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Transfer of CD8+ T Cell Memory Using Bcl-2 as a Marker

Alexis Dunkle,1 Ivan Dzhagalov,1 Claire Gordy, and You-Wen He

The processes that regulate T cell memory generation are important for therapeutic design and the immune response to disease. However, what allows a subset of effector T cells to survive the contraction period to become memory cells is incompletely understood. The Bcl-2 family is critical for T cell survival, and Bcl-2 has been proposed to be important for the survival of memory cells. However, previous studies have relied on double-knockout models, potentially skewing the role of Bcl-2, and the use of Bcl-2 as a marker in adoptive transfer experiments, a method required to confirm the memory potential of cell subsets, has not been possible because of the intracellular localization of the protein. In this study, we present a novel Bcl-2 reporter mouse model and, to our knowledge, show for the first time that a distinct subset of effector T cells, and also a subset within the CD127hiKLRG1lo memory precursor effector cell population, retains high Bcl-2 expression at the peak of the CD8+ T cell response to Listeria monocytogenes. Furthermore, we show that Bcl-2 correlates with memory potential in adoptive transfer experiments using both total responding CD8+ T cells and memory precursor effector cells. These results show that even within the memory precursor effector cell population, Bcl-2 confers a survival advantage in a subset of effector CD8+ T cells that allows differentiation into memory cells and cement Bcl-2 as a critical factor for T cell memory.

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emory T cell induction is critical for efficient responses to disease and for therapeutic efforts such as vaccination. During an acute response, Ag-specific T cells undergo expansion and contraction phases that ultimately lead to the formation of a stable memory population. However, what allows the survival of the memory cells while the other ~90% of effector T cells die is still unclear. A number of factors, including signal strength, inflammation, and cytokines, have been proposed to contribute to the formation of T cell memory (reviewed in Ref. 1). Similarly, a number of surface molecules have been proposed to have functional and/or identifying roles in the cells fated to become memory.

Expression of the IL-7Rα subunit (CD127) has been shown to mark effector cells fated to become memory cells, which is consistent with the evidence that IL-7 is important for memory cell survival (2, 3). However, some data have cast doubt on the true importance of CD127 and IL-7 signaling in memory formation because cells in an IL-7−/− environment exhibit relatively normal CD127 modulation and establishment of memory (4). Other studies showed that CD127 expression alone is not sufficient for the establishment of T cell memory, calling CD127 expression a “permissive” rather than an “instructive” feature (5, 6). CD127 has been used with another surface marker, killer cell lectin-like receptor G1 (KLRG1), to distinguish memory precursor effector cells (MPECs) from short-lived effector cells (SLECs) at the peak of the immune response (7). The factors that promote MPEC survival are still unclear, but MPECs have been shown to express higher levels of Bcl-2 (7, 8).

Because of the important roles of the antiapoptotic proteins of the Bcl-2 family in T cell survival, these proteins are strong candidates for regulating T cell memory. Both Bcl-2 and Mcl-1 are expressed in memory T lymphocytes, whereas Bcl-xL is upregulated in effector cells (9–13). Using a conditional knockout model, Bcl-xL was shown to be dispensable for T cell memory (14). The roles of Mcl-1 and Bcl-2 in memory formation have been more difficult to assess using knockout models because of the importance of these proteins in thymocyte and naive T cell survival (11, 12, 15–18). An examination of Bim−/− Bcl-2−/− cells (which partially escape Bcl-2−/−−induced death in the thymus and periphery due to the loss of one allele of Bim) showed that Bcl-2 was not required for memory to lymphocytic choriomeningitis virus (LCMV) infection (19), but the decreased Bim and/or the lymphopenic environment may have affected the requirement for Bcl-2 in this system. A follow-up study concluded that the Bim/Bcl-2 balance is indeed important for the survival of effector cells to become memory (8). While making great strides toward confirming a role for Bcl-2 in T cell memory, it is important to note that these studies largely relied on a system in which Bim expression was altered either through genetic manipulation or by pharmacologic agents, and previous studies have suggested that alterations in Bim, including loss of a single allele, can shift the balance between Bcl-2 and Mcl-1 in terms of their importance in T cell survival (20). Because of this interplay between many of the Bcl-2 family members, alternate approaches are needed to assess the in vivo role of Bcl-2 under endogenous gene expression conditions.

Although previous studies have implicated a role for Bcl-2 in T cell memory, it has been difficult to directly assess its importance because of the lack of appropriate conditional knockout models. Furthermore, because Bcl-2 is an intracellular protein, it cannot be used to define populations for the live-cell sorting and subsequent functional studies that are essential for determining the memory potential of effector T cells. In this study, we introduce a Bcl-2 reporter mouse that allows not only the analysis of Bcl-2 expression in effector T cells in the context of infection but also the sorting of these cells to determine the memory potential of Bcl-2–
expressing cells. Using this model, we identify effector CD8+ T cells that express relatively high levels of Bcl-2 both within the total responding population and, notably, within the MPEC subset. Furthermore, we show that Bcl-2 expression correlates with the establishment of memory to the bacterial pathogen Listeria monocytogenes in both the total effector CD8+ T cell pool and within the MPEC population. These studies confirm the important role of the prosurvival protein Bcl-2 in the formation of T cell memory and provide a useful model for future studies on Bcl-2 and T cell memory, including studies on vaccination and the response to disease.

Materials and Methods

Generation of Bcl-2YFP and OT1YFP mice

The modifications of the bacterial artificial chromosome (BAC) were performed as described by Sparwasser et al. (21) with minor modifications. The original shuttle vector, pLD53.RecA, was a gift from Dr. G. Eberl (Institut Pasteur, Paris, France), and the original insert was removed by Ncol and Ascl digestion and replaced with the recombinase cassettes. The cassettes were generated by overlap PCR using Pfu Ultra High-Fidelity Polymerase (Stratagene, La Jolla, CA). The annealing temperatures were usually 60°C.

The Bcl-2 yellow fluorescent protein (YFP) recombination cassette was designed to insert the cDNA sequence of a membrane-targeted version of YFP at the translational start of Bcl-2, deleting the first 72 nt of exon 1 of Bcl-2. The two ~1-kb-long flanking fragments (box A and box B) were amplified from BAC RP23-405G16 with the primers 5' TTG GCG CCC CTC TCG GAG TTA ATG CAG-3' and 5' GCT CGT AGT GCG ATG ATG AAC CTG AAT CGC CAG-3'. The membrane-targeted version of YFP was amplified from the pLD53.RecA vector using the primers 5'-GCA GCT TCT TTT CGG GGA AGG ATG CTG TGT ATG AGA AGA ACC-3' and 5'-CAT CAG CAT CCG CCA AGC TGA TTC AGT CGC-3'. The three PCR products were gel purified and used as templates in an overlap PCR to generate the Bcl-2 YFP recombination cassette, which was ligated into pLD53.RecA as described above to obtain the shuttle vector pBcl-2 YFP. The vector content was confirmed by sequencing.

BAC DNA was linearized with Ncol, and the band of the correct size was excised from the gel and electroeluted. The DNA was diluted to 2 ng/μl with microinjection buffer and was injected into the pronucleus of FVB/N fertilized eggs. The progeny were screened for the successful integration of the BAC by PCR. To detect YFP, the primers for screening integration were 5'-CGT TTC GTA GAA CGG GTT G-3' and 5'-CGG TGG TGG AGA TGA ACT TC-3'. The primers for detection of the BAC ends were as follows: 5'-BAC end, 5'-GCT CTG GAG TGA ATC ATA CCA CGA CAG-3', 5'-GGC ATG ATG AAC CTG AAT GCC CAG-3', 5'-BAC end, 5'-GGC TCA CCC ACT AGT CAA TTC GGC 3'-5'-GAA GCC TGC ATG TCC GCC TCC TCC-3'. The Bcl-2YFP mice were crossed onto the C57BL/6 background or the C57BL/6 variant expressing the congeneric marker CD45.1 for at least 5 generations before analysis experiments and for at least 15 generations for transfer experiments. To generate OT1YFP mice, Bcl-2YFP mice were bred to OT1 mice, and the progeny were screened for both YFP and the OT1 TCR Vα2 by flow cytometry. C57BL/6, CD45.1, and OT1 mice were all obtained from The Jackson Laboratory (Bar Harbor, ME).

Flow cytometry

Organs (thymus and spleen) were removed and reduced to a single-cell suspension in FACS buffer (2% FBS in PBS). Fls were blocked by incubating with 25–50% 24G2 hybridoma supernatant in FACS buffer for 10 min on ice. Surface molecules were stained by adding 0.25–1 μl fluorescein isothiocyanate-conjugated Abs per 106 cells in a 100- to 200-μl volume of FACS buffer. Cells were stained on ice for at least 15 min then washed and resuspended in FACS buffer with or without propidium iodide (Sigma-Aldrich) at 10 μg/ml. Flow cytometry was performed on a FACSscan or a FACSQuant cytomter (BD Biosciences, San Jose, CA), and the results were analyzed using FlowJo software (TreeStar, Ashland, OR). All Abs to surface molecules (CD4, CD8, B220, Mac-1, Gr-1, CD25, CD44, CD127, KLRG1, and Vε2) were from eBioscience or BioLegend (both San Diego, CA). Although YFP could be detected in both the FITC and the PE channel, unsaturated YFP staining used to obtain the compensation between these channels such that the signal could be detected in only one channel.

For intracellular staining of Bcl-2 and Ki67, the single-cell suspensions that had been stained for surface markers were washed with FACS buffer and resuspended in 100 μl FACS buffer. The cells were fixed with 100 μl 4% paraformaldehyde (EMD Biosciences, San Diego, CA) in PBS for 20 min at 4°C, permeabilized with 250 μl 0.1% saponin (Sigma-Aldrich) in FACS buffer for 20 min at 4°C, and incubated for 1 h in ice with an anti-Bcl-2–PE Ab (BD Biosciences) at 1:5 dilution or an anti–Ki67–eFluor 450 Ab (eBioscience) at 1:40 dilution in 100 μl 0.1% saponin in FACS buffer. The cells were washed with 0.1% saponin in FACS buffer and were resuspended in 250 μl 0.1% saponin in FACS buffer for data acquisition.

Infection of Bcl-2YFP mice with L. monocytogenes–OVA

For the initial analysis of Bcl-2 expression, Bcl-2YFP mice were infected directly. For the sorting experiments, 50,000–100,000 OT1 YFP cells were transferred by i.v. injection into congeneically marked recipients 1 d before infection. Briefly, spleens from OT1YFP mice were removed and made into a single-cell suspension. RBCs were lysed by incubation in ACK buffer for 1–2 min at room temperature. Cells were resuspended in 2% PBS in FCS for CD8+ T cell enrichment prior to sorting. CD8+ T cells were enriched using the EasySep CD8+ T Cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada) following the manufacturer’s instructions. Cells were resuspended in PBS for injection (200 μl/mouse).

Recombinant L. monocytogenes expressing chicken OVA (LM-OVA) was a gift from Dr. M. Bevan (University of Washington, Seattle, WA). Frozen stocks of the LM-OVA were grown in brain–heart infusion broth (Difco) supplemented with 5 μg/ml erythromycin (Sigma-Aldrich). Bacterial culture samples were grown to midlog phase as measured by OD600, aliquoted, and frozen at −80°C. Doses were confirmed by spreading bacterial samples on brain–heart infusion agar (Difco) plates. Immediately prior to infection, the bacteria were thawed, rinsed, and diluted in PBS for i.v. injection (100–200 μl/mouse). For the primary infections and the recall infections of recipient mice from transfer experiments, a dose of 106 CFU/mouse was used. For the recall infections in Bcl-2YFP mice, a dose of 107 CFU/mouse was used.

Analysis of effector CD8+ T cells

Seven or 8 d postinfection, spleens were removed and prepared to a single-cell suspension in 2% FBS in PBS as above. Cells were incubated with 24G2 hybridoma supernatant for 10 min on ice to block Fls with free mouse IgG1 (BD Biosciences) for 10 min. Then, a prepared mixture of OVA peptide (American Peptide Company, Sunnyvale, CA) bound to DimeroX (an H-2Kb-IgG1 fusion protein; BD Biosciences; bound to OVA overnight in PBS at 37°C) with secondary Ab (PE-anti-mouse IgG1; BD Biosciences) was added to the cells along with Abs to surface molecules. Staining was allowed to proceed for 30–90 min, and cells were analyzed on a FACSCanto cytometer (BD Biosciences).

Sorting and adoptive transfer of OT1YFP CD8+ T cell populations

Day 7 or 8 splenocytes were prepared and enriched for CD8+ T cells as described above. Typically, three to four mice were pooled for each experiment. Enriched CD8+ T cells were stained with an anti–KLRG1 (all from BioLegend) for at least 20 min. Cells were sorted on a FACSDiva sorter (BD Biosciences). After sorting, cells were washed once with PBS and resuspended in PBS for i.v. injection into the tail vein of naive, congeneically marked recipient mice. Approximately 10,000 cells from each population were transferred (see figure legends for the cell number transferred for each experiment). In some experiments, 5 × 106 unsorted, enriched CD8+ cells were transferred as a positive control.

Memory analysis of recipient mice

After transfer of OT1YFP cells, at least 5 wk were allowed to pass before secondary challenge of the recipient mice. A secondary LM-OVA infection and T cell analysis were performed as described above with the exception that the analysis was performed on day 4 or 5. Splenic cellularity was determined by staining enriched CD8+ T cells from each group with an anti–congenic marker CD45.1/CD45.2, the OT1 TCR subunit Vα2, CD127, and KLRG1 (all from BioLegend) for at least 20 min. Cells were sorted on a FACSDiva sorter (BD Biosciences). After sorting, cells were washed once with PBS and resuspended in PBS for i.v. injection into the tail vein of naive, congeneically marked recipient mice. Approximately 10,000 cells from each population were transferred (see figure legends for the cell number transferred for each experiment). In some experiments, 5 × 106 unsorted, enriched CD8+ cells were transferred as a positive control.
percentages observed during sorting and was used as the denominator to calculate recovery/expansion.

**Short-term analysis of recipient mice**

Splenocytes from 8 to 12 donor mice were pooled on day 7 of infection, and enriched CD8+ cells were sorted into populations V-VIII as described previously. A total of 25,000–50,000 cells (4- and 7-d experiments) or 100,000 cells (5-wk experiments) were transferred by i.v. injection (200 µl in PBS). Because of the rarity of the sorted populations and the unavoidable loss of some volume of the cell preparation, only one to four mice could be included per group per experiment. The data shown for 4- and 7-d experiments are the combination of three separate experiments. The number of donor cells recovered and the percent recovery were calculated as described above, and YFP fluorescence was detected by flow cytometry.

**Statistical analyses**

The data were analyzed using Prism software versions 5 and 6 (GraphPad Software, La Jolla, CA). The significance of the data was assessed using an ANOVA analysis.
unpaired Student t test or, for comparisons across multiple groups, a one-way ANOVA with Tukey’s multiple comparison test. p < 0.05 was considered to indicate statistical significance. In expression level assays in which the different groups from within an individual mouse were compared, a paired analysis was used.

**Results**

**Bcl-2<sup>YFP</sup> reporter mouse**

To more closely examine the regulation and importance of Bcl-2 during an immune response, we generated a reporter mouse in which the gene encoding YFP was inserted into the translation initiation site of the Bcl-2 locus on a BAC transgene (Bcl-2<sup>YFP</sup> mice; Fig. 1A). The BAC including the Bcl-2 gene spans ~250 kb due to the presence of a large intron between exons 2 and 3. To confirm that the complete Bcl-2<sup>YFP</sup> BAC was inserted (and thus any potential gene control regions located on the BAC), we used PCR to detect both the Bcl-2<sup>YFP</sup> fusion gene and both ends of the endogenous Bcl-2 (Fig. 1C). Furthermore, YFP expression mirrored known patterns of Bcl-2 expression in thymocyte populations (Fig. 1D) and in T and B cells and granulocytes in the spleen (Fig. 1E). For example, Bcl-2 is expressed in most of the CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells but not in the Mac1<sup>+</sup>Gr-1<sup>+</sup> granulocytes.

**Analysis of Bcl-2 (YFP) expression in effector and memory CD8<sup>+</sup> T cells responding to LM-OVA**

Using YFP as a marker, we confirmed that both naive and memory CD8<sup>+</sup> T cells expressed high levels of Bcl-2 (Fig. 2A). To examine the regulation of Bcl-2 during an immune response to infection, we used an infection model in which Bcl-2<sup>YFP</sup> mice were infected with a sublethal dose of LM-OVA, which induces a dominant CD8<sup>+</sup> T cell response to the peptide OVA<sub>257–264</sub> presented by MHC class I H-2K<sup>b</sup>. Consistent with previously reported patterns of Bcl-2 expression (9, 10), most activated (CD44<sup>hi</sup>) OVA-specific CD8<sup>+</sup> T cells downregulated YFP compared with naive cells 7 d postinfection (Fig. 2B). However, a small population (typically ~15–20%) of effector cells expressed high levels of Bcl-2 at this time point (Fig. 2B). A similar trend was observed in memory CD8<sup>+</sup> T cells responding to a recall challenge (Fig. 2C). Whereas previous studies examined gene expression on a population level, the YFP reporter system identified this previously unrecognized subpopulation of high Bcl-2–expressing effector cells, which we hypothesized were the cells that ultimately survive to establish CD8<sup>+</sup> T cell memory.

**Effector CD8<sup>+</sup> T cells with higher Bcl-2 have greater memory potential**

To determine whether there is a difference in the potential of Bcl-2–expressing populations to become memory cells, we sorted effector T cells based on Bcl-2 expression and transferred these cells to naive animals. We avoided using K<sup>b</sup>-OVA binding to identify OVA-specific cells because K<sup>b</sup>-OVA binding to the TCR could have biological effects in responding T cells. Therefore, we crossed Bcl-2<sup>YFP</sup> mice onto a congenically marked (CD45.1<sup>+</sup>/CD45.2<sup>+</sup>) OT<sup>1</sup> TCR-transgenic background (OT1 identified as CD45.1<sup>+</sup>V<sup>A<sub>2</sub></sup>+) cells. Then, we gated four populations based on Bcl-2 expression (populations I–IV), each consisting of ~15–20% of the total and together representing the full range of Bcl-2 expression (Fig. 3A). Consistent with previous observations that the MPEC population expresses higher Bcl-2 than SLECs (7, 8), the Bcl-2<sup>hi</sup> population (I) was most enriched for MPECs, and the percentage of MPEC cells decreased with Bcl-2 levels with the exception of the Bcl-2<sup>lo</sup> cells (IV) (Fig. 3B). A postsort analysis (data not shown) indicated that population IV contained a higher percentage of contaminating host cells, which would appear in the MPEC gate due to the similarities of the MPEC markers with resting T cell markers, than the other three populations.

After at least 5 wk, mice transferred with populations I–IV were challenged with LM-OVA to assess the establishment of memory. A flow cytometry analysis revealed that as the Bcl-2 level of the donor cells decreased so did the memory potential (Fig. 3C). Bcl-2<sup>hi</sup> cells (population I) consistently yielded the highest number of memory cells, whereas Bcl-2<sup>lo</sup> cells (population IV) did not yield a notable response (Fig. 3C). The effect of Bcl-2 on memory outcome was statistically significant as assessed by ANOVA (p = 0.0143; Fig. 3C). In one experiment (shown in Fig. 3C), there was a trend toward a graded decrease in memory potential with...
decreasing Bcl-2, and in a second experiment, we only detected
donor cells in the mice that received population I (Bcl-2hi) (data
not shown). These results indicate that the highest Bcl-2 ex-
pressers have the capacity to efficiently become memory cells.

Memory potential correlates with Bcl-2 expression in MPECs
Although the data above indicated that higher levels of Bcl-2
correspond to increased memory potential, it remained possible
that the differences between populations I–IV in establishing
memory were due to the differences in MPEC percentages be-
tween these populations, not Bcl-2 expression per se. Therefore, in
a set of preliminary experiments, we separated effector OT1YFP
cells into SLECs, which were uniformly Bcl-2lo, and Bcl-2hi and
Bcl-2lo MPECs using different gating strategies (Supplemental
Fig. 1A). Five to 10 million unsorted CD8+ T cells were trans-
ferred into separate mice as a positive control, and we could detect
an expansion of the memory population (as measured by the
calculated number of OT1YFP cells recovered/the number trans-
ferred) in the mice that received unsorted CD8+ T cells (Supple-
mental Fig. 1B). Consistent with the results of studies in CD8+
T cells using lymphocytic choriomeningitis virus as a pathogen
(7), we did not detect an appreciable expansion of donor cells
upon secondary challenge in mice transferred with the SLEC
population (Supplemental Fig. 1B). Within the MPEC population,
different gating strategies yielded different results. When we
assigned the Bcl-2hi and Bcl-2lo gates based on what appeared to
be a distinct break in YFP expression (top 15% versus bottom
69%), the Bcl-2lo population was better at conferring memory
(Supplemental Fig. 1B, left panel). However, when we defined
these populations to include the highest tertile and the lowest
tertile, the high population appeared to be better (Supplemental
Fig. 1B, right panel). An intermediate gating strategy yielded
intermediate results (Supplemental Fig. 1B, center panel). These
results suggest that Bcl-2int-hi MPECs, specifically those repre-
senting the top 20–50% of MPECs, may have the best memory
potential.

To further define the relation between Bcl-2 expression levels
and memory potential within the MPEC population, we divided
MPECs into equal-sized populations based on Bcl-2 expression and
compared the memory potential between high and low expressors
(Fig. 4A). A postsort analysis confirmed that all of the sorted
MPEC populations were 96% pure for OT1YFP cells and dem-

FIGURE 3. Memory potential of effector T cell populations sorted based on YFP (Bcl-2) expression. (A) Sorting of effector T cells. CD8-enriched spleno-
cytes gated on effector (CD44hi) donor (CD45.1+Vα2−) cells were sorted into populations I–IV based on YFP expression. Numbers represent the percentage of the
parent population within the gate. (B) Percentage of MPECs in the sorted populations (numbers indicate percent of parent). (C) Number of donor cells detected
in secondary recipients after recall challenge. The number of donor (CD8+CD44hiCD45.1+Vα2+) cells was calculated by multiplying the frequency by the
total number of splenocytes (only live cells included). The mean + SD for each group is shown. Data are representative of two independent experiments (four
mice per group each). Significance was assessed using an ANOVA (*p = 0.0143) with Tukey’s multiple comparisons test (*p < 0.05).
cells, to obtain a “snapshot” of the level of proliferation ongoing in each population. Interestingly, the percent of cells that was Ki67+ was inversely correlated to YFP expression, indicating that the cells with the highest levels of Bcl-2 had the lowest level of proliferation at this timepoint (Fig. 4B). These data are consistent with numerous reports that Bcl-2 has a negative effect on cell cycle progression (22–25) and indicate that any advantage we observe for Bcl-2high cells in the memory response is likely due to survival, not enhanced proliferation.

Upon recall challenge and analysis of the mice that were transferred with these populations, we observed that MPECs with higher Bcl-2 had significantly greater memory potential than Bcl-2low MPECs (Fig. 4C). Because our earlier results suggested that the highest Bcl-2 expressors of the MPEC population may be less fit to become memory, we selected Bcl-2int-high (population VI) cells to represent Bcl-2 “high” cells for a comparison with Bcl-2low cells (population VIII). Interestingly, the trend that the Bcl-2 highest MPECs are somewhat less fit to become memory was observed in two of three experiments in which we compared all four populations V–VIII (Supplemental Fig. 3). Taken together, these results indicate that Bcl-2 can be used as a functional marker of memory potential, even within the MPEC population.

**Engraftment and YFP expression of sorted cells immediately following transfer and before recall**

To confirm that all sorted populations responded equally to the ex vivo procedures and that the generation of memory was not due to any differences in short-term survival following sorting/transfer, we transferred greater numbers of cells from populations V–VIII (as shown in Fig. 4) and assessed engraftment 4 and 7 d as well as 5 wk after transfer. Although the cell numbers were quite small, we could detect a small population of Vα2+ (OT1 TCR) CD45.2+ (or CD45.1+) cells in the spleen at all time points (Fig. 4A). The number of cells recovered and the percent recovery (number of cells normalized to number transferred) were indistinguishable between populations at days 4 and 7 (Fig. 5B, 5C). The number of cells remaining 5 wk after transfer matched the trend observed in the memory response, although the differences were not statistically significant, likely because of the small number of cells detected (Fig. 5B, 5C, right panel). These data indicate that differences in long-term, but not short-term, survival of the different populations are responsible for the differences in memory responses. Strikingly, relative YFP fluorescence between the groups did not change over the week following injection (Fig. 5D), indicating that the populations as defined by Bcl-2 expression are stable over this time period. However, at the later time point, the YFP expression within each group was more variable and some groups were indistinguishable, indicating that those cells that did survive likely altered Bcl-2 expression to return some baseline over time.

**Discussion**

We have shown that the dynamic regulation of Bcl-2 during the effector phase of the immune response is important for establishing T cell memory. Using the OT1YFP LM-OVA infection system, we found that a subset of cells retains high Bcl-2 expression at the
peak of the immune response, an observation that was not possible in previous studies using gene expression techniques, and that this population contains a high percentage of the CD127hiKLRG12 MPEC population. Using adoptive transfer, we confirmed the observations of other groups that the CD127hiKLRG12 population is indeed enriched for memory precursors. Our results indicate that high Bcl-2 expression can be used as an alternative marker to CD127/KLRG1 to mark effector CD8+ T cells with high memory potential. Although MPECs have been shown to express higher levels of Bcl-2 than SLECs, using the reporter mouse, we identified a gradient in Bcl-2 expression even within the MPEC population and showed that Bcl-2 can be used as a marker of memory potential within the MPEC population. The identification of the subset of MPECs with the highest memory potential is important because it will allow a more refined comparison of the phenotypic and functional characteristics of memory precursor cells in the future to identify subtle changes that promote memory development.

Intriguingly, based on the different results obtained with different gating strategies (Supplemental Figs. 1, 2), it appears that the MPECs that express intermediate-high, but not the highest, levels of Bcl-2 may be the best at establishing memory. In two of three experiments comparing populations V-VIII, it was not the MPEC-Bcl-2hi population (V), but the MPEC-Bcl-2med-hi population (VI), that yielded the highest recovery of memory cells, whereas MPEC-Bcl-2low cells were consistently poor memory cells (Supplemental Fig. 2). This was consistent with the results of the preliminary experiment in which we tested different MPEC gates (Supplemental Fig. 1) and with the prerecall numbers of transferred cells (Fig. 5). Although initially these results seem to contrast the results of the experiments in which effector T cells

FIGURE 5. Short-term analysis of transferred OT1YFP populations. OT1YFP cells from 8 to 12 donor mice infected with LM-OVA were pooled on day 7 and sorted into populations V–VIII as shown in Fig. 4. The splenocytes of recipient mice were examined 4 and 7 d as well as 5 wk after transfer. (A) Representative flow cytometry plots for the identification of transferred cells. (B) Total number of recovered donor cells per spleen. The percentage of donor cells (CD8+CD44hiCD45.1+Vα2+) was multiplied by the total number of splenocytes, and the mean + SD is shown. For transfers of 4 and 7 d, three experiments of n = 1–2 mice/group were combined; for 5 wk, a single experiment (n = 4) is shown. The only significant differences observed between groups were with the mice that did not receive cells (“no cells”, ANOVA). (C) Percent recovery of donor cells. Because the number of cells transferred slightly varied between experiments, the number of cells recovered was normalized to the number transferred, and the mean + SD is shown. (D) YFP fluorescence. The YFP MFI of donor cells was determined for each recipient mouse. The mean + SD is shown.
(not gated on MPECs) were transferred (Fig. 3), we must consider the fact that the higher Bcl-2 population (population I) in these experiments was more enriched for MPECs. The population I gate likely encompasses a relatively large percentage of the MPEC curve (corresponding to population V and population VI), and therefore, the gating strategy used for total effector cells does not distinguish between MPEC-Bcl-2\textsuperscript{hi} and MPEC-Bcl-2\textsuperscript{med-hi} cells.

The trend that MPEC-Bcl-2\textsuperscript{hi} cells may be less fit is an interesting observation that should be addressed in future studies. It is likely that factors other than Bcl-2 are required for memory formation, and the highest Bcl-2–expressing cells may be less fit when other factors are considered. One potential explanation for this phenomenon is the differences in proliferation between the different populations. Consistent with a negative role for Bcl-2 in proliferation, expressing high Bcl-2, while allowing some proliferation, we showed that proliferation in MPECs was inversely correlated with Bcl-2 expression. Intuitively, it is rational that those cells programmed for long-term survival are less proliferative at the effector phase of the response. However, it is possible that the best cells strike a balance between survival and proliferation, expressing high Bcl-2, while allowing some proliferation. Our system can be used to identify other factors that may contribute to memory by comparing MPEC-Bcl-2\textsuperscript{hi} and MPEC-Bcl-2\textsuperscript{med-hi} cells.

Taked together, our data show that relatively high levels of Bcl-2 lead to memory in CD8\textsuperscript{T} cells. The system presented in this study will provide a useful model to examine factors that influence T cell memory. As discussed above, comparing the populations defined in this study could identify cell-intrinsic factors important for memory. Furthermore, using this system, we can identify cell-extrinsic factors that are important for memory by altering the pretransfer or recipient environment. This model not only allows an assessment of memory formation but also offers a readout of pretransfer or recipient environment. This model not only allows extrinsic factors that are important for memory by altering the for memory. Furthermore, using this system, we can identify cell-extrinsic factors that are important for memory by altering the pretransfer or recipient environment. This model not only allows an assessment of memory formation but also offers a readout of pretransfer or recipient environment.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

Supplemental Figures

**Figure S1: Analysis of memory potential of SLEC and MPEC populations using OT1YFP mice.** A. Sample gating for sorting of SLEC and MPEC populations with Bcl-2. Primary recipients (CD45.1+) received CD45.2+OT1YFP cells one day before infection with 10^4 CFU LM-OVA. After 7-8 days, cells were sorted by gating CD44^hiOT1YFP cells (OT1 YFP cells identified by CD45.2+ with or without the addition of the OT1 TCR marker, Vα2+). SLEC and MPEC populations were gated based on CD127 and KLRG1, and the MPEC population was further sorted based on Bcl-2 expression. The numbers on the FACS plots indicate the percentage of the parent population within each gate. B. Five days after secondary challenge, the spleens of recipient mice were analyzed, and the total number of OT1 YFP cells recovered per spleen was calculated based on the percentage of CD45.2+Vα2+ cells detected. This was normalized to the number of cells transferred to assess the fold expansion of the cells at the memory time-point. For the unsorted cells, the number of OT1 cells transferred was calculated based on the percentage of the total CD8+ population. The numbers above the MPEC columns indicate the percentage of the MPEC population that was sorted (e.g., 15|69 indicates that the top 15% was included in the MPEC^hi gate and the bottom 69% was included in MPEC^lo gate as shown in A).
Figure S2: Post-sort analysis of MPEC populations. Post-sort analysis of populations V-VIII sorted as shown in Figure 4. Purity (CD45.1⁺) is shown as a percentage (top row). For YFP and CD127 (middle, bottom), the number is the mean fluorescence intensity (MFI) of the population.
Figure S3: Memory potential of all four MPEC populations. Cells were gated and transferred as described in Fig. 4. Five days after secondary challenge, the spleen was analyzed by flow cytometry to detect donor cells in mice that received all four MPEC populations. The number of OT1 YFP cells per spleen was calculated, and the mean ± SD of the mice within each group (four mice per group) is shown. Significance was determined by ANOVA (right, p = 0.1146; center, **p = 0.0072; left, **p = 0.0029) followed by Tukey’s multiple comparisons test (*p<0.05, **p<0.01).