Cutting Edge: Caspase-11 Limits the Response of CD8^+ T Cells to Low-Abundance and Low-Affinity Antigens

Tessa Bergsbaken and Michael J. Bevan

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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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Inflammatory caspases, including caspase-11, are upregulated in CD8⁺ T cells after Ag-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 with wild-type cells, including increased production of IFN-γ and reduced expression of CD62L. Casp11⁻/⁻ T cells specific for endogenous Ags were more readily deleted than wild-type cells. These data indicate that 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.

Caspase-11 in mice and caspase-4 and caspase-5 in humans are members of the family of inflammatory caspases that also includes caspase-1 and caspase-12 (1). Unlike other caspases, caspase-11 is expressed at low levels in resting cells and is upregulated upon activation via a type I IFN–dependent process (2). Caspase-11 can bind directly to intracellular LPS, resulting in caspase-11 processing and activation (3) and leading to downstream events that can include caspase-1 activation, cell death, and inflammatory cytokine production and release (1). Caspase-11 can also act in its proform to regulate actin dynamics; caspase-11 promotes actin depolymerization by facilitating interactions among actin-interacting protein 1, coflin, 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 compared with naive 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 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 with 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 follicular helper cells (11, 12) and low concentrations of high-affinity peptide favoring Foxp3 expression (13).

We 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-Ag, the increased sensitivity of Casp11⁻/⁻ cells results in more rapid deletion in tissues in which the Ag 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 The Jackson Laboratory, Nur77-GFP, OT-I, iFABP-tOVA 232-6 (14), and Casp11⁻/⁻ (15) mice were maintained at the University of Washington and used in accordance with Institutional Animal Care and Use Committee guidelines.

Mice received 1–2 × 10⁴ naïve OT-I T cells and were immunized with 2 × 10⁸ Listeria monocytogenes expressing OVA (LM-OVA), 150 μg OVA, or 1–2 × 10⁶ dendritic cells (DCs) pulsed with LPS and 1 μM N4, Q4, or T4 peptide, as previously described (16). Mice were pulsed with 1 mg BrdU i.p. 2.5 h before sacrifice. OT-I T cells were stimulated ex vivo with peptide for 4 h in the presence of brefeldin A prior to intracellular cytokine staining.

Department of Immunology and Howard Hughes Medical Institute, University of Washington, Seattle, WA 98109

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Address correspondence and reprint requests to Dr. Tessa Bergsbaken, Department of Immunology and Howard Hughes Medical Institute, University of Washington, Seattle, WA 98109. E-mail address: berget@uw.edu

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Abbreviations used in this article: DC, dendritic cell; IEL, intestinal epithelium; LM-OVA, Listeria monocytogenes expressing OVA; MLN, mesenteric lymph node; WT, wild-type.

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Flow cytometry and cell sorting
Cells were stained with the indicated Abs (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 FACSAnia.

RT-PCR
RNA was isolated using a QIAGEN RNeasy Kit, per the manufacturer’s instructions. Quantitative RT-PCR was performed using the Quantitect SYBR Green RT-PCR Kit (QIAGEN) and the following primers: casp11F: 5′-CCTGAAGTTCCAAGGCTT-3′; casp11R: 5′-CCTTTTGTTA-CGGCCATT-3′; actbF: 5′-GGGCTTATTCCTCCATCG-3′; and actbR: 5′-CCATTTGTTAACAATGCCCATG-3′.

In vitro stimulations
Splenocytes were pulsed with 1 nM to 1 μM N4, Q4, or T4 peptide or no peptide at 37°C for 40 min and washed thoroughly with media. A total of 5 x 10^5 splenocytes were mixed with 1–2 x 10^5 naïve OT-I T cells. T cells were labeled with 2 μM CFSE (Invitrogen) where indicated.

Statistical analysis
All graphs depict mean ± SD. The two-tailed Student 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 the development of CD8+ T cells by sorting double-negative, double-positive, and CD8 single-positive thymocytes, as well as naïve CD8+ peripheral T cells. Caspase-11 was upregulated as thymocytes matured and increased further after cells entered the periphery (Fig. 1A). We also examined whether caspase-11 expression was upregulated after Ag-dependent activation in the periphery. Naive OT-I T cells, which express a TCR specific for the OVA peptide SIINFEKL, were transferred into mice that were then infected with LM-OVA or immunized with OVA protein. Activated OT-I T cells were sorted, and caspase-11 expression was analyzed. Caspase-11 was upregulated an additional 5-fold in activated OT-I T cells in response to both protein immunization and infection (Fig. 1B) compared with naïve cells.

To analyze the function of caspase-11 during the activation of CD8+ T cells, caspase-11-sufficient (wild-type [WT]) and Caspase-11−/− OT-I T cells were transferred into mice, followed by challenge with LM-OVA or OVA protein. There were equal numbers of WT and Caspase-11−/− OT-I T cells 7 d after LM-OVA challenge (data not shown). However, after OVA immunization, Caspase-11−/− cells were present at higher numbers than WT OT-I T cells as early as 4 d after immunization, and this difference became more pronounced at 6 d (Fig. 1C).

Previous reports indicated that caspase-11 is able to regulate the migration of T cells (4), and we hypothesized that failure of Caspase-11−/− T cells to migrate out of the spleen might explain their increased relative to WT cells. However, we saw no increase in the ratio of WT/Caspase-11−/− CD8+ T cells in the blood or inguinal lymph node, suggesting that WT cells were not exiting the spleen more readily than Caspase-11−/− cells (data not shown). These data suggest that caspase-11 limits the

**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 caspase-11 expression was examined by quantitative RT-PCR and normalized to actB. Data are pooled from two mice, representative of two experiments. (B) A total of 10^5 naïve OT-I T cells was transferred into mice, followed by LM-OVA infection or OVA immunization. OT-I T cells were sorted at day 7 for LM-OVA or day 6 for OVA immunization, and caspase-11 expression was analyzed as in (A). Data are pooled from three independent experiments with three mice/group. (C) A total of 10^4 WT and Caspase-11−/− OT-I T cells was cotransferred into mice, followed by i.p. immunization with 150 μg OVA, and the ratio of WT/Caspase-11−/− cells was determined at the indicated time points. Data in (C) are pooled from two experiments. *p < 0.05, **p < 0.005, ***p < 0.0005. ns, not significant.

**FIGURE 2.** Caspase-11 limits CD8+ T cell accumulation in response to low-abundance and low-affinity ligands in vitro. WT and Caspase-11−/− OT-I T cells were labeled with CFSE and stimulated with splenocytes pulsed with the indicated concentrations of N4 or T4 peptides. (A) CFSE dilution 2 d after peptide stimulation (open graphs) or in unstimulated controls (shaded graphs), values indicate the percentage of OT-I T cells that have undergone division, as determined by flow cytometry. Data are pooled from three independent experiments with three mice/group. (B) Ratio of WT/Caspase-11−/− OT-I T cells. Data are from a single experiment and are representative of three experiments. *p < 0.05, **p < 0.0005.
accumulation of CD8+ T cells in response to protein immunization but has little effect 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 that the increased numbers of Casp11+/− OT-I T cells after protein immunization, but not LM-OVA infection, could be due to their enhanced response to 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 various concentrations of a high-affinity ligand (SIINFEKL, N4). 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. 2). In response to low concentrations of N4, we saw an increased percentage of CFSElo cells in the Casp11+/− population compared with WT (Fig. 2A) and increased accumulation of Casp11+/− cells (Fig. 2B). When a lower-affinity ligand (SIITEEKL, T4) was used, we also observed an increase in proliferation and outgrowth of Casp11+/− cells compared with WT cells (Fig. 2). 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, because WT and Casp11+/− cells have equal access to Ag, 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 that 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 SIITFEKL (Q4) to pulse DCs. Consistent with what we observed in vitro, we saw increased accumulation of Casp11+/− cells compared with WT cells after both N4-DC and Q4-DC immunization, and the difference was enhanced with lower-affinity Ag (Fig. 3A). BrdU incorporation at 3 d 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 that 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 d after Q4-DC immunization. OT-I T cells were restimulated in vitro with high and low concentrations of N4 peptide, and the production of IFN-γ and IL-2 was measured. A similar percentage of WT and Casp11+/− cells produced IFN-γ after stimulation with both high and low concentrations of N4 (Fig. 3C, 3D). However, the percentage of IFN-γ+ 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 with WT cells (Fig. 3E). These data indicate that Casp11+/− T cells expand more readily in response 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 peptides for 12 h, and the fold change in GFP expression in the GFPhi population over unstimulated WT OT-I T cells was compared. We found that 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;
caspase-11 expression is upregulated in CD8* T cells as they mature, and its functions negatively regulate TCR signaling following antigenic stimulation, resulting in decreased expansion and effector function, especially in response to low-level Ag stimulation. The stimulus for caspase-11 upregulation in this setting is still unclear. Our data suggest that it is not a type I IFN-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 IFN production, and protein immunization. Reactive oxygen species also stimulate caspase-11 upregulation (19), and TCR stimulation results in a metabolic shift that generates reactive oxygen species 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. Naive 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 Ag 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 Ags are used (21, 22). Lower expression of the TCR, CD3, and other surface molecules associated with robust TCR activation were implicated in reduced signaling (21–23). In addition, protein tyrosine phosphatases that negatively regulate TCR signaling are upregulated in CD8* memory T cells (22). Our data suggest that caspase-11 represents an additional regulatory mechanism for tuning the sensitivity of maturing T cells to both foreign and endogenous Ags.

Overall, these data indicate that caspase-11 is upregulated as T cells become more Ag 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 sensi-
tivity to low-affinity Ags, allowing increased expansion and enhanced effector function without the risk of developing autoimmune.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplemental Figure 1: *Casp11<sup>−/−</sup>* OT-I T cells have increased levels of F-actin compared to WT OT-I T cells after stimulation with low affinity peptides.

(A-B) WT and *Casp11<sup>−/−</sup>* OT-I T cells were stimulated with 1µM N4 or T4 peptide for 60 minutes. F-actin was stained using Alexa Fluor 647 phalloidin. (A) Representative histograms of F-actin levels after N4 (*top panel*) or T4 (*lower panel*) stimulation. (B) MFI of F-actin in WT and *Casp11<sup>−/−</sup>* OT-I T cells, lines connect samples from the same well. Representative of two experiments. (C) WT Nur77-GFP OT-I T cells were stimulated with 1µM T4 for 100 minutes. Nur77-GFP<sub>hi</sub> and Nur77-GFP<sub>lo</sub> cells were gated (*left panel*) and the level of F-actin compared (*right panel*).