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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 CD127\(^{hi}\)KLRG1\(^{lo}\) 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. *The Journal of Immunology*, 2013, 190: 000–000.
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 Bcl-2+ 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-2**<sup>YFP</sup> and OT1**<sup>YFP</sup> 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 NotI and Ascl digestion and replaced with the recombination 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 GCC CGC CGC CCT TCG GAG TTT AAT CAG-3'/5'-CAT CCT TCC CGG CAA AGA AGC TTC-3' and 5'-GCT ACG AGT GGG ATG CTG GAG ATG-3'/5'-ATG CGG CCG CCA AGA TGA TTA GCT ATG AAT TCC AGG-3'. The membrane-targeted version of YFP was amplified from the pLD53.ReCa vector using the primers 5'-CGT TTC GGA AAG CGC GTT GG-3' and 5'-CAT CTC CAG CAT CCG ACTG-3'/5'-AGC TTT AGC TTA CTT GGA ATG-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 the modified bacterial artificial chromosome (BAC) DNA was linearized with NruI, and the band of the correct size was excised from the gel and electroeluted. The DNA was diluted to 2 μg/ml with microinjection buffer and was injected into the pronucleus of FVB/N fertilized eggs. The progeny was screened by PCR for the successful integration of the BAC by PCR. To detect YFP, the primers for screening were 5'-CGT TTC GGA AGC CGC GTT GG-3' and 5'-CGG TGG TGC AGA TGA ACT TCC-3'. The primers for detection of the BAC ends were as follows: 5'-BAC end, 5'-GCT CTC GAG TGA ATA CCA CGA CGA-3'/5'-GCG ATG ATT AAC CTG AAT CGC CAG-3'; 5'-BAC end, 5'-GGC CTA CCC ACT AGT CAA TGG GGC 3'/5'-GAA GGC TTC ATG CCG GCC TCC TTC TCC-3'. The Bc-2<sup>YFP</sup> mice were crossed onto the C57BL/6 background and were designated by guest on July 28, 2017 http://www.jimmunol.org/ Downloaded 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). FcRs 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 fluoro-rescently labeled Ab per 10<sup>6</sup> 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 2% paraformaldehyde (Sigma-Aldrich) at 2 μg/ml. Flow cytometry was performed on a FACScan or a FACSCanto cytometer (BD Biosciences, San Jose, CA), and the results were analyzed using FlowJo software (Tree Star, Ashland, OR). All Abs to surface molecules (CD4, CD8, B220, Mac-1, Gr-1, CD44, CD45, KLRC1, and Vo2) were from eBioscience or BioLegend (both San Diego, CA). Although YFP could be detected in both the FITC and the PE channel, unstained YFP was used to assess 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 μm 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-2**<sup>YFP</sup> mice with *L. monocytogenes*–OVA

For the initial analysis of Bcl-2 expression, Bcl-2<sup>YFP</sup> mice were infected directly. For the sorting experiments, 50,000–100,000 OT1<sup>YFP</sup> cells were transferred by i.p. injection into congenically marked recipients 1 d before infection. Briefly, spleens from OT1<sup>YFP</sup> 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 PBS 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 supplemented with 5 μg/ml erythromycin (Sigma-Aldrich). Bacterial culture samples were grown to midlog phase as measured by OD<sub>600</sub>-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 responses of recipient mice from transfer experiments, a dose of 10<sup>6</sup> CFU/mouse was used. For the recall infections in Bcl-2<sup>YFP</sup> mice, a dose of 10<sup>4</sup> 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 FcRs with free mouse IgG1 (BD Biosciences) for 10 min. Then, a prepared mixture of OVA peptides (American Peptide Company, Sunnyvale, CA) bound to DimerX (an H-2<sup>K</sup>-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 OT1**<sup>YFP</sup>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 the T cell–specific congenic marker CD45.1/CD45.2, the OT1 TCR subunit Vo2, CD127, and KLRC1 (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, congenically 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 × 10<sup>4</sup> unsorted, enriched CD8+ cells were transferred as a positive control.

**Memory analysis of recipient mice**

After transfer of OT1<sup>YFP</sup> 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 counting cells using a Countess cell counter (Invitrogen). For the flow cytometric analysis, 50,000 total events were collected to assess the percentage of CD8+ cells in the spleen (only live cells were included based on propidium iodide exclusion). Then, a high number of live CD8+ cells was collected to determine the frequency of the relatively rare donor cells. The total number of OT1<sup>YFP</sup> cells per spleen was calculated based on the observed frequencies and the total cell number. The percentage recovery or fold expansion of the donor cells was calculated by dividing the calculated number of OT1<sup>YFP</sup> cells in the spleen by the number of cells transferred. For mice that received unsorted CD8+ cells, the number of OT1<sup>YFP</sup> cells transferred was determined based on the
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

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**FIGURE 1.** Bcl-2<sup>YFP</sup> reporter mouse. (A) Schematic of BACs containing Bcl-2 and the YFP insert. (B) PCR of BAC or founder mice tail DNA to detect the Bcl-2<sup>YFP</sup> allele and the 3' end of the BAC. The founder with the highest level of Bcl-2 expression established the Bcl-2<sup>YFP</sup> line. (C) Bcl-2 versus YFP fluorescence in the thymus. (D) YFP expression in thymocytes stained with CD4/CD8 to distinguish double-negative (DN), double-positive (DP), and single-positive (SP) populations and CD44/CD25 to separate DN subsets. YFP fluorescence in each gated population is shown. (E) YFP expression in CD4<sup>+</sup>, CD8<sup>+</sup>, B220<sup>+</sup> (B cells), and Mac-1<sup>+</sup>Gr-1<sup>+</sup> (granulocytes) cells in the spleen. For (D) and (E), open histograms represent splenocytes from mice that do not contain the YFP transgene (negative control), and shaded histograms represent Bcl-2<sup>YFP</sup> cells.
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-2YFP 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-2YFP (data not shown). Using thymocytes, we confirmed that YFP expression directly parallels that of 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+ T cells, CD8+ T cells, and B cells but not in the Mac1+Gr-1+ granulocytes.

Analysis of Bcl-2 (YFP) expression in effector and memory CD8+ T cells responding to LM-OVA
Using YFP as a marker, we confirmed that both naive and memory CD8+ 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-2YFP mice were infected with a sublethal dose of LM-OVA, which induces a dominant CD8+ T cell response to the peptide OVA257–264 presented by MHC class I H-2Kb. Consistent with previously reported patterns of Bcl-2 expression (9, 10), most activated (CD44hi) OVA-specific CD8+ 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+ 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+ T cell memory.

Effector CD8+ 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+OVA–binding to identify OVA–specific cells because K+–OVA binding to the TCR could have biological effects in responding T cells. Therefore, we crossed Bcl-2YFP mice onto a congenically marked (CD45.1/2+) background (OT1YFP). To avoid complications from directly infecting the OT1YFP mice (because of the overwhelming number of Ag–specific cells and the lack of CD4+ cells), 50,000–100,000 OT1YFP CD8+ T cells were transferred to wild-type recipient mice 1 d before infection. This number of cells, while supraphysiologic, has been used in similar studies, and in our Bcl-2YFP system, the phenotype of the responding CD8+ T cells was similar to that observed in the endogenous responders when Bcl-2YFP mice were infected directly (data not shown).

Cells were first gated on CD8+CD44hiOT1YFP (OT1 identified as CD45.1+Vα2+) 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-2hi population (I) was most enriched for MPECs, and the percentage of MPEC cells decreased with Bcl-2 levels with the exception of the Bcl-2lo 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-2hi cells (population I) consistently yielded the highest number of memory cells, whereas Bcl-2lo 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 expressers 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 between 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 transferred 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 transferred) in the mice that received unsorted CD8+ T cells (Supplemental 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, left panel). An intermediate gating strategy yielded intermediate results (Supplemental Fig. 1B, center panel). These results suggest that Bcl-2int-hi MPECs, specifically those representing 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 demonstrated the difference in the YFP mean fluorescence intensity (MFI) between the populations (Supplemental Fig. 2). Interestingly, the CD127 MFI increased along with YFP expression, but the dynamic range of CD127 was much smaller than that of YFP expression (Supplemental Fig. 2). Whether this is because of a difference in the actual range of expression levels or rather in the limitations of the detection of the signal is not clear.

To better characterize our sorted populations, we examined Bcl-2 and Ki67 expression in the corresponding OT1YFP effector cells. As expected, the MFI of Bcl-2 protein staining correlated with the assigned group as defined by YFP expression, confirming that the downregulation of the reporter, YFP, accurately matched that of the protein Bcl-2 in effector cell subpopulations and did not represent an artifact of the reporter system (data not shown). To determine the relative proliferative capacity of the different populations, we stained the cells for Ki67, a marker of dividing cell proliferation.
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-hi (population VI) cells to represent Bcl-2 “high” cells for a comparison with Bcl-2lo cells (population VIII). Interestingly, the trend that the Bcl-2highest 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.

**Fig. 4.** Memory potential of Bcl-2 “high” and “low” MPEC populations. (A) Sorting of OT1YFP cells on day 7 of LM-OVA infection. Following OT1YFP pretransfer and LM-OVA infection, CD8+-enriched splenocytes were gated as shown (CD8+CD45.1+CD44hiVα2+). MPECs (CD127hiKLRG12) were split into populations V–VIII based on Bcl-2-YFP expression. The numbers indicate the percentage of the parent population within the gate. (B) Ki67 expression in YFP-sorted MPECs. OT1YFP cells were transferred, and mice were infected with LM-OVA as for sorting. On day 7 following infection, cells were stained for surface markers and Ki67. The percent Ki67+ cells (mean ± SD) is shown for each group (as gated in [A]) within individual mice. Significance was calculated using a paired ANOVA (****p < 0.0001) with Tukey’s multiple comparisons (NS p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). (C) Analysis of mice that received Bcl-2 high and low populations (VI and VIII, respectively) 5 d after recall challenge. The number of OT1YFP cells per spleen was calculated, and the mean ± SD of the mice within each group (four mice per group) is shown (**p < 0.01 using Student t test). Data are representative of three independent experiments.

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 CD127hiKLRG1− MPEC population. Using adoptive transfer, we confirmed the observations of other groups that the CD127hiKLRG1− 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-2mid-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
(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{highest} 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 cell cycle progression, 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.

Taken together, our data show that relatively high levels of Bcl-2 lead to memory in CD8\(^+\) 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 the effects of environmental changes specifically on Bcl-2, which we have proven to be an important modulator of T cell memory.

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Disclosures
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