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Antigen-Free Adjuvant Assists Late Effector CD4 T Cells To Transit to Memory in Lymphopenic Hosts

F. Betul Guloglu,*1 Jason S. Ellis,*2 Xiaoxiao Wan,* Mermagya Dhakal,* Christine M. Hoeman,*3 Jason A. Cascio,* and Habib Zaghouani*†

The events controlling the transition of T cells from effector to memory remain largely undefined. Many models have been put forth to account for the origin of memory precursors, but for CD4 T cells initial studies reported that memory T cells derive from IFN-γ–nonproducing effectors, whereas others suggested that memory emanates from highly activated IFN-γ–producing effectors. In this study, using cell proliferation, expression of activation markers, and production of IFN-γ as a measure of activation, we defined two types of effector CD4 T cells and investigated memory generation. The moderately activated early effectors readily transit to memory, whereas the highly activated late effectors, regardless of their IFN-γ production, develop minimal memory. Boosting with Ag-free adjuvant, however, rescues late effectors from cell death and sustains both survival and IFN-γ cytokine responses in lymphopenic hosts. The adjuvant-mediated memory transition of late effectors involves the function of TLRs, most notably TLR9. These findings uncover the mechanism by which late effector CD4 T cells are driven to transit to memory and suggest that timely boosts with adjuvant may enhance vaccine efficacy.

Immune memory is one of the cardinal features of the immune system and provides the fundamental basis for vaccination against microbes (1). Due to the low frequency at which memory precursors arise and the lack of memory markers, the events that control transition of effector cells into memory remain largely undefined. Despite these limitations, progress has been made and many models have been put forth to explain the origin of memory precursors (1–7). For CD4 T cells, it has been shown that memory T cells derive from effectors producing low amounts of IFN-γ (8). Recently, however, observations were reported indicating that effector CD4 T cells producing significant IFN-γ can transit to memory (9, 10). These findings suggest that IFN-γ may not be the only factor involved in the transition from effector to memory (2). An alternative hypothesis has been put forth postulating that effector CD4 T cells that surpass a minimum required activation threshold and do not fully commit to terminal differentiation have a potential to differenate into memory (11–13). However, despite the inclusive nature of this premise, it remains difficult to explain the decreasing potential model, which suggests that effector cells progressively lose their memory potential along the way to terminal differentiation (6, 14). Thus, the origin of CD4 T cell memory remains a matter of debate, and further studies to bring about insight on the development of memory are justified. In this study, we used the DO11.10 TCR transgenic mouse and devised a transfer model that increases the frequency of effector T cells with potential for transition to memory (10) and investigated the development of memory in the context of the activation status of effector CD4 T cells as defined by cell division, expression of activation markers, and production of effector cytokines. Accordingly, OVA-specific DO11.10 CD4 T cells were labeled with the intracellular fluorescent dye, CFSE (15), transferred into BALB/c mice, and the hosts were immunized with OVA323–339 peptide (OVAp) emulsified in CFA. Three days later, expression of activation markers and intracellular cytokine production were analyzed ex vivo on CFSE-demarked effector cell divisions. These analyses revealed two levels of activation at the effector stage, and the cells were classified into moderately activated early effectors and highly activated late effectors. The two types of effectors were then sorted on the basis of CFSE dilution, and each cell division was transferred into naive hosts for parking. Four months later, memory precursors from each cell division were analyzed prior to any rechallenge with OVAp, and the generation of memory responses was evaluated upon challenge of the host mice with OVAp in CFA. The findings indicate that the moderately activated early effectors readily yield memory. However, the highly activated late effectors display little memory, but stimulation with Ag-free adjuvant rescues the cells from apoptosis and sustains expansion, survival, and increased cytokine production that culminate in significant memory responses. Interestingly, the adjuvant effect on the late effectors is mediated through TLR, most notably TLR9. Overall, the findings explain the discrepancies concerning the ontogeny of CD4 T cell memory and advocate for timely administration of adjuvants for the development of effective vaccines.
Materials and Methods

**Mice and Ags**

DO11.10 Rag2-/- transgenic mice expressing a TCR specific for OVAp were purchased from Taconic Laboratories (Hudson, NY). BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Rag2-deficient BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were used in accordance with the guidelines of the institutional animal care and use committee. Influenza virus hemagglutinin (HA) aa residues 110–120 peptide, which is immunogenic in BALB/c mice (H-2b) as OVAp, was used as a negative control (10). All peptides were purchased from EZBiolab.

**CFSE labeling and immunization**

Naive splenic DO11.10 CD4+ T cells were isolated using magnetic beads (Miltenyi Biotec). The cells (10 x 10^6 cells/ml) were then incubated with 10 μM CFSE at 37°C for 10 min and washed once with ice-cold DMEM-10% FCS and once with PBS. CFSE-labeled T cells (10 x 10^6 cells/mouse) were adoptively transferred i.v. into BALB/c mice. Two days later, the hosts were immunized s.c. with 125 μg OVAp in 200 μl PBS/CFA (1/1v). The hosts were sacrificed 72 h later, and their draining lymph nodes (LNs) were harvested.

**Sorting of effector T cells**

LN effector T cells from BALB/c hosts were first analyzed for the number of divisions on the basis of CFSE dilution. The divisions D2 through D8 were sorted individually on the basis of CFSE and injected into BALB/c or Rag2-/- mice. Effector T cell divisions were isolated on the basis of IFN-γ production by using a mouse IFN-γ Secretion Assay-Cell Enrichment and Detection Kit (Miltenyi Biotec) to label IFN-γ-producing cells and MoFlo XDP (Beckman Coulter) to sort the cells.

**Ex vivo analysis of effector T cells**

The LN cells from BALB/c hosts were stained for surface molecules without any Ag stimulation. For analysis of intracellular cytokines, a brief 4-h stimulation with OVAp (10 μM) or PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of 10 μg/ml brefeldin A was performed, as described (10, 16).

**Analysis of recall responses by effector T cells**

Individually sorted effector divisions (D2 to D8) were injected to naive BALB/c mice (5 x 10^6 per mouse), and, 1 d later, the hosts were immunized s.c. with a suboptimal dose of OVAp (20 μg per mouse) in 200 μl PBS/CFA (1/1v). IFN-γ responses from draining LNs were analyzed at different days (4, 6) following immunization. Briefly, LN cells (0.1, 0.3, and 0.9 x 10^6 cells/well) were incubated with 10 μM OVAp for 24 h, and IFN-γ was detected in culture supernatants by ELISA. Immunized mice with no transfer of effector T cells were used as baseline control for IFN-γ responses. HA peptide was used as a negative control for in vitro stimulation.

**Analysis of memory precursor responses**

Each effector division (D2 to D8) was sorted based on CFSE intensity, and 5 x 10^6 cells were adoptively transferred i.v. into Rag2-/- hosts.

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**FIGURE 1.** The effector phase includes moderately activated early and highly activated late cells that develop different recall IFN-γ responses. CFSE-labeled naive DO11.10 T cells were transferred into BALB/c mice, and the hosts were immunized with OVAp in CFA. Three days later, the LN cells were harvested, and the number of cell divisions, the frequency of cytokine responders, and expression of activation markers were analyzed. (A) Shows the effector divisions as measured by CFSE dilution and presented in dot plot and histogram format. (B) Shows intracellular and surface staining of cell divisions with isotype controls (left panel) and anti-IFN-γ and anti-CD62L Abs, respectively (right panel). (C) Shows percentage of CD4 T cells producing cytokines and mean fluorescence intensity for the indicated markers compared with isotype control. (D) The LN cells in each effector division were sorted and transferred into naive BALB/c mice (5 x 10^6 cells/mouse), and the hosts were immunized with a suboptimal dose of OVAp in CFA. The mice were sacrificed at days 4 and 6, and the LN cells were stimulated with 10 μM OVAp and IFN-γ responses were measured by ELISA. Immunized mice with no transfer of effector T cells were used as baseline control for IFN-γ responses. The top plot shows the mean of IFN-γ response of each division at days 4 and 6. Each point represents the mean ± SE. The bottom plot shows a summary of data comparing the mean ± SE memory IFN-γ response of early (D2, D4, and D5) and late (D6, D7, and D8) divisions at the peak of their responses. The results are representative of four independent experiments. ***p < 0.005.
for long-term parking. The splenic cells were harvested after 4-mo parking, and 0.5 × 10^6 cells/well were stimulated with OVAp or control HA peptide for 24 h. IFN-γ was detected in culture supernatants by ELISA.

### Analysis of memory response

The Rag2^-/- hosts were immunized s.c. with a suboptimal dose (0.25 μg) of OVAp/CFA (10) after 4-mo parking, and the splenic and LN IFN-γ responses were analyzed at day 3 postimmunization. LN (0.1 × 10^6 cells/well) and spleen (SP) (0.5 × 10^6 cells/well) cells were stimulated with the indicated amounts of OVAp or control HA peptide for 24 h. IFN-γ was detected in culture supernatants by ELISA. As memory control, DO11.10 naive T cells were transferred into Rag2^-/- mice and, 1 d later, the hosts were immunized with 125 μg OVAp/CFA and their responses were analyzed 4 mo later. Another memory control group was Rag2^-/- mice recipient of all divisions (D2 to D8) that were parked for 4 mo.

### Flow cytometry and Abs

Surface and intracellular staining was carried out as previously described (10), and data were acquired using Cyan ADP Analyzer (Beckman Coulter) or FACSCalibur (BD Biosciences). Fluorescence analysis was performed using Summit 5.2 software (Beckman Coulter). The Abs used for surface staining include KJ1-26-PE/Cy5, -FITC, and -allophycocyanin; anti-CD44-PE/Cy5, -allophycocyanin, anti-CD25-PE, and -allophycocyanin; and anti-CD127-allophycocyanin, anti-CD4-PECy7, and anti-CD3-PE. For intracellular staining, we used anti-IFN-γ-allophycocyanin and -PE and anti-IL-2-PE. KJ1-26 is an anti-clonotypic mAb that binds the TCR expressed on DO11.10 T cells. To analyze cell death of CFSE-labeled effector T cells, LN cells were cultured for 42 h with OVAp (10 μM) and stained with annexin V.

### IFN-γ measurement by ELISA

The capture (purified, R4-6A2) and detection (biotinylated, XMG1.2) rat anti-mouse IFN-γ Abs were purchased from BD Pharmingen. Streptavidin reagent (Sigma-Aldrich) was used as template to determine TLR expression by real-time PCR.

### Analysis of TLR expression by real-time PCR

RNA was extracted from naive, effector, and memory T cells with TRI reagent (Sigma-Aldrich) and used as template to determine TLR expression by real-time PCR. The assay used the Power SYB Green RNA-to-CT 1-Step kit (Applied Biosystems) and run on a Step One Real-Time PCR System (Applied Biosystems). The primers used to detect TLR mRNA expression are as follows: TLR2 forward, 5'-ACCGAAACCCCTAGACAAACTGGTCA-3'; and TLR2 reverse, 5'-AACACGGGTTTGCAGTGTCGCTG-3'. TLR3 forward, 5'-CCTGCGTTGCGAGTTGAAACACT-3', and TLR3 reverse, 5'-AAGAAGGGGCAGGCTGTCGA-3'; TLR4 forward, 5'-ATCATGGACTGTTCATCTCCTTC-3', and TLR4 reverse, 5'-GGAAGGCGTGGTACTGTTTCTG-3'.

### Statistics

Statistical differences between groups were calculated using GraphPad Prism 5. Unpaired t test (Figs. 2A, D, F) and one-tailed paired t test (Figs. 4C, 5, 6, 7A, 8) were used for statistical analysis.
**Results**

*Highly activated late effectors develop diminished IFN-γ recall responses relative to moderately activated early effectors*

The study of the initial events controlling transition from effector to memory is hampered by the low frequency at which memory precursors arise and the lack of specific memory markers. Recently, TCR transgenic cell transfer models were used to increase the frequency of effector T cells with potential for transition to memory and proved suitable for tracking the initial events underlying the development of memory (8, 10). These and other investigations, however, yielded discrepancies as to the origin of memory when IFN-γ was used to gauge the activation status of effector T cells (8–10). In this study, we sought to define the activation status of effector T cells on the basis of the number of cell divisions the effector T cells undergo upon exposure to Ag, the expression of activation markers, and the production of IFN-γ cytokine, and to determine transition to memory. Accordingly, TCR transgenic CD4 T cells specific for OVA were purified from the SP of naive DO11.10 mice, labeled with the cell division marker CFSE, and transferred to naive BALB/c mice, and the hosts were immunized s.c. with OVA in CFA, as we previously described (10). The draining LN CD4 T cells were then analyzed ex vivo for cell division, expression of activation markers, and cytokine production. As illustrated in Fig. 1, the cells have undergone 8 divisions with peak 1 as undivided cells and peak 9 as cells that divided 8 times (Fig. 1A). We refer to peak 1 through 9 as division (D) 1 through (D) 9, and because D1 and D9 have very few cells, they will not be considered for investigation of transition to memory. This limitation is related to the number of cells to transfer into the BALB/c hosts, and further increase in cell number might require higher dose of Ag for priming, which could interfere with memory differentiation (18, 19). Moreover, when the LN cells were stained ex vivo for intracellular IFN-γ and production of the cytokine was analyzed per cell division, there was an increase in IFN-γ production as the cells progressed in cell division (Fig. 1B, right panel). The isotype control did not show any significant IFN-γ production (Fig. 1B, left panel). As shall be seen below, the cells not only divided and produced IFN-γ, but also expressed activation markers, indicating a loss of naive phenotype and acquisition of effector status. The results for IL-2 production and expression of activation markers are presented in a graph format alongside IFN-γ (Fig. 1C). The findings indicate that the effector phase can be divided into moderately activated early divisions (D2–5) and highly activated late divisions (D6–8). The early division T cells had lower levels of CD25 and CD62L, and only a small percentage of the cells had IFN-γ and IL-2 cytokines (Fig. 1B, 1C). The late division T cells had an overall higher CD25 expression, and a significant percentage produced IFN-γ and IL-2. CD62L expression varied among the divisions, possibly reflecting TCR signal strength (20). CD44 expression was higher on the early divisions, but decreased in the late effectors, which could be related to survival and T cell memory development (21). Overall, the effector phase can be divided into moderately activated early effectors (D2–5) and highly activated late effectors (D6–8).

To further characterize the activation status of the effector cells, each division was sorted based on CFSE dilution and transferred

**FIGURE 3.** Late effectors display survival disadvantage relative to early effectors. The BALB/c LN effectors were sorted from each division, and the early (D4–5) and late (D6–8) effectors were transferred into Rag2–/– mice. Four months later, the SP cells were harvested, and in (A) the percentage and absolute number of surviving CD4+KJ1-26+ cells were measured by flow cytometry and their IFN-γ responses were evaluated by ELISA. (B) Unseparated LN cells were cultured for 42 h in the presence of OVA and then stained with annexin V. The cells were then gated on CFSE, and annexin V binding was analyzed on each division. The bars show the mean percentage of annexin V+ cells for early (D4–5) and late (D6–8) effectors. (C) Prior to transfer, the CFSE-positive D2–8 effectors were sorted and stimulated with PMA and ionomycin (P+I), labeled, and analyzed for IFN-γ production (left panel), and IFN-γhigh and IFN-γlow cells of D6–8 and D4–5 cells were separated and reanalyzed for IFN-γ to confirm the purity of separation. (D) Hosts of the sorted IFN-γhigh and IFN-γlow late effectors were sacrificed 6 wk posttransfer, and the percentage, absolute number, and IFN-γ production by the CD4+KJ1-26+ splenic cells were measured as in (A) and compared with D4–5 early effectors. The graphs represent the mean of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.005.
into naive BALB/c mice, and the hosts were challenged with a suboptimal dose of OVAp in CFA. The LN cells were then harvested and stimulated with graded concentrations of OVA peptide (triangles) or 10 μM HA peptide (rectangles) as control, and IFN-γ responses were measured by ELISA. (B) The LN from the CFA (gray bars) and no CFA (black bars) groups were harvested after 4- or 16-wk parking, and the cells were analyzed for IL-7R expression and apoptosis by 7-aminoactinomycin D using flow cytometry. (C) Naive CFSE-labeled DO11.10 T cells were transferred into BALB/c mice, and the hosts were immunized with 125 μg OVA/CFA. Three days later, the LN effectors were isolated and retransferred into Rag2−/− hosts. One day later, one group of mice was given CFA, and the other was left without CFA. After 120 d of parking, the SP cells were harvested, and the percentage of KJ1-26+ CD4+ T cells was estimated among total splenic cells. The graph represents the mean of four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.005.
IFN-γ, but leads to unresponsiveness of highly activated late effectors perhaps due to apoptosis and/or restoration of a resting state, which could impact transition to memory (1).

**Transition to memory is more effective for early compared with late effectors**

The fact that the late effectors could not develop significant in vivo recall responses could translate into defective transition to memory (22). To test this premise, the early and late effector divisions were individually transferred to Rag2<sup>−/−</sup> hosts and subjected to a long-term (4-mo) parking without Ag. The rationale for parking the effector cells in a Rag2<sup>−/−</sup> host stems from the following reasoning: the lymphopenic environment should sustain survival of memory precursors that arise at a very low frequency (23–27), thereby making the readout of memory responses feasible. Second, the effector cells of all divisions are subjected to identical survival mechanisms in the absence of Ag, and any difference in the memory responses will solely reflect how each cell division affects memory development. The validation of this model was shown in our earlier work, in which memory precursors were shown to give rise to rapid and robust IFN-γ responses as compared with control naive T cells (10). The Rag2<sup>−/−</sup> hosts were then used to test the effectors for generation of memory precursors prior to immunization with Ag and the development of memory responses upon rechallenge with suboptimal dose of OVAp. The findings show that, prior to any reimmunization, the early effectors (D2, D4, and D5) gave rise to significant IFN-γ–producing memory precursors, whereas the late effectors (D6–8) had a much lower precursor response (Fig. 2A). Note D3 had a low precursor response as was previously defined (10). Again, whereas these findings parallel with the in vivo recall responses (Fig. 1D), they are surprising, as highly activated effector cells yielded limited memory precursors. Upon challenge with OVAp/CFA, the LN responses analyzed on day 3 postimmunization were reflective of the precursor pattern, as the early effectors yielded higher memory responses than late effectors (Fig. 2B). After 4 d, though, memory responses began to develop for late effectors (data not shown). When the response of combined early effector divisions (D2, D4, D5) was compared with the response of late effector divisions (D6–8), there was a significantly higher memory IFN-γ response by early versus late LN effectors (Fig. 2C). A similar trend was observed with splenic memory responses (Fig. 2C). Also, highly activated cells that have undergone ≥8 divisions did not yield significant IFN-γ memory responses (Supplemental Fig. 1). Overall, the early effectors are more efficient than the late effectors in transitioning to memory as they develop rapid and robust cytokine responses to a suboptimal dose of Ag, which is the hallmark of memory.

**Late effectors display survival disadvantage relative to early effectors**

Late effectors developed poor IFN-γ responses upon recall with Ag and were unable to generate significant memory responses. It is thus logical to envision that the late effectors may manifest a survival disadvantage and yield limited memory precursors. To test for survival, the D6–8 effectors were isolated and transferred into Rag2<sup>−/−</sup> hosts, and, after 4-mo parking, the percentage and absolute number of KJ-126<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells in the SP were evaluated by flow cytometry and compared with D4–5 early effectors, which, unlike D2 or D3, generate unconditional memory responses (10). The results indicate that the late effectors persisted at a much lower frequency than the early effectors, as the percentage among total splenic cells was 0.4 ± 0.04 for D6–8 as compared with 0.9 ± 0.13 for D4–5, and the absolute number of KJ-126<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> cells per SP was 43,000 ± 5,000 for D6–8 versus 69,000 ± 5,000 for D4–5 (Fig. 3A). Moreover, when the splenic cells were stimulated with OVAp, the precursors generated from the late effectors produced significantly lower amounts of IFN-γ (0.3 ± 0.09 ng/ml for D6–8 versus 3.8 ± 0.9 ng/ml for D4–5), as tested by ELISA (Fig. 3A). When the amount of IFN-γ was divided by the number of T cells (Fig. 3A, bottom panel), there was less cytokine per cell in the late versus early effectors (3.2 ± 0.7 ng/ml for D6–8 versus 29.8 ± 5.6 ng/ml for D4–5). Thus, the late effectors yielded lower numbers of long-lived memory precursors producing low levels of IFN-γ. This survival disadvantage is most likely related to increased apoptosis among late effectors because analysis of cell death indicated that D6–8 effectors had a slightly higher rate (13.2 ± 0.9%) of apoptosis relative to D4–5 early effectors (8.8 ± 1.1%) (Fig. 3B). It was previously shown that IFN-γ–producing effector cells do not persist because they undergo cell death in an IFN-γ–dependent paracrine manner (28, 29). Because within the late effectors some cells produce more IFN-γ than others, it is possible that late effectors producing high levels of IFN-γ undergo apoptosis at a significantly higher level than late effectors producing low levels of IFN-γ. To explore this possibility, sorted D2–8 LN effector T cells from BALB/c hosts recipient of CFSE-labeled naive DO11.10 T cells and immunized with OVA/CFA were shorty stimulated with PMA and ionomycin, and the late effector T cells producing high (D6–8 IFN-γ<sup>high</sup>) and low (D6–8 IFN-γ<sup>low</sup>) IFN-γ were sorted on the basis of CFSE dilution and IFN-γ intensity (see boxes in Fig. 3C, left panels). Low IFN-γ–producing D4–5 early effectors were also sorted for control purposes; the purity of the effectors was assessed (Fig. 3C, right panels); and the cells were transferred into Rag2<sup>−/−</sup> hosts for parking. Subsequently, the percentage, absolute number, and IFN-γ response of the surviving KJ1-26<sup>+</sup> T cells were determined. As indicated in Fig. 3D, the high IFN-γ–producing late effectors (D6–8<sup>high</sup>) were very low in percentage (0.4 ± 0.16% of CD4+KJ1-26+) whereas the late effectors (D6–8<sup>low</sup>) produced significantly lower amounts of IFN-γ (0.3 ± 0.09 ng/ml for D6–8 versus 29.8 ± 5.6 ng/ml for D4–5).
for D6–8high versus 2.2 ± 0.5% for D4–5) and absolute cell number (8.7 ± 4.3 × 10^3 cells for D6–8high versus 38.8 ± 4.3 × 10^3 cells for D4–5) and did not produce detectable memory IFN-γ cytokine by ELISA. The low IFN-γ–producing late effectors (D6–8low), although yielding significant cell percentage and number relative to D6–8high effectors (1.2 ± 0.3% and 27.2 ± 10.9 × 10^3 cells for D6–8low), remained inefficient at producing memory IFN-γ relative to memory T cells arising from D4–5 early effectors (0.7 ± 0.3 ng/ml for D6–8low versus 2.3 ± 0.1 ng/ml for D4–5). The frequency and absolute number of KJ1-26+ cells in blood, bone marrow, LNs, and lungs showed the same trend (data not shown). These findings suggest that high IFN-γ–producing late effectors have a survival disadvantage relative to both low IFN-γ–producing late effectors and early effector cells. Overall, late effectors are not effective in the generation of memory because the IFN-γ–producing cells are short-lived and possibly dividing less, whereas the long-lived counterparts remain poor IFN-γ producers compared with early effectors.

**Late effectors require continuous contact with adjuvant for survival and generation of effective memory**

To begin investigation of the mechanism underlying the differential survival among early and late effectors, we sought to determine whether CFA plays a role in transition of the effectors into memory cells. The rationale for this stems from the observation that effector T cells that remain parked in the immune host recipient of OVAp/CFA regimen develop a strong memory response (10). To this end, naive DO11.10 T cells were transferred into naive Rag2^-/- and BALB/c mice, and the hosts were immunized with OVAp/CFA. Rag2^-/- mice were used for a 4-mo rest (parking of the effector cells without isolation or transfer into a new host), and BALB/c mice were used to separate effector T cells that were then transferred into Rag2^-/- hosts for parking. After 4 mo, the splenic cells from both groups were harvested and stimulated with graded concentrations of OVAp or control HA peptide, and IFN-γ–precursor responses were measured. The results show that the effector cells that remained in the host developed a strong memory response, whereas the effectors that were transferred into a new host had minimal IFN-γ–response (Fig. 4A). Moreover, when the LNs from both hosts were harvested after 4 or 16 wk parking and the cells were analyzed for IL-7R expression and apoptosis, the effectors that remained in the immune host displayed significantly higher IL-7R expression (84.7 ± 3.5% versus 33.7 ± 2.8% on week 4, and 90 ± 1.2% versus 73 ± 0.5% on week 16) and survival rate (66.5 ± 3.9% versus 38.5 ± 7.6% on week 4, and 81.7 ± 2.4% versus 48 ± 2.1% on week 16), which is indicative of a better transition into memory (Fig. 4B). This could be due to more efficient IL-2 signaling during priming leading to earlier re-expression of IL-7R on memory precursors (Supplemental Fig. 2). These observations suggested that continuous contact with CFA

**FIGURE 7.** TLR9 ligand supports transition of late effectors into memory. (A) Early (D4–5) and late (D6–8) effectors were isolated from BALB/c mice (as in Fig. 3) and transferred into Rag2^-/- hosts. On days 1, 4, 7, and 10 after cell transfer, the hosts received TLR2, TLR4, or TLR9 ligand (optimal regimens), and the percentage and IFN-γ production by the CD4^+KJ1-26^ cells were analyzed 3 wk after the last TLR ligand injection. Each bar represents mean of four independent experiments. (B and C) The hosts were transferred with separated IFN-γ–low or IFN-γ–high cells from D6–8 effectors or unseparated D4–5 cells (as in Fig. 3), and the hosts were given TLR9 ligand as in (A) and splenic cells were harvested 3 wk after the last TLR injection. The graphs in (B) show the percentage, absolute number, and IFN-γ production by the CD4^+KJ1-26^ cells, whereas in (C) the plots show intracellular IFN-γ. Each bar represents the mean of at least three independent experiments. (D) Purified naive, effector, and memory DO11.10 T cells were cultured in the presence of media alone (open bars), media supplemented with TLR9 ligand (black bars), or media from APC culture stimulated with TLR9 ligand (gray bars). Seventy-two hours later, live cells were counted using trypan blue exclusion. For these assays, LN effector T cells (D2–8) were isolated on the basis of CFSE, whereas naive T cells and memory precursors (from Rag2^-/- hosts recipient of D2–8 LN effectors) were sorted based on KJ1-26, anti-CD3, and anti-CD4 staining. Purified splenic APCs were cultured simultaneously, but separately, from T cells in the presence of TLR9 ligand (CpG–oligodeoxynucleotide 1866) 50 μg/ml). Twelve hours after the initiation of culture, APC supernatant was transferred to T cell culture. Each bar represents the mean of at least four independent experiments. The cell numbers were relative to culture without TLR ligands (set at 1 × 10^6). *p < 0.05, **p < 0.01, ***p < 0.005.
perhaps influences transition to memory and prompted us to test Ag-free adjuvant for such a premise. Accordingly, when LN DO11.10 CD4 T effectors were transferred from BALB/c mice into Rag2−/− hosts that were given CFA, a high percentage of KJ1-26+CD4+ cells among total splenic cells was found after 4-mo parking (Fig. 4C). This percentage and the absolute number were significantly higher relative to Rag2−/− hosts that were not given CFA (4.7 ± 1.2% and 5.4 ± 1.7 × 10^5 cells for CFA versus 2.2 ± 0.16% and 2.1 ± 0.2 × 10^5 cells for no CFA). Similar results were obtained when the effector T cells were generated in Rag2−/− mice and transferred into new Rag2−/− hosts for parking and exposure to CFA (Supplemental Fig. 3). Overall, parking of effector T cells in CFA-containing environment increases survival of the cells and transition to memory.

To determine the specific type of effectors that respond to CFA adjuvanticity to generate effective memory, we sorted the early and late effectors and transferred them into Rag2−/− mice, and the hosts were given Ag-free CFA. Subsequently, the frequency and absolute number of memory cells and their ability to produce IFN-γ were evaluated. The results presented in Fig. 5 show that the percentage and absolute number of both early and late effector-derived memory splenic cells increased significantly when the hosts were given CFA. The IFN-γ response, which was minimal for D6–8-derived memory precursors, also increased for both types of effectors. Even the nonresponsive D3 and the LN-conditioned D2 divisions (10) had increased memory precursors and IFN-γ memory responses (Fig. 5A). More important, the otherwise short-lived unresponsive late effectors (D6–8) that yielded minimal long-lived cells relative to D4–5 effectors in the absence of CFA (0.5 ± 0.14% for D6–8 versus 1.2 ± 0.38% for D4–5) have become responsive when the mice were given CFA boost and developed long-lived memory cells like the D4–5 early effectors (2.02 ± 1.0% for D6–8 versus 3.7 ± 1.2% for D4–5) (Fig. 5B). Similar results were observed at the level of IFN-γ production as the amount of cytokine increased from 0.37 ± 0.14 ng/ml with no CFA to 5.1 ± 1.8 ng/ml with CFA for late effectors. Although this is half the amount observed with the early effectors, the index of IFN-γ increases when CFA is given was much higher for late effectors (ratio of CFA/no CFA 15.6 ± 9.5 for late effectors versus 5.4 ± 2.2 for early effectors). Overall, late effectors acquire the ability to develop long-lived cells that produce significant IFN-γ when the hosts are given CFA.

**Adjuvant effects on transition to memory involve TLRs, most notably TLR9**

CFA adjuvanticity most likely works through TLRs (30). The improved survival and IFN-γ production attributes may have resulted from TLR-mediated adjuvanticity. Indeed, when the CFSE+ LN effector T cells (D2–8) were parked in naive Rag2−/− mice that were given CFA, the high percentage of KJ1-26+CD4+ cells among total splenic cells was found after 4-mo parking (Fig. 4C). This percentage and the absolute number were significantly higher relative to Rag2−/− hosts that were not given CFA (4.7 ± 1.2% and 5.4 ± 1.7 × 10^5 cells for CFA versus 2.2 ± 0.16% and 2.1 ± 0.2 × 10^5 cells for no CFA). Similar results were obtained when the effector T cells were generated in Rag2−/− mice and transferred into new Rag2−/− hosts for parking and exposure to CFA (Supplemental Fig. 3). Overall, parking of effector T cells in CFA-containing environment increases survival of the cells and transition to memory.

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**Adjuvant effects on transition to memory involve TLRs, most notably TLR9**

CFA adjuvanticity most likely works through TLRs (30). The improved survival and IFN-γ production attributes may have resulted from TLR-mediated adjuvanticity. Indeed, when the CFSE+ LN effector T cells (D2–8) were parked in naive Rag2−/− hosts and exposed to different TLR ligands, the survival and IFN-γ responses increased with some, but not all TLR ligands tested (Fig. 6). Specifically, although the percentage of KJ1-26+ CD4+ T cells among total splenic cells was not significantly affected by any of the TLR ligands, the number of CD4 T cells increased significantly with TLR9 ligand (1 ± 0.1 × 10^5 cells for PBS versus 1.8 ± 0.4 × 10^5 cells for TLR9), and IFN-γ responses were significantly higher with TLR4 and TLR9 ligands (0.86 ± 0.2 ng/ml for PBS versus 2.4 ± 0.44 ng/ml for TLR4 and 2.7 ± 0.45 ng/ml for TLR9). Thus, the restoration of transition to memory by CFA is most likely due to TLR-mediated stimulation.

Because both TLR4 and TLR9 ligands increased IFN-γ memory responses of effectors, we sought to determine whether such an effect operates at the level of cell survival or amplification of IFN-γ secretion. To test this, D4–5 early and D6–8 late effectors were sorted and transferred into Rag2−/− mice, and the hosts were given TLR4 and TLR9 ligands. TLR2 ligand was included as a control because it had no significant effect on the percentage or IFN-γ response of memory precursors (Fig. 6). As indicated in Fig. 7A, whereas all of the TLR ligands increased the IFN-γ memory responses of D4–5 early effectors (0.9 ± 0.2 ng/ml for PBS versus 5.7 ± 1.6 ng/ml for TLR4 and 3.4 ± 0.8 ng/ml for TLR9), only TLR9 ligand augmented both the survival (0.3 ± 0.04% for PBS versus 0.7 ± 0.06% for TLR9) and IFN-γ memory responses of late effectors (0.18 ± 0.14 ng/ml for PBS versus 3.6 ± 1.2 ng/ml for TLR9). In addition, the TLR9 effects manifest in both IFN-γhigh and IFN-γlow late effectors (Fig. 7B, 7C). Indeed, TLR9 ligand increased the survival of IFN-γhigh late effectors leading to amplification of IFN-γ secretion (0.4 ± 0.06% and 2.3 ± 1.3 × 10^5 cells for PBS versus 0.7 ± 0.03% and 6.1 ± 0.7 × 10^5 cells for TLR9). However, TLR9 ligand did not increase the percentage or number of memory CD4 T cells, but augmented the frequency of IFN-γ-producing cells among IFN-γlow late effectors (Fig. 7B, 7C). It was previously shown that both T cells and APCs can express TLR9, and TLR9 ligand can improve the survival of activated T cells through MyD88 pathway in T cells (31). To determine whether TLR9 ligand exercises its effect directly on rested memory T cell precursors or through APCs, KJ1-26+ CD4+ CD3+ T cells were sorted from naive DO11.10 mice (naive), LN of OVA/pCFA-immunized BALB/c mice that include CFSE-labeled effector cells (effector), and from SP of Rag2−/− hosts that contain long-term parked memory DO11.10 T cells (memory). The sorted cells were then cultured for a short-term with or without TLR9 ligand to test its effect on the survival of T cells.
The results show that TLR9 ligand can exert its effect directly on the memory T cells because incubation of sorted T cells with TLR9 ligand significantly increased their survival relative to incubation without TLR9 ligand (1 ± 0.05 × 10^6 cells for medium alone versus 4.2 ± 0.7 × 10^6 cells for TLR9) (Fig. 7D). Supernatant from TLR9-stimulated APCs also had an effect on T cell survival. TLR9 ligand had no direct effect on naive or effector T cells (Fig. 7D) despite the fact that both memory and naive T cells express TLR9 (Fig. 8). Interestingly, effector T cells were unresponsive to TLR9 ligand under any culture condition. This could be explained by temporary downregulation of TLR9 on effector T cells (Fig. 8). Such downregulation was more prominent on late effector CD4 T cells (data not shown). Moreover, the results in Fig. 8 indicate that this temporary regulation of TLR gene expression is not unique to TLR9, but also observed with other receptors. These results suggest that memory precursors are programmed to respond to TLR ligands directly as well as through factors secreted by APCs to promote better survival and provide advantage over newly primed naive cells. In vivo exposure of D6–8 late effectors to CpG increases the frequency of IL-2–producing IFN-γ–positive memory T cells (Supplemental Fig. 4). Thus, it is possible that IL-2 increases resistance to apoptosis (32) by diminishing withdrawal of growth/survival factors and increasing antiapoptotic molecules such as bcl-xl, c-FLIP, and bcl-2, as was suggested previously (33).

Discussion
The study presented in this report puts forth plausible evidence as to the events controlling the transition of T cells from effector to memory. Indeed, much of our current understanding of memory development stands on two disparate observations, one of which claims that moderation in T cell activation favors transition to memory, whereas the second argues that development of memory ensues when the effector cells are undergoing heightened activation (8, 9). In this study, we provide evidence that both early and late effectors, which display moderate and robust activation, respectively, are endowed with potential for the generation of memory. However, the latter require sustained effector function to transit to memory. Indeed, D4–5 early effectors, which display a lower level of activation, readily generate memory as shown in other studies (34, 35) and bode well with the postulate that a threshold level of activation is required for transition to memory (11, 36). Similarly, the results concur with earlier findings suggesting that transition to memory is more prominent with low IFN-γ–producing effectors (8). However, differently from this model, data shown in this work imply that highly activated late effectors, regardless of their IFN-γ production, could not efficiently transit to memory unless a boost with Ag-free CFA was made. The underlying mechanism for this phenomenon is that the late effectors are susceptible to apoptosis, and CFA, through TLR activation, diminishes cell death and possibly increases cell division. In fact, we demonstrate that TLR9 is most prominent in this model for acquisition of survival and proliferation attributes by high IFN-γ producers as well as amplification of cytokine production among low IFN-γ–producing late effectors. Interestingly, this effect can occur either by direct interaction of TLR9 ligand with the T cells, as was shown previously (37, 38), or through induction of soluble factors by APCs. These observations support the findings indicating that high IFN-γ producers transit to memory (9) when transferred into infected mice as the T cells could be responding to TLR-mediated viral adjuvancy (9, 39). Timely administration of Ag-free adjuvant may be critical as the effector T cells may need to terminate Ag-dependent activation and restore a resting state in order for adjuvant to sustain transition to memory. Preliminary experiments indicated that adjuvant does not induce re-expression of IL-7R (data not shown), suggesting that other yet-to-be-defined pathways are involved in the contraction and maintenance of memory precursors. It is possible that TLR9 operates through the apoptotic/cell cycle pathways and involves genes such as Bmi-1, mcl-1, c-flip, Noxa, CD40L, ICOS, OX-40, PD-1, CD95, and other TNFR family members that were suggested to be taking part in maintenance of memory cells (32, 40–42). Essentially, the findings bode well with reports suggesting that transition to memory is intrinsically programmed early during priming of naive T cells (43), and that adjuvant assists effectors to reach their full potential for transition to memory. Finally, given that both TCR signaling strength and duration of contact between T cells and APCs control the level of expansion, differentiation, and survival of effectors (40, 44), the interplay between Ag-free adjuvant and these parameters remains to be defined.

Overall, the findings support a model in which both moderately activated early effectors and highly activated late effectors have potential to transit to memory. However, the highly activated late effectors, except perhaps the terminally differentiated cells (45), will require Ag-free adjuvant stimulation through TLRs to transit to memory. Whereas these observations resolve the discrepancies among high and low IFN-γ producers and moderate versus hyperactive effectors as progenitors for memory development, there is an added implication for the role of TLR ligands in sustaining better memory responses. It is thus logical to envision vaccination strategies incorporating Ag-free adjuvant boosts for the development of effective memory. In addition, different microbes may require different combinations of TLR ligands and TLR–independent pathways for the development of strong memory responses (46–51).

Disclosures
The authors have no financial conflicts of interest.

References


