



Published in final edited form as:

*J Immunol.* 2007 July 1; 179(1): 36–40.

## Cutting Edge: Migration to Nonlymphoid Tissues Results in Functional Conversion of Central to Effector Memory CD8 T Cells<sup>1</sup>

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### Abstract

Memory CD8 T cells, essential for defense against intracellular pathogens, are heterogeneous with respect to phenotype and function. Constitutively lytic effector memory cells primarily reside in nonlymphoid tissues, whereas secondary lymphoid tissues contain functionally quiescent central memory cells. However, the mechanism by which functionally distinct memory populations are maintained is unknown. In this study, we show that resting CD8 memory cells modified their functional abilities upon entry into nonlymphoid tissues, as exemplified by the induction of granzyme B and lytic activity. Contemporaneously, the costimulator CD27 was down-regulated. These findings hold important implications for memory cell lineage development and tissue-specific immunity.

Seminal studies provide evidence for the existence of two subsets of memory CD8<sup>+</sup> T cells, central memory cells (T<sub>CM</sub>)<sup>3</sup> which express the receptors CCR7 and CD62L, allowing efficient homing to lymph nodes, and effector memory cells (T<sub>EM</sub>) which lack these homing receptors (1,2) and therefore reside mainly in nonlymphoid tissues (3,4). T<sub>CM</sub> cells and T<sub>EM</sub> cells also differ in both their proliferative ability and effector functions with T<sub>EM</sub> having a lower proliferative capacity than T<sub>CM</sub> (5,6). T<sub>CM</sub> cells lack immediate effector function whereas CD8 T<sub>EM</sub> cells express molecules necessary for lytic activity such as perforin and granzyme B and display direct ex vivo effector function (1,2,4). More recently, it has become evident that some memory CD8 T cells express a mixed CD62L<sup>-</sup> CCR7<sup>+</sup> phenotype that differs from the phenotypes of classical T<sub>EM</sub> and T<sub>CM</sub> cells, implying further heterogeneity within the memory lineage (7). In addition it has been demonstrated that virus-specific intraepithelial lymphocytes in the gut resemble neither T<sub>CM</sub> nor T<sub>EM</sub> CD8 T cells isolated from spleen or blood suggesting that anatomic location may play a role in local memory differentiation (8). Furthermore, the lack of expression of CD27, a member of the TNF receptor family, has emerged as an indicator of constitutive effector function in human and mouse memory CD8 T cells (1,9–11).

<sup>1</sup>This work was supported by National Institutes of Health Grant DK45260.

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### Disclosures

The authors have no financial conflict of interest.

<sup>3</sup>Abbreviations used in this paper: T<sub>CM</sub>, central memory T cells; B6, C57BL/6; LM-ova, *Listeria monocytogenes* expressing ova; VSV-ova, vesicular stomatitis virus encoding ovalbumin; T<sub>EM</sub>, effector memory T cells; MFI, mean fluorescence intensity.

In terms of function, it has been shown that for CD8 T cells, nonlymphoid derived memory cells exhibit constitutive effector levels of lytic activity whereas splenic memory cells, despite a CD62L<sup>-</sup> phenotype, are poorly lytic directly ex vivo (4). The basis for this dichotomy is unknown but memory CD8 T cells have the ability to traffic between lymphoid and nonlymphoid tissues as a part of a common blood-borne pool (12–14). Thus, functional differences between memory T cells in distinct locations could be due to the existence of subsets of effector memory cells that preferentially home to specific tissues or alternatively due to the modulation of functional activities upon entry of blood-borne memory cells into nonlymphoid tissues. Therefore, we undertook studies to assess whether entry of nonlytic memory cells into nonlymphoid tissues resulted in functional modifications. Our results indicate that memory cell phenotype and function are plastic and are dynamically modulated as a result of migration.

## Materials and Methods

### Mice and infections

C57BL/6J (*Ptprc<sup>b</sup>* = CD45.2) mice were purchased from The Jackson Laboratory and C57BL/6 (*Ptprc<sup>a</sup>* = CD45.1) mice from Charles River through the NCI program. B6.SJL-*Ptprc<sup>a</sup> Pepc<sup>b</sup>*/BoyJ-Tg(TcraTcrb)1100Mjb/J-B6.129S7-*Rag1<sup>tm1Mom</sup>* (CD45.1 OT-I-RAG<sup>-/-</sup>) were bred at University of Connecticut Health Center. Mice were infected i.v. with VSV-ova ( $1 \times 10^6$  pfu) or  $1 \times 10^3$  cfu of LM-ova (15,16).

### Isolation of T cell subsets and adoptive transfer

Lymphocytes were isolated from lymphoid and nonlymphoid tissues as previously described (4). In some experiments, spleen cells were isolated using the same technique as that employed for isolation of nonlymphoid cells. This isolation protocol did not result in alterations of the expression levels of CD27 or CD62L (data not shown). CD27<sup>high</sup>/CD62L<sup>high</sup>, CD27<sup>high</sup>/CD62L<sup>low</sup>, and CD27<sup>low</sup>/CD62L<sup>low</sup> populations were purified by sorting using a FACSVantage SE (BD Biosciences). The purity of sorted samples ranged between 91% and 99% and cell populations were adoptively transferred i.v.

### Analysis of intracellular granzyme B expression

For intracellular detection of granzyme B, cells were stained with antihuman granzyme B or an isotype control for 45 min at RT.

### Measurement of cytolytic activity

Cytolytic activity was measured using [<sup>51</sup>Cr]sodium chromate-labeled EL4 cells (an H-2<sup>b</sup> thymoma) with or without the addition of 10 μg/ml of the VSV-N protein-derived peptide RGYVYQGL. Serial dilutions of sorted effector cells were incubated in 96-well round-bottom microtiter plates with  $2.5 \times 10^3$  target cells for 5 h at 37°C. Percent specific lysis was calculated as:  $100 \times [(cpm \text{ released with effectors} - cpm \text{ released alone}) / (cpm \text{ released by detergent} - cpm \text{ released alone})]$ .

## Results and Discussion

### Preferential expression of granzyme B by nonlymphoid memory CD8 T cells correlates with CD27 levels

To determine whether antimicrobial CD8 memory T cells were phenotypically and functionally distinct in different tissues, we compared expression levels of CD27, CD62L, and granzyme B (Fig. 1). Granzyme B is involved in lymphocyte-mediated killing of target cells by CTL (17,18). We have previously shown that unlike tissue-resident cells, splenic

memory cells are poorly lytic indicating that there is not a strict correlation between CD62L expression and lytic activity (4). Therefore, we employed CD27 expression in conjunction with CD62L expression to further dissect memory cell subsets, since studies have shown that the CD27<sup>low</sup> CD8<sup>+</sup> T cell subset is primarily composed of cells preferentially expressing perforin and exhibiting CTL activity (1,10,19). More recently, it has been shown that influenza virus-specific CD27<sup>low</sup> T cells are present in the lung after secondary infection and that the loss of CD27 coincides with high granzyme B expression (10). To generate memory cells, CD45.1 OT-I cells were transferred into B6 CD45.2 mice that were then infected i.v. with LM-ova (15,16), rested for 46 days and secondarily infected with VSV-ova (20). Lymphocytes were isolated 70 days later from the spleen, lung, and liver and the phenotype and granzyme B expression of the Ag-specific T cells was examined. At this time point, small populations of memory CD8 T cells in the spleen and lung expressed CD62L, whereas liver memory cells generally lacked this receptor (Fig. 1). CD27 levels were heterogeneous, with distinct CD27<sup>high</sup> and CD27<sup>low</sup> memory cell subsets in the spleen and lung whereas liver memory cells were largely CD27<sup>low</sup>. Interestingly, granzyme B expression was lowest in splenic memory cells and was substantially higher in lung and liver memory cells (Fig. 1). These data are also consistent with the recent demonstration that CD27 down-regulation correlates with the expression of cytotoxic effector molecules such as granzyme B (10).

### Granzyme B is up-regulated on CD8 T<sub>CM</sub> cells after entry into nonlymphoid tissues

To determine whether memory cells altered their phenotype upon entry into nonlymphoid tissues, we transferred sorted CD62L<sup>low</sup>/CD27<sup>high</sup> memory cells from the spleen to naive mice and analyzed the transferred cells 4 days later. These cells expressed very low levels of granzyme B before transfer (Fig. 2A). Splenic memory cells after transfer retained high levels of CD27 (mean fluorescence intensity (MFI) 986) and low levels of CD62L (MFI 75) and granzyme B (MFI 23). However, upon entry into nonlymphoid tissues CD27 expression was down-regulated in the lung (MFI 418) and even more so in the liver (MFI 130), whereas CD62L expression remained low. Also, granzyme B expression was induced in the transferred cells in both the lung (MFI 90) and the liver (MFI 149) (Fig. 2A). We also followed the fate of transferred CD62L<sup>high</sup>/CD27<sup>high</sup> cells (T<sub>CM</sub>). Before transfer, this population did not express detectable granzyme B (Fig. 2B). After transfer, CD27 expression remained unchanged in the spleen (MFI 1106), but was substantially down-regulated in the lung (MFI 393) and liver (MFI 153). CD62L expression remained high in the spleen and lung but was down-regulated to some extent in the liver. Granzyme B expression was considerably up-regulated in both the lung (MFI 61) and liver (MFI 122) (Fig. 2B). Thus, T<sub>CM</sub> can give rise to functional T<sub>EM</sub>. It should be noted that in all of the transfer experiments, recovery of transferred cells was 3–10% of total input cell number (data not shown), in agreement with estimates of naive and memory cell survival after transfer (21), suggesting that the results obtained were not due to minor contaminants that preferentially survived and migrated to specific tissues.

T cells down-modulate CD27 after interaction with the CD27 ligand CD70 in vitro and in vivo (22,23). To analyze whether the observed down-regulation of CD27 was due to CD70 engagement, we transferred CD27<sup>high</sup> splenic memory cells and simultaneously administered a blocking anti-CD70 mAb (24). Four days posttransfer lymphocytes were isolated from the spleen, lung, and liver and CD27 expression was examined (Fig. 2C). In both the lung and the liver, the level of CD27 on transferred memory cells was down-regulated compared with the cells in the spleen and blocking CD70 had no effect on CD27 levels. The amount of anti-CD70 mAb employed effectively blocked the primary CD8 T cell response to LM infection indicating the mAb was active (data not shown). These results suggested that down-modulation of CD27 expression on migrating memory cells was not

CD70-mediated but was the result of as yet unidentified factors acting to reduce CD27 levels.

### Splenic memory cells acquire lytic activity after entry into nonlymphoid tissues

The presence of granzyme B is not an absolute indicator of the ability of a CD8 T cell to mediate target cell lysis since other molecules are also required for CTL activity. Therefore, we measured the direct lytic activity of transferred splenic memory CD8 T cells. Enriched CD8 T cells from spleens of mice previously infected with VSV were adoptively transferred into naive mice and 4 days later sorted splenic and lung tetramer<sup>+</sup> CD8 T cells were assayed for their CTL capacity. Transferred memory cells isolated from the spleen exhibited low levels of lytic activity (Fig. 3), as did the starting population before transfer (data not shown). In contrast, memory cells obtained from the lung exhibited substantial levels of lytic activity, inducing 10–15% specific lysis at a very low E:T ratio (0.25:1) (Fig. 3). Thus, the induction of granzyme B expression correlated with up-regulation of cytolytic activity in migrating memory CD8 T cells.

### Nonlymphoid T<sub>EM</sub> cells modulate CD27 expression but retain granzyme B following transfer

Our results indicated that T<sub>CM</sub> cells could convert to functional T<sub>EM</sub> cells upon entry into nonlymphoid tissue. However, whether this phenomenon represented a “revolving door” through which T<sub>EM</sub> cells exiting from nonlymphoid tissues reverted to T<sub>CM</sub>-like cells was unknown. To test this possibility, CD62L<sup>low</sup>/CD27<sup>low</sup>GrB<sup>high</sup> memory CD8 T cells isolated from the liver were transferred to naive animals and 4 days later were analyzed. Interestingly, transferred CD62L<sup>low</sup>/CD27<sup>low</sup> liver memory cells isolated from the spleen exhibited heightened CD27 levels as compared with their nonlymphoid counterparts in the lung and liver where CD27 expression was similar to that of the cells before transfer (Fig. 4). However, granzyme B levels remained high in transferred memory cells isolated from all tissues. Thus, in this case CD27 expression levels did not apparently correlate with lytic activity. Although at present we do not have an explanation for this finding, given the observed heterogeneity in CD27 expression (Fig. 1), it is perhaps likely that CD27 expression levels may not precisely correlate with granzyme B expression. It is also possible that a period longer than 4 days is required to observe down-regulation of lytic molecules such as granzyme B and this may depend on protein half-life. Alternatively, under normal circumstances lytic memory cells may not exit nonlymphoid tissues in large numbers. Nevertheless, the results again indicated the short-term mutability of the memory cell population with respect to phenotype.

Our data indicated that CD27 but not CD62L levels correlated in most cases with increased levels of granzyme B. Furthermore, CD62L<sup>low</sup>/CD27<sup>low</sup>/GrB<sup>high</sup> memory CD8 T cells appeared to represent terminally differentiated cells whereas both CD62L<sup>low</sup>/CD27<sup>high</sup>/GrB<sup>low</sup> and CD62L<sup>high</sup>/CD27<sup>high</sup>/GrB<sup>neg</sup> memory CD8 T cells were functionally flexible. Although it has been suggested that CD27 is lost from CD8 T cells only after repetitive antigenic stimulation (10), we found that down-regulation of CD27 can occur in the absence of Ag, as shown by adoptive transfer of OT-I memory CD8 T cells into naive hosts. However, all memory CD8 T cells we examined expressed CD27 at some level leaving open the possibility that further loss of CD27 may occur following repetitive antigenic stimulation. In addition to modulation of CD27 levels, migration of nonlytic memory CD8 T cells to nonlymphoid tissues triggered the induction of the cytolytic effector molecule, granzyme B and resulted in the induction of lytic activity. These data indicated that memory T cell phenotype and function are not fixed and are dependent on location. Although it remains unclear whether there is a subset of T<sub>EM</sub> cells that preferentially home to nonlymphoid tissues, we would predict that this is not the case as all populations of purified

memory T cells homed to the different tissues and up-regulated granzyme B expression. Since cells entering both the lung and liver were functionally modulated, common factors involved in effector molecule regulation must exist in at least these tissues, if not others. It should be noted that our studies utilized secondary memory cells, which may exhibit distinct characteristics as compared with primary memory CD8 T cells. Our original experiments of mouse T<sub>CM</sub> and T<sub>EM</sub> studied primary and secondary memory cells (4) and as reiterated here, showed that splenic primary and secondary memory CD8 T cells contain at least a subpopulation of cells with a T<sub>EM</sub> phenotype that display negligible levels of lytic activity. Recent reports indicate that lytic activity, granzyme B expression and protective capacity of memory CD8 T cells increase with the number of booster immunizations (25,26). Nonetheless, a subset of T<sub>CM</sub> is apparent after multiple immunizations, particularly when the lack of CD62L expression is not used as criteria to identify T<sub>EM</sub>. The length of time after immunization is another important criteria to consider when assessing memory CD8 T cells since phenotype and perhaps function can change over time as a result of population dynamics (26–28). In any case, our data favor the hypothesis that one consequence of traversing the endothelium to enter parenchymal tissues is induction of lytic activity. This phenomenon may be regulated by T cell interactions with chemokines and adhesion molecules that are necessary for extravasation of memory T cells from blood vessels to extralymphoid sites. A pathway such as this could represent a regulatory mechanism for ensuring optimal activity of memory T cells in the appropriate locale.

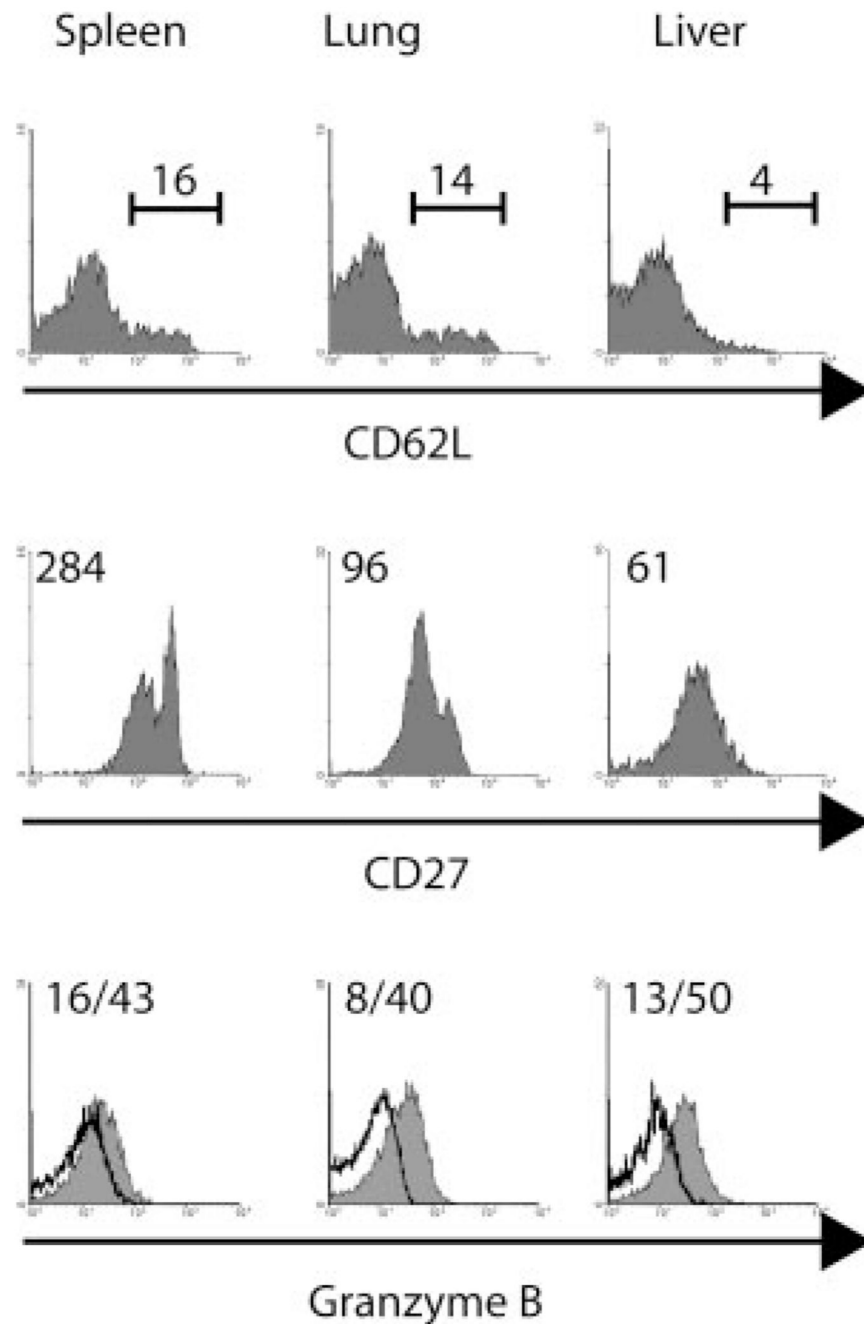
## Acknowledgments

We thank Diane Gran for FACS sorting.

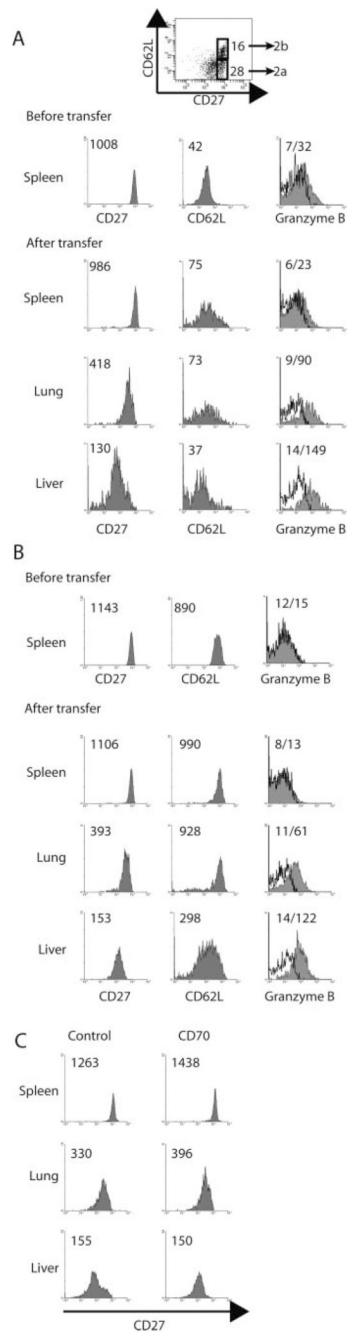
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**FIGURE 1.**

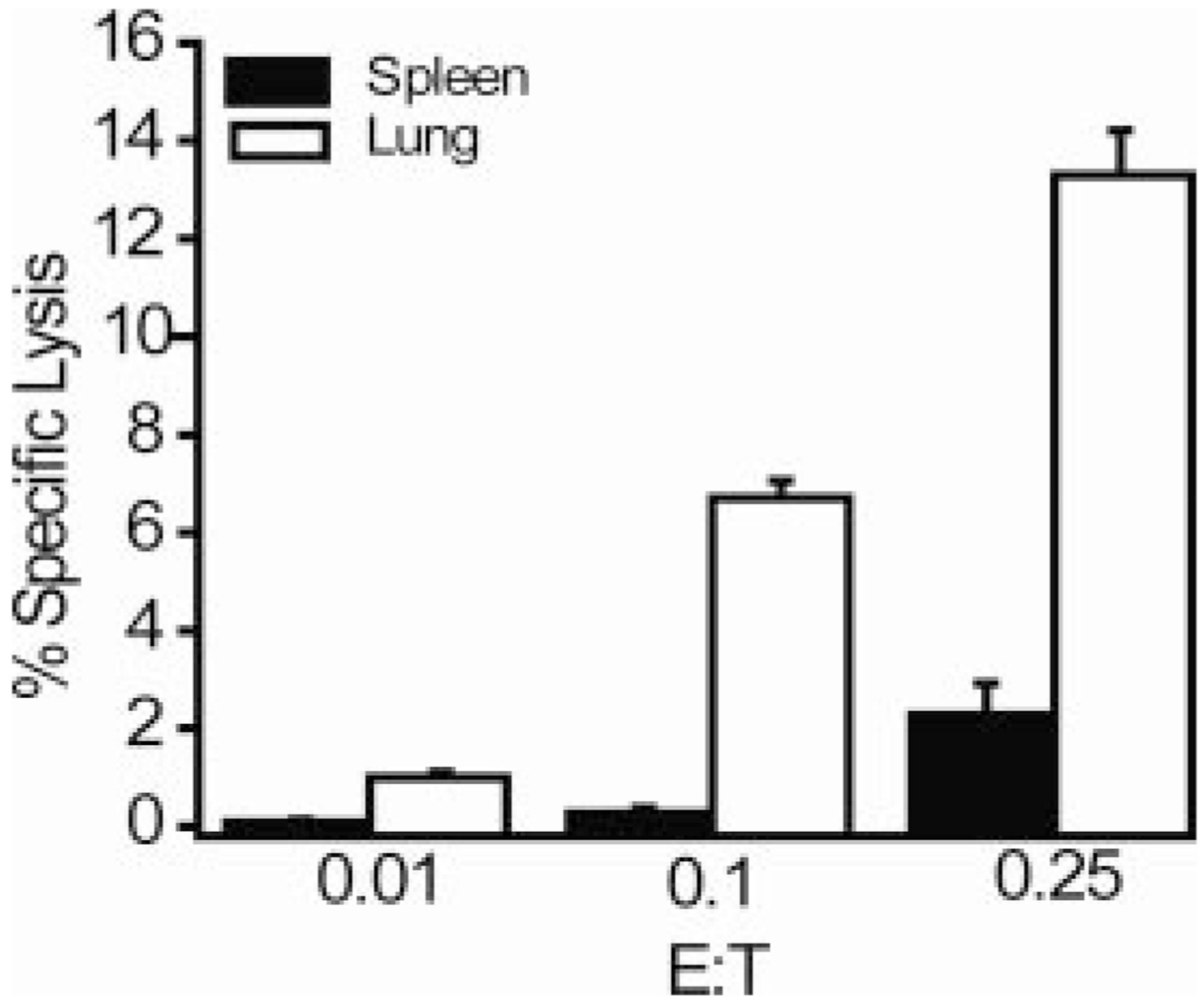
Phenotype and granzyme B expression of splenic and nonlymphoid memory CD8 T cells.  $1 \times 10^3$  CD45.1 OT-I-RAG<sup>-/-</sup> splenocytes were transferred into CD45.2 mice and 1 day later the mice were infected with  $1 \times 10^3$  cfu of LM-ova i.v. and 46 days post infection re-infected with  $1 \times 10^6$  pfu VSV-ova i.v. 70 days later the level of CD62L, CD27 and GrB expression of the OT-I memory CD8 T cells was measured by flow cytometry. Values indicate the mean fluorescent intensity (MFI) of staining for the population. For GrB staining the MFI is shown for the isotype control and test, respectively. Data are representative of 3–4 mice analyzed from two experiments.

**FIGURE 2.**

Splenic CD8 T<sub>CM</sub> down-regulate CD27 and acquire granzyme B after entry into nonlymphoid tissues. OT-I transferred CD45.2 mice were infected with  $1 \times 10^3$  CFU of LM-ova i.v. 48 days later mice were re-infected with  $1 \times 10^6$  PFU VSV-ova i.v., and 97 days later, splenocytes were isolated, enriched for CD8 T cells, donor cells were sorted into either CD62L<sup>low</sup>/CD27<sup>high</sup> cells (A) or CD62L<sup>high</sup>/CD27<sup>high</sup> cells (B) and transferred to naive CD45.2 mice ( $1 \times 10^6$  and  $5 \times 10^5$ , respectively). Four days later lymphocytes were isolated from the spleen, lung, and liver, and the level of CD27, CD62L, and granzyme B expression by the transferred OT-I T cells was determined. C, OT-I transferred mice were infected with LM-ova i.v.; 46 days later mice were infected with VSV-ova i.v., and 58 days later



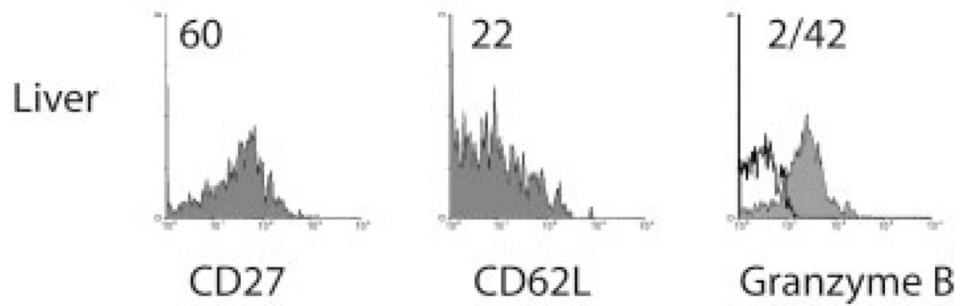
splenocytes were isolated, enriched for CD8 T cells, donor cells were sorted, and  $8.5 \times 10^5$  CD27<sup>high</sup> cells were transferred to naive CD45.2 mice. On days 0, 1, and 2, mice were treated with either 250  $\mu$ g of anti-CD70 (FR70) or control rat IgG. Four days later, lymphocytes were isolated from the spleen, lung and liver, and the level of CD27 was determined on the transferred OT-I T cells. Values indicate the MFI of staining for the population. For GrB staining the MFI is shown for the isotype control and test, respectively. Data are from one representative animal of 1–4 mice analyzed from four experiments ( $n = 9$ ).



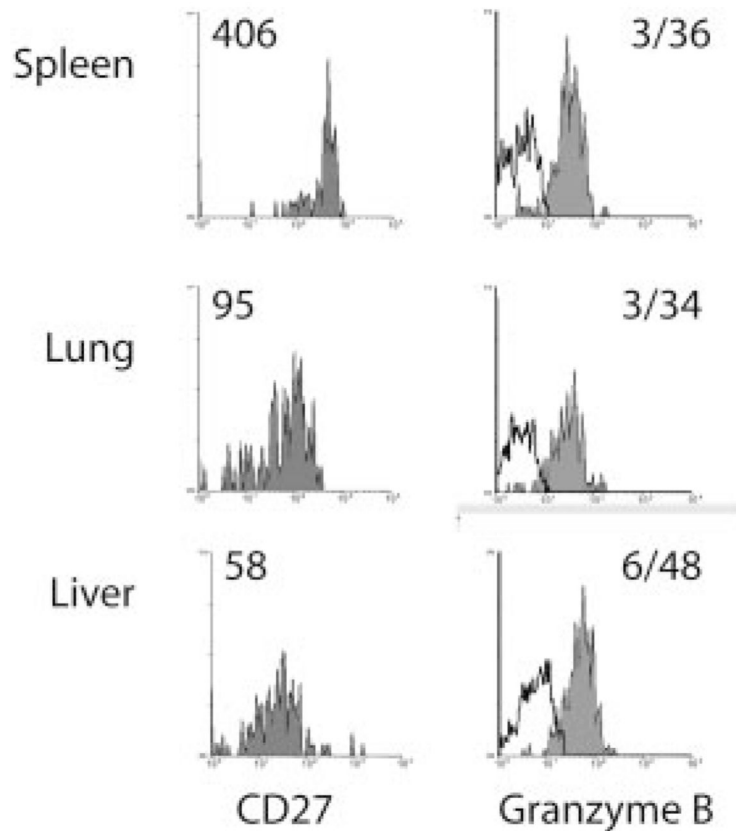
**FIGURE 3.**

Splenic CD8 T<sub>M</sub> acquire lytic activity after entry into nonlymphoid tissues. CD45.2 B6 mice were infected with  $1 \times 10^5$  PFU of VSV-Indiana, rested for 71 days and infected with  $1 \times 10^5$  VSV-New Jersey. Approximately 9 mo after the secondary infection, CD8 T cells were enriched from the spleens and transferred to CD45.1 B6 mice; 4 days posttransfer tetramer<sup>+</sup> CD8 T cells were sorted from the spleen and lungs and set up in a CTL assay. Experiments were performed twice with similar results, and the data are the mean of three mice from one experiment.

## Before transfer



## After transfer

**FIGURE 4.**

Nonlymphoid  $T_{EM}$  cells modulate CD27 expression but retain granzyme B following transfer. OT-I transferred CD45.2 B6 mice were infected with LM-ova and 48 days later were infected with VSV-ova i.v.; 97 days later, CD62L<sup>low</sup> liver memory T cells were sorted and  $1 \times 10^5$  OT-I cells were transferred to naive CD45.2 B6 recipients, and 4 days posttransfer CD27, CD62L, and granzyme B expression of OT-I cells from the spleen, lung, and liver was determined. Values indicate the MFI of staining for the population. For GrB staining, the MFI is shown for the isotype control and test, respectively. Data are from one representative animal of two mice analyzed from two experiments.