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CD4 Help Regulates Expression of Crucial Genes Involved in CD8 T Cell Memory and Sensitivity to Regulatory Elements

Laetitia Rapetti, Sylvain Meunier, Christiane Pontoux, and Corinne Tanchot

The role of CD4 help during CD8 memory differentiation has been clearly demonstrated in different experimental models. However, the mechanisms involved to mediate CD4 help and the extent of its effects remain largely unknown. Using gene analysis at a single cell level, which allows the study of gene expression in terms of frequency, intensity and coexpression, we show that unhelped CD8 T cells harbor severe defects in the expression of crucial genes involved in proliferation, survival, and cytotoxic functions. The three main characteristics of CD8 memory differentiation described so far. Importantly, during secondary response, unhelped CD8 T cells exhibit blockade in all cytotoxic pathways (perforin, Fas ligand, IFN-γ), demonstrating the highly ubiquitous effect of CD4 help. Secondly, restimulated unhelped CD8 T cells extinguish the majority of their stimulated genes, showing that CD4 help favors the persistence of gene expression. Indeed, during secondary response, unhelped CD8 T cells exhibit a profile very similar to naive T cells, demonstrating that no instructive program has been imprinted in these cells. Finally, unhelped CD8 T cells exhibit a higher sensitivity to immunoregulatory genes during secondary immune response. Therefore, these results characterize the multiple effects of CD4 help on CD8 memory differentiation and provide important insights for the understanding of protective memory responses. The Journal of Immunology, 2008, 181: 299–308.
Materials and Methods

Mice
C57BL/6 mice; CD3-e-deficient (CD3-e/-) mice (30); female Rag2-deficient (Rag2/-) mice, expressing a TCR-αβ transgenic specific for the male HY Ag, restricted to MHC class 1 Db (31); and Rag2/-CD40/-transgenic (Tg)3 mice were all bred at the Center for the Development of Advanced Experimental Techniques (Orléans, France).

Immunization protocols
Female mice were immunized with male cells by adoptive transfer. Briefly, female CD3-e/- mice were irradiated and conjoined with 0.5 × 106 male bone marrow (BM) cells together with 4.5 × 106 female BM cells from CD3-e/- mice. Three days later, 0.5 × 106 purified lymph node (LN) CD8 Thy1.2 TCR anti-HY Tg T cells (from Rag2/- or CD40/- female mice) were injected with an equal number of purified LN CD4 T cells (from C57BL/6 female mice) into chimeric CD3-e/- mice. For primary response, naive CD8 T cells isolated from LN of Rag2/- TCR Tg mice were injected. For secondary responses, CD8 T cells isolated 2 mo after transfer from LN of the first hosts were purified and injected into a new set of chimeric mice. T cell purification was performed by negative selection, using a mix of mAbs (BD Pharmingen) recognizing B cells, macrophages, dendritic cells, and CD8 T cells (for CD4 T cell purification) or CD4 T cells (for CD8 T cell purification) and coated with Dynabeads (Dynal Biotech).

At different time points after immunization, the number of CD8 Tg T cells recovered from the spleen, a pool of LN, BM, and liver was determined and referred to as the number of CD8 Tg cells/mouse.

Abs and immunofluorescence analysis
For cell staining the following mAbs were used: biotin-labeled anti-CD127 (IL-7Rα) and anti-CD62 L-selectin (CD62L) streptavidin-allophycocyanin; PerCP-labeled anti-CD4 and anti-CD8; PE-labeled anti-CD69, anti-CD90.2, anti-IFN-γ, and anti-TNF-α (BD Pharmingen); and FITC-labeled anti-CD3.70 (anti-TCR-α Tg) and anti-CD90.1 were used. For TGF-β staining, a anti-mouse TGF-β Ab (Cell Signaling Technology) was revealed by a Alexa Fluor 488 Ab (Invitrogen). Flow cytometry was performed with a FACSCalibur cytometer, and data files were analyzed using CellQuest Pro software (BD Biosciences).

Ex vivo and in vitro cytokine secretion
Ex vivo intracellular staining was used to determine cytokine expression. For early time points of secondary response, we determined the IFN-γ production by CD8 Tg T cells, using a described protocol (32). Briefly, cells were incubated (20 min) at 37°C with collagenase D (20 μg/ml) and brefeldin A (10 μg/ml). Then a classical procedure for intracellular staining was used (3). For in vitro intracellular staining from day 4 after immunization, in vitro stimulation was required and performed. Briefly, purified CD8 Tg T cells were incubated 4 h at 37°C with female APCs and 2.5 μg of anti-CD3 mAbs (clone 2C11) in the presence of brefeldin A (10 μg/ml). Cells were stained using a procedure as previously described (3). ELISA was conducted with purified CD8 Tg T cells incubated for 24 h with female APCs and 2.5 μg of anti-CD3 mAbs (clone 2C41). Cytokine secretion was determined in culture supernatants by ELISA kit (R&D Systems).

Evaluation of Ag load
We determined male Ag load remaining in hosts during primary and secondary responses by two independent methods that gave comparable results. Firstly, a system of “sensor cells” was used. A total of 0.5 × 106 naïve Thy1.1+ CD8 Tg T cells were injected 24 h before animals sacrifice. At different time points after immunization, thus mimicking the spread of viral or bacterial infection. In the presence of male cells, these cells have a high capacity for cell divisions, allowing male cells to grow in host mice at early time points after immunization, thus mimicking the spread of viral or bacterial infection. In the presence of CD4 Tg T cells, HY Tg CD8 Tg T cells differentiate efficiently into memory CD8 cells. When deficient in CD40 expression, CD8 Tg T cells do not receive helper signals and thus do not differentiate into memory CD8 cells (3).

Copies per male and female cells, as reporter gene. Primers used were the following: ZfY-1, (forward) 5'-ggatagtcagagccctgttg-3' and (reverse) 5'-tggagttggtcaagtacctacg-3'; hprt, (forward) 5'-aagctctcgataggttgg-3' and (reverse) 5'-tacattagctgacagcaggg-3'. Primers were designed to achieve similar amplification efficiency and produce amplicons of similar size. Briefly, DNA was extracted from 2 × 106 spleen cells. Due to the small number of male cells among total cells, a different dilution of genomic DNA was used to detect ZfY-1 and hprt. A 5 ng genomic DNA was used for hprt gene and 20 ng for ZfY-1, allowing us to determine an optimal threshold cycle (Ct) comprising of between 20 and 22 for hprt gene and 24 and 36 for ZfY-1 gene. For each sample, real-time PCR was performed in triplicate and in the same run for both genes to avoid extrinsic variation. The number of male cells per million total cells was calculated using the following: 2 × 2^(Ct(Ct-hprt)-(Ct(hprt/d/hprt)) × 10^d, where d is the dilution of genomic DNA.

Real-time multiple parametric single cell RTPCR
This technique described by Peixoto et al. (29), permits the simultaneous quantification of 19 different mRNA molecules at single cell level. All primers were previously referred and designed to achieve similar amplification efficiency with no competition phenomenon. Amplons present similar size. This technique has been proved not to induce any bias in quantification process and presented a range of sensitivity, comprising of between 2 and 10^3 mRNA copies of each gene. Briefly, spleen cells were processed using a FACSVantage with an automatic cell deposition unit (BD Biosciences). Cells were collected in individual PCR tubes containing 5 μl of PBS-diethyl pyrocarbonate 0.1% and store at −80°C. After a lysis step, specific reverse transcription followed by semi-nested PCR was performed. The first round of PCR amplified the 19 genes with equal efficiency. A second round of PCR, performed on first round products, was used to detect separately each expressed gene and to select positive cells. An aliquot from first round PCR product was finally quantified by real-time PCR for each positive single cell. To ensure that amplifications performed on different experiments could be directly compared, we included an internal standard for quantitative evaluations. It is an in vitro-synthesized RNA (granzyme A) of a know number of molecules, ranging across all expression levels and undergoing the same reverse transcription and PCR amplification process that allowed evaluation of both reverse transcription and PCR efficiency.

Statistical analysis
Statistical analysis was performed with GraphPad software. The two-tailed Fischer’s exact test was used for the percentage of cell expressing gene and the two-tailed Mann-Whitney U test for mRNA quantification and CD8 T cell expansion. These tests compare two unpaired populations and are adequate to evaluate small samples. The amounts are comprised between n = 60 and 120 cells, depending on the day of mRNA quantification, and represent n = 12 mice per time point for CD8 T cell expansion. Results were considered significantly different at a value for p < 0.05. All differences mentioned are statistically significant.

Results
To elucidate the role of CD40 expression in efficient CD8 memory T cell differentiation, we used a well described noninfectious immunization system directed against the male Ag. This experimental system relies on adoptive transfer of naïve T cells to empty host. However, it is well demonstrated by us and other groups that naïve HY CD8 Tg T cells did not display lymphopenia-induced proliferation after transfer to empty hosts (3, 33–35). Consequently, these cells do not divide nor display memory phenotype or function, upon transfer to T cell-deficient mice (3, 33–35). Thus, in this system, stimulation with the male Ag is strictly required for in vivo proliferation and differentiation, favoring the study of Ag-driven responses in T cell-deficient mice, where homeostatic proliferation could not create any bias. BM cells were used as a source of male cells. These cells have a high capacity for cell divisions, allowing male cells to grow in host mice at early time points after immunization, thus mimicking the spread of viral or bacterial infection. In the presence of CD4 Tg T cells, HY Tg CD8 Tg T cells differentiated efficiently into memory CD8 cells. When deficient in CD40 expression, CD8 Tg T cells do not receive helper signals and thus do not differentiate into memory CD8 cells (3).
Therefore, this model provides a powerful system to study changes associated with defective and efficient memory CD8 T cell differentiation.

**CD40 deficiency on CD8 T cells impedes optimal expansion during secondary responses**

Because CD8 memory T cells are characterized by higher proliferative capacity, we studied the expansion capacity of CD8CD40^{+/+} and CD8CD40^{-/-} T cells during primary and secondary responses. During the primary response, the CD8CD40^{+/+} and CD8CD40^{-/-} kinetics of T cell accumulation and decline were overlapping (Fig. 1A, left) and the cell numbers recovered at the steady state were similar (10.2 ± 2.1 × 10^6 vs 9.0 ± 2.6 × 10^6). However, the two populations behaved very differently during the secondary response (Fig. 1A, right). As expected (36), CD8CD40^{+/+} T cells expanded earlier and more vigorously in secondary than in primary response (Fig. 1A). The proliferative response peaked at day 7 in the secondary response (16 ± 2.1 × 10^6) compared with day 9 in the primary response (14.2 ± 2.9 × 10^6). In sharp contrast, the expansion of CD8CD40^{-/-} T cells was not improved during the secondary response (Fig. 1A, right) and exhibited a similar profile to the primary response in the early time points (Fig. 1A). Moreover, cell persistence during the late secondary memory phase was deeply compromised. Indeed, 11.5 ± 1.1 × 10^6 CD8CD40^{+/+} T cells and 4.8 ± 1.5 × 10^6 CD8CD40^{-/-} T cells were recovered at day 60 (Fig. 1A, right). Thus, compared with CD40 competent CD8 T cells, CD40-deficient CD8 T cells exhibited similar primary response but a defective secondary response.

We further addressed whether the differences in cell expansion during secondary responses were due to different homing capacities. At day 1 after injection, the same total number of CD8 T cells was recovered (0.11 ± 0.02 × 10^6 for CD8CD40^{+/+} T cells vs 0.13 ± 0.04 × 10^6 for CD8CD40^{-/-} T cells). Moreover, both populations distributed equally well in the different organs studied (Fig. 1B). The majority of both CD8 populations were recovered from the spleen (>40%). Some 15–20% were recovered from the LN and BM and 10% from the liver. Similar results were obtained at later time points (data not shown).

We also studied whether CD40 deficiency on CD8 T cells modified central memory (CCR7^{hi}CD62L^{hi}) vs effector memory (CCR7^{lo}CD62L^{lo}) T cell differentiation (37). Because effector memory T cells have less proliferative capacity than central memory T cells upon Ag restimulation (37), we could argue that the lower expansion of CD8CD40^{-/-} T cells was due to differences in the rate of central/effector memory T cell differentiation. CD62L expression on CD8 T cells, which allows central memory to effector memory T cell distinction, was studied in spleen, LN, BM, and liver (Fig. 1C). The CD62L expression profile was very similar for both CD8CD40^{+/+} T cells and CD8CD40^{-/-} T cells in all organs studied during the steady state of the primary response (Fig. 1C, left), throughout the secondary response (data not shown), and at the end of the secondary response (Fig. 1C, right). The majority of CD8 T cells expressed CD62L in lymphoid organs (spleen and LN), whereas both CD8 populations display a effector memory T cell phenotype in the liver (CD62L^{lo}).

In conclusion, although CD8CD40^{-/-} T cells had no defect in terms of migration or central memory/effector memory T cell differentiation, they presented a major defect in their capacity to proliferate and survive in secondary response. These results demonstrated that CD40 deficiency on CD8 T cells precludes the development of enhanced proliferative capacity during the secondary response, which is an important property of memory T cells.

**CD40 deficiency on CD8 T cells prevents rapid elimination of Ag during the secondary response**

We have previously shown that CD8CD40^{-/-} T cells were able to eliminate Ag because male cells were undetectable 2 mo after immunization (3). In this experiment, we investigated whether CD40 deficiency on CD8 T cells could modify the kinetics of Ag elimination during both primary and secondary responses.

Two different methods were used to study elimination of male Ag. We first studied the expression of CD69 on a new set of naive CD8 Tg T cells (sensor population) injected 24 h before analysis to detect any residual Ag persistence (28, 33). We compared the Ag elimination by CD8CD40^{+/+} T cells during the primary and the secondary responses (Fig. 2A, left). In both cases at day 9, the CD69 expression by sensor cells was comparable to the basal expression (5%), suggesting that the male Ag was no longer present or not of sufficient quantity to activate a new set of naive CD8 T cells. However, the control of Ag load occurred earlier in the secondary than in primary response: the Ag load stopped accumulating by day 2 (compared with days 3–4 in primary response), and never reached the level observed in the primary response. Finally, a 2-day delay in the control of the Ag load was observed between the primary and secondary response.

**FIGURE 1.** Impaired expansion of CD8CD40^{-/-} T cells during secondary response. A. Total number of CD8 Tg T cells recovered from the spleen, LN, BM, and liver of individual mice at different time points after primary (squares) or secondary (circles) immunization with 0.5 × 10^6 CD8 T cells. Results show the comparison of CD8CD40^{+/+} and CD8CD40^{-/-} T cells on primary (left) and secondary (right) responses. Data show the average ± SD of three mice per group and are representative of four independent experiments. *p < 0.05; **p < 0.01; and ***p < 0.001 represent significant statistical differences between both CD8 populations. B, T cells recovery at day 1 after secondary immunization of CD8CD40^{+/+} (left) or CD8CD40^{-/-} (right) T cells in the spleen, LN, BM, and liver. C, Cell surface expression of CD62L on CD8CD40^{-/-} T cells (thin line histogram) or CD8CD40^{+/+} T cells (thick line histogram) recovered at the steady state of the primary (left column) or secondary (right column) responses in spleen, LN, BM, and liver. CD62L profile on naive CD8 T cells (dotted line histogram) is shown as a positive control. In B and C are representative of four independent experiments with three to four mice per group.
FIGURE 2. Delayed Ag elimination by CD8CD40−/− T cells during secondary response. A, Percentage of activated (CD69+CD44+) sensor cells during primary (squares) and secondary (circles) responses of CD8CD40+/+ (left) or CD8CD40−/− (right) T cells. These data show the mean percentage ± SD of sensor cells expressing CD69 at different time points and are representative of four independent experiments. B, Male Ag quantification using genomic Zfy-1 DNA real-time PCR. Results show the number of male cells detectable per million splenic cells during primary and secondary responses of CD8CD40+/+ (left) and CD8CD40−/− (right) T cells. A smaller scale response due to the lower level of expression of Zfy-1 at days 7 and 9 is shown (inset). The results are representative of four different experiments using n = 3 mice per group and at each time point. Real-time PCR was performed in triplicate for each sample.

FIGURE 3. Gene expression analysis at the single cell level at day 4 of the secondary response. A, Representation of the pattern of gene expression by single-sorted CD8 T cells 4 days after secondary immunization of CD8CD40+/+ or CD8CD40−/− T cells. Each line represents one cell and each column one gene. This analysis allows the determination of the percentage of each gene present in each population (vertical readout) and to study the coexpression of different genes into the same cell (horizontal readout). Finally for each cell expressing a particular gene, it is possible to quantify the number of mRNA molecules present inside the cell. B, Quantitative mRNA expression in the fraction of positive cells. For each gene, individual mRNA (circles) and mean mRNA (horizontal bar) are represented for CD8CD40+/+ (left line, purple) and CD8CD40−/− (right line, pink) T cells. These data represent 30 of 120 cells obtained for each gene in each population. Similar studies with equivalent single cell analysis were performed at each time point.
Unhelped CD8 T cells exhibit a defect in several cell surface receptor genes involved in memory cell survival

To determine whether the lower efficiency of CD8CD40−/− T cells during the secondary response could be related to an intrinsic defect during CD8 memory T cell differentiation, we measured the differential mRNA expression of key genes involved in memory differentiation. We compared the gene expression of CD8CD40+/+ with CD8CD40−/− T cells at the steady state of the primary response and during the secondary response (days 2, 4, 7, 15, and 60) by real-time RT-PCR performed at the single cell level (28, 29). Day 0 represents the steady state of the primary response (day 60). The selected genes were related to effector and regulatory functions as illustrated on day 4 of secondary responses in Fig. 3. As shown on 30 of a minimum 120 cells analyzed per group, this technique allows us to determine the frequency of gene expression within each group, and more interestingly the level of expression and coexpression within the population expressing the gene of interest (Fig. 3).

Focusing on the memory T cells higher capacity for survival, we studied whether CD8CD40−/− T cells develop any defect in the expression of cytokine receptor genes, such as IL-7Rα, IL-15Rα, and IL-21Rα, during the secondary response (39–42) (Fig. 4). We found a down-regulation of IL-7Rα mRNA upon restimulation in both CD8 populations (Fig. 4A). This down-regulation was exacerbated in CD8CD40−/− T cells compared with CD8CD40+/+ T cells. The percentage of IL-7Rα mRNA-positive cells was 2-fold lower at day 4 in CD8CD40−/− T cells compared with CD8CD40+/+ T cells. Moreover, IL-7Rα mRNA re-expression was significantly delayed in CD8CD40−/− T cells because the percentage of cells expressing IL-7Rα mRNA remained low at day 7, whereas already up-regulated in CD8CD40+/+ T cells. From days 15 to 60, the percentage of CD8 T cells expressing the IL-7Rα mRNA was identical in both populations. No significant difference was observed in the mean number of mRNA molecules, except at day 15 when the number was increased in CD8CD40+/+ T cells, suggesting better survival during the contraction phase (Fig. 4B). These results were confirmed at the cell surface level because the IL-7Rα molecule was down-regulated in both populations at day 3, but the re-expression was severely delayed in CD8CD40−/− T cells compared with CD8CD40+/+ T cells (Fig. 4C). IL-7Rα re-expression was observed as soon as day 7 in CD8CD40+/+ T cells compared with day 30 for CD8CD40−/− T cells.

For IL-15Rα and IL-21Rα, only gene expressions have been studied because the available Abs are not strong enough to discriminate between both CD8 populations. The percentage of cells expressing IL-15Rα mRNA strongly increased at day 4 in CD8CD40+/+ T cells but not in CD8CD40−/− T cells (Fig. 4D, left). A significantly reduced expression of IL-15Rα mRNA was also observed at the steady state of the secondary response. The number of IL-15Rα mRNA molecules was also strongly increased at day 7 in CD8CD40+/+ T cells (Fig. 4D, right). The percentage of cells expressing IL-21Rα mRNA was also constantly lower during the early time points of the secondary response and was extinguished at the steady state in CD8CD40−/− T cells, whereas CD8CD40+/+ T cells maintained expression (Fig. 4E, left). The number of IL-21Rα mRNA molecules in expressing cells was, however, similar in both populations (Fig. 4E, right). Interestingly, the percentage of cells coexpressing IL-15Rα and IL-21Rα at day 4 was 24.3% in CD8CD40+/+ T cells compared with 12.6% in CD8CD40−/− T cells (p = 0.03, data not shown), suggesting the latter are less sensitive to the synergistic effect of the IL-15 and IL-21 cytokines (42). Together, these results suggest that the absence of CD4 signaling on CD8 T cells (due to CD40 deficiency) has a deleterious impact on genes involved in cell survival, during both the effector phase and steady state of the secondary response.

Unhelped CD8 T cells show defects in expression of genes involved in cytotoxicity during secondary response

CD8 T cells can kill target cells by different pathways including the perforin, Fas/Fas ligand (FasL), and IFN-γ pathways. Effective cytotoxicity by the perforin pathway requires coexpression of perforin with granzyme A or granzyme B in the same cell (43). Thus,
we determined the percentage of cells coexpressing these two sets of molecules in each cell (Figs. 5A and 3). At the steady state of the primary response, the percentage of cells coexpressing genes required to kill by the perforin pathway was similar in CD8CD40+/+ and CD8CD40−/− T cells. During the secondary response, the percentage of perforin pathway-expressing cells drastically increased until day 4 in CD8CD40+/+ T cells (70.6%), but in contrast remained stable or even decreased in CD8CD40−/− T cells between day 2 and 4 (29.4%). At day 7, the percentage in CD8CD40−/− T cells finally increased to levels reached on day 4 with CD8CD40+/+ T cells. However, at the steady state of the secondary response, the cytotoxic potential of CD8CD40−/− T cells was severely reduced compared with CD8CD40+/+ T cells (9.9% and 27.2% of residual perforin pathway-expressing cells, respectively). Importantly, the percentage of killing by the perforin pathway of CD8CD40+/+ T cells was twice that observed at the end of the primary response (27.2% vs 14%, p = 0.022). In conclusion, CD8CD40−/− T cells exhibited a 3-day delay in expressing similar perforin activity during the crucial phase of Ag elimination and were not maintained efficiently at steady state in CD8CD40−/− compared with the CD8CD40+/+ T cells. Moreover, the increase in granzyme A and granzyme B mRNA in expressing cells was drastically higher in CD8CD40+/+ T cells (2- to 3-fold higher) compared with CD8CD40−/− T cells at days 2 and 4, respectively, but not significantly different for perforin (Fig. 5B). Our results demonstrate that CD8CD40−/− T cells exhibit a clear defect in the perforin pathway, both in terms of kinetics and strength of cytotoxic responses generated.

Although it has been shown that the perforin pathway is the major cytotoxic pathway, CD8 T cells can also kill via Fas/FasL interactions (44). The percentage of cells expressing FasL was identical in both populations at the steady state of the primary response (Fig. 5C). During the secondary response, this percentage increased more drastically in CD8CD40+/+ T cells than in CD8CD40−/− T cells till day 4 (76% vs 57% at day 2 and 80% vs 53% at day 4). Between days 7 and 15, CD8CD40−/− T cells reached the same percentage of FasL-expressing cells as CD8CD40+/+ T cells. Finally, the percentage of cells expressing FasL decreased in both populations until day 60, but this reduction was more severe for CD8CD40−/− T cells. There was, however, no statistical difference in the number of mRNA molecules in both populations over time (Fig. 5D). Thus, our results demonstrate that CD8CD40−/− T cells are impaired in FasL pathway.

An other important pathway of killing for CD8 T cells is their capacity to secrete the IFN-γ cytokine (45). Although the frequency of IFN-γ mRNA-expressing cells was similar in both populations until day 4 after immunization (30%) (Fig. 6A), the number of mRNA molecules coding for IFN-γ was much reduced for CD8CD40−/− T cells (1200 vs 400 molecules at day 4) (Fig. 6B). It has been described that secondary responses induce permanent effector gene expression (28). This effect was the case for CD8CD40+/+ T cells that maintained a high frequency of IFN-γ mRNA-expressing cells from day 7 onward. In contrast, CD8CD40−/− T cells did not display this memory imprinting. The percentage of IFN-γ mRNA-expressing cells dropped drastically after day 4, to a similar percentage found in primary response. We confirmed these results at the protein level, performing intracellular staining ex vivo (Fig. 6C). At day 2, no significant IFN-γ expression was detected in either cell population. However at day 4, 57% of CD8CD40+/+ T cells, whereas only 20% of CD8CD40−/− T cells, expressed the protein, and the mean fluorescence intensity was also superior for CD40-competent compared with CD40-deficient CD8 T cells. After day 4, the IFN-γ protein was no longer detected directly ex vivo and restimulation for 4 h in vitro with anti-CD3 Ab was performed (Fig. 6D). As previously published, CD8CD40−/− T cells isolated at the steady state of the primary response were severely impaired in their capacity to produce IFN-γ compared with CD8CD40+/+ T cells (3). During the early time point of the secondary response, CD8CD40+/+ T cells regained the capacity to produce IFN-γ upon restimulation (days 7 and 15) but lost it over time because at the steady state only 20% of CD8CD40−/− T cells produced IFN-γ compared with 72% for CD8CD40+/+ T cells. Finally, we measured IFN-γ secretion by ELISA after in vitro restimulation of both populations (Fig. 6E). Although CD8CD40−/− T cells isolated at the steady state of the primary response secreted high levels of IFN-γ cytokine, IFN-γ
levels of CD8CD40−/− T cells were lower. CD8CD40+/− T cell IFN-γ production was greater in the secondary than the primary response, whereas CD8CD40−/− T cells maintained a very low level of IFN-γ secretion, similar to that found in primary cells. Our results demonstrate that CD8CD40−/− T cells exhibit a clear defect in IFN-γ production.

Finally, we have studied the TNF-α gene, which has pleiotropic effects including inflammatory and killing activities (46). The percentage of TNF-α mRNA-expressing cells increased similarly during early phase of secondary response in both CD8CD40+/− and CD8CD40−/− T cells (Fig. 6G). However, within the positive fraction, CD8CD40+/− T cells exhibited defective production of TNF-α both at the mRNA expression level (mean mRNA = 388 vs 186, p < 0.05, for CD8CD40+/− vs CD8CD40−/− T cells, respectively, at day 4 of secondary response) and the protein expression level after a short in vitro restimulation (Fig. 6F).

In conclusion, CD8CD40+/− T cells are dramatically impaired in their cytotoxic potential both qualitatively and quantitatively. CD8CD40−/− T cells remain capable of cytotoxic activity but at significantly lower levels compared with CD8CD40+/− T cells. Thus, the absence of CD40 expression on CD8 T cells altered the cytotoxic capacity developed by memory T cells both in terms of kinetics and intensity in all cytotoxic pathways considered.

CD8CD40−/− T cells exhibit a higher level of immunoregulatory genes

Finally, to address whether memory T cell differentiation modifies CD8 T cell sensitivity to inhibitory mechanisms, such as TGF-β or IL-10 production, the mRNA expression of the TGF-β receptor type II subunit (TGF-βRII), TGF-β, and the IL-10R α-chain (47, 48) were analyzed. We observed that the percentage of cells expressing TGF-βRII was higher among CD8CD40−/− T cells than CD8CD40+/− T cells during the effector phase of the secondary response (Fig. 7A). Similarly, the percentage of CD8 T cells expressing IL-10Rα was higher in CD8CD40−/− T cells during the initial phase of the secondary response (until day 7). This higher percentage of IL-10Rα-expressing cells was associated with a 3-fold higher number of IL-10Rα mRNA molecules in CD8CD40−/− T cells at day 4 (Fig. 7B). We detected a high but identical percentage of TGF-β-expressing cells in both populations until day 15 (Fig. 7C, left). However, the number of TGF-β mRNA molecules was 2-fold higher at day 4 in CD8CD40−/− T cells (Fig. 7C, right). These results were confirmed at the protein level by intracellular staining ex vivo (Fig. 7D). We observed a similar up-regulation of TGF-β in both populations at day 2; however, higher levels were seen in CD8CD40−/− T cells than in CD8CD40+/− T cells.
All together these results strongly suggest an increased susceptibility of CD8CD40−/− T cells to inhibitory signaling during the effector phase of the secondary response.

**Discussion**

The generation of efficient CD8 memory T cells is a differentiation process in which the presence of CD4 T cells is an absolute requirement (3–7). We have demonstrated that CD8CD40−/− T cells do not differentiate into memory cells in a noninfectious model (3). Similar conclusions are more controversial in infectious models. Two reports studying *Listeria Monocytogenes* genes responses show no role for CD40/CD40L interactions in either the primary or the secondary response (50, 51), whereas many others infectious studies describe a deficient secondary response in the absence of CD40/CD40L interactions (13, 52–55). A study by Lee et al. (56) suggests a role for CD40 on APCs but not on CD8 T cells during secondary response in a model of influenza immunization. The accumulation of CD8CD40−/− T cells was studied in the lung during the primary response and at day 6 after boosting. No differences were observed between CD8CD40−/− T cells and CD8CD40+/+ T cells at these time points. In agreement with these studies, we found no role for CD40 expression by CD8 T cells concerning the primary response. The CD8CD40−/− T cells expanded and eliminated the Ag as well as CD8CD40+/+ T cells. However, when we evaluated more extensively the secondary response of CD8 T cells, we found the CD8CD40−/− T cell response to be severely impaired. Major differences were found at earlier time points, with CD8CD40−/− T cell numbers peaking much later than CD8CD40+/+ T cell numbers. Thus, the less impressive differences observed at day 6 in the influenza system mimic the results we observed at day 7, when CD8CD40−/− T cells regain some effector function but to a level comparable to the primary responses. Therefore, the role of CD40 signaling remains to be readdressed kinetically in infectious models to definitively exclude its importance in secondary responses.

Although many studies have contributed to an increased understanding of CD8 memory T cell generation (1, 2, 8, 17, 20–26), the molecular mechanisms underlying the superior function of CD8 memory T cells compared with defective primed CD8 T cells remain largely unknown. In this study, we extend our previous results showing that CD8CD40−/− T cells have not imprinted the instructive signals responsible for memory T cell differentiation. The secondary response of CD8CD40−/− T cells mimics a primary response in terms of expansion and kinetics of Ag clearance. The study of effector gene expression in secondary responses explains these functional deficiencies and demonstrates the highly ubiquitous effect of CD4 help on CD8 memory differentiation. CD8CD40−/− T cells are dramatically impaired in their cytotoxic potential, affecting all cytotoxic pathways, both qualitatively and quantitatively compared with CD8CD40+/+ T cells. This correlates with the delay in the control of Ag load in the secondary response. The expression of cytokine receptors (IL-7Rα, IL-15Rα, IL-21Rα) is also impaired in the absence of CD40 expression at the early phase of secondary immunization, which may explain the fewer number of CD8CD40−/− T cells recovered at the end of the secondary response due to their lower survival potential.

Importantly, at early time points of the secondary response, the percentage of CD8CD40−/− T cells expressing genes involved in down-regulation of the immune response (i.e., TGF-βRII, IL-10Rα, and TGF-β) is constantly higher than the percentage of CD8CD40+/+ T cells expressing these genes. The TGF-β cytokine has been shown to inhibit both proliferation and differentiation of CTL by acting on perforin, FasL, and IFN-γ pathways (57, 58). All of these pathways are down-regulated at day 4 in...
CD8CD40$^{+/+}$ T cells. One possible hypothesis we are currently investigating is that the higher sensitivity of CD8CD40$^{+/+}$ T cells to regulatory elements is responsible for their lower functional capacity.

We and others have demonstrated that the program of differentiation into memory cells is established during the primary response (4, 28, 59). However, we found no difference in gene expression, for all genes studied, between helped and unhelped T cells at the end of the primary response. These data suggest that these genes are not directly implicated in the program of differentiation into memory cells. In particular, IL-7Rα and CD62L expression are identical for both CD8 populations in agreement with some but not all studies (13–17, 60, 61). The frequency of Tg CD8 T cells, persistence of Ag, type of viral infection, and the lymphoid organ studied may partly explain the discrepancy observed in the different experimental settings. The discrepancy could also reflect nonoverlapping mechanisms of CD4 help depending on the immuno- genic Ag. Determining what the mechanisms are that underlie memory differentiation will help to identify why unhelped CD8 responses are defective. Differences in transcription factors, repressor genes, chromatin remodeling, or gene methylation and alterations of the CD40 signaling pathway or other members of the TNF/TNFFR family could be responsible for altered CD8 T cell responses and need to be further investigated (8, 9, 17, 20–26).

Interestingly, it is striking to observe that although CD8CD40$^{+/+}$ memory T cells maintain a gene expression profile allowing for heightened response on Ag re-exposure, CD8CD40$^{−/−}$ T cells extinguish the majority of their stimulated genes. At the end of the secondary response CD8CD40$^{−/−}$ T cells have shut down most of the relevant activated genes (perforin, granzyme A, granzyme B, FasL, IFN-γ, IL-15Rα, IL-21Rα, TGF-βRII, IL-10Rα, and TGF-β), demonstrating that any instructive program has not been imprinted in these CD8 T cells.

Finally, heterogeneity within the memory T cell pool was observed all along the kinetics of the secondary responses in both CD8CD40$^{+/+}$ and CD8CD40$^{−/−}$ T cells (as exemplified at day 4 in Fig. 3). This observation is in agreement with recent studies demonstrating that CD8 effector T cells were shown to be remarkably heterogeneous, rather than homogeneous, when screened for the simultaneous expression of multiple effector molecules (28, 62, 63). These observations confirm several theories and mathematical models that have postulated gene expression to be stochastic (64–66). It is tempting to speculate that functional heterogeneity within the memory T cell pool may enhance the efficiency of memory T cell responses against pathogens.

Overall, our results demonstrate that the absence of CD4 help signaling due to the lack of CD40 engagement on CD8 cells leads to profoundly defective programming of CD8 memory T cells. As suggested previously, CD8 memory should no more be defined as a passive phenomenon of effector cell persistence following antigenic encounter, but as a complex differentiation process. The ability to mount an efficient primary response is definitely not a signature for the generation of long-lived efficient memory cells.

Importantly, this work provides important insights about the mechanisms associated with CD4 help in the generation of efficient secondary responses. We demonstrate the ubiquitous role of CD4 help during the development of proper CD8 memory T cells that participate to 1) the enhancement of all cytotoxic pathways, but also 2) the persistence of target effector gene expression and 3) the regulation of immunoregulatory elements. These data have major implications concerning the inhibition of tumor cell growth and the development of effective vaccination strategy.

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Disclosures
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