4, 5]. In the presence of CD4 T cells, the CD8 T cell expansion followed a classical pattern with a peak at Day 7 and then a contraction (up to Day 14) and resting phase (Fig. 2A). When aCD40ab was used instead of CD4 T cells, no differences were observed between the two groups (Fig. 2A). Therefore, in a full CD40-competent environment, aCD40ab can efficiently substitute CD4 help in regard to CD8 T cell expansion.

In CD40− chimeras, CD8 T cell expansion was observed in the presence of CD4 T cells (Fig. 2B), but the number of CD8 T cells recovered at Day 7 was decreased significantly, compared with their CD40+ counterparts ($6.5\times10^6\pm1.1\times10^6$ vs. $13.8\times10^6\pm2.7\times10^6$; $P<0.01$; Supplemental Fig. 1). In CD40− chimeras injected with aCD40ab, CD8 T cell expansion was severely impaired and similar to the lethargic population: the aCD40ab treatment had no effect, as expected. The next step was to determine whether CD40 agonist signals were mediated through APCs and/or CD8 T cells.

In CD8 CD40− chimeras, the expansion of CD8CD40$^{-/-}$ T cells, injected with CD4 T cells or with aCD40ab, was similar to CD40+ chimeras (Fig. 2C). In APC CD40− chimeras receiving CD4 T cells, the expansion remained similar to CD40+ chimeras as well (Fig. 2D). The number of CD8 T cells recovered at Day 7 was reduced slightly but not statistically different compared with CD40+ chimeras (Supplemental Fig. 1). Conversely, CD8 T cell expansion was severely impaired in APC CD40− chimeras receiving aCD40ab (Fig. 2D). At Day 7, the number of cells was fourfold reduced compared with CD40+ chimeras ($3.5\times10^6\pm1.3\times10^6$ vs. $13.9\times10^6\pm2.9\times10^6$; $P<0.01$; Supplemental Fig. 1). Of note, the expansion of CD8 T cells in this group was significantly higher compared with the lethargic cells ($3.5\times10^6\pm1.3\times10^6$ vs. $1.2\times10^6\pm0.25\times10^6$; $P<0.05$; Supplemental Fig. 1), showing that some signals were mediated through CD40 expression by CD8 T cells.

Collectively, our data demonstrate that direct CD40 signaling on APCs is important for CD8 T cell expansion but can be bypassed by other CD4 help signals.

To further explore the role of CD40 signaling on CD8 T cell expansion and their following migration in the periphery, we studied the number of CD8 T cells recovered from the liver (**Fig. 3**). The number of lethargic cells remained very low all along the primary response (Fig. 3A). On the contrary, in CD40+ chimeras injected with CD4 T cells or aCD40ab, the number of CD8 T cells strongly increased until Day 7 (Fig. 3A). Overall, the numbers of CD8 T cells recovered upon coinjection with CD4 T cells were similar in all studied groups (Fig. 3A–D). This demonstrated that the deficiency of CD40 on APCs and/or CD8 did not impact T cell migration capacity in an otherwise full CD4 help-competent environment. In CD8 CD40− chimeras injected with aCD40ab, no differences were observed, as well compared with CD40+ chimeras (Fig. 3C and A, respectively). On the contrary, in CD40− and APC CD40− chimeras, the numbers of CD8 T cells were reduced dramatically and comparable with those observed for lethargic cells. These results confirm the predominant role of CD40 expression on APCs to induce CD8 T cell expansion and conse-

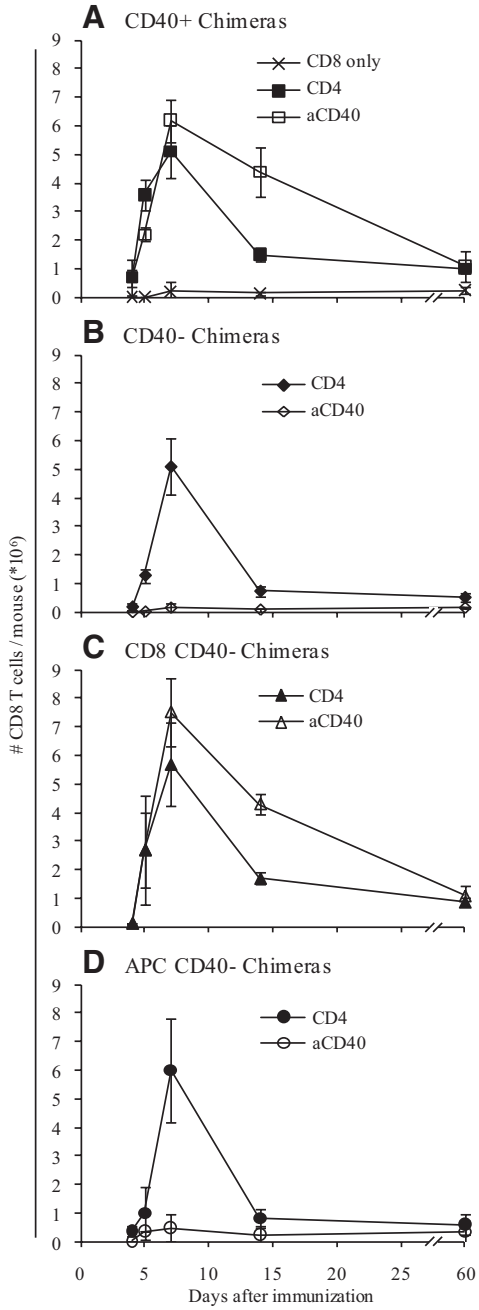

Figure 3. Effect of CD40 deficiency on CD8 T cell migration. Total number of CD8 T cells recovered from the liver of individual mice at different time-points after immunization. (A) CD8 T cells recovered from CD40+ chimeras. CD8 T cells were injected alone (×) or coinjected with CD4 T cells (■) or aCD40ab (□). (B) CD8 T cells recovered from CD40− chimeras. CD8 T cells were coinjected with CD4 T cells (♦) or aCD40ab (◇). (C) CD8 T cells recovered from CD8 CD40− chimeras. CD8 T cells were coinjected with CD4 T cells (▲) or aCD40ab (△). (D) CD8 T cells recovered from APC CD40− chimeras. CD8 T cells were coinjected with CD4 T cells (●) or aCD40ab (○). Data show the average ± SEM of three mice/group and are representative of six independent experiments.

quent migration to peripheral tissues. Interestingly, in CD40+ and CD8 CD40− chimeras injected with aCD40ab, the contraction phase of CD8 T cells was delayed compared with their counterpart injected with CD4 T cells, suggesting that aCD40ab could provide more prolonged survival signals than CD4 T cells. However, few CD8 T cells remained in the liver at the end of the primary responses in all chimeras.

The differences in CD8 T cell expansion observed among the groups injected with CD4 T cells were not a result of a defect in CD4 T cell expansion, as the numbers of CD4 T cells recovered did not significantly differ for these groups in lymphoid organs (Supplemental Fig. 2) and the liver (data not shown). No differences were found as well in the numbers of B cells recovered from lymphoid organs and liver of different chimeras (injected with CD4 T cells or aCD40ab; data not shown). Thus, the administration of aCD40ab has no impact on the survival or the toxicity toward the B cells in the settings used.

CD40 signaling on APCs is involved in the acquisition of CD8 T cell effector functions in the early phase of the immune responses

To assess the in vivo cytotoxic capacity of CD8 T cells, we quantified the male antigen load by real-time PCR (**Fig. 4**). The male antigen was eliminated almost completely at Day 7 and totally undetectable at Day 14 in all groups, including the lethargic ones. This demonstrates that CD4 help is not strictly required for the development of CD8 cytotoxic functions leading to antigen elimination. However, CD4 T cells induced a faster kinetic of antigen elimination in CD40+ chimeras, as the number of male cells detected was, respectively, two- and threefold lower at Days 4 and 5 compared with the lethargic one (Fig. 4). No differences were observed in the presence of aCD40ab compared with the presence of CD4 T cells, demonstrating that CD40 signaling bypassed CD4 help completely. In contrast, the kinetic of antigen elimination in CD40− chi-

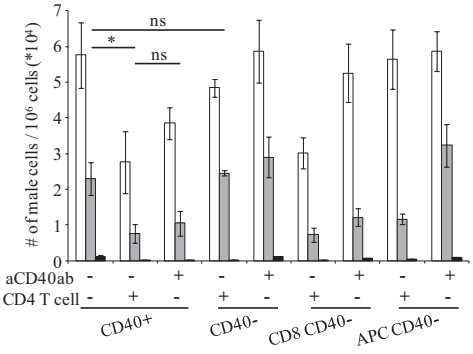

Figure 4. Delayed antigen elimination under CD40 deficiency. Male antigen quantification using genomic Zfy-1 DNA real-time PCR. Results show the number of male cells detected/million splenic cells. The histograms represent the quantification of male cells in the different chimeras at Day 4 (white histograms), Day 5 (gray histograms), and Day 7 (black histograms). Each sample was performed in triplicate. Data show the average ± SEM of three mice/group and are representative of three independent experiments.

meras injected with CD4 T cells or aCD40ab was similar to the lethargic group, suggesting that CD4 help could not bypass CD40 deficiency on the APCs and CD8 T cells. No differences were observed between CD8 CD40− chimeras and their CD40+ counterparts. Thus, aCD40ab stimulation on the APCs alone is sufficient to allow rapid antigen elimination by CD8 T cells. In APC CD40− chimeras injected with CD4 T cells or aCD40ab, the level of antigen load was similar to the lethargic one at Day 4. However, at Day 5, only the chimera injected with aCD40ab showed a delay in antigen elimination. Altogether, this suggests that CD4 help involves mainly CD40 signaling on APCs to allow rapid antigen elimination.

To further analyze the effect of CD40 signaling on CD8 T cell effector functions, we investigated their ability to express IFN-γ after a short in vitro restimulation (**Fig. 5**). Only one-third of lethargic CD8 T cells expressed IFN-γ (~35%±6% at Days 5 and 7), and they did not significantly improve their IFN-γ expression thereafter (Fig. 5A). In CD40+ chimeras, no differences were observed between CD4 T cells and aCD40ab stimulation. Approximately 55% of CD8 T cells are able to express IFN-γ as soon as Day 4, and this percentage increased constantly thereafter (Fig. 5A). In fact, CD8 T cells isolated from all groups of mice injected with CD4 T cells have a similar profile of IFN-γ expression until Day 7, demonstrating that CD40 signaling through APCs or CD8 T cells was not required strictly during the effector phase (Fig. 5A–D). The production of IFN-γ by CD8 T cells in CD40− or APC CD40− chimeras injected with aCD40ab was, however, altered and similar to the lethargic group (Fig. 5A, B, and D), whereas its production by CD8 T cells from CD8 CD40− chimeras was similar to that of CD40+ chimeras (Fig. 5C and A, respectively). Therefore, during the effector phase of the immune response, CD40 expression on APCs is important to induce IFN-γ expression by CD8 T cells but could be bypassed by other CD4 helper signaling.

CD40 signaling on APCs modulates the kinetics of IL-7Rα and CD62L expression on CD8 T cells

We finally evaluated the regulation of IL-7Rα and CD62L expression, which includes two important markers of the effector CD8 T cell phase. The expected down-regulation of IL-7Rα on CD8 T cells shortly after activation was observed in all groups at Day 3 (data not shown). However, kinetic of its re-expression differed among them. The IL-7Rα expression on lethargic CD8 T cells remained low at Day 7 (**Fig. 6A** and Supplemental Fig. 3A). In CD40+ chimeras, injected with CD4 T cells or aCD40ab, the re-expression of IL-7Rα was observed as soon as Day 5, and ~65% of CD8 T cells re-expressed it at Day 7. In CD40− chimeras injected with CD4 T cells, CD8 T cells initiated IL-7Rα re-expression by Day 5, but the re-expression was delayed slightly at Day 7 compared with CD40+ chimeras (53.4%±0.4 vs. 67.4±3.3; $P<0.05$). In the presence of aCD40ab, the IL-7Rα re-expression was strongly delayed and comparable with the profile observed in lethargic cells. In CD8 CD40− chimeras, injected with CD4 T cells or aCD40ab, CD8 T cells initiated IL-7Rα re-expression by Day 5, but the re-expression was delayed slightly at Day 7 compared with CD40+ chimeras (and similar to CD40− chimeras injected with CD4 T cells). CD40 signaling on CD8 T cells thus contrib-

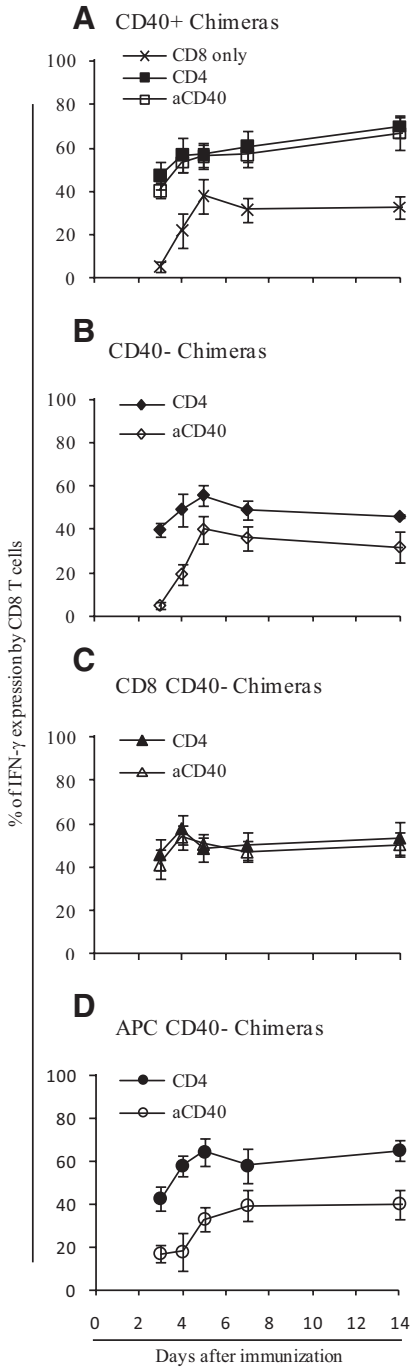

Figure 5. CD40 deficiency altered IFN-γ expression during effector phase. Intracellular expression of IFN-γ by splenic CD8 T cells after 2 h of in vitro restimulation. (A) Percentage of CD8 T cells expressing IFN-γ from CD40+ chimeras. CD8 T cells were injected alone (×) or coinjected with CD4 T cells (■) or aCD40ab (□). (B) Percentage of CD8 T cells expressing IFN-γ from CD40− chimeras. CD8 T cells were coinjected with CD4 T cells (♦) or aCD40ab (◇). (C) Percentage of CD8 T cells expressing IFN-γ from CD8 CD40− chimeras. CD8 T cells were coinjected with CD4 T cells (▲) or aCD40ab (△). (D) Percentage of CD8 T cells expressing IFN-γ from APC CD40− chimeras. CD8 T cells were coinjected with CD4 T cells (●) or aCD40ab (○). Data show the average ± SEM of three mice/group and are representative of six independent experiments.

utes mildly to IL-7Rα re-expression. In APC CD40− chimeras, injected with CD4 T cells, the kinetic of IL-7Rα re-expression was identical to CD40+ chimeras. In the presence of aCD40ab, defects in receptor dynamics were comparable with those observed in CD40− chimeras (Fig. 6A), showing that CD40 signaling on the APCs is important. In conclusion, injection of aCD40ab is sufficient to ensure re-expression of IL-7Rα when APCs expressed CD40. However, in the absence of CD40 expression by the APCs, CD4 T cells allow a faster IL-7Rα expression, suggesting that CD4 help may be provided in a CD40-independent pathway.

Regarding CD62L expression, in all chimeras injected with CD4 T cells, the expected transitory down-regulation on CD8 T cells after activation was observed at Day 5 and maintained at Day 7 (Fig. 6B and Supplemental Fig. 3B). On the contrary, the down-regulation of CD62L was severely delayed on lethargic cells and still not detected at Day 5. In CD40+ and CD8 CD40− chimeras injected with aCD40ab, the CD62L expression profile was similar to those injected with CD4 T cells. In contrast, in APC CD40− and CD40− chimeras injected with aCD40ab, the CD62L expression profile mimicked that of lethargic cells (Fig. 6B). Collectively, these results demonstrate that CD40 on APCs participates in the down-regulation of CD62L. However, other signals from CD4 T cells can overcome this deficiency.

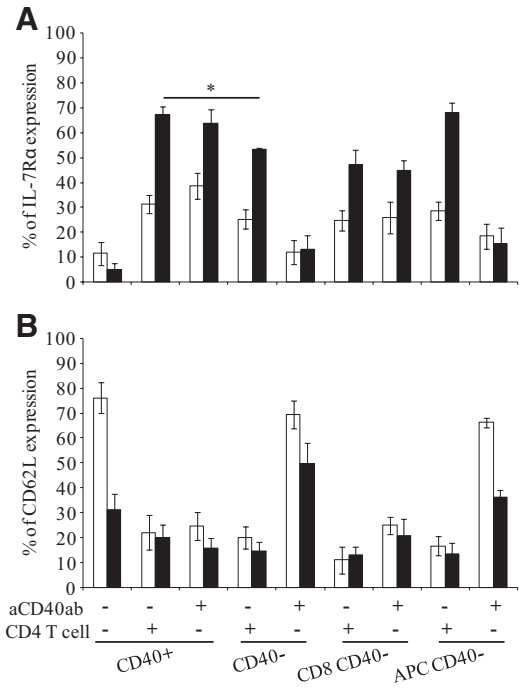

Figure 6. Effect of CD40 deficiency on IL-7Rα and CD62L expression. Percentage of IL-7Rα $^{+}$(A) and CD62L^{+} (B) on gated, splenic CD8 T cells recovered from the different chimeras at Day 5 (white histograms) and Day 7 (black histograms). Data show the average ± SEM of three mice/group and are representative of six independent experiments.

CD40 signaling on CD8 T cells is crucial for memory differentiation

We studied the number and the phenotype of CD8 T cells recovered from the different chimeras at the late phase of the primary response. CD8 T cell numbers were equivalent in all groups except for the lethargic group and the CD40− group injected with aCD40ab, which exhibited reduced CD8 T cell numbers (Fig. 2 and Supplemental Fig. 4). Additionally, CD8 T cells isolated at Day 60 all expressed IL-7Rα at a higher level than naïve cells (Supplemental Fig. 3A). CD8 T cells from all chimeras also progressively re-expressed CD62L and at Day 60; ~90% of them were CD62L^{high} (Supplemental Fig. 3B). Therefore, irrespective of CD4 help and/or CD40 signaling, CD8 T cells surviving the contraction phase harbored the same profile of IL-7Rα and CD62L expression.

However, the quantity and the phenotype of CD8 T cells did not necessarily reflect their quality (functional properties). For instance, CD62L and CCR7 molecules were described originally in humans to discriminate between effector memory T cells and T_{CM}, the later lacking effector functions [34]. Subsequent experiments in mice reported, however, that T_{CM} cells differed from the original description of human T_{CM} cells in that they retained effector functions [35]. Therefore, to assess the quality of CD8 T cells generated at the end of the primary response in the different settings of CD4 help, we monitored their potential for high cytokine production and cell division upon secondary challenge, two important hallmarks of CD8 memory T cells.

We first studied IFN-γ expression by CD8 T cells at Day 60 after a short in vitro restimulation (**Fig. 7**A). The percentage of lethargic CD8 T cells expressing IFN-γ did not increase after the contraction phase (Fig. 7A), whereas its expression by CD8 T cells from CD40+ chimeras, injected with CD4 T cell or aCD40ab, was strongly increased. Thirty-eight percent ± 7% of lethargic cells compared with 81% ± 6.2% of CD8 T cells in CD40+ chimeras injected with CD4 T cells expressed it ($P<0.01$). Therefore, aCD40ab stimulation can fully substitute CD4 help to induce a high capacity of cytokine productions by CD8 T cells. Conversely, CD8 T cells from CD40− chimeras, injected with CD4 T cells or aCD40ab, did not improve their capacity to express IFN-γ after the contraction phase (Fig. 7A). As the ability to maintain a high capacity of cytokine productions is a hallmark of memory T cells [1–3], these results demonstrate that CD40 signaling on APCs and/or on CD8 T cells is involved in memory differentiation. In CD8 CD40− chimeras, injected with CD4 T cells or aCD40ab, the percentage of CD8CD40$^{-/-}$ T cells expressing IFN-γ did not increased after the contraction phase (Fig. 7A). For example, in chimeras injected with CD4 T cells, 55% ± 4.7% of CD8 T cells in CD8 CD40− chimeras expressed IFN-γ compared with 81% ± 6.2% in CD40+ chimeras ($P<0.01$). Therefore, CD40 on CD8 T cells plays a crucial and nonredundant function in long-term IFN-γ expression. In APC CD40− chimeras, the IFN-γ expression by CD8 T cells was not altered in the presence of CD4 T cells (Fig. 7A). CD8 T cells constantly increased their capacity to express IFN-γ throughout the immune response. At Day 60, 75% ± 6.7% of cells expressed IFN-γ compared with 81% ±

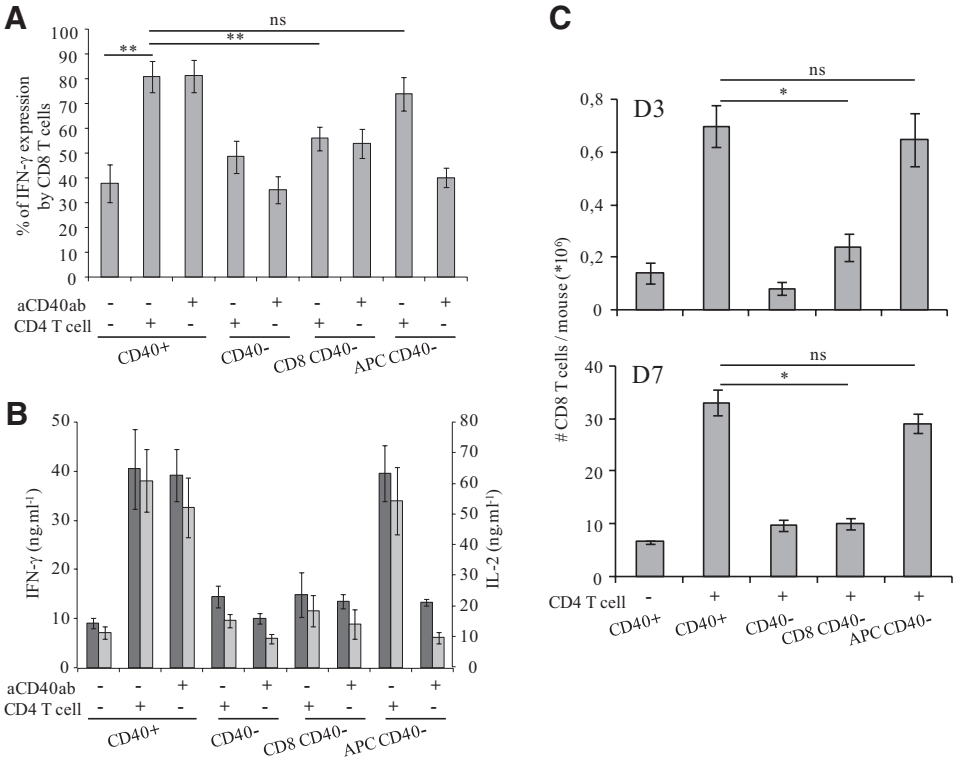

Figure 7. CD40 deficiency altered the memory differentiation. (A) Intracellular expression of IFN-γ by splenic CD8 T cells at Day 60, after 2 h of in vitro restimulation. (B) IFN-γ and IL-2 secretion (ELISA) by CD8 T cells at Day 60, after 24 h of in vitro restimulation. Data show the average ± SEM of three mice/group and are representative of six independent experiments. (C) Total number of CD8 T cells recovered from the spleen and LN of individual mice at Days 3 (D3; upper graph) and 7 (D7; lower graph) after secondary immunization (as described in Materials and Methods) in the presence of CD4 T cells. The secondary response of lethargic CD40+ CD8 T cells is also shown. Data show the average ± SEM of three mice/group and are representative of two independent experiments.

6.2% in CD40+ chimeras ($P<0.57$). Therefore, CD40 on APCs is not involved in the high capacity of IFN-γ production by CD8 T cells. Importantly, in the presence of aCD40ab, the capacity of CD8 T cells to express IFN-γ was impaired drastically and was similar to the expression by lethargic cells (Fig. 7A). Thus, the unique stimulation of CD40 through CD8 T cells is not sufficient to induce memory differentiation.

To further analyze CD8 T cell memory differentiation, the secretion of IFN-γ and IL-2 by CD8 T cells isolated at Day 60 was determined after in vitro restimulation (Fig. 7B). As shown previously [4, 5], lethargic CD8 T cells secreted very low levels of both cytokines. In CD40+ chimeras, stimulated with CD4 T cells or aCD40ab, CD8 T cells secreted similarly high levels of IFN-γ and IL-2, showing that aCD40ab is sufficient to substitute CD4 help. Besides, CD40 signaling is important, as in CD40− chimeras, CD8 T cells were unable to secrete high levels of IFN-γ and IL-2 in the presence of CD4 T cells or aCD40ab. In CD8 CD40− chimeras, stimulated with CD4 T cells or aCD40ab, a three- to fourfold diminution of IFN-γ and IL-2 secretions was observed when compared with CD40+ chimeras (Fig. 7B). In APC CD40− chimeras, CD8 T cells stimulated with CD4 T cells secreted similarly high levels of IFN-γ and IL-2, whereas a fourfold decrease was measured upon aCD40ab stimulation. These results confirm the prominent role of CD40 expression on CD8 T cells, for their differentiation into memory cells.

Finally, we performed in vivo secondary responses for all chimeras injected with CD4 T cells and analyzed CD8 T cell secondary expansion capacity (Fig. 7C). CD8 T cells, which exhibited a defect in cytokine secretions (from CD40− and CD8 CD40− chimeras or lethargic CD8 T cells), have severely altered expansion at Days 3 and 7 upon secondary immunization compared with CD8 T cells that secreted high levels of cytokines (from CD40+ and APC CD40− chimeras). Therefore, although CD8 T cells from CD40− and CD8 CD40− persisted to a relatively high number at the end of the primary response, they were not endowed with high cell-division capacity.

Altogether, these results demonstrate that CD40 expression on CD8 T cells is required to enhance cytokine secretions and cell divisions of CD8 T cells upon secondary challenge, therefore allowing their differentiation into memory cells, whereas CD40 expression on APCs is less involved in this process.

DISCUSSION

Among the multiple mechanisms of CD4 help discovered so far, the CD40–CD40L interactions play an important role in CD8 immune responses [30]. Accordingly, researchers have used agonist aCD40ab to increase CD8 T cell responses to bypass CD4 help in a number of immune models [30, 36]. In several cancer models, for example, aCD40ab can induce direct apoptosis of CD40-expressing tumor cells and may prove a promising tool in cancer treatments [37]. However, its administration may have unexpected consequences. Well-documented reviews disclose the side-effects of aCD40ab treatments [21, 30, 38]. The administration of aCD40ab in a number of cancer models provokes the expression of several angiogenic factors promoting tumor growth [22–25]. Moreover, sustained systemic treatment with aCD40ab often engenders toxicity

[30]. Dissecting the exact impact of aCD40ab on the various cell targets may provide novel strategy to prevent the development of side-effects. In this study, we designed an experimental system allowing restricted expression of CD40 on APCs or CD8 T cells, and we carefully distinguished the primary effector immune response from further differentiation into memory CD8 T cells.

In agreement with many earlier studies, we observed that aCD40ab could efficiently substitute CD4 help during the primary response [13–16]. However, complete substitution required a full CD40-competent environment: CD40 deficiency on APCs or CD8 T cells resulted in altered responses. Importantly, we found that the different parameters of CD8 responses (cell division, antigen clearance, effector functions, and memory differentiation) did not follow the same requirements regarding CD40 expression (**Table 2**).

During the early phase of the primary response, in CD8 CD40− chimeras, the sole injection of aCD40ab is sufficient to obtain an immune response similar to the one observed in CD40+ chimeras, as assessed by expansion, antigen elimination, IL-7Rα, CD62L, and IFN-γ expression. In contrast, the injection of aCD40ab into APC CD40− was ineffective, confirming a major role of CD40 expression on APCs during primary responses. Importantly, when APCs are $CD40^{-/-}$, the injection of CD4 T cells fully bypassed CD40 deficiency, demonstrating that CD40 expression on APCs is not strictly required. Finally, in CD40− chimeras, CD4 T cells did not fully restore the immune response, reinforcing the synergistic role of CD40 expression on APCs and CD8 T cells. These results suggest that some helper signals were CD40-dependent and not redundant with other signals provided by CD4 T cells (Table 2). This is in accordance with an earlier study describing a CD40-independent pathway of CD4 help on APC activation, as well as direct CD4–CD8 T cell communication to deliver helper signals to CD8 T cells [39].

A different picture emerged during the late phase of immune response, whereas CD8 T cells progressively acquired memory properties. Studying intracellular IFN-γ expression and IFN-γ and IL-2 productions after in vitro restimulation (mimicking the secondary response), we showed the important and nonredundant role of CD40 expression by CD8 T cells on memory generation (Table 2). Indeed, when CD8 T cells are CD40-deficient, neither the stimulation of the APCs by aCD40ab nor by CD4 T cells could overcome the defects in IFN-γ and IL-2 productions by CD8 T cells. Conversely, when APCs are $CD40^{-/-}$, enhanced IFN-γ and IL-2 secretions by CD8 T cells were observed in the presence of CD4 help but not upon aCD40ab stimulation. Importantly, CD8 T cells exhibiting a default in cytokine secretions also had a severely altered expansion upon in vivo secondary immunization compared with CD8 T cells that secreted high levels of cytokines. These results demonstrate that CD40 expression on CD8 T cells is fundamental to allow memory generation. However, as shown by the defects observed in the APC CD40− chimera injected with aCD40ab, CD40 expression on CD8 T cells is not strictly sufficient for CD8 T cell differentiation. Other CD40-independent signals provided by CD4 T cells are also required to allow CD8 memory differentiation. Overall, this demonstrates that CD8 T cells must go through different checkpoints to differentiate into memory cells. One may hypothesis that some CD4 help signaling, mainly through the APCs, is necessary to drive efficient effector phase. This transition to an effector stage may be a prerequisite for further memory differentiation but may not be sufficient. Some other CD4 help signaling, mainly through CD40 on CD8 T cells, is necessary to fulfill complete memory differentiation.

Among the signals provided by CD4 help that are CD40-independent, other members of the TNF–TNFR are good candidates. Numerous studies have demonstrated the important role of TRANCE, CD70, OX40, and 41BB on CD8 expansion, survival, and/or acquisition of effector functions [40–44]. Interestingly, a report about the role of OX40–OX40L interaction in CD4 T cell survival reached a similar conclusion to our observations with CD40–CD40L interactions [45]. The authors demonstrated that OX40 and OX40L could be expressed on activated APCs and CD4 T cells. The deficiency of OX40L, on APCs or CD4 T cells, induced a significant reduction in T cell proliferation [45].

Our study confirms and extents the important role of CD40–CD40L interactions in noninfectious immune responses. The implication of these interactions is more controversial in pathogen models [46-54]. For example, the CD8 response against LCMV infection is mostly described as CD40–CD40L-dependent [47, 48]. In the Listeria model, depending on the authors, the response is CD40–CD40L-dependent [49,

TABLE 2. Schematic view of the CD40 signaling and CD4 help

	CD40 signaling			
	CD40 on APC	CD40 on CD8	CD40 on APC and CD8	CD4 help signals by CD40-independent signaling
Early phase of primary response	Important but redundant	Modest contribution and redundant	Synergistic action and not fully redundant	Partially rescued expansion and effectors fonctions
Late phase of primary response	Modest contribution and redundant	Strictly necessary and not redundant	Strictly necessary and not redundant	Could not overcome CD40 deficiency by CD8 T cells

CD40 signaling versus CD4 help signals are highlighted here. The impact of CD40 signaling during the early phase (effector phase) and the late phase of the primary response (memory differentiation) is distinguished. It is also mentioned whether CD40 signaling is redundant, i.e., could be replaced by other signals provided by CD4 T cells or strictly necessary. The impact of CD4 T cell help by CD40-independent signals is also shown.

50] or independent [51, 52]. These discrepancies remain to be elucidated but could be related to the infectious doses, the pathogens used, or the timing and kinetics of the response. Only few studies have dissected the role of CD40 expression on APCs and CD8 T cells in response to pathogen infection [52, 53]. No crucial role of CD40 expression on CD8 T cells has been detected in these studies, neither during primary nor secondary responses. Accordingly, in primary response, we showed that CD40 expression on CD8 T cells was not involved. In these infectious models, secondary responses were assessed only for CD8 expansion at one time-point of the immune response. Similar expansions were found between $CD40^{-/-}$ and $CD40^{+/+}$ CD8 T cells in these analyses. However, in a previous report, we made an extensive analysis of the secondary responses of $CD8CD40^{+/+}$ and $CD8CD40^{-/-}$ T cells [29]. We found that the secondary response of $CD8CD40^{-/-}$ T cells was severely altered compared with $CD8CD40^{+/+}$ T cells. Defects in proliferation were found at early time-points, but the most striking observation was the profound cytotoxic defaults of $CD8CD40^{-/-}$ T cells [29]. Thus, discrepancies between these and our studies may rely on the different readouts used and the time-point considered. Interestingly, a recent study supported a role of CD40 expression on CD8 T cells in a viral model [54]. Upon certain viral infections, the APCs expressed CD40L, suggesting that CD40–CD40L interactions could be bidirectional on APCs, CD4, and CD8 T cells. Most importantly, CD40 deficiency on CD8 T cells inhibited the killing of the infected target cells, demonstrating that CD40 expression on CD8 T cells could play a role in certain viral models as well [54]. Finally, using a model of *Leishmania donovani* infection in susceptible BALB/c mice, Martin's group [55] reported that $CD40^{+}$ CD8 T cells executed CD40-dependent cytotoxicity on $CD4^{+}$ $CD25^{+}$ regulatory T cells. They demonstrated that CD40 signaled through Ras, PI3K, and PKC, resulting in NF-κB-dependent induction of the cytotoxic mediators granzyme and perforin. These data sustained our previous study, where we found that $CD8CD40^{-/-}$ T cells have severely altered perforin and granzyme B cytotoxic functions compared with $CD8CD40^{+/+}$ T cells [29].

Further studies are therefore required to evaluate the role of CD40 signaling on CD8 T cells in response to pathogen infections. With respect to the numerous mechanisms of CD4 help described so far, it is, however, tempting to speculate that CD4 helper signals will differ depending of the kind of CD8 immune responses (viral, bacterial, antitumoral, autoimmune) [3, 9–11]. Concerning primary immune responses, it is generally admitted in many viral and bacterial systems that CD40 signaling appears redundant (and CD4 T cells not required) during primary responses, as pathogen-derived products are recognized directly by TLRs on the APCs, allowing CD40-independent (and CD4 T cell help-independent) APC maturation [51, 52]. For those primary responses that are CD4 T cell help-independent, it is, however, well-demonstrated that CD4 T cells were required to allow memory differentiation [7, 8]. It is still unsettled through which mechanism CD4 T cells will help CD8 T cells in these infectious models. One may finally question whether if the infectious products that bypass CD40 signaling to allow efficient primary response are the same that would allow CD8 memory differentiation. As it becomes obvious that distinct pathogens differently modulate DC costimulatory capacity, they may also differently modulate CD8 T cell responses [56]. In this regard, it is interesting to note that CD8 T cells can receive cosignals from some but not all TLR [56-58]. Notably, it has been shown that direct signaling through TLR2 on CD8 T cells increased their functional properties [58].

Altogether, our results reveal a complex crosstalk among APCs, CD4 T cells, and CD8 T cells. CD4 T cells provide CD40-dependent and independent signals to allow complete CD8 T cell effector and memory differentiation. Our data confirm the potential benefit of aCD40ab agonist therapy to improve memory differentiation. However, they suggest that future trials would have to take into account the whole complexity of CD4 T cell help: the cell subsets involved, the molecules targeted, and their kinetics of expression to achieve maximal efficacy, while reducing counteracting and toxic effects.

AUTHORSHIP

S.M. planned and did experiments, drew the figures, and contributed to the paper redaction. L.R. performed the initial experiments, designed the quantitative PCR experiments, and contributed to the paper redaction. L.B., C.P., and A.L. contributed to the experiments. C.T. supervised the project, designed the experiments, and wrote the paper.

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DISCLOSURES

The authors declare no financial or commercial conflict of interest.

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