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Cutting Edge: Caspase-11 limits the response of CD8+ T cells to low abundance and low affinity antigens

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Abstract

Inflammatory caspases, including caspase-11, are upregulated in CD8$^+$ T cells after antigen-specific activation, but little is known about their function in T cells. We report that caspase-11-deficient (*Casp11*$^{-/-}$) T cells proliferated more readily in response to low affinity and low abundance ligands both *in vitro* and *in vivo* due to an increased ability to signal through the TCR. In addition to increased numbers, *Casp11*$^{-/-}$ T cells had enhanced effector function compared to wild-type cells including increased production of IL-2 and reduced expression of CD62L. *Casp11*$^{-/-}$ T cells specific for endogenous antigens were more readily deleted than wild-type cells. These data indicate caspase-11 negatively regulates TCR signaling possibly through its ability to regulate actin polymerization, and inhibiting its activity could enhance the expansion and function of low affinity T cells.

Introduction

Caspase-11 in mice and caspases-4 and -5 in humans are members of the family of inflammatory caspases, that also includes caspases-1 and -12(1). Unlike other caspases, caspase-11 is expressed at low levels in resting cells and upregulated upon activation via a type I interferon-dependent process(2). Caspase-11 can bind directly to intracellular LPS resulting in caspase-11 processing and activation(3), leading to downstream events that can include caspase-1 activation, cell death, and inflammatory cytokine processing and release(1). Caspase-11 can also act in its proform to regulate actin dynamics; caspase-11 promotes actin depolymerization by facilitiating interactions between actin interacting protein 1 (Aip1), cofilin, and F-actin(4). Accordingly, caspase-11-deficient (*Casp11*$^{-/-}$) cells have altered migration and display reduced fusion of lysosomes to pathogen containing vacuoles(4, 5).

Surprisingly, activation-dependent expression of caspase-11 is also observed in CD4$^+$ and CD8$^+$ T cells; both effector and memory T cells have increased expression of caspase-11 when compared to naïve cells(6–8). Caspase-11 processing was not observed in activated T cells, but T cell migration was affected, with *Casp11*$^{-/-}$ cells migrating less efficiently into lymphoid tissues(4). Modulation of actin polymerization by caspase-11 could regulate additional aspects of T cell biology, including TCR signaling(9). The strength of signals

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received through the TCR can have affects on the phenotype and function of T cells after activation. Activation of $CD8^+$ T cells with high affinity peptides results in increased expansion and effector function compared to stimulation with lower affinity peptides(10). The strength of TCR stimulation can also affect the phenotype of $CD4^+$ T cells, with $CD4^+$ T cells receiving higher levels of stimulation preferentially developing into T_{FH} cells(11, 12) and low concentrations of high affinity peptide favoring FoxP3 expression(13).

We have addressed the role of caspase-11 in the activation and function of $CD8^+$ T cells and found that *Casp11*$^{-/-}$ cells proliferate more readily in response to suboptimal levels of TCR stimulation, leading to a larger effector and memory pool and increased effector function in response to both low abundance and low affinity ligands *in vivo*. However, in the presence of low concentrations of self antigen, the increased sensitivity of *Casp11*$^{-/-}$ cells results in more rapid deletion in tissues in which the antigen is expressed. These data indicate that in addition to promoting cell death and inflammatory cytokine production, caspase-11 acts as a negative regulator of TCR signaling and limits the expansion and function of T cells in response to low abundance or low affinity TCR ligands.

Materials and Methods

Mice and immunizations

C57BL/6 and Nur77-GFP mice were purchased from Jackson Laboratory. Nur77-GFP, OT-I, iFABP-tOVA 232-6(14), and *Caspase-11*$^{-/-}$(15) mice were maintained at the University of Washington and used in accordance with Institutional Animal Care and Use Committee guidelines.

Mice received 1–2x10^4 naïve OT-I T cells and were immunized with 2x10^3 LM-OVA, 150μg ovalbumin, or 1–2x10^6 DCs pulsed with LPS and 1μM N4, Q4, or T4 peptide as previously described(16). Mice were pulsed with 1mg BrdU i.p. 2.5 hours before sacrifice. OT-I T cells were stimulated *ex vivo* with peptide for 4 hours in the presence of Brefeldin A prior to intracellular cytokine staining.

Flow cytometry and cell sorting

Cells were stained with the indicated antibodies (BD Biosciences and eBioscience) or Alexa Fluor 647 phalloidin (Life Technologies), flow cytometry was performed on a FACSCanto (BD), and data were analyzed using FlowJo software (TreeStar). For cell sorting, thymocytes were stained with CD4, CD8α, and TCRβ, and effector OT-I T cells were stained using CD8 and CD45.1. Cells were sorted on a FACS Aria.

RT-PCR

RNA was isolated using a Qiagen RNeasy kit per manufacturers instructions. Quantatative RT-PCR was performed using the Quantitect SYBR green RT-PCR kit (Qiagen) and the following primers: casp11F: 5′-CCTGAAGAGTTCACAAGGCTT-3′; casp11R: 5′-CCTTTCGTGTACGGCCATTG-3′; actbF: 5′-GGCTGTATTCCCCTCCATCG-3′, actbR: 5′-CCAGTTGGTAACAATGCCATGT-3′.

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In vitro stimulations

Splenocytes were pulsed with 1nM-1μM N4, Q4, or T4 peptide or no peptide at 37°C for 40 minutes, then washed thoroughly with media. $5x10^4$ splenocytes were mixed with $1–2x10^4$ naïve OT-I T cells. T cells were labeled with 2μM CFSE (Invitrogen) where indicated.

Statistical analysis

All graphs depict mean ± SD. Two-tailed Student's *t* test was used to determine statistical significance.

Results

Caspase-11 limits CD8+ T cell expansion after protein immunization

Caspase-11 is upregulated in a variety of cell types undergoing activation, including $CD8^+$ T cells(6). We examined the levels of caspase-11 mRNA during development of $CD8^+$ T cells by sorting double negative, double positive, and CD8 single positive thymocytes as well as naïve $CD8^+$ peripheral T cells. *Casp11* was upregulated as thymocytes matured and further increased after cells entered the periphery (Fig. 1A). We also examined whether *casp11* expression was upregulated after antigen-dependent activation in the periphery. Naïve OT-I T cells, which express a TCR specific for the ovalbumin peptide SIINFEKL, were transferred into mice which were then infected with *Listeria monocytogenes* expressing ovalbumin (LM-OVA) or immunized with ovalbumin protein. Activated OT-I T cells were sorted and *casp11* expression analyzed. Ca*sp11* was upregulated an additonal 5-fold in activated OT-I T cells in response to both protein immunization and infection (Fig. 1B) compared to naïve cells.

To analyze the function of caspase-11 during the activation of $CD8^+$ T cells, caspase-11-sufficient (WT) and $Casp11^{-/-}$ OT-I T cells were transferred into mice followed by challenge with LM-OVA or ovalbumin protein. There were equal numbers of WT and $Casp11^{-/-}$ OT-I T cells 7 days after LM-OVA challenge (data not shown). However, after ovalbumin immunization, $Casp11^{-/-}$ cells were present at higher numbers than WT OT-I T cells as early as 4 days after immunization and this difference became more pronounced at 6 days (Fig. 1C). The increased number of $Casp11^{-/-}$ compared to WT cells was maintained at memory timepoints (Fig. 1C). Previous reports indicated that caspase-11 is able to regulate the migration of T cells(4), and we hypothesized that failure of $Casp11^{-/-}$ T cells to migrate out of the spleen might explain their increase relative to WT cells. However, we saw no increase in the ratio of WT to $Casp11^{-/-}$ $CD8^+$ T cells in the blood or inguinal LN suggesting WT cells were not exiting the spleen more readily than $Casp11^{-/-}$ cells (data not shown). These data suggest that Caspase-11 limits the accumulation of $CD8^+$ T cells in response to protein immunization but has little affect on their migration out of central lymphoid organs.

Caspase-11 limits CD8+ T cell accumulation in response to low abundance and low affinity ligands

We hypothesized the increased numbers of $Casp11^{-/-}$ OT-I T cells after protein immunization and not LM-OVA infection could be due to their enhanced response to

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ligands of low abundance. This was addressed *in vitro* by examining the expansion of WT and *Casp11*$^{-/-}$ OT-I T cells in response to splenocytes pulsed with varying concentrations of a high affinity ligand (SIINFEKL, N4). We found that in response to high concentrations of N4, WT and *Casp11*$^{-/-}$ OT-I T cells diluted CFSE similarly and WT cells showed a mild defect in accumulation (Fig. 2A, Fig. 2B). In response to low concentrations of N4, we saw an increased percentage of CFSE^{lo} cells in the *Casp11*$^{-/-}$ population compared to WT (Fig. 2A) and increased accumulation of *Casp11*$^{-/-}$ cells (Fig. 2B). When a lower affinity ligand (SIITFEKL, T4) was used, we also observed an increase in proliferation and outgrowth of *Casp11*$^{-/-}$ compared to WT cells (Fig. 2A, Fig. 2B). These data indicate that there is a cell intrinsic enhancement in the ability of *Casp11*$^{-/-}$ T cells to proliferate in response to low levels of antigenic stimulation, as WT and *Casp11*$^{-/-}$ cells have equal access to antigen, cytokines, and other factors in this *in vitro* setting.

Casp11$^{-/-}$ CD8$^+$ T cells primed with low affinity ligands in vivo display enhanced proliferation and effector function

Higher levels of TCR signaling can lead to differences in effector function, and we hypothesized the increased TCR sensitivity of *Casp11*$^{-/-}$ cells may result in enhanced effector function. To address this, mice were primed with DCs pulsed with N4 or T4 and the expansion of WT and *Casp11*$^{-/-}$ OT-I T cells was examined. T4-DC immunization was not sufficient to activate all transferred cells (data not shown); therefore, we used the intermediate affinity peptide SIIQFEKL (Q4) to pulse DCs. Consistent with what we observed *in vitro*, we saw increased accumulation of *Casp11*$^{-/-}$ cells compared to WT cells after both N4-DC and Q4-DC immunization and the difference was enhanced with lower affinity antigen (Fig. 3A). BrdU incorporation at 3 days after Q4-DC immunization revealed that *Casp11*$^{-/-}$ OT-I T cells proliferated more than WT cells (Fig. 3B). No difference was observed in Annexin V staining (data not shown), indicating accumulation of *Casp11*$^{-/-}$ cells was due to enhanced proliferation and not decreased sensitivity to cell death.

Cytokine production by *Casp11*$^{-/-}$ OT-I T cells relative to controls was examined 4 days after Q4-DC immunization. OT-I T cells were restimulated *in vitro* with high and low concentrations of N4 peptide, and the production of IFNγ and IL-2 was measured. A similar percentage WT and *Casp11*$^{-/-}$ cells produced IFNγ after stimulation with both high and low concentrations of N4 (Fig. 3C, 3D). However, the percentage of IFNγ$^+$ cells producing IL-2 was increased in *Casp11*$^{-/-}$ OT-I T cells in response to low concentrations of N4 (Fig. 3C, 3D). We also examined the expression of CD62L after Q4-DC immunization and found that *Casp11*$^{-/-}$ OT-I T cells had reduced expression compared to WT cells (Fig. 3E). These data indicate that *Casp11*$^{-/-}$ T cells expand more readily to *in vivo* peptide-DC stimulation and have enhanced effector function including an increased ability to produce IL-2.

Enhanced TCR signaling in *Casp11*$^{-/-}$ CD8$^+$ T cells

We hypothesized that caspase-11 modulated the strength of the TCR signal received during priming. This was measured in WT and *Casp11*$^{-/-}$ OT-I T cells using a Nur77-GFP reporter(17). *Casp11*$^{-/-}$ and WT cells had similar GFP expression in the absence of peptide stimulation (data not shown). WT and *Casp11*$^{-/-}$ Nur77-GFP OT-I T cells were stimulated with N4, Q4, or T4 peptide for 12 hours, and the fold change in GFP expression in the

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GFP^{hi} population over unstimulated WT OT-I T cells was compared. We found *Casp11*$^{-/-}$ T cells had significantly increased GFP expression over a range of peptide affinities (Fig. 4A) and concentrations (data not shown). Additionally, more cells in the *Casp11*$^{-/-}$ population responded to low affinity ligands; a higher percentage of *Casp11*$^{-/-}$ became Nur77-GFP^{hi} within 12 hours of stimulation (Fig. 4B). By 24 hours post activation, this phenotype was less apparent (data not shown), suggesting a difference in the rate of activation rather than the inability of a subset of WT cells to respond. This data could be explained by increased expression of the TCR and associated molecules in *Casp11*$^{-/-}$ T cells; however, naïve WT and *Casp11*$^{-/-}$ OT-I T cells had similar surface expression of Vα2, Vβ5, CD3, and CD8α (data not shown). These data indicate TCR signaling in response to low abundance/affinity ligands is inhibited by caspase-11, resulting in weaker TCR signaling overall and delayed activation.

Caspase-11 has been shown to positively regulate actin depolymerization, leading to increased levels of F-actin in *Casp11*$^{-/-}$ cells(4,5), and we speculated this could lead to enhanced TCR signaling in *Casp11*$^{-/-}$ T cells. Using phalloidin staining, we verified increased F-actin levels in *Casp11*$^{-/-}$ compared to WT OT-I T cells after stimulation with low affinity ligands (Supplemental Fig. 1A, 1B). In addition, onset of Nur77-GFP expression in WT OT-I T cells after T4 stimulation correlated with higher levels of F-actin when compared to Nur77-GFP^{lo} cells (Supplemental Fig. 1C).

More efficient deletion of self-reactive *Casp11*$^{-/-}$ T cells

To determine if self-reactive *Casp11*$^{-/-}$ T cells respond more strongly to endogenous ligands, we co-transferred WT and *Casp11*$^{-/-}$ OT-I T cells into mice expressing ovalbumin under the control of the fatty acid binding promoter (iFABP-tOVA), which leads to ovalbumin production by mature enterocytes of the small intestine(14). At 6–7 days post-transfer, *Casp11*$^{-/-}$ OT-I T cells outnumbered WT cells in the intestinal epithelium (IEL) and mesenteric lymph node (MLN) (Fig. 5A, 5B). Thirty days after transfer, OT-I cell numbers were significantly lower in the MLN as has been reported(14, 18). At this time, 50% of mice had no OT-I T cells of either genotype in the MLN, and the remaining mice had equal numbers of WT and *Casp11*$^{-/-}$ cells (Fig. 5A, 5B). Notably, in the IEL compartment at this late timepoint, WT OT-I T cells outnumbered *Casp11*$^{-/-}$ cells (Fig. 5A, 5B). These data suggest the sensitivity of *Casp11*$^{-/-}$ OT-I T cells to low abundance endogenous ligands allowed preferential early expansion and increased numbers in the intestinal tissue relative to WT cells; however, *Casp11*$^{-/-}$ OT-I T cells were more efficiently deleted in the intestine over time.

Discussion

Caspase-11 expression is upregulated in CD8^{+} T cells as they mature and functions to negatively regulate TCR signaling following antigenic stimulation, resulting in decreased expansion and effector function, especially in response to low level antigen stimulation. The stimulus for caspase-11 upregulation in this setting is still unclear. Our data suggests it is not type I interferon-dependent process, which is observed in other cell types. Upregulation of caspase-11 is equivalent during priming with *Listeria*, which results in robust type I

interferon production, and protein immunization. Reactive oxygen species (ROS) have also been demonstrated to stimulate caspase-11 upregulation(19), and TCR stimulation results in a metabolic shift that generates ROS that could account for the upregulation of *Casp11* during T cell activation(20).

The stepwise increase in the expression of caspase-11 by CD8$^+$ T cells correlates with the reduced sensitivity of the TCR. Naïve CD8$^+$ T cells that have recently left the thymus are more sensitive to TCR signals than those that have undergone maturation in the periphery(21), and antigen sensitivity leading to cell division is further reduced in CD8$^+$ memory cells(22). Reduced TCR signaling in maturing T cells is more readily apparent when low abundance and low affinity antigens are used(21, 22). Lower expression of the TCR, CD3, and other surface molecules associated with robust TCR activation have been implicated reduced signaling (21–23). In addition, protein tyrosine phosphatases that negatively regulate TCR signaling are also upregulated in CD8$^+$ memory T cells(22). Our data suggests caspase-11 represents an additional regulatory mechanism for tuning the sensitivity of maturing T cells to both foreign and endogenous antigens.

Overall, these data indicate caspase-11 is upregulated as T cells become more antigen experienced and acts as a negative regulator of TCR signaling particularly in response to low abundance and low affinity TCR ligands. Caspase-11 can regulate actin polymerization which is known to play a critical role in regulating TCR signaling(9). These data suggest that modulating the function of caspase-11 in T cells could be used in the context of immunization to regulate their sensitivity to low affinity antigens, allowing increased expansion and enhanced effector function without the risk of developing autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IEL	intestinal epithelium
MLN	mesenteric lymph node
WT	wild-type

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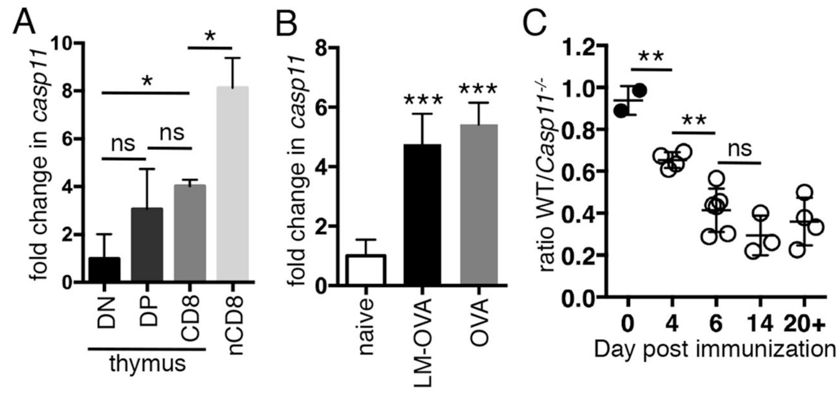

Figure 1. Caspase-11 expression is induced in CD8+ T cells during development and priming and limits the size of the CD8+ T cell pool after protein immunization

(**A**) Cells were sorted from C57BL/6 mice and *casp11* expression was examined by qRT-PCR and normalized to *actB*. Data are pooled from 2 mice, representative of 2 experiments. (**B**) 10^4 naïve OT-I T cells were transferred into mice followed by LM-OVA infection or ovalbumin immunization (OVA). OT-I T cells were sorted at day 7 for LM-OVA or day 6 for OVA immunization and *casp11* expression was analyzed as in (**A**). Data pooled from 3 independent experiments with 3 mice/grp. (**C**) 10^4 WT and *Casp11*$^{-/-}$ OT-I T cells were cotransferred into mice followed by i.p. immunization with 150μg OVA and the ratio of WT/*Casp11*$^{-/-}$ cells was determined at the indicated timepoints. Data in (**C**) are pooled from 2 experiments. *ns, not significant, *p<0.05, **p<0.005, ***p<0.0005*

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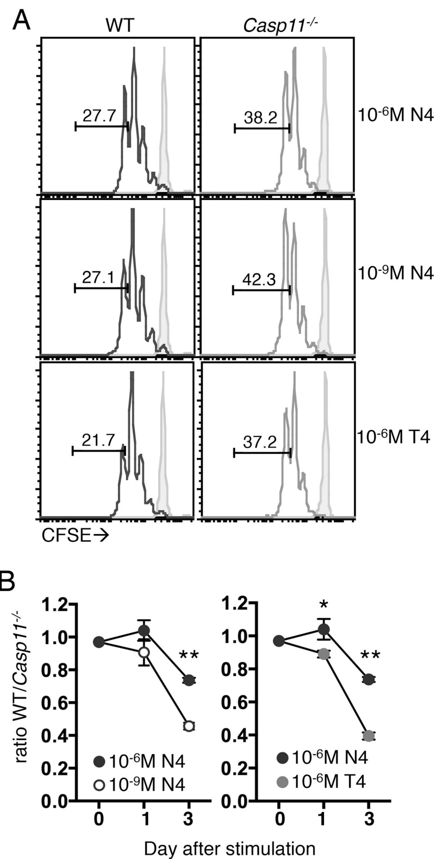

Figure 2. Caspase-11 limits CD8+ T cell accumulation in response to low abundance and low affinity ligands *in vitro*

WT and *Casp11*$^{-/-}$ OT-I T cells were CFSE labeled and stimulated with splenocytes pulsed with the indicated concentrations of N4 or T4 peptides. (**A**) CFSE dilution 2 days after peptide stimulation (open histograms) or in unstimulated controls (shaded histograms), values indicate the percentage of OT-I T cells that have undergone 4 or more divisions. (**B**) Ratio of WT to *Casp11*$^{-/-}$ OT-I T cells. Data are from a single experiment and representative of 3 experiments. $*p<0.05$, $**p<0.0005$

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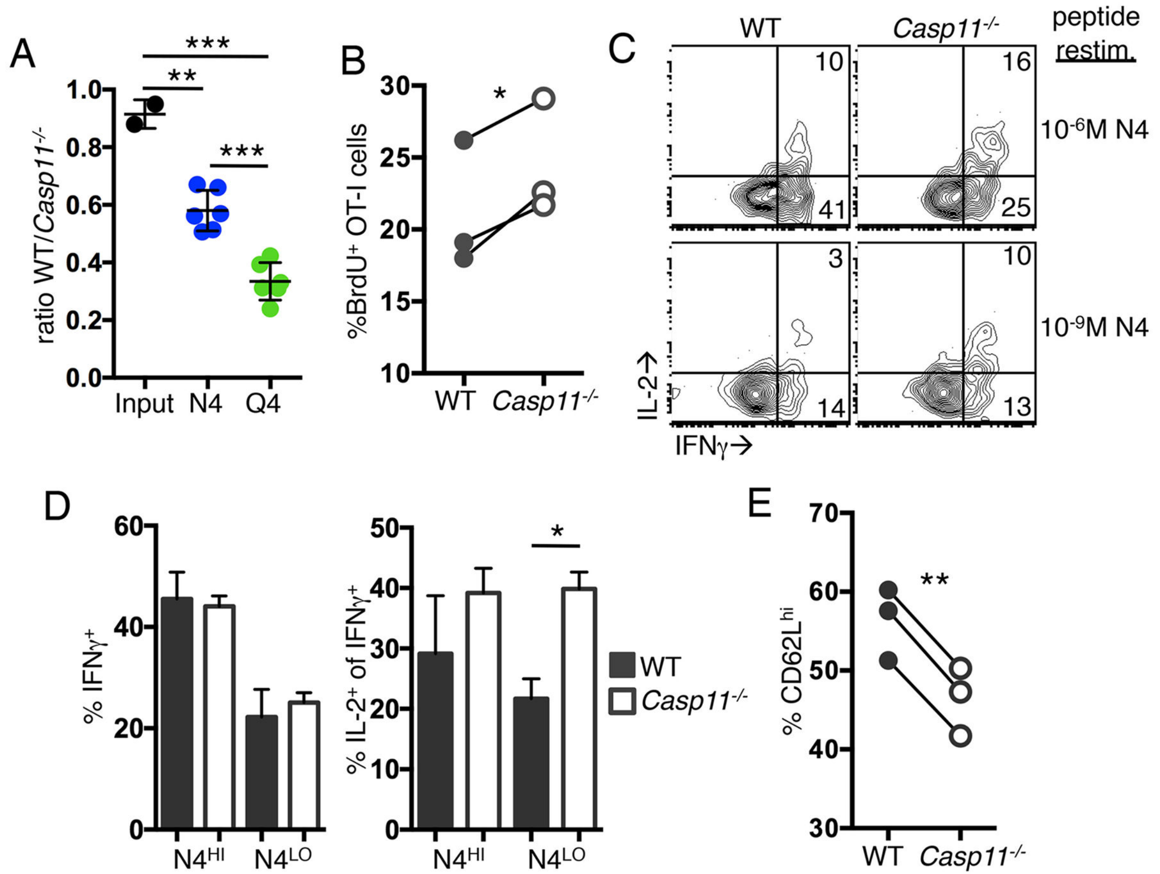

Figure 3. *Casp11*$^{-/-}$ CD8 T cells primed with low affinity ligands *in vivo* display enhanced proliferation and effector function

C57BL/6 mice received $1\text{x}10^4$ WT and *Casp11*$^{-/-}$ OT-I T cells and $1–2\text{x}10^6$ N4 or Q4 peptide pulsed DCs. (**A**) The ratio of WT:*Casp11*$^{-/-}$ OT-I T cells in the spleen on day 4 after immunization. Data are pooled from 2 experiments. (**B–E**) Mice were immunized with Q4-DCs and BrdU incorporation was measured on day 3 (**B**) and cytokine production after restimulation with the indicated concentration of N4 peptide on day 4 (**C–D**). CD62L expression on day 4 (**E**). Data are from a single experiment and representative of 2–3 experiments. *p<0.05, **p<0.005, ***p<0.0005

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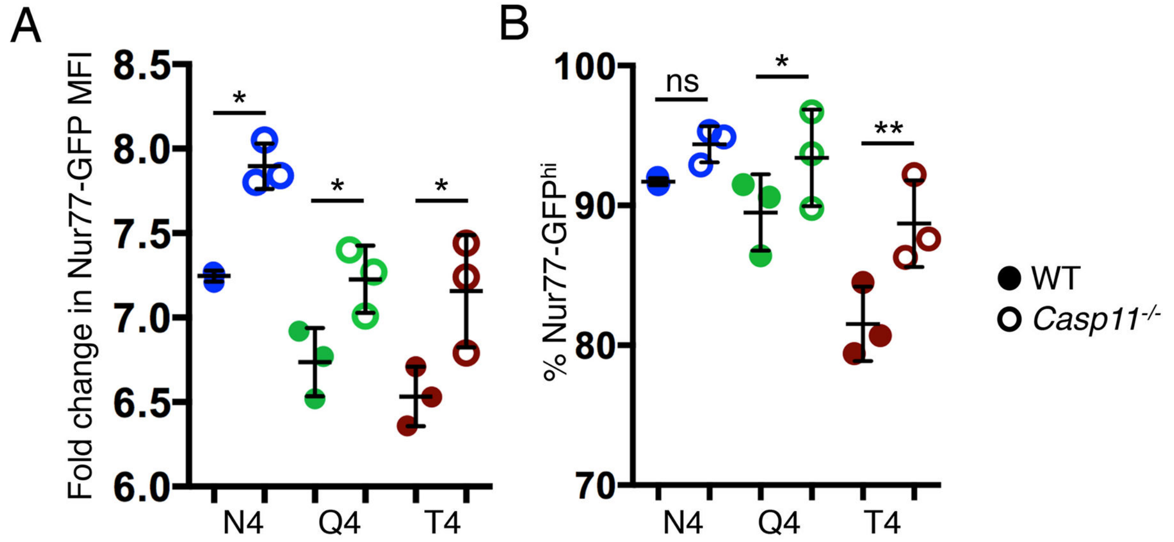

Figure 4. Enhanced TCR signaling in *Casp11*$^{-/-}$ T cells after *in vitro* stimulation

WT Nur77-GFP and *Casp11*$^{-/-}$ Nur77-GFP OT-I T cells were stimulated for 12 hours with unpulsed splenocytes or splenocytes pulsed with 1μM N4, Q4, or T4. The GFP MFI of unstimulated WT cells was set to 1.0. (**A**) The fold change in the MFI of GFP^{hi} peptide pulsed WT and *Casp11*$^{-/-}$ cells relative to unstimulated cells. (**B**) The percentage of cells that are Nur77-GFP^{hi} after stimulation. Data are from one experiment, representative of 2 experiments. *ns, not significant, *p<0.05, **p<0.005*

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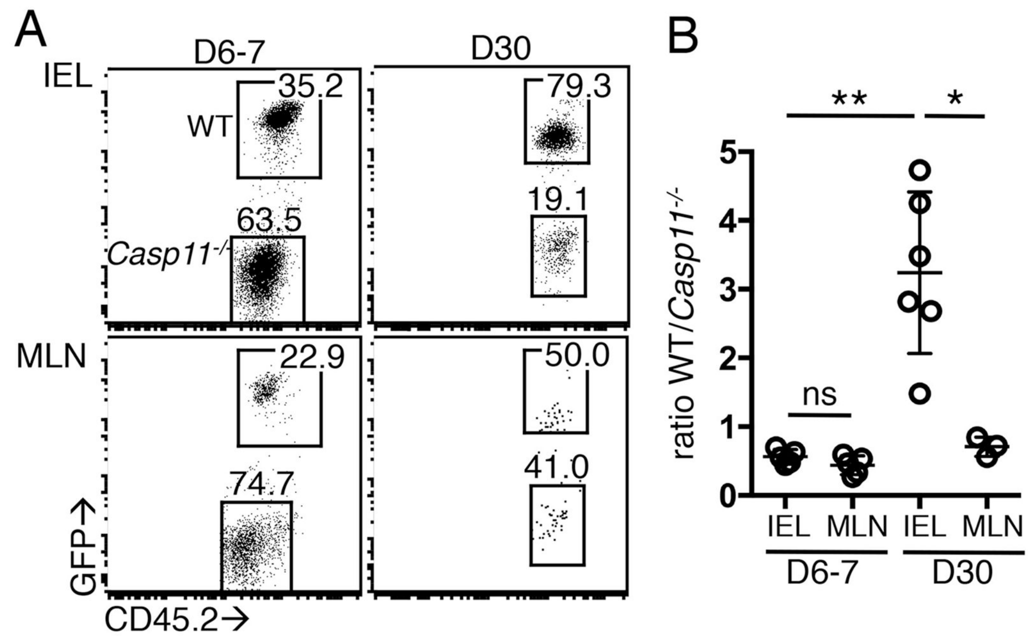

Figure 5. Self-reactive *Casp11*$^{-/-}$ T cells do not persist after transfer

CD45.1^{+} iFABP-OVA mice received 0.5–1x10^{6} GFP^{+} WT and CD45.2^{+} *Casp11*$^{-/-}$ OT-I T cells IEL and MLN were examined 6–7 and 30 days post transfer. (**A**) Representative plots showing WT and *Casp11*$^{-/-}$ OT-I cells of total transferred cells. (**B**) Ratio of WT to *Casp11*$^{-/-}$ OT-I T cells. Data are pooled from 3 independent experiments. $*p<0.05$, $**p<0.005$