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Lack of IL-7 and IL-15 signaling affects interferon-γ production by, more than survival of, small intestinal intraepithelial memory CD8$^+$ T cells

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Survival of antigen-specific CD8$^+$ T cells in peripheral lymphoid organs during viral infection is known to be dependent predominantly on IL-7 and IL-15. However, little is known about a possible influence of tissue environmental factors on this process. To address this question, we studied survival of memory antigen-specific CD8$^+$ T cells in the small intestine. Here, we show that 2 months after vaccinia virus infection, B8R$_{20–27}$/H2-K^b tetramer$^+$ CD8$^+$ T cells in the small intestinal intraepithelial (SI-IEL) layer are found in mice deficient in IL-15 expression. Moreover, SI-IEL and lamina propria lymphocytes do not express the receptor for IL-7 (IL-7Rα/CD127). In addition, after in vitro stimulation with B8R$_{20–27}$ peptide, SI-IEL cells do not produce high amounts of IFN-γ neither at 5 days nor at 2 months postinfection (p.i.). Importantly, the lack of IL-15 was found to shape the functional activity of antigen-specific CD8$^+$ T cells, by narrowing the CTL avidity repertoire. Taken together, these results reveal that survival factors, as well as the functional activity, of antigen-specific CD8$^+$ T cells in the SI-IEL compartments may markedly differ from their counterparts in peripheral lymphoid tissues.

Introduction

Gastrointestinal mucosa represents a major site of entry as well as initial replication for many viral and bacterial pathogens (including HIV). Massive depletion of CD4$^+$CCR5$^+$ memory T cells occurs in the mucosal tissues within the first 2 weeks of HIV-1 infection [1, 2]. In connection with this, vaccines providing protection against gastrointestinal infectious diseases must be able to induce long-term mucosal immune responses [3–9]. Previously, we demonstrated that long-lasting protection against mucosal viral transmission could be accomplished by CD8$^+$ CTLs that must be present at the mucosal site of antigen exposure, although some mucosal memory CTLs may be induced even after systemic vaccination [10–16]. This protective effect was ablated when CD8$^+$ cells were depleted in vivo, and required that CTLs were present in the gut mucosa, whereas splenic CTLs alone were unable to protect against mucosal viral challenge [10]. Thus, unless Abs are able to completely prevent viral entry, induction of local long-lasting mucosal CD8$^+$ CTLs in the gut should be considered essential to design a protective mucosal vaccine.

The maintenance of antigen-specific CD8$^+$ T cells, their homeostatic proliferation and survival during the memory phase of immune responses are known to be predominantly dependent on IL-15 and IL-7, respectively [17–26]. Other cytokines elicited by infectious (or tumor) antigens may work during the primary response as endogenous adjuvants, and could contribute to the survival of long-lived memory CD8$^+$ T cells [27–30]. Based on proliferative potential, cytokine production, and surface phenotype, memory CD8$^+$ T cells can be divided into effector memory (EM) and central memory (CM) cells [31, 32]. One approach to

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discriminate between these two subsets is based on the detection of surface IL-7Rα/CD127 and CD62L; EM cells have $CD127^+$ $CD62L^-$ phenotype, whereas CM cells should be $CD127^+CD62L^+$. In peripheral lymphoid tissues, both $CD8^+$ T-cell memory subsets may be found, but in the case of nonlymphoid tissues, EM $CD8^+$ T cells are expected to be more prevalent due to the differences in migratory pathways used [33]. Mechanisms of $CD8^+$ CTL survival in mucosal tissues, in particular, in the small intestinal intraepithelial (SI-IEL) and lamina propria (SI-LP) are not well understood yet [33–35]. Here, we addressed a role that IL-15 and IL-7 play for the survival of memory IEL $CD8^+$ T cells in the SI in mice that were mucosally vaccinated with modified vaccinia ankara (MVA). We found that 2 months after MVA was intrarectally (i.r.) administered, virus-specific $B8R_{20–27}$/H2-K^b tetramer (B8R tetramer)$^+$ $CD8^+$ T cells were present in the SI-IEL. Surprisingly, these cells in WT mice were mainly $CD127^-CD62L^-$, in sharp contrast to the B8R tetramer$^+$ $CD8^+$ T cells found in the spleens of the same animals (EM and CM). Additionally, $B8R_{20–27}$-specific CTLs isolated from the SI-LP were $CD127^-CD62L^-$ as well. Moreover, such long-living $CD127^-$ $CD8^+$ T cells were found in the SI-IEL isolated from IL-15 KO mice, indicating that some $CD8^+$ T-cell subpopulation is able to survive within the gut epithelial layer in the absence of both IL-7 and IL-15 signaling. To find out which surface markers could be associated with residual survival of memory $CD8^+$ CTLs in the gut, cells from WT and IL-15 KO mice were analyzed by flow cytometry for surface expression of CD11a, CD11c, NKG2D, and CD8αα homodimer. We found that none of these markers was expressed by the majority of the gut memory $CD8^+$ CTLs.

Thus, we may conclude that the antigen-specific memory $CD8^+$ CTLs residing in the SI-IEL compartment may be maintained for a long-term independently of signaling via IL-7 and IL-15. Their survival may be dependent on soluble and/or cell contact signals which substitute for cytokines specific predominantly for lymphoid tissues. Identifying these tissue-specific prosurvival mechanisms may be crucial for the development of new strategies for mucosal vaccination.

Results

Absence of IL-15 affects the antigen-specific $CD8^+$ T-cell response in mucosally vaccinated mice

In order to estimate quantitative and qualitative effect of IL-15 of the antigen-specific $CD8^+$ T-cell response, WT and IL-15 KO mice were i.r. inoculated with MVA. As a readout, we assessed MVA-specific CTL expansion and contraction as well as their functional activity during acute (day 5) and memory (2 months) phases postinfection (p.i.), respectively. To enhance sensitivity of our model, especially in IL-15 KO animals, we measured immune response against the immunodominant vaccinia virus $B8R_{20–27}$ epitope [36]. By flow cytometry, in WT mice during the acute phase (5 days) of infection, a robust immune response was found, with high relative percentages of B8R tetramer$^+$ $CD8^+$ T cells in the spleen as well as in the SI-IEL (Fig. 1 and Table 1). By day 13, their frequencies both in the SI-IEL and spleen decreased approximately two-fold compared with their level at day 5, and contracted to as low as 1–2% by 2 months after immunization. In sharp contrast, in IL-15 KO mice, the presence of B8R tetramer$^+$ $CD8^+$ T cells was substantially lower compared with WT mice, throughout the experiment (compare WT vs IL-15 KO: spleen, 5 days – 20.62 ± 1.12 vs 9.97 ± 2.03, $p = 0.0101$; 13 days – 10.70 ± 0.78 vs 1.47 ± 0.15, $p = 0.0003$; 2 months – 1.73 ± 0.12 vs 1.53 ± 0.09, $p > 0.05$; SI-IEL, 5 days – 23.83 ± 1.58 vs 3.34 ± 0.29, $p = 0.0002$; 13 days – 13.39 ± 0.68 vs 3.20 ± 0.75, $p = 0.0006$; 2 months – 1.59 ± 0.17 vs 1.19 ± 0.36, $p > 0.05$). Difference in kinetics between WT and IL-15 KO mice was more evident when we estimated the absolute numbers of B8R tetramer$^+$ $CD8^+$ T cells in the spleen and the SI-IEL (Fig. 1; compare WT vs IL-15 KO: spleen, 5 days – 1.49 ± 0.09 ($\times 10^6$) vs 0.15 ± 0.03 ($\times 10^6$), $p = 0.0002$; 13 days – 0.64 ± 0.03 ($\times 10^6$) vs 0.11 ± 0.03 ($\times 10^6$), $p = 0.0003$; 2 months – 0.09 ± 0.01($\times 10^6$) vs 0.03 ± 0.01($\times 10^6$), $p = 0.0017$; SI-IEL, 5 days – 1.76 ± 0.07($\times 10^6$) vs 0.08 ± 0.02 ($\times 10^6$), $p < 0.0001$; 13 days – 0.77 ± 0.04 ($\times 10^6$) vs 0.15 ± 0.01($\times 10^6$), $p < 0.0001$; 2 months – 0.43 ± 0.02 ($\times 10^6$) vs 0.05 ± 0.01($\times 10^6$), $p = 0.0001$).

It is well known that IL-15 is a crucial cytokine necessary not only for the survival of memory $CD8^+$ T cells, but also for their functional activity. To ascertain functional avidity, we quantitated IFN-γ production by splenocytes and SI-IEL cells (by ELISpot assay). We found that splenocytes from WT animals (Fig. 2A, upper panel; Fig. 2B and C show normalized and net IFN-γ response as percentage of max. and total response, respectively) showed IFN-γ production in response to titrated amounts of $B8R_{20–27}$ peptide. This response was at peak during acute phase (5 days) after immunization, and declined with time. In contrast, in IL-15 KO mice the magnitude of response to the highest dosage of $B8R_{20–27}$ peptide (1 μM; characterizes total responders to peptide epitope) was significantly lower (compare WT vs IL-15 KO: 5 days – 1358.67 ± 311.10 vs 430.00 ± 164.62, $p = 0.0577$). Furthermore, the immune response in IL-15 KO mice decreased with time more abruptly, as it was already markedly diminished at 13 days after vaccination (WT vs IL-15 KO: 506.67 ± 116.09 vs 35.33 ± 5.24, $p = 0.0072$). Importantly, in IL-15 KO mice we detected almost complete lack of $CD8^+$ T cells having high functional avidity (recognizing 1 pM of $B8R_{20–27}$ peptide), which was already evident during the acute phase (compare WT vs IL-15 KO: 5 days – 110.33 ± 6.06 vs 43.33 ± 5.81, $p = 0.0624$; 13 days – 61.33 ± 3.53 vs 21.00 ± 2.65, $p = 0.0059$; 2 months – 41.00 ± 2.31 vs 10.00 ± 0.58, $p = 0.0002$). Thus, IL-15 was found to be important not only for the maintenance of antigen-specific $CD8^+$ T cells during the memory phase (2 months p.i.), but also for the generation of cells with full functional capacity (high functional avidity).

In contrast to that which was found in the spleen, IFN-γ production in the SI-IEL compartment from the WT animals was barely detectable 2 months after immunization (Fig. 2, lower panel; compare 5 days vs 2 months 1 μM: 666.67 ± 370.33 vs 18.33 ± 3.18). Also, this response was characterized by skewing

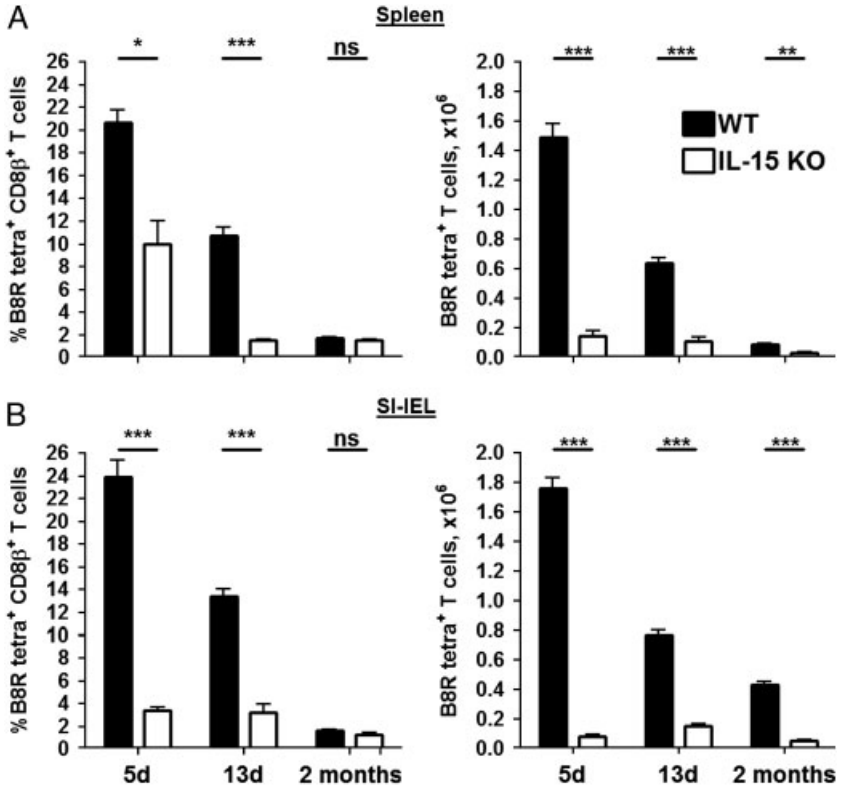


Figure 1. Kinetics of $B8R_{20-27}$/H2-K^b tetramer$^+$ $CD8^+$ T cells in the spleen and SI-IEL of C57BL/6 and IL-15 KO mice. (A) Relative percentages and (B) absolute numbers of B8R tetramer$^+$ $CD8^+$ T cells recovered from the spleen and SI-IEL of WT and IL-15 KO mice are presented. Lymphocytes were isolated 5 days, 13 days, and 2 months after i.r. immunization with MVA 10^7 pfu/mouse. Cells were stained with PE-conjugated $B8R_{20-27}$/H2-K^b tetramer together with anti-CD8α (spleen) or anti-CD8β (SI-IEL) mAbs. Data shown are the mean+SD of three animals per group. Experiments were performed twice with comparable results. Data were analyzed by unpaired Student's *t*-test. *$p<0.05$, **$p<0.01$, ***$p<0.0001$, ns: not significant.

to the presence of cells recognizing high and middle concentrations of $B8R_{20-27}$ peptide (1 μM and 100 pM) that further distinguishes them from the splenic counterparts (compare spleen vs SI-IEL, 5 days: 1 μM – 1358.67±311.10 vs 666.67±370.33; 100 pM – 506.67±116.09 vs 421.33±239.88; 1 pM – 41.00±4.00 vs 0.90±0.17). Strikingly, although B8R tetramer$^+$ $CD8^+$ T cells can be detected in SI-IEL in IL-15 KO animals, these cells had almost completely lost capacity to produce IFN-γ even at the peak of infection (Fig. 2; compare 5 days vs 13 days: 1 μM – 7.90±4.55 vs 6.53±3.31, $p>0.05$).

Physical avidity and TCR expression disparate with functional avidity of CTLs from intestinal tissues

Based on the ELISpot data, we reasoned that physical avidity of TCR B8R tetramer$^+$ $CD8^+$ T cells might affect their functional capacity. To check this possibility, we assessed by flow cytometry the level of TCR expression on B8R tetramer$^+$ $CD8^+$ T cells (Fig. 3A shows staining of naïve splenoctes and SI-IEL cells and Fig. 3B shows the strategy for gating cells as exemplified on WT cells 5 days p.i.). We found that two uneven populations with high and low TCR expression were present both in the spleen and in the SI-IEL (Fig. 3C). Interestingly, during the acute and memory phases, B8R tetramer$^+$ $CD8^+$ T cells in the spleen from both WT and IL-15 KO animals had comparable percentage of cells with high TCR expression (WT vs IL-15 KO: 72 and 65% and 70 and 66%, respectively). In contrast, a different pattern was found in the SI-IEL, whereas at the acute-phase CTLs in both mouse strains highly expressed TCR at comparable, but still lower frequency compared with the spleen (WT vs IL-15 KO: 55 and 50%, respectively); however, during the memory phase the cells from WT but not IL-15 KO expressed the TCR at the same or even higher level (WT vs IL-15 KO: 72 vs 32%, respectively). In fact, among memory CTLs in the SI-IEL of the IL-15 KO animals, the majority of B8R tetramer$^+$ $CD8^+$ T cells expressed TCR at low levels (compare day 5 vs 2 months: 49 vs 67%, respectively). Thus, MVA-specific SI-IEL cells from the IL-15 KO mice but not from WT animals had selective tissue-specific decline in the frequency of B8R tetramer$^+$ cells that expressed TCR at high level, which was progressing from the acute to the memory phase. The extensive and sustained TCR downregulation in the absence of IL-15 may be linked to a higher activation status of these cells and may be associated with a lower production of IFN-γ in IL-15KO animals. Additionally, by using $B8R_{20-27}$/H-$2K^b$ tetramer, we measured mean fluorescence intensity (MFI) of

Table 1. Phenotype of memory $B8R_{20-27}$-specific $CD8^+$ T cells in the SI-IEL and spleen from WT and IL-15 KO mice[a)]

Tissue	Marker	Time after infection			
		5 Days		2 Months	
		WT	IL-15 KO	WT	IL-15 KO
SI-IEL	$CD127^+CD62L^-$	1.62±0.16	2.23±0.38	ND	ND
	$CD127^+CD62L^+$	ND	1.17±0.64	ND	2.93±1.13
	$CD11c^+CD11a^-$	1.11±0.48	11.27±0.71$^{\#}$	17.90±1.99	12.70±1.46
	$CD11c^+CD11a^+$	7.90±0.49	17.50±1.04$^{\#\#}$	3.33±0.52	3.12±0.19
	$CD11c^-CD11a^+$	71.00±3.51	37.63±1.45*	ND	ND
	$NKG2D^+$	1.40±0.21	8.40±0.70**	11.37±0.45	10.40±2.45
SI-LP	$CD127^+CD62L^-$	0.07±0.07	0.07±0.03	ND	NEC
	$CD127^+CD62L^+$	ND	ND	ND	NEC
	$CD11c^+CD11a^-$	0.47±0.15	NEC	14.70±0.85	NEC
	$CD11c^+CD11a^+$	24.60±1.70	NEC	ND	NEC
	$CD11c^-CD11a^+$	61.00±2.08	NEC	ND	NEC
	$NKG2D^+$	0.32±0.08	21.00±1.91	20.73±1.69	NEC
Spleen	$CD127^+CD62L^-$	1.73±0.07	1.53±0.05	73.33±3.28	66.90±3.77
	$CD127^+CD62L^+$	2.20±0.30	1.63±0.08	15.00±2.52	19.67±1.45
	$CD11c^+CD11a^-$	6.58±0.36	2.56±0.59	1.03±0.09	0.23±0.15
	$CD11c^+CD11a^+$	71.77±3.26	67.40±2.91	42.33±2.03	43.23±1.75
	$CD11c^-CD11a^+$	15.73±0.94	16.07±0.55	40.53±1.81	42.77±1.97
	$NKG2D^+$	70.97±2.00	61.13±3.21	80.30±1.31	72.43±3.35

a) Lymphocytes from WT and IL-15 KO animals were isolated at 5 days and 2 months after i.r. immunization with MVA 10^7 pfu/mouse. Four-color flow cytometry was performed. Cells from tissues were stained with PE-conjugated $B8R_{20-27}$/H2-K^b tetramer together with CD8α mAbs. Then, cells were gated on tetramer$^+$ $CD8^+$ T cells, and further analyzed for the expression of CD127, CD62L, CD11a, CD11c, and NKG2D markers expression. Data shown are the mean percent of gated cells and SEM of three animals per interval and are one representative experiment of two experiments performed with comparable results. ND, not detected; NEC, not enough cells: due to the paucity of the total isolated LP cells as well as recovered $B8R_{20-27}$/H2-K^b tetramer$^+$ CTLs; $^{\#}p = 0.00057$; $^{\#\#}p = 0.00434$; $^*p = 0.00487$; $^{**}p = 0.00623$. Text shaded in grey denotes the data with significant differences in percentages of different surface markers between WT vs. IL-15 KO animals.

vaccinia virus-specific CTLs stained with different concentrations of tetramer, which reflects the relative physical TCR avidity [37, 38]. For this, we isolated tissues from the WT animals 5 days after i.r. infection with MVA. Comparison of the curves for $CD8^+$ T cells from the SI-IEL vs spleen (Fig. 4, left panel) revealed that the MVA-specific CTLs expressed TCR with rather high physical avidity for $B8R_{20-27}$. Interestingly, the LP cells, which are anatomically adjacent to the intraepithelial lymphocyte (IEL), had slightly lower TCR avidity, but still it was much higher compared with the spleen. Similar results were obtained when we depicted the same data as a % of max. MFI level, determining an average tetramer dilution factor giving 50% max. MFI (Fig. 4, right panel): spleen – 1:60, SI-LP – 1:80, and SI-IEL – 1:200.

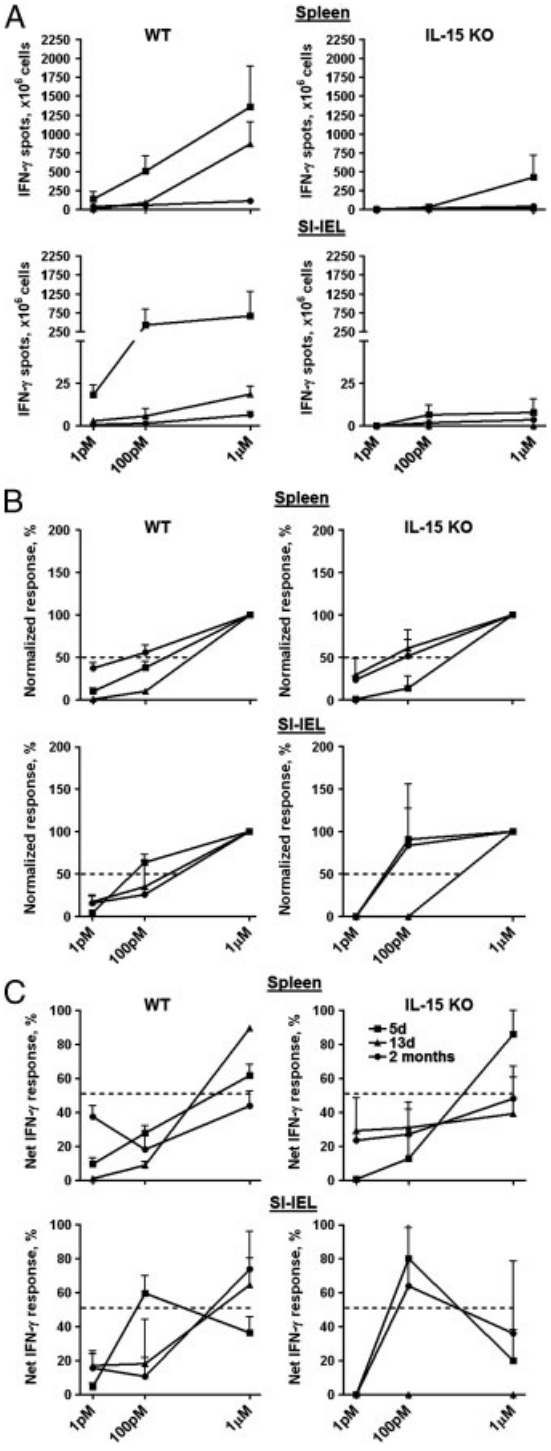
Altogether, we may conclude that data on functional and physical avidity of MVA-specific CTLs isolated from spleen (lymphoid tissue) correspond to each other, whereas in the SI-IEL and SI-LP (nonlymphoid tissues) they do not. It may imply that not only cell-intrinsic features (physical avidity) may shape CTL functional activity, but cell-extrinsic parameters as well, especially in case of nonlymphoid tissues (cytokine milieu, costimulatory molecules). Our data on MFI of tetramer$^+$ cells in IEL and LP compared with spleen in the memory phase cannot answer this question.

Memory antigen-specific $CD8^+$ T cells in SI-IEL and SI-LP from IL-15 KO mice lack IL-7Rα/CD127 expression

It is known that both IL-7 and IL-15 are of crucial importance for homeostatic proliferation of memory $CD8^+$ T cells [17, 24, 39]. In particular, IL-15 signals are considered to be more important for homeostatic proliferation of memory $CD8^+$ T cells, whereas IL-7 signals are responsible for their survival [31]. However, what defines prosurvival conditions for memory $CD8^+$ T cells in different nonlymphoid tissues, including the gut, is not well understood. At least for the cells from the SI-LP, it was shown that they may survive due to signals provided by nonhematopoietic cells via CD70 [40]. As was recently demonstrated by Jiang et al. [41], the residual c-Myc-deficient CD8αα TCRαβ IEL cells display reduced proliferation and increased apoptosis, which correlate with significantly decreased expression of IL-15 receptor subunits and lower levels of the antiapoptotic protein Bcl-2 [41]. Thus, c-Myc controls the development of CD8αα TCRαβ IEL cells from thymic precursors apparently by regulating IL-15 receptor expression and consequently Bcl-2-dependent survival [41]. It is well accepted that IEL lymphocytes are slower proliferating cells compared with other lymphoid cells. In many experimental systems, IEL lymphocytes alone did not show activation-induced proliferation, but they

significantly inhibited the proliferation of activated lymph node T cells in a cell number-dependent manner [42].

To determine whether the cells from the SI-IEL comply to the same prosurvival strategy, we first assessed by flow cytometry whether the B8R tetramer$^+$ CD8$^+$ T cells express CD127 (IL-7Rα). Memory CD8$^+$ T cells in the spleens in both mouse strains developed toward EM (CD127$^+$CD62L$^-$) and CM (CD127$^+$ CD62L$^+$) subsets at comparable frequencies (Fig. 5 and Table 1).



Surprisingly, however, very few memory B8R tetramer$^+$ CD8$^+$ T cells from the SI-IEL both in WT and in IL-15 KO mice (Fig. 5 and Table 1) expressed CD127. The same was true for the gut cells analyzed during the acute phase as well. In addition, SI-LP lymphocytes from WT mice were also negative for CD127 expression during the entire experiment, and in IL-15 KO animals it was proved at least at the acute phase (Fig. 5 and Table 1). Thus, memory B8R-specific CD127$^{lo/-}$CD8$^+$ T cells residing in the SI-IEL compartment can be found not only in WT mice but also in the IL-15 KO.

To evaluate whether residual IL-7Rα/CD127$^+$ lymphocytes in the SI-IEL were still able to transduce signals from IL-7, we isolated SI-IEL lymphocytes, and treated them in vitro with recombinant murine IL-7 cytokine (rmIL-7; 200 ng/mL, 20 min at 37°C), followed by subsequent staining for intracellular phosphorylated STAT5 protein. As CTLs from naïve and antigen-experienced mice contain comparable low frequency of CD127$^+$ cells (our unpublished data) and due to the paucity of antigen-specific CD8$^+$ CTLs recoverable from SI-IEL compartment especially at the memory stage, we used SI-IEL cells from naïve mice. As a control, we used splenocytes. As shown in Fig. 6A and B, a much smaller proportion of total SI-IEL lymphocytes was able to mediate IL-7-dependent STAT5 phosphorylation compared with total splenocytes (solid black and grey lines, respectively). The relative percentage of IL-7Rα/CD127$^+$ cells almost completely corresponded to the frequency of IL-7-treated STAT5 (Y694)$^+$ cells both in the SI-IEL (5±0.8 and 4.5±0.3, respectively) and in the spleen (16.0±0.7 and 20.1±1.3, respectively). Thus, these results confirm that the cells from the spleen as well as SI-IEL express functional receptor for IL-7, but the proportion of TCRαβ$^+$ CD8αβ$^+$ SI-IEL cells that express functional IL-7Rα/CD127 is miniscule and much smaller than in the spleen (Fig. 6B).

Expression of CD11a, CD11c NKG2D, and CD8αα homodimer on gut memory CD8$^+$ T cells requires IL-15

The prevalence during both acute and memory phases after immunization of MVA-specific CTLs lacking IL-7 receptor in the SI-IEL from both WT and even IL-15 KO animals suggested that during the memory phase, SI-IEL lymphocytes might survive by using alternative pathways without IL-7 or IL-15 signaling. Until

Figure 2. Functional avidity of B8R-specific CD8$^+$ T cells for IFN-γ production in the spleen and SI-IEL of C57BL/6 and IL-15 KO mice. (A–C) Splenic (upper panels) and SI-IEL (lower panels) populations were isolated from WT and IL-15 KO animals 5 days, 13 days, and 2 months after infection, and treated with B8R$_{20–27}$ peptide to perform ELISpot for IFN-γ production. B8R$_{20–27}$ peptide (1 μM, 100 pM, and 1 pM) was added directly to the tissue leukocyte populations, which were placed in triplicates (*Materials and methods*). (A) Absolute numbers of responders/10^6 total leucocytes to different peptide concentrations are shown. (B) Normalized data calculated against the response to 1 μM of peptide are shown. Dotted line corresponds to 50%max.response (max.response = 1 μM peptide concentration). (C) Percentage of responders out of total response is shown [53]. Data are the mean+SD of three animals per group. Experiments were performed twice with comparable results.

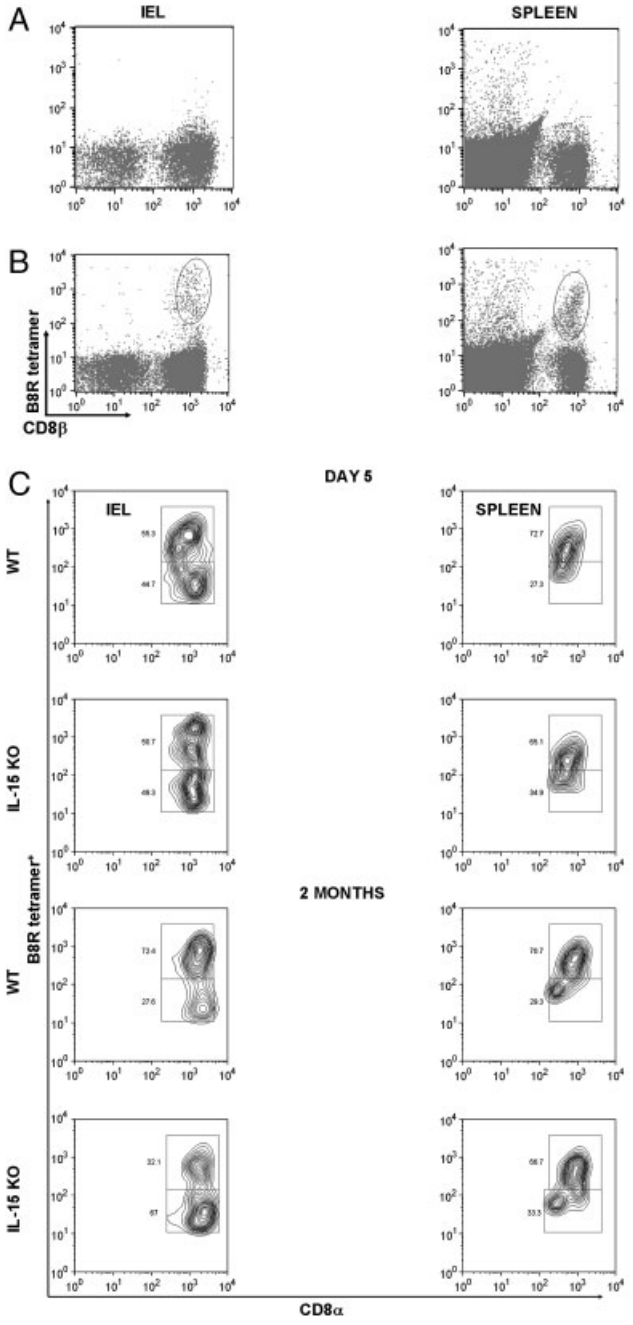


Figure 3. TCR expression level on B8R-specific CTLs from SI-IEL and spleen isolated during acute and memory phase after i.r. MVA-immunization. Cells from WT and IL-15 KO mice were isolated during acute and memory phases as described in the *Materials and methods*, and stained with PE-conjugated $B8R_{20-27}/H2\text{-}K^b$ tetramer. Both splenic and SI-IEL cells were costained with anti-CD8α and CD8β (spleen) mAbs. Then, B8R tetramer$^+$ CD8β$^+$-gated CTLs were plotted against CD8α. The level of B8R tetramer staining is shown. (A) The control staining of lymphocytes from naïve animals was performed and a very low background was detected. (B) Originally, cells were gated on B8R tetramer$^+$ CD8β$^+$ T cells as exemplified on WT cells, day 5 p.i. (C) TCR level expression on B8R tetramer$^+$ CTLs from spleen and SI-IEL is shown on density plots and presented as percentage out of total B8R tetramer$^+$ CTLs. Data from a representative staining are shown. Comparable results were found in three mice studied.

recently, several attempts have been taken to phenotype memory CD8$^+$ T cells in the SI-IEL in WT as well as TCR transgenic mice [34, 43]. Based on these reports, we decided to study the expression of plausible candidates (β2-integrins CD11a and CD11c, and NKG2D) that could be associated with the gut specific survival of memory CTLs.

At 5 days after immunization, B8R tetramer$^+$ CD8$^+$ T cells in the SI-IEL from WT and, to a lesser extent, in IL-15 KO animals, abundantly expressed CD11a integrin (Fig. 7, upper panel; Table 1), and a few of them were CD11a$^+$CD11c$^+$ (7.90 ± 0.49 vs 17.50 ± 1.04 for WT vs IL-15 KO, $p = 0.00434$; whereas the majority were CD11a$^+$CD11c$^-$: 71.00 ± 3.51 vs 37.63 ± 1.45, $p = 0.00487$). The same pattern was observed for the MVA-specific CTLs from the LP (Table 1 and data not shown). In sharp contrast, B8R tetramer$^+$ CD8$^+$ T cells from the spleen in both mouse strains revealed an inverse pattern of expression, where the CD11a$^+$CD11c$^+$ subset was dominant, and CD11a$^+$ CD11c$^-$ was subdominant. It is interesting that 2 months after immunization, the majority of B8R tetramer$^+$ CD8$^+$ T cells in the SI-IEL lost surface CD11a expression, and few of them were positive for CD11c both in WT and in IL-15 KO mice (Fig. 7 and Table 1). In contrast, in the spleen the vast majority of these cells was expressing CD11a$^+$ (>80%) being either single-positive (CD11a$^+$) or double-positive (CD11a$^+$, CD11c$^+$) (Table 1). Thus, although at early stages both CD11a and CD11c may be induced on the SI-IEL population, during the memory phase they were downregulated irrespective of the presence or absence of IL-15 in vivo, and thus these markers could not be associated with their survival.

Apart from the β2-integrins, the NKG2D molecule is also known to transduce costimulatory and/or prosurvival signals after binding to a number of its ligands expressed by the gut epithelial cells. At the acute stage, few B8R tetramer$^+$ CD8$^+$ T cells in the SI-IEL and SI-LP from the WT mice expressed NKG2D, whereas in IL-15 KO mice this frequency was modestly increased (Fig. 8, upper panel; Table 1, compare WT vs IL-15 KO: 1.40 ± 0.21 vs 8.40 ± 0.70, $p = 0.00623$). In contrast, in the spleen from both mouse strains, most antigen-specific CD8$^+$ T cells were positive for NKG2D (Table 1), although in IL-15 KO mice their frequency was slightly lower. When the cells were checked during the memory phase, we saw that in all tissues (SI-IEL, spleen, and SI-LP) from WT mice, especially in the gut, the relative percentage of NKG2D$^+$ tetramer$^+$ CD8$^+$ T cells had increased compared with the acute phase (Fig. 8, lower panel; Table 1) but was still much smaller than that found in the spleen. In contrast, in IL-15 KO animals, the percentage of NKG2D$^+$ cells did not change with time.

Along with that, previously it was shown that some of the memory CTLs may express CD8αα homodimer, which may ligate an MHC-class I-like molecule, thymus leukemia (TL) antigen, known to be abundantly expressed on the basolateral membrane of mouse intestinal epithelium [44, 45]. When we checked appearance of CD8αα homodimer within the B8R tetramer$^+$ CD8$^+$ T cells from the SI-IEL, we found that whereas 5 days after infection SI-IEL cells expressed CD8αα homodimer in both mouse

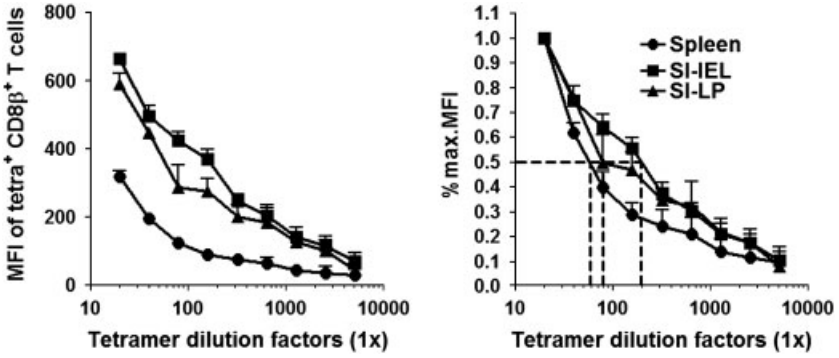


Figure 4. Small intestinal B8R-specific IEL lymphocytes have high physical avidity for vaccinia virus $B8R_{20–27}$ epitope. Cells from spleen (circles), SI-IEL (squares), and SI-LP (triangles) were isolated from WT mice 5 days after i.r. immunization with 10^7 pfu MVA, and stained with anti-CD8β Abs and B8R tetramer at the indicated dilutions. Left, mean fluorescence intensities (MFI) of $CD8\beta^+$ T cells representative of three mice per tissue from two comparable experiments are shown. Right, normalized MFI of $CD8\beta^+$ T cells presented as a percentage out of max.MFI level.

strains (Fig. 9, upper panel), spleen cells did not express it at all. Interestingly, the frequency of CD8αα homodimer-positive cells in IL-15 KO mice was approximately three-fold higher compared with WT animals. During the memory phase, no changes in relative percentage of $CD8\alpha\alpha^+$ cells were seen in either mouse strains when compared with day 5, nor were positive cells found in the spleens.

Thus, memory B8R $tetramer^+$ $CD8^+$ T cells residing in the SI-IEL compartment in both WT and IL-15 KO animals modestly express CD127, CD11c, and NKG2D, but not CD11a. The relative frequency of CD8αα homodimer-positive cells in the SI-IEL did not change with time, and has mouse-strain-specific features. In contrast, in both WT and IL-15 KO mice, the vast majority of memory B8R $tetramer^+$ $CD8^+$ T cells from the spleen expressed CD11a and NKG2D, and some of them were positive for CD11c.

CD103 may also be involved in the maintenance of memory cells at mucosal sites. However, a study by Masopust et al. concluded that memory CTL from the IEL are positive for CCR9, and less so for CD103 [46].

Altogether, the data obtained allow us to conclude that although memory CTLs can be found in the SI-IEL, which factors contribute to their survival in the absence of signaling via IL-7, IL-15, CD11a and/or CD11c integrins, NKG2D, and CD8αα homodimer remains unknown.

Discussion

The development of protective immunity is intrinsically connected to the route of infection [46–50]. However, generation and survival of antigen-specific $CD8^+$ T cells after i.r. immunization is not well understood [10, 51, 52]. In this study, we continued to characterize: (i) the kinetics of $CD8^+$ T-cell responses in the gut after i.r. immunization, (2) the dependency of functional activity and survival of antigen-specific $CD8^+$ T cells from the SI-IEL compartment on IL-7 and IL-15 signals.

We found that the immune response in the spleen and SI-IEL compartment from WT mice was characterized by induction of B8R $tetramer^+$ $CD8^+$ T cells that paralleled each other during both expansion and contraction phases (Fig. 1). Response against the immunodominant vaccinia virus $B8R_{20–27}$ epitope (also present in the MVA), may elicit as high as 12% total splenic CTLs or up to 10×10^6 vaccinia-virus-specific CTLs [36]. These data perfectly fit to our results (Fig. 1). However, we saw that the number of IFN-γ-producing cells (Fig. 2) was much lower for the SI-IEL compartment despite the substantial presence of B8R $tetramer^+$ $CD8^+$ T cells. As was shown here and in our recent study [53], memory $B8R_{20–27}$-specific $CD8^+$ T cells in the SI-IEL compartment modestly produced IFN-γ, but with low avidity, whereas in the spleen they were characterized by populations with low-, middle-, and high-functional avidity. In addition, $B8R_{20–27}$-specific $CD8^+$ T cells from the LP were previously shown to be uniquely enriched in the high-avidity cells [53]. Thus, after mucosal immunization, $CD8^+$ T cells with different functional avidity were distributed unequally in different mucosal and systemic sites. As shown in the current study, even greater changes in functional activity were found in IL-15 KO mice, in which T cells tended to quickly loose their IFN-γ-producing capacity. Strikingly, in the SI-IEL compartment from IL-15 KO animals even at the peak of immune response a very modest response was detected (Fig. 2, day 5).

As functional avidity may be directly linked to the physical TCR avidity and high TCR expression level, we compared them for B8R $tetramer^+$ CTLs from the SI-IEL vs spleen. Interestingly, we found that the modest functional activity of $B8R_{20–27}$-specific $CD8^+$ T cells from the WT SI-IELs did not correspond tightly to the TCR expression level by the B8R $tetramer^+$ $CD8^+$ T cells (Fig. 3). In particular, although at the acute phase (day 5) the SI-IEL from the WT mice had a pronounced in vitro IFN-γ production, however, only half of them were characterized by high TCR level expression. In contrast, during the memory phase, although they had hardly detectable functional activity, still the vast majority of them expressed TCRs at high level that was even increased compared with the acute phase. In sharp contrast, although SI-IEL cells from the IL-15 KO animals had very low-functional activity, the frequency of B8R $tetramer^+$ $CD8^+$ T cells with high TCR expression was comparable with that found in the WT mice. Strikingly, during the memory-phase SI-IEL

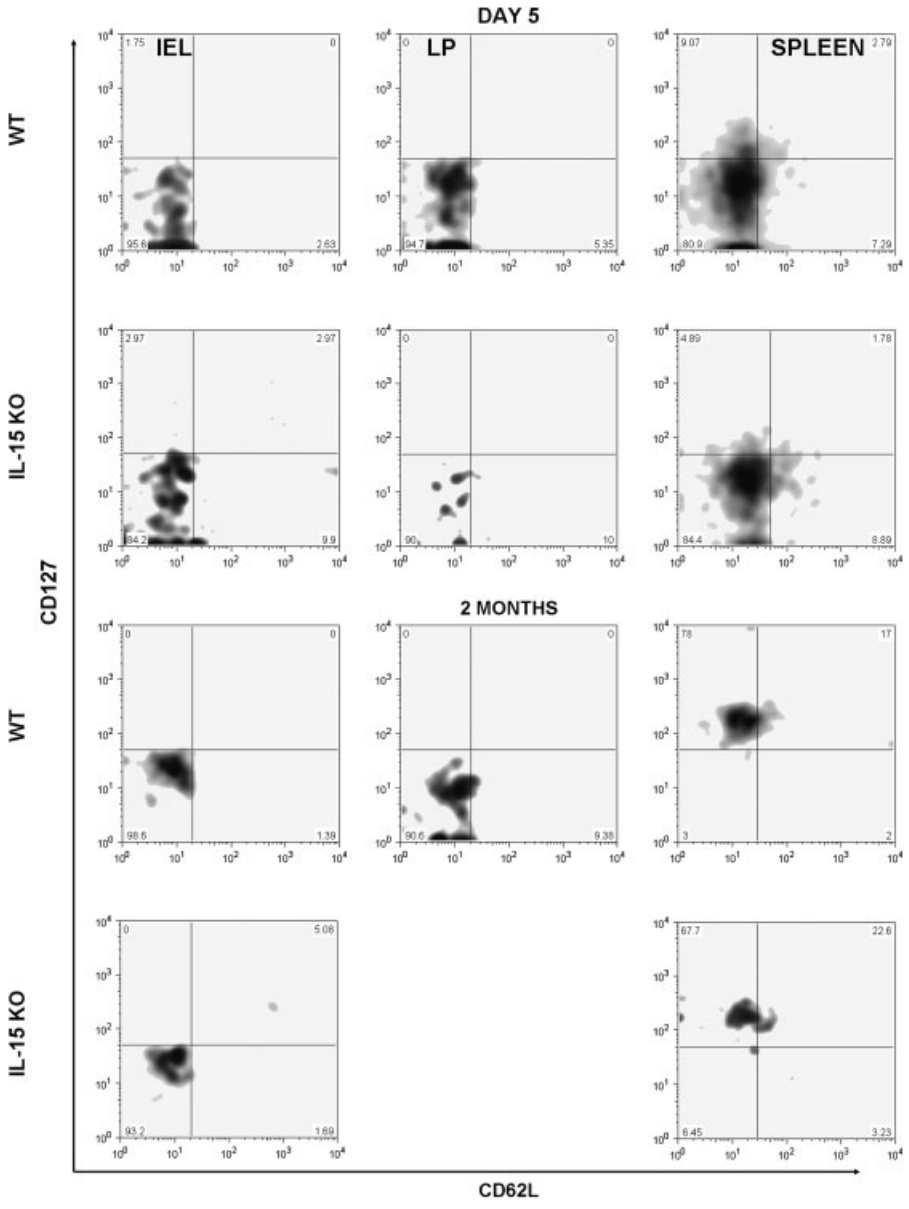


Figure 5. Memory B8R tetramer$^+$ CD8$^+$ T cells in SI-IEL from WT and IL-15 KO mice express CD127 at low levels. Lymphocytes from WT and IL-15 KO animals were isolated at 5 days and 2 months after i.r. immunization with MVA 10^7 pfu/mouse. Four-color flow cytometry was performed. Cells from IEL, LP, and spleen were stained with PE-conjugated B8R tetramer together with anti-CD8α mAbs. Then, cells were gated on tetramer$^+$ CD8$^+$ T cells, and further analyzed for CD127 and CD62L marker expression. Due to the paucity of cells isolated from the LP of IL-15 KO mice at 2 months p.i., these data are not presented. One out of the two representative experiments is shown.

lymphocytes from IL-15 KO mice revealed an inverse proportion of high-to-low TCR expression subsets, where the latter comprised up to 67% compared with 27% for the WT mice. However, due to the fact that the physical avidity of B8R tetramer$^+$ CTL in the SI-IEL was even higher compared with the spleen (Fig. 4; WT mice), we may conclude that assessment of both functional and physical avidities may be of special importance for antigen-specific CD8$^+$ T cells residing in nonlymphoid tissues. Altogether, IL-15 turned out to participate not only in expansion of memory CD8$^+$ T-cell precursors (Fig. 1), but also in the development of their full functional activity, which was evident during the acute phase of infection (decreased number of total and high-avidity responders). In particular, the lack of IL-15 in vivo mostly affected functional activity/development of middle-to-high avidity CD8$^+$ T cells and had less impact on low-avidity T cells, consistent with our earlier finding that IL-15 promotes induction of high-avidity CTLs [54].

A special role for IL-7 and IL-15 for survival and homeostatic proliferation of memory CTLs has been documented in numerous studies [18–23, 25, 35, 55, 56]. As we were able to detect memory CTLs in the SI-IEL from the IL-15 KO animals, we decided to check if their survival was linked to CD127 expression and IL-7 signaling. We showed by flow cytometry that during the acute phase the vast majority of B8R tetramer$^+$ CD8$^+$ T cells isolated from SI-IEL both in the IL-15 KO and in the WT mice were negative for CD127 expression (Fig. 5), consistent with the data published elsewhere [34]. Additionally, we found that the memory CTLs from LP were negative for CD127 as well (Fig. 5), suggesting that some alternative prosurvival factor(s) specific to the epithelial layer of the small intestine might exist. Such

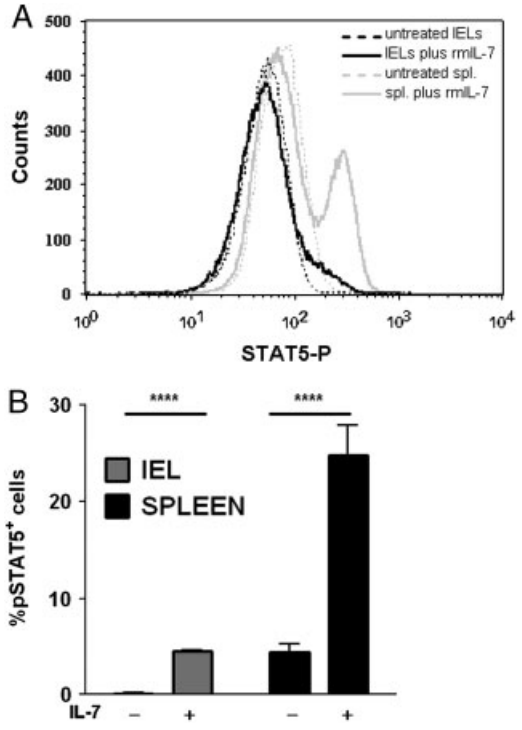


Figure 6. Residual CD127/IL-7Rα expression on naïve CD8$^+$ T cells from WT SI-IEL induces IL-7-specific STAT5 phosphorylation. Lymphocytes from naïve WT animals were isolated as described in the *Materials and methods*. Then cells were either treated with recombinant murine IL-7 in DPBS/1%BSA (rmIL-7; 200 ng/mL, 20 min at 37°C) or kept untreated. (A) Histograms for intracellular phospho-STAT5 (Y694) expression on total splenoctes and SI-IEL cells before and after adding rmIL-7 representative of two mice per tissue from two comparable experiments are shown. (B) Relative percentages of IL-7-induced intracellular STAT5 (Y694)$^+$ total splenic and IEL populations from naïve WT mice. Isolated cells were treated in vitro with IL-7 (*Materials and methods* section). Data show representative example from one out of the two experiments. Error bars indicate mean+SD. Data were analyzed by Mann–Whitney test, ****$p<0.0001$.

assumption is substantiated by the finding that even in naïve C57BL/6 animals some nonspecific "memory-like" TCRβ$^+$ CD8αβ$^+$ T cells may be detected in the SI-IEL, which express CD127/IL-7Rα at very low levels [57]. Here, we proved that despite the low frequency of CD127$^+$ SI-IEL cells, these few cells were capable of transducing signals from IL-7 in vitro, thus confirming the functionality of CD127/IL-7Rα on the gut CD8αβ$^+$ T cells from the IEL (Fig. 6). However, the vast majority of memory CD8$^+$ T cells within intestinal epithelium were found to survive independently of CD127 expression, thus ruling out IL-7 as a necessary survival factor in this tissue. In line with this, previously it was shown that other cytokines, transducing signals via the common γ_c-chain/CD132, may influence SI-IEL development. In particular, the lack of signaling via IL-2, IL-4, IL-7, IL-9, IL-15 affected only TCRαβ$^+$CD8αα$^+$ and TCRγδ$^+$CD8αα$^+$ IEL subsets [58–62], seemingly having no major impact on TCRαβ$^+$ CD8αβ$^+$ IEL cells. On the other hand, selective overexpression of either IL-7 [63] or IL-15 [64] by the gut epithelium did not result in increased frequency of CD8αβ$^+$ T cells in the SI-IEL, possibly because TCRαβ$^+$ SI-IEL cells in naïve mice express few if any receptors for IL-2, IL-4, IL-7, IL-15 [65]. Similarly, splenic TCRαβ$^+$ CD8αβ$^+$ T cells in naïve mice do not entirely depend on the IL-15 signaling as they still contain CD44^{lo}CD122^{lo} precursors of "conventional" TCRαβ$^+$CD8αβ$^+$ T cells that were described in IL-15 KO mice, thus, arguing for their IL-15-independent maintenance [66]. In fact, additional molecules might have had even bigger impact on their prosurvival program (e.g. *itk* and IRF-1 etc.; [67, 68]). Based on this, we reasoned that in contrast to the role for IL-7 and IL-15 documented in peripheral lymphoid tissues, some other cytokine/cell-contact factors might be involved in long-term maintenance of memory CD8αβ$^+$ T cells in nonlymphoid tissues (intestinal epithelium; [69]). Although some memory intraepithelial CD8$^+$ T cells can survive in the small intestine in IL-15 KO mice after mucosal vaccination with MVA, these mice will be significantly less protected against mucosal challenge with WR virulent vaccinia virus compared with WT mice. Some studies demonstrated already that IL-15 plays an important role in protection, especially in early activation of memory CD8$^+$ CTL after reinfection [70].

As was shown above, not only survival but also functional activity of the gut memory CTLs was compromised in the IL-15 KO mice. It is known that the local tissue microenvironment (cytokine, chemokine, TLR–ligands, cell–cell contacts) can significantly influence the pathway of antigen presentation that elicits proliferation and differentiation of CD8$^+$ T cells, affecting cytokine profile and memory responses [71–76]. Inclusion of cytokines and other biological adjuvants into vaccine formulas can facilitate skewing immune responses both quantitatively and qualitatively in desirable directions [3, 76]. As a result of such strategy, previously we found a synergistic effect of cytokines and mucosal adjuvants for the induction of mucosal and systemic CD8$^+$ T-cell responses together with protective immunity against mucosal viral challenge [12, 50]. Moreover, such protection was mediated by CD8$^+$ T cells and was associated with their presence at the mucosal site [77, 78]. Our studies provided a better understanding of the effects that local microenvironment (cytokines, cell contact signals) might have on generation of memory CTL, particularly at mucosal sites. An important factor for the development of effective memory CD8$^+$ T cells is the presence of tissue-specific prosurvival factors. Our current study demonstrated that the mechanisms of CTL survival in mucosal tissues (small intestinal epithelium) may differ from those present in peripheral lymphoid tissues.

As memory CTL which reside in the gut SI-IEL are nonmigrating cells [46] and cannot recirculate with the pool of systemic memory CTLs, they are unable to obtain prosurvival signals produced within lymphoid tissues, e.g. IL-7 and IL-15. Thus, it is important to pinpoint what additional factors may contribute to their survival. Apart from different signaling molecules involved in this process, it is also plausible that the gut memory CTLs might merely depend on the abundancy of nutrient factors supplied from food, in particular, glucose. It is worth mentioning that in response to high-glucose concentration some gut epithelial cell types are able to produce IL-10 [79], that may also contribute to IEL-cell survival especially when they are deprived of growth factors [80].

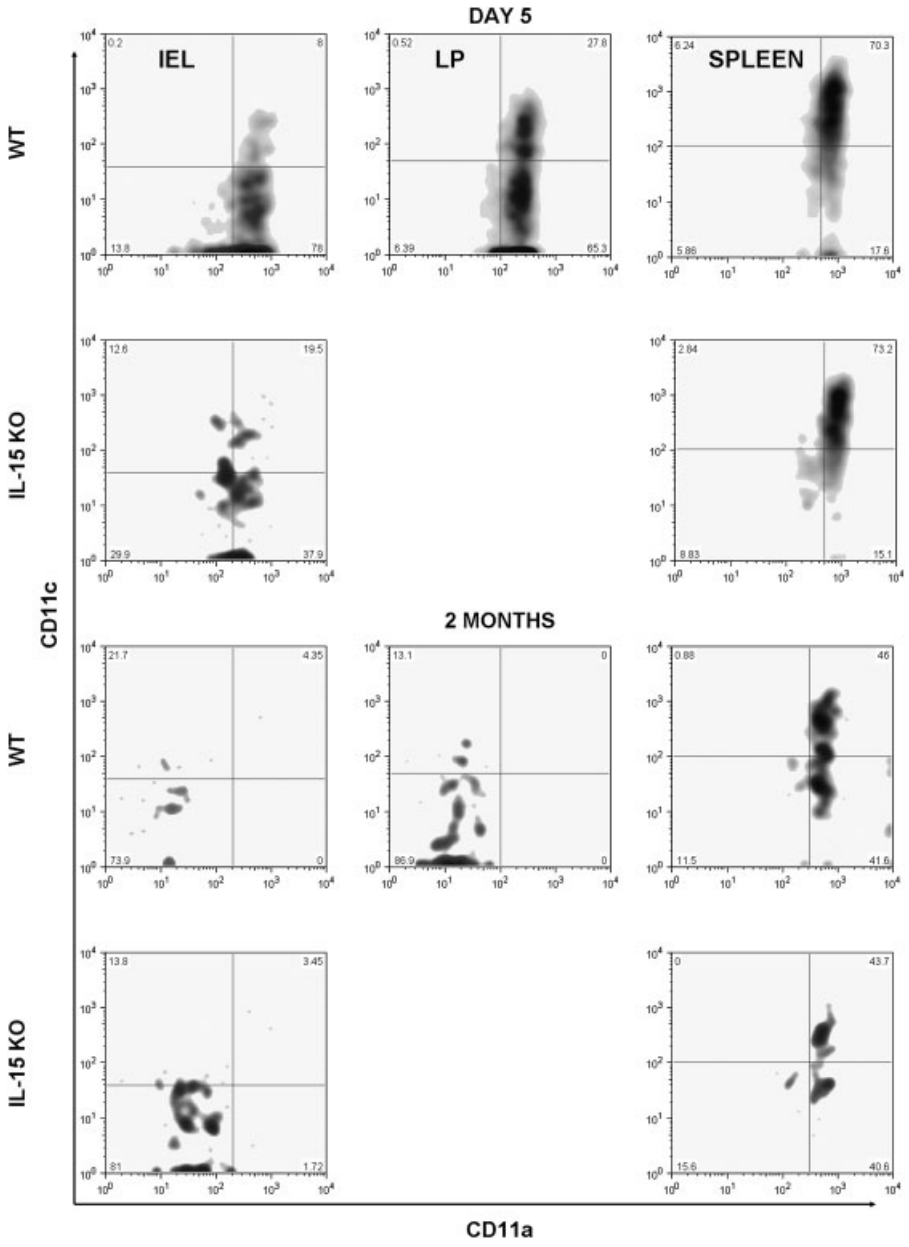


Figure 7. Memory B8R tetramer$^+$ CD8$^+$ T cells in the SI-IEL from WT and IL-15 KO mice express CD11a and CD11c at low levels. Lymphocytes from WT and IL-15 KO animals were isolated at 5 days and 2 months after i.r. immunization with MVA 10^7 pfu/mouse. Four-color flow cytometry was performed. Cells from IEL, LP, and spleen were stained with PE-conjugated B8R tetramer together with anti-CD8α mAbs. Then, cells were gated on tetramer$^+$ CD8$^+$ T cells, and further analyzed for CD11a and CD11c marker expression. Due to the paucity of isolated cells from the LP lymphocytes of IL-15 KO mice at 5 days and 2 months p.i., these data are not presented. One out of the two representative experiments is shown.

Despite the fact that currently no cytokine/cell contact molecule(s) responsible for the survival of TCRαβ$^+$CD8αβ$^+$ SI-IEL cells has been identified, still it is plausible to deduce that somehow they may integrate signals which elicit constitutive expression of antiapoptotic factors including cIAP-1, XIAP, bcl-x_L, and Mcl-1 as it was found in naïve animals [81]. In fact, antigen-specific memory TCRαβ$^+$CD8αβ$^+$ from both spleen and SI-IEL do express bcl-2 at high levels [34].

Besides classic surface markers that help to distinguish between naïve and memory CD8$^+$ T cells [31], little is known about the expression of the activating NKG2D molecule on the CD8$^+$ T cells in the SI-IEL. As a number of its ligands are expressed by the gut epithelium, such ligation might be involved in providing prosurvival signals to the antigen-specific CTLs [82]. Previously, NKG2D expression was described only on a subset of splenic memory CD8$^+$ T cells [83], which transduces activating signals by complexing with either DAP10, the only adaptor protein expressed on CD8$^+$ T cells, and/or with DAP12 [83]. DAP10 under certain circumstances is considered to transduce costimulatory signals in CD8$^+$ T cells, whereas DAP12 has direct stimulatory activity in NK

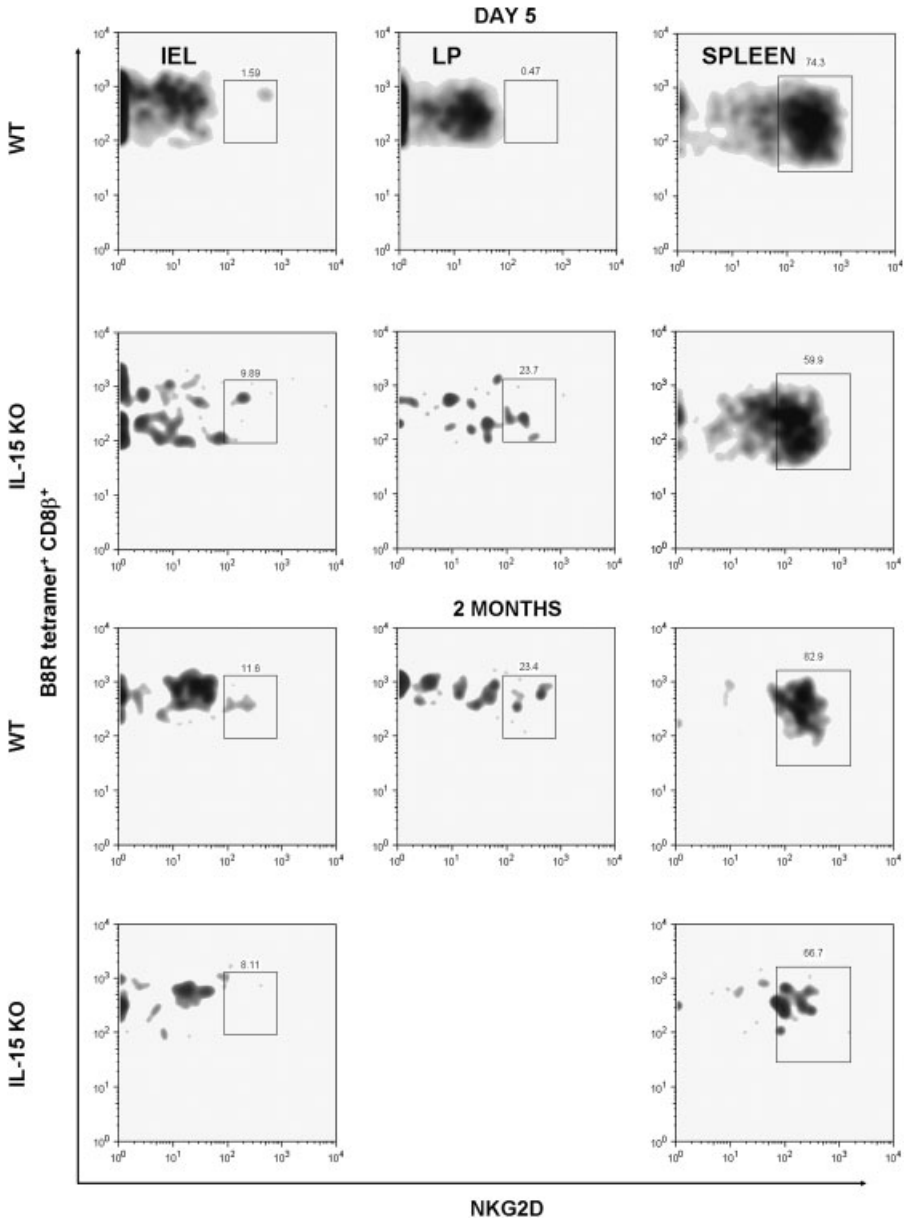


Figure 8. Memory B8R tetramer$^+$ CD8$^+$ T cells in SI-IEL from WT and IL-15 KO mice express NKG2D at low levels. Lymphocytes from WT and IL-15 KO animals were isolated at 5 days and 2 months after i.r. immunization with MVA 10^7 pfu/mouse. Triple-color flow cytometry was performed. Cells from IEL, LP, and spleen were stained with PE-conjugated B8R tetramer together with anti-CD8β mAbs. Then, cells were gated on tetramer$^+$ CD8$^+$ T cells, and further analyzed for NKG2D marker expression. Due to the paucity of isolated cells from the LP of IL-15 KO mice at 2 months p.i., these data are not presented. One out of the two representative experiments is shown.

cells. CD8$^+$ T cells normally do not express DAP12; however, during in vitro stimulation with IL-2, at least CD8$^+$CD44^{lo} T cells do express DAP12 [84]. Importantly, in naïve mice neither TCRαβ$^+$ nor TCRγδ$^+$ SI-IEL cells express NKG2D, i.e. that only cytokine-specific and/or antigen-specific activation may be responsible for NKG2D expression [65, 83]. Thus, we speculated that during antigen-specific stimulation, signaling via NKG2D in CD8$^+$ SI-IEL cells might substitute for the lack of prosurvival IL-7 and IL-15. We found that the frequency of NKG2D$^+$ cells was increased in IEL (and LP) from IL-15 KO mice at memory phase compared with acute phase (Fig. 8). We may suppose that at least, in part, NKG2D expression may be associated with CTL survival both in the SI-IEL and in the SI-LP compartments.

Additionally, we investigated the expression on B8R tetramer$^+$ CD8$^+$ T cells of CD8αα homodimer (Fig. 9), which binds to the TL antigen, known to be abundantly expressed on the basolateral membrane of mouse intestinal epithelium [45]. Due to the fact that the CD8αα homodimer was shown to be transiently expressed on activated CTLs, we thought it might contribute to the survival and differentiation of CD8 memory

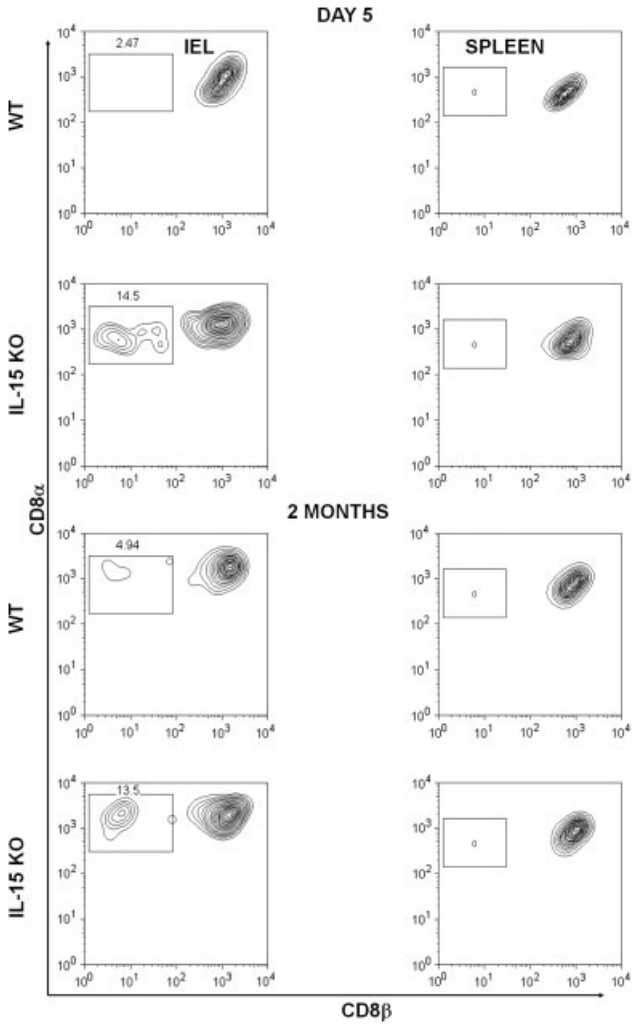


Figure 9. Memory B8R tetramer$^+$ CD8$^+$ T cells in SI-IEL from IL-15 KO mice contain a higher frequency of CD8αα-homodimer$^+$ cells than WT mice. Splenocytes and SI-IEL cells from WT and IL-15 KO animals were isolated at 5 days and 2 months after i.r. immunization with MVA 10^7 pfu/mouse. Triple-color flow cytometry was performed. Cells were stained with PE-conjugated B8R tetramer together with anti-CD8α and anti-CD8β mAbs. Then, cells were gated on tetramer$^+$ CD8$^+$ T cells, and further analyzed for the expression of CD8αα-homodimer. One out of the two representative experiments is shown.

T-cell precursors. Although the interaction of CD8αα homodimer with TL antigen may be of some importance in antigen-experienced CTL, however, it was not involved in survival of nonspecific CD8αα$^+$ TCRαβ$^+$ and CD8αα$^+$ TCRγδ$^+$ IEL cells [85]. In our study, we saw that during both acute and memory phases the frequency of CD8αα$^+$ B8R tetramer$^+$ CTLs was increased only in the SI-IEL from IL-15 KO but not from WT mice (Fig. 9). Collectively, around 16 and 21% of total memory CTL in the SI-IEL from WT and IL-15 KO mice were positive for either NKG2D or CD8αα, which could bind to the gut epithelium-specific ligands (Figs. 8 and 9). These results raise the possibility that somehow memory CTL from SI-IEL compartments were shifting their programs toward higher frequencies of NKG2D$^+$ and CD8αα$^+$ that might be associated with their survival in the absence of IL-7 and IL-15 signaling.

Altogether, our findings emphasize that the local tissue environment in the intestinal mucosa is unique, and shapes and organizes very specific populations of different protective lymphocytes and allows CD8$^+$ T-cell survival without IL-7 or IL-15 signaling. Furthermore, an environmental condition in the intestinal epithelium does not promote IFN-γ production by SI-IEL cells long after vaccination, whereas in the adjacent LP they are enriched with high avidity IFN-γ-producing CD8$^+$ CTLs [53]. What factors contribute to the development of prosurvival conditions that facilitate the maintenance of antigen-specific T cells in the gut mucosa and their unique pattern of functional activity remains to be investigated.

Materials and methods

Mice

Female C57BL/6 mice were purchased from the Frederick Cancer Research Center (Frederick, MD). IL-15 KO mice on the H2-K^b C57BL/6 genetic background were purchased from Jackson Laboratories.

Viruses and immunization protocol

MVA, developed by A. Mayr, University of Munich, Germany [86], was propagated and titrated in chicken embryo fibroblast cells. This virus was a gift of Dr. Bernard Moss, Dr. Patricia Earl, and Dr. Linda Wyatt (NIAID). For immunization, mice were injected i.r. with 10^7 pfu MVA as described previously [87].

Cell purification: Isolation of SI-IEL and LP lymphocytes, and lymphocytes from the spleen

Spleens were aseptically removed and single-cell suspensions were prepared by gentle passage of the tissue through sterile screens. SI-IEL cells and LP lymphocytes from mice were isolated as described previously with a minor modification [52].

Flow cytometry

Flow cytometry was performed by using a FACScalibur, and data were further analyzed with CellQuest software (BD Biosciences) [88]. The following mAbs were used: FITC-conjugated anti-CD62L (clone 53–5.8; obtained from BD Biosciences), Allophycocyanin (APC)-conjugated anti-CD127 (clone A7R34); APC-conjugated anti-NKG2D (clone CX5), APC-conjugated anti-CD8β (clone CT-CD8b; all obtained from eBiosciences). Soluble tetrameric B8R$_{20–27}$/H2-K^b complex was conjugated to PE-labeled streptavidin (made by the NIH Tetramer Core Facility).

IL-7 bioassay

Lymphocytes from naïve WT animals were either treated with recombinant murine IL-7 (rmIL-7; PeproTech) in DPBS/1% BSA or kept untreated, and incubated for 20 min at 37°C. To verify rmIL-7-specific signaling, cells were stained for intracellular phosphorylated STAT5 protein with antiphospho STAT5 (Y694) Alexa Fluor®-488 mAbs according to the manufacturer (clone 47; BD™ PhosFlow) and studied by flow cytometry. Additionally, cells were stained with mAbs against APC-conjugated anti-CD127 (clone A7R34; eBiosciences).

IFN-γ ELISpot

IFN-γ ELISpot was performed as described previously [89]. Cells were plated in triplicates in a volume of 200 μL, 0.2×10^6/well, to which we directly added titrated the amounts of $B8R_{20–27}$ peptide (TSYKFESV), which is an immunodominant poxvirus CTL epitope restricted by H-$2K^b$ [36]. Spots were counted in AID ELISpot Reader (Cell Technology). Data are presented as the mean and SEM of three animals per interval. These experiments were performed twice with comparable results.

Statistical analysis

Statistical comparisons were assessed by unpaired Student's *t*-test and Mann–Whitney test by using GraphPad Prism version 5.00, GraphPad Software (San Diego, CA; www.graphpad.com).

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **B8R tetramer:** $B8R_{20–27}$/H2-K^b tetramer · **CM:** central memory · **EM:** effector memory · **IEL:** intraepithelial lymphocyte · **i.r.:** intrarectally · **LP:** lamina propria · **MVA:** Modified Vaccinia Ankara · **p.i.:** postinfection · **SI-IE:** small intestinal intraepithelial · **SI-LP:** small intestinal lamina propria · **TL:** thymus leukemia

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