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Caspase-8 and c-FLIP_L Associate in Lipid Rafts with NF- κ B Adaptors during T Cell Activation*

Ravi S. Misra^{‡,1}, Jennifer Q. Russell[‡], Andreas Koenig[‡], Jennifer A. Hinshaw-Makepeace[‡], Renren Wen[§], Demin Wang[§], Hairong Huo[¶], Dan R. Littman[¶], Uta Ferch^{||}, Jurgen Ruland^{||}, Margot Thome^{**2}, and Ralph C. Budd^{‡,3}

[‡]Immunobiology Program, Department of Medicine, University of Vermont College of Medicine, Burlington, Vermont 05405-0068

[§]Blood Research Institute, Department of Microbiology and Molecular Genetics, Blood Center of Wisconsin, Milwaukee, Wisconsin 53201-2178

[¶]Howard Hughes Medical Institute, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, New York 10016

^{||}Department of Medicine (Hematology/Oncology), Technical University of Munich, Munich, Germany

^{**}Institute of Biochemistry, University of Lausanne, BIL Biomedical Research Center, 1066 Epalinges, Switzerland

Abstract

Humans and mice lacking functional caspase-8 in T cells manifest a profound immunodeficiency syndrome due to defective T cell antigen receptor (TCR)-induced NF- κ B signaling and proliferation. It is unknown how caspase-8 is activated following T cell stimulation, and what is the caspase-8 substrate(s) that is necessary to initiate T cell cycling. We observe that following TCR ligation, a small portion of total cellular caspase-8 and c-FLIP_L rapidly migrate to lipid rafts where they associate in an active caspase complex. Activation of caspase-8 in lipid rafts is followed by rapid cleavage of c-FLIP_L at a known caspase-8 cleavage site. The active caspase-c-FLIP complex forms in the absence of Fas (CD95/APO1) and associates with the NF- κ B signaling molecules RIP1, TRAF2, and TRAF6, as well as upstream NF- κ B regulators PKC θ , CARMA1, Bcl-10, and MALT1, which connect to the TCR. The lack of caspase-8 results in the absence of MALT1 and Bcl-10 in the active caspase complex. Consistent with this observation, inhibition of caspase activity attenuates NF- κ B activation. The current findings define a link among TCR, caspases, and the NF- κ B pathway that occurs in a sequestered lipid raft environment in T cells.

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³To whom correspondence should be addressed: Given Medical Bldg., Burlington, VT 05405-0068. Tel.: 802-656-2286; Fax: 802-656-3854; ralph.budd@uvm.edu..

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Activation of caspases has traditionally been associated with oligomerization of death receptors and induction of cell death (1). It has been determined more recently that caspase activity is also required for the induction of proliferation by primary naïve human and murine T cells (2–4). Inhibition of caspase activity by the pan-caspase blocker, Z-VAD-fmk,⁴ or the caspase-8-specific blocker, IETD-fmk, greatly reduced production of IL-2 and proliferation following TCR ligation (2, 3). It was subsequently shown that a non-functional mutation in the *caspase-8* gene in humans (5, 6) or the deletion of *caspase-8* in murine T cells (7) resulted in an immunodeficiency syndrome characterized by markedly decreased production of IL-2 and proliferative capacity of T cells.

It remained uncertain from these studies how caspase activation was linked to T cell antigen receptor (TCR) ligation, what were the regulatory protein(s) and substrate(s) of caspase activity, and whether this required the presence of the death receptor Fas (CD95/APO1). Equally puzzling was how the activation of caspase-8 might be limited to avoid induction of apoptosis. We recently observed that following T cell activation, cleavage occurs of certain known caspase substrates, such as c-FLIP-Long form (c-FLIP_L) and RIP1, although another caspase-8 substrate associated with cell death, Bid, was not cleaved (4). This suggested that active caspase-8 may become sequestered in a specific site within T cells after activation.

We considered the possibility of c-FLIP_L as both an activator of caspase-8 and an early caspase-8 substrate after T cell activation. c-FLIP_L is homologous to caspase-8 in containing two death effector domains, but bears a mutation in the caspase domain, which renders it enzymatically inactive (8). Following ligation of Fas, c-FLIP_L is co-recruited with caspase-8 to the death-inducing signal complex (1, 8). In addition, c-FLIP_L is able to directly heterodimerize with and activate caspase-8 in its full-length form, independently of Fas (9–11). c-FLIP_L, furthermore, contains a caspase cleavage site at Asp³⁷⁶ that yields processed p43 FLIP (12, 13). Assuch, c-FLIP_L may be an early caspase-8 substrate during T cell

⁴The abbreviations used are:

Z	benzyloxycarbonyl
fmk	fluoromethyl ketone
c-FLIP _L	c-FLIP-Long form
TCR	T cell antigen receptor
MBCD	methyl- β -cyclodextran
IL	interleukin
MALT1	mucosa-associated lymphoid tissue translocation protein 1
CARMA1	caspase-recruitment domain membrane-associated guanylate kinase protein 1
PBS	phosphate-buffered saline
TRAF	tumor necrosis factor receptor-associated factor
LAT	linker for activation of T cells

activation. c-FLIP_L also associates with RIP1 and TRAF2 to promote activation of NF- κ B (14). Mice overexpressing c-FLIP_L in the T cell compartment manifest augmented IL-2, and enhanced proliferation (15, 16). Collectively, c-FLIP_L may provide an important link in our understanding of how TCR stimulation activates caspase-8, what is the caspase-8 substrate(s), and how caspase activity may link to NF- κ B activation.

Recent reports indicate that the *para*-caspase, mucosa-associated lymphoid tissue translocation protein 1 (MALT1), B cell lymphoma protein 10 (Bcl-10), and caspase-recruitment domain membrane-associated guanylate kinase protein 1 (CARMA1) form a complex that is required for TCR-mediated NF- κ B activation (17–22). MALT1 is regarded as a *para*-caspase as it contains a caspase-like domain, although actual caspase activity has not been demonstrated as MALT1 does not bind any known caspase substrates (23). Given the similar phenotypes between caspase-8-deficient mice (7) and mice deficient for MALT1 (20), Bcl-10 (21, 22), or CARMA1 (17–19), we explored a potential link between caspase-8 activation and the MALT1 complex. Because CARMA1, Bcl-10, and MALT1 localize to lipid rafts upon TCR stimulation, we considered that the active caspase complex we had previously defined in activated T cells (15) might form in lipid rafts and be connected with CARMA1, Bcl-10, and MALT1.

In the current study, we demonstrate that resting T cells manifest low to negligible levels of active caspase-8, but this increases following activation of T cells in association with lipid rafts. TCR-induced caspase-8 activation occurs rapidly and in the absence of Fas expression. Concomitant with cleavage of c-FLIP_L to p43 FLIP is the association of the active caspase complex with several members of the NF- κ B activation pathway, including PKC θ , CARMA1, Bcl-10, MALT1, as well as RIP1, TRAF2/6, and the IKK $\alpha/\beta/\gamma$ complex. These findings provide new insights into the mechanisms of TCR-induced caspase activation, and how this links to NF- κ B pathway.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 wild-type mice, B6 transgenic mice expressing c-FLIP_L in the T cell compartment (c-FLIP_L-Tg) (16), their non-transgenic littermate controls, B6lpr mice, NF- κ B reporter mice (24), and T cell conditional caspase-8-deficient (*tcasp8*^{-/-}) mice (7) were housed and bred in the University of Vermont animal facility and used at 2–6 months of age. The animal facility is AALAC-approved, and protocols were approved by the University of Vermont College of Medicine IACUC. Original breeding pairs of C57BL/6 and B6lpr mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Bcl-10-deficient mice (21) were housed at The Blood Center of Wisconsin and MALT1-deficient mice (22) were housed at the Technical University of Munich. The NF- κ B-luciferase reporter mice have the luciferase gene controlled by two copies of KB sequences from the IgK enhancer (25).

T Cell Purification

Spleen and lymph node cells were isolated and disrupted through nylon mesh in RPMI 1640 with 25 mM Hepes (MediaTech, Herndon, VA) containing 5% (v/v) bovine calf serum

(HyClone, Logan, UT). Erythrocyte lysis of splenocytes was performed using Geys solution. T cells were purified using negative selection by incubating splenocytes with anti-MHC II (M5/114/15/2), anti-CD11b (M1/70), anti-NK1.1 (PK136), and anti-B220 (RA3-6B2) on ice for 30 min. Cells were washed three times and rocked with goat anti-mouse and goat anti-rat conjugated magnetic beads at a 10:1 ratio of beads to cell (Qiagen, Valencia, CA) at 4 °C for 45 min. Magnetic depletion was used to remove bead-bound cells. Cells were washed and resuspended in culture medium (RPMI 1640, 2.5 mg/ml glucose (Sigma), 10 mg/ml folate (Invitrogen), 110 µg/ml pyruvate (Invitrogen), 5×10^{-5} M 2-mercaptoethanol (Sigma), 292.3 µg/ml glutamine (Invitrogen), 100 units/ml penicillin-streptomycin (Invitrogen), and 5% fetal calf serum).

T Cell Culture

C57BL/6 and c-FLIP_L-Tg T cells were activated in culture medium by plate-bound anti-CD3 (10 µg/ml, clone 145-2C11), anti-CD28 (clone 37.51) ascites (1:500), and recombinant human IL-2 (50 units/ml, Cetus, Emeryville, CA) for 2 days. Cells were then removed from anti-CD3 and supplied with fresh medium plus IL-2. For short-term activation studies, T cells were stimulated with soluble anti-CD3 (20 µg/ml) cross-linked with goat anti-hamster IgG (50 µg/ml, Caltag, Invitrogen) for 30 or 60 min. Purified CD8⁺T cells were activated with anti-CD3 (5 µg/ml) and anti-CD28 (1:500 dilution of ascites). To measure NF-κB-luciferase activity, anti-CD3/CD28-activated T cells from NF-κB-luciferase transgenic mice were harvested after 48 h, washed with PBS, and lysed. The lysates were then analyzed using luciferin (Promega Corp., Madison, WI) and measured in a luminometer for 10 s. Four measurements were made for each sample. Results are presented as the mean (± S.E.) with background subtracted.

CFSE Dye Labeling of Cells

Cells were washed with PBS plus 0.1% bovine serum albumin, and then mixed with 5 µM CFSE (Molecular Probes) in PBS plus 0.1% bovine serum albumin. Cells were labeled at 10^7 /ml for 10 min at 37 °C. Cells were then washed three times. A portion of the cells was incubated overnight at 37 °C to serve as a non-cycling control for flow cytometry, and the remaining cells were stimulated.

Immunoblot Analysis

T cells were lysed in buffer containing 0.2% Nonidet P-40, 20 mM Tris-HCl (pH 7.4) (American Bioanalytical, Natick, MA), 2 mM sodium orthovanadate (Sigma), 10% glycerol (Fisher), 150 mM NaCl (Sigma), complete protease inhibitor (Roche Diagnostics), and 20 µM z-VAD-fmk (MP Biomedicals). Protein concentration was determined by Bradford assay (Bio-Rad). Protein lysates were boiled for 5 min in loading buffer containing 2-mercaptoethanol and separated using SDS-PAGE on 8, 10, or 12.5% gels. Proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad) and blocked using 4% milk in Tris-buffered saline plus 0.1% Tween 20 (American Bioanalytical) at room temperature for 1 h. Membranes were incubated at 4 °C overnight in milk containing one of the following primary detection antibodies: paxillin (clone 165, BD Biosciences), cholera toxin B subunit (Sigma), Bcl-10 (clone H-197, Santa Cruz Biotechnology, Santa Cruz, CA),

CARMA1 (catalog number 3189, ProSci, Poway, CA), caspase-8 (a kind gift of Dr. Andreas Strasser, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), IKK α (catalog number 2682, Cell Signaling Technology, Beverly, MA), IKK β (catalog number 2684, Cell Signaling Technology), IKK γ (clone FL-419, Santa Cruz), LAT (catalog number 06-807, Upstate Biotechnology, Lake Placid, NY), RIP1 (clone 38, BD Biosciences), TRAF2 (catalog number 592, MBL, Woburn, MA), TRAF6 (catalog number 597, MBL), FLIP (clone Dave-2, Axxora, San Diego, CA), and MALT1 (C terminus-specific, a generous gift from Dr. Vishva Dixit, Genentech, South San Francisco, CA). Immunoreactive proteins were visualized using horseradish peroxidase-labeled conjugates (Santa Cruz; Southern Biotech, Birmingham, AL; Biomedica, Foster City, CA; and Jackson ImmunoResearch) and developed using LumiGLO (KPL, Gaithersburg, MD). Immunoblot membranes were treated with azide, washed, and reprobed with another antibody to ensure valid comparisons among the same set of samples.

Biotin-VAD-fmk Treatment and Caspase Precipitation Assay

T cells were incubated in culture medium plus recombinant human IL-2 with 10 μ M biotin-VAD-fmk (MP Biomedicals) at 37 °C for 15 min. As a negative control, a portion of T cells was incubated with 100 μ M non-biotinylated z-VAD-fmk prior to incubation with biotin-VAD-fmk (not shown). Cells for unfractionated lysates were lysed in buffer containing 20 μ M biotin-VAD-fmk. 600 μ g of unfractionated lysate or 800 μ l of cytosolic and lipid raft fractions (see below) were precleared by rocking with 40 μ l of Sepharose 6B-agarose beads (Sigma) at 4 °C for 2 h. Supernatants were then rocked with 60 μ l of streptavidin-Sepharose beads (Zymed Laboratories Inc. and Invitrogen) at 4 °C overnight. Beads were washed 5 times in lysis buffer without protease inhibitor, then boiled in loading buffer. Beads were removed by centrifugation and immunoblot analysis was then performed on supernatants.

Lipid Raft and Cytosolic Fraction Isolation

As described previously, T cells were washed twice in cold PBS, then lysed in TNE buffer (5 mM iodoacetic acid (Pierce), 150 mM NaCl, 15 mM EDTA (Sigma), 10 mM Tris-HCl (pH 7.4)) supplemented with 0.5% Triton X-100, and 100 mM Na₂CO₃ (Sigma) for 30 min on ice (26). Lysates were then sonicated three times for 10 s each and rested on ice for 10 min. Lysates were mixed with Opti-Prep™ sucrose substitute (Sigma) to a final concentration of 40%. OptiPrep was diluted to 30 or 5% by mixing with TNE buffer. The 40% OptiPrep/cell lysate mixture was placed in an ultracentrifuge tube and the 30 and 5% solutions were layered over the 40% solution. Gradients were centrifuged overnight at 4 °C at 200,000 \times g. Twelve sequential 1-ml fractions were taken, starting with the top layer. A portion of each fraction was used for active caspase precipitation and another portion was used for immunoblot analysis.

Caspase Activity Assay

Relative caspase activities were determined using the Apo-ONE Caspase-3/7 Assay (Promega). Fresh or day 4 effector T cells were resuspended in culture medium at 10×10^6 /ml. Cells were serially diluted in culture medium and then mixed with 100 μ l of caspase reagent (DEVD-rhodamine) according to the manufacturer's protocol. Spectrophotometric

readings were taken over a range of times. The data shown represent readings at 5 h for fresh and 90 min for effector populations.

Cholesterol Depletion

Fresh and day 4 wild-type effector T cells were washed with serum-free medium, resuspended in serum-free media containing glucose, 2-mercaptoethanol, and 1.5% fatty acid-free bovine serum albumin (Sigma), and warmed for 10 min at 37 °C. Cells were then treated with methyl- β -cyclodextran (MBCD, Sigma) at a final concentration of 20 mM for 10 min at 37 °C. Cells were washed with fatty acid-free bovine serum albumin media, and lipid rafts isolated. Fresh cells were then activated using soluble anti-CD3 cross-linked with goat anti-hamster IgG for 60 min. Following MBCD treatment fresh, 60-min stimulated and effector T cells were washed with room temperature PBS prior to lysis in biotin-VAD-fmk-containing buffer.

RESULTS

Active Caspase-8 and c-FLIP Are Found in Lipid Rafts of Activated T Cells

The levels of total cellular caspase-8 are equivalent between resting and effector T cells, and caspase-8 activation following TCR stimulation does not result in cell death but rather, is necessary to initiate cell cycling (3, 4). To achieve separation of caspase-mediated death from proliferation, we considered whether TCR-initiated caspase-8 activation might occur in a sequestered location. Because many signaling events triggered by TCR activation occur in lipid rafts, and lipid rafts are stable through the effector T cell stage (27), we examined the possible migration of caspase-8 and c-FLIP_L to this compartment. Cell lysates were mixed with OptiPrep sucrose substitute and overlaid with a discontinuous OptiPrep gradient followed by ultracentrifugation overnight (see “Experimental Procedures” for details). Twelve 1-ml fractions were taken and immunoblots performed for paxillin, a non-raft cytosolic component, and the ganglioside, GM1, which localizes in part to lipid rafts in resting T cells (Fig. 1A) (28). Based on these reference molecules, fractions 3 and 4 were observed to contain lipid rafts, whereas fractions 9–12 contained the cytosolic components (Fig. 1A, *top panels*).

Examination of fresh wild-type T cells revealed that essentially all caspase-8 and c-FLIP_L were localized to the non-raft cytosolic fractions. Fresh c-FLIP_L-Tg T cells contained a small but reproducibly detectable amount of caspase-8 and c-FLIP_L in the lipid raft fractions. This parallels previous observations that resting c-FLIP_L-Tg T cells manifest spontaneous caspase activity (15), and that c-FLIP_L heterodimerizes with and activates caspase-8 (10, 29). In contrast to fresh T cells, day 4 effector T cells from both wild-type and c-FLIP_L-Tg T cells manifested within the lipid raft fraction increased an amount of full-length and processed caspase-8 and c-FLIP_L. Both caspase-8 and c-FLIP_L contain known caspase-8 cleavage sites in their C-terminal domains that yield processed p43 caspase-8 and p43 FLIP (10). Notably, the relative proportion of processed to uncleaved caspase-8 and c-FLIP was substantially higher in the lipid raft fraction compared with the cytosolic component of effector T cells (Fig. 1A).

These findings suggested that active caspase-8 might be enriched in the raft fraction. To examine this more definitively, wild-type fresh and day 4 effector T cells were treated with biotin-VAD-fmk prior to lysis and then fractionated into raft and cytosolic components. Protein content in lipid rafts from fresh and effector populations was comparable (data not shown). Fractions were then individually precipitated with streptavidin-Sepharose beads, separated by SDS-PAGE, and immunoblotted to reveal active caspase-8. As shown in Fig. 1B, no active caspase-8 was detected in fresh T cells in either the raft or cytosolic fractions. However, in day 4 effector T cells, both active full-length caspase-8 and p43 caspase-8 were precipitated exclusively from lipid raft fraction 4 (Fig. 1B), even though the vast majority of caspase-8 still resided in cytosolic fractions 10 and 11 (Fig. 1A, lower left panels). In addition, c-FLIP_L co-precipitated with the active caspase complex in lipid rafts, the majority of which was processed to p43 FLIP despite the reverse ratio in the whole cell lysate (Fig. 1B, lower panel). This is consistent with the view that caspase activity was concentrated in the lipid raft fraction. Thus, c-FLIP_L and caspase-8 migrate to lipid rafts following TCR ligation, and this accompanies the activation of caspase-8 and cleavage of c-FLIP_L.

NF- κ B Signaling Molecules Migrate to Lipid Rafts and Associate with Active Caspases

We (15) and others (30) have previously observed that processed p43 FLIP can associate with the adaptor proteins, TRAF2 and RIP1, to promote activation of NF- κ B. An additional pathway linking TCR triggering to NF- κ B activation has been defined more recently to include PKC θ , CARMA1, MALT1, and Bcl-10 (31). Given that caspase activity is necessary for optimal NF- κ B signaling in T cells (6, 15), we examined whether members of the TCR-mediated NF- κ B signaling machinery, PKC θ , CARMA1, MALT1, and Bcl-10, co-localized with active caspase-8 and c-FLIP in lipid rafts. Lipid raft and cytosolic fractions were thus prepared from wild-type fresh and day 4 effector T cells and immunoblotted for molecules linked to the NF- κ B pathway. As with caspase-8 and c-FLIP_L, the major portion of most of these molecules was located in the cytosolic compartment of fresh T cells and they were not precipitated with biotin-VAD-fmk (data not shown). In contrast, day 4 effector T cells contained within the lipid raft compartment a portion of total cellular PKC θ , CARMA1, MALT1, and Bcl-10 (Fig. 2A, left panels). The lipid rafts of effector T cells also contained portions of adaptor proteins RIP1, TRAF2, and TRAF6, as well as members of the IKK complex, IKK α , IKK β , and IKK γ (Fig. 2A, left panels). Of note is that the size of TRAF2 as well as TRAF6 differed between the lipid raft and cytosolic fractions. This might represent differences in the known ubiquitination of these molecules (32–34), as a spread of low to higher molecular weight forms of TRAF2 and TRAF6 were faintly visible in the lipid raft fractions. Thus, lipid rafts from effector T cells contained much of the machinery for activation of NF- κ B in close proximity with active caspase-8.

Because several members of the NF- κ B pathway co-localized with caspase-8 in lipid rafts, we examined whether these molecules actually associated with active caspases. Effector T cells were labeled with biotin-VAD-fmk and then lipid raft preparations made. Fractions 3, 4 (raft), and 10, 11 (cytosolic) were subjected to precipitation with avidin-Sepharose. PKC θ , CARMA1, MALT1, Bcl-10, FADD, RIP1, TRAF2, TRAF6, IKK α , IKK β , and IKK γ were all observed exclusively in active caspase precipitates from lipid rafts, and not in the cytosolic fraction (Fig. 2A, right panels), even though the major portion of nearly all these

proteins resided in the cytosolic fraction (Fig. 2A, *left panels*). As negative controls, neither linker for activation of T cells (LAT, raft localized) (35, 36) nor paxillin (cytosol localized) was present in the active caspase complex (Fig. 2A, *lower right panels*). Further specificity of the assay was demonstrated by the ability of a 10-fold excess of non-biotinylated z-VAD-fmk to compete for binding of biotin-VAD-fmk to active caspases, and hence for the ability to co-precipitate associating molecules such as MALT1 and Bcl-10 (Fig. 2B). Although MALT1 is a *para*-caspase, studies have shown that it does not possess caspase activity and does not itself bind Z-VAD-fmk (37). Formation of the active caspase complex was also independent of Fas expression, as the same associations were observed in Fas-deficient *lpreffector* T cells (Fig. 2C). Thus, active caspase-8 and NF- κ B signaling proteins interact in lipid rafts of effector T cells.

Caspase-8 Is Required for Association of MALT1 and Bcl-10 in the Active Caspase Complex

Because biotin-VAD-fmk will bind to all active caspases, we investigated to what extent the presence of caspase-8 was specifically required for the association of MALT1 and Bcl-10. For these studies we used mice bearing a conditional deletion of caspase-8 in the T cell compartment. *tcasp8*^{-/-} mice contain homozygous caspase-8/loxP/loxP sites and are crossed with lck-Cre mice (7). T cells from these mice are known to be hyporesponsive to TCR signals. T cells from *tcasp8*^{-/-} mice or wild-type mice were stimulated with anti-CD3/CD28 plus IL-2. On day 4 biotin-VAD-fmk lysates were made, precipitated with avidin-Sepharose, and immunoblotted for caspase-8, MALT1, and Bcl-10. Although whole cell lysates from caspase-8-deficient T cells contained similar levels of MALT1 and Bcl-10 as wild-type T cells (data not shown), neither of these molecules was detectable in the biotin-VAD-fmk precipitates from caspase-8-deficient T cells (Fig. 3A).

Because Bcl-10 and MALT1 associate via an immunoglobulin-like domain within MALT1 (31), we further examined the dependence of recruitment of MALT1 and Bcl-10 to the active caspase complex on the presence of the other protein. Day 4 effector T cells were generated from Bcl-10-deficient and MALT1-deficient mice and their respective normal littermate controls. Active caspases were precipitated from biotin-VAD-fmk-labeled lysates, and immunoblots performed for caspase-8, MALT1, and Bcl-10. T cells from Bcl-10-deficient mice, MALT1-deficient mice, and their respective wild-type controls expressed similar levels of total caspase-8 in whole cell lysates, as well as relatively similar amounts of active caspase-8 in avidin-Sepharose precipitates (Fig. 3, *Band C*). As before, Bcl-10 and MALT1 were both associated with active caspases in wild-type T cells. However, Bcl-10-deficient T cells had no detectable MALT1 associated with active caspases (Fig. 3B, *right panels*). Similar findings were observed in a second experiment. This was consistent with the ability of MALT1 to directly associate with Bcl-10 (31, 38), which was confirmed by co-immunoprecipitation of MALT1 with Bcl-10 (data not shown). By contrast, MALT-deficient effector T cells still manifested Bcl-10 in the active caspase complex (Fig. 3C, *right panels*). Thus, Bcl-10 links MALT1 to the active caspase complex in effector T cells.

The Active Caspase-8 Complex Forms Rapidly following TCR Ligation

We next examined how quickly caspase-8 activation occurs after TCR stimulation and how rapidly NF- κ B signaling proteins associate with active caspases. Purified T cells were analyzed either unstimulated or following stimulation with soluble cross-linked anti-CD3 for 30 or 60 min. Each population was then treated with biotin-VAD-fmk for an additional 15 min prior to lysis. Active caspases were precipitated from whole cell lysates using identical amounts of protein (600 μ g), and immunoblots performed for caspase-8, MALT1, and Bcl-10. As noted earlier, unstimulated T cells possessed very little active caspase-8. In several experiments this ranged from undetectable to the low level shown in Fig. 4A. This may reflect either some activation or death of T cells during their purification, or more likely the low levels of homeostatic proliferation of T cells in normal mice (39). Nonetheless, caspase-8 activation rapidly increased within 30 min of TCR stimulation (Fig. 4A). The levels of associated MALT1 and Bcl-10 also rapidly increased proportionally with the levels of active caspase-8. These data reveal that active caspase-8 rapidly complexes with members of the NF- κ B signaling pathway following TCR ligation.

The quick activation of caspase-8 was reflected in the rapid increase of total caspase activity within the first couple of cell cycles following TCR ligation. Wild-type lymph node cells were labeled with the membrane dye CFSE and stimulated for 2 days with anti-CD3/CD28 and then transferred to new wells containing medium plus IL-2 for an additional 24 h to avoid activation-induced cell death. Under these conditions dead cells comprised less than 1% of T cells. Day 3 effector T cells were then sorted based on the number of cell divisions, and caspase activity was determined. Undivided T cells possessed negligible levels of caspase activity, comparable with naïve T cells (Fig. 4B) (4). By contrast, substantial levels of caspase activation were generated within 1–2 cell divisions, and this increased only minimally with further cell divisions. These findings are consistent with the early appearance of an active caspase complex following T cell activation, and with persistence of an active caspase complex in effector T cells. Furthermore, early caspase activity was necessary for full activation of NF- κ B. Using mice that transgenically express the NF- κ B binding site linked to a luciferase reporter (24, 25), caspase blockade with z-VAD-fmk attenuated NF- κ B activity in activated T cells (Fig. 4C). The findings were consistent in three experiments.

Cholesterol Depletion of Lipid Rafts Disrupts Formation of the Active Caspase-8 Complex

Because the active full-length caspase-8 that rapidly forms in lipid rafts after T cell activation is sustained in day 4 effector T cells and associates with NF- κ B signaling molecules, we tested whether disruption of lipid rafts would inhibit the activation of caspase-8. Day 4 effector T cells were treated or not treated with the cholesterol depleting agent MBCD for 10 min, lysed, and then fractionated into lipid raft and cytosolic compartments. Analysis of cell fractions revealed a decrease within the lipid raft fraction of LAT and caspase-8, but no change in the cytosolic location of paxillin (Fig. 5A). To disrupt lipid raft formation prior to activation, fresh T cells were pretreated or not treated with MBCD for 10 min followed by TCR stimulation for 60 min using crosslinked anti-CD3. In a similar manner, day 4 effector T cells were treated or not treated with MBCD for 10 min in the absence of further TCR stimulation. Each population was then incubated with biotin-

VAD-fmk for an additional 15 min to label active caspases, and lysed. An equivalent amount of input protein (600 μ g) from each population was used to precipitate active caspases and then immunoblotted for caspase-8, MALT1, Bcl-10, and caspase-3. The data show a clear reduction of full-length active caspase-8 from fresh T cells pretreated with MBCD prior to TCR stimulation for 60 min, as well as from day 4 effector T cells treated with MBCD before lysis (Fig. 5B). Likewise, the association of MALT1 and Bcl-10 with active caspases decreased upon disruption of lipid rafts. No active caspase-3 was detectable in fresh T cells after 60 min of TCR activation, regardless of MBCD treatment, indicating that caspase-8 activation precedes caspase-3 activation. In contrast, day 4 effector T cells did contain active caspase-3, consistent with previous studies (2, 4, 40). Furthermore, because pro-caspase-3 is activated by its cleavage, the level of active cleaved caspase-3 was not altered by MBCD treatment (Fig. 5B). This also served as a control that biotin-VAD-fmk was still able to label and precipitate active caspases in the presence of MBCD. Thus, activation of full-length caspase-8 is dependent on the continuous presence of intact lipid rafts.

DISCUSSION

The current findings demonstrate that following T cell activation there is rapid migration and activation of caspase-8 within lipid rafts, and in association with c-FLIP_L. Increased expression of c-FLIP_L enhances the level of active caspase-8 in T cells through the previously shown heterodimerization of c-FLIP_L with caspase-8 (10, 29). Furthermore, c-FLIP_L is at least one immediate substrate for caspase-8 in this process. The active caspase complex also associates with several members of the NF- κ B signaling pathway independently of Fas expression. This parallels previous observations that caspase activity in T cells is necessary for activation of NF- κ B (6). Collectively, these results suggest a model in which TCR-induced caspase-8 activation occurs in a sequestered environment that limits the degree of caspase-8 activation as well as restricts the cleavage of caspase-8 substrates to those involved with NF- κ B activation and not with cell death.

The TCR-induced association of c-FLIP_L with active caspases suggests a role for c-FLIP_L in the optimal activation of full-length caspase-8. This is consistent with structural studies showing that c-FLIP_L contains an activation loop that interacts with the enzymatic pocket of caspase-8 (10). c-FLIP_L is then rapidly cleaved by caspase-8 to form processed p43 FLIP (9, 12, 41). Evidence of p43 FLIP is apparent shortly after T cell activation (3, 4) and p43 FLIP associates more avidly with RIP1 and TRAF2 than does full-length c-FLIP_L (14, 15, 30). Consistent with these observations, induction of NF- κ B activity by c-FLIP_L requires caspase activity, whereas p43 FLIP activates NF- κ B independently of caspase activity (30). An additional caspase cleavage product of c-FLIP_L consisting of the 22-kDa N terminus has also been reported recently to interact directly with IKK γ (42). Collectively these findings provide at least one explanation for the requirement of caspase-8 enzymatic activity in T cell activation, rather than caspase-8 merely functioning as a scaffolding protein. Further support for the concept that caspase-8 activation is necessary for T cell proliferation comes from studies of humans bearing an enzymatically inactive caspase-8. T cells from these individuals proliferate minimally to TCR stimulation and manifest diminished activation of NF- κ B (5, 6). Reconstitution of caspase-8-deficient Jurkat T cells with wild-type caspase-8,

but not inactive mutant caspase-8, restored NF- κ B activation upon anti-CD3/anti-CD28 stimulation (6). Similar defective T cell proliferation was observed in mice that lack caspase-8 in the T cell compartment (7). These findings are consistent with our observations that in the absence of caspase-8 in T cells, MALT1 and Bcl-10 were not observed in the active caspase complex.

The active caspase complex that forms in lipid rafts during T cell activation may serve as a matrix to both spatially limit the degree of caspase activation, as well as to nucleate several intermediaries of the NF- κ B pathway. This complex includes PKC θ , CARMA1, MALT1, Bcl-10, RIP1, TRAF2/6, and IKK $\alpha/\beta/\gamma$. Mice deficient for PKC θ , CARMA1, Bcl-10, or MALT1 all have deficient TCR-mediated NF- κ B signaling (17, 20–22, 43). Related findings by Su *et al.* (6) demonstrated the association of Bcl-10 and the IKK complex with active caspases in TCR-stimulated human T cells. Our findings extend those observations by showing the additional association in this complex of c-FLIP_L, CARMA1, and RIP1, and the lack of a requirement for Fas.

We also observed that in effector T cells TRAF2 and TRAF6 are associated with active caspases. This is consistent with recent findings by Bidère *et al.* (44) that TRAF6 associates with caspase-8 in lipid rafts. Furthermore, RNA silencing of TRAF2 or TRAF6 expression in Jurkat T cells caused decreased IKK activation in response to anti-CD3/anti-CD28 stimulation (45). TRAF6 and Bcl-10 are responsible for Lys⁶³ubiquitination of IKK, which is essential for TCR-mediated NF- κ B signaling (45–48). Moreover, MALT1 contains two putative TRAF6 binding sites that, when mutated, lead to a decrease in MALT1-mediated IKK activation (45). TRAF2/6, MALT1, and IKK members migrate to lipid rafts following TCR stimulation, and expression of raft-targeted IKK γ is sufficient to directly drive I κ B β phosphorylation and NF- κ B signaling (49). The active caspase complex we describe resides exclusively in lipid rafts and contains many of the components necessary for TCR-mediated NF- κ B signaling.

It is clear that a significant portion of active caspase-8 in stimulated T cells is in its full-length form within lipid rafts and represents only a very small portion of total cellular caspase-8. This is consistent with molecular modeling of caspase-8 with c-FLIP_L (10, 11). A previous study of lipid rafts in activated T cells did not observe relocation of caspase-8 to lipid rafts (50). We too were initially unimpressed by the amount of caspase-8 that could be detected in the lipid raft fraction of day 4 effector T cells, and this was even less apparent at early time points. It was only after we included the additional step of selectively precipitating only the active caspase fraction using biotin-VAD-fmk that we observed the dramatic difference between active caspase-8 in the lipid raft fraction and its absence in the cytosolic fraction, despite the vast majority of total caspase-8 residing in the cytosolic fraction. Lipid raft integrity was also essential for both promoting and maintaining caspase-8 activation as well as the associated NF- κ B signaling molecules. By contrast, activation of pro-caspase-3 requires its cleavage, which is irreversible, and is thus maintained following disruption of lipid rafts. Furthermore, active caspase-3 was not detected after 60 min of TCR stimulation, whereas active fulllength caspase-8 was observed. These data indicate that caspase-3 is not likely responsible for activating full-length caspase-8 nor for the association of MALT1 and Bcl-10 to active caspase complexes, and are also consistent with the studies

showing normal T cell activation in caspase-3-deficient mice (51). Thus, confining active caspases to lipid rafts may protect effector T cells from unrestricted proteolysis of target proteins that are normally cleaved during Fas-mediated apoptosis, such as Bid (1, 4, 40), whereas at the same time promoting cleavage of other substrates, such as c-FLIP_L (4, 12, 41), that can promote NF- κ B activation.

Although the active caspase complex begins to form in lipid rafts within 30 min of TCR ligation, it clearly persists to the day 4 effector T cell stage. Previous studies revealed that naïve T cells have relatively small lipid rafts at the plasma membrane, whereas they contain a considerable intracellular raft component (52, 53). Upon TCR activation, surface lipid rafts increase in size and stability, and contain greater concentrations of selected signaling molecules (53–55). For example, a portion of total cellular Lck shifts from an intracellular localization in naïve T cells to the plasma membrane in association with CD8 in effector T cells, and this association is maintained in memory T cells (56). In another study, effector CD4⁺ T cells at day 5 continued to exhibit CD4, LAT, Fyn, and Lck in the lipid raft fractions, indicating that several signaling molecules remain stably associated with lipid rafts for prolonged periods of time (27). Our results showing the active caspase complex in day 4 effector T cells support these findings and are consistent with the known persistence of NF- κ B activity in effector T cells (27). However, once caspase-8 and c-FLIP_L association is disrupted, as with cholesterol depletion of lipid rafts, the activity of caspase-8 is greatly reduced, despite no change in total cellular levels of caspase-8. The biological role for maintaining active caspase-8 in the effector T cell stage is uncertain at present. One possibility may be to maintain NF- κ B signaling molecules complexed in a subcellular location in proximity to the TCR signaling machinery, thereby decreasing the lag time between repeated TCR ligation and NF- κ B signaling. Another possibility is that continued caspase activity may be important to ensure that T cells undergo apoptosis if repeatedly stimulated during their effector stage. Activation-induced cell death might drive the intermediate levels of effector caspases to a point that is incompatible with cell survival. This would resemble the findings with c-FLIP_L-Tg T cells in which augmented caspase activity results in both more rapid cell cycling as well as more rapid cell death (29).

At present it is uncertain how the active caspase complex is recruited to lipid rafts. One possibility would be the migration of caspase-8 to death receptors following TCR ligation. Although Fas also migrates to lipid rafts during the induction of cell death by Fas ligand (26), we found TCR-induced caspase activation and recruitment of the NF- κ B signaling molecules to be independent of Fas. We have also not detected Fas in the active caspase complex in wild-type T cells.⁵We are currently investigating whether other death receptors, such as TNFR1 or TRAIL receptors, are required for caspase activation in T cells. A somewhat similar complex involving RIP1 and TRAF2 has been observed shortly following stimulation of TNFR1 to activate NF- κ B, which later recruits caspase-8 to promote cell death (57). A recent study has suggested that TRAF6 is responsible for localization of caspase-8 to lipid rafts (44). An additional candidate would be CARMA1, which contains a PDZ domain that is responsible for recruitment of MALT1 and Bcl-10 to lipid rafts (31).

⁵J. Q. Russell, unpublished observations.

The exact role for c-FLIP_L in modulating cell death and NF- κ B signaling remains unclear. Transient transfection of c-FLIP_L was shown to induce spontaneous activation of NF- κ B in a caspase-8-dependent manner (30). By contrast, c-FLIP-deficient T cells manifested the normal ability to activate NF- κ B (58). Furthermore, transfection of c-FLIP_L into some cell lines inhibited Fas ligand-mediated secretion of IL-8 and other known NF- κ B target genes (59). Caspase blockade did not inhibit IL-8 production in response to Fas ligation, indicating that caspase activity may not be necessary for the expression of certain NF- κ B-responsive genes following Fas ligation, where caspase activation is strong, but is required for TCR-induced NF- κ B signaling where caspase activation is substantially less (59). Non-apoptotic effector T cells maintain moderate levels of caspase activity compared with the high levels of caspase activity generated following Fas stimulation (4). In the case of TCR-mediated caspase-8 activation, c-FLIP_L likely plays an important role in caspase-8 activation, and p43 FLIP may more effectively recruit NF- κ B signaling molecules than full-length c-FLIP_L (9–11). In contrast, when Fas is ligated, caspase-8 activation can be inhibited by c-FLIP-mediated competition with caspase-8 for recruitment to FADD, and thus potentially blocking the recruitment of NF- κ B signaling molecules (60). Further studies are necessary to resolve these apparent discrepancies between active caspase complexes formed following Fas *versus* TCR ligation.

The current model suggests certain predictions regarding how c-FLIP_L regulates T cell activation. One is that c-FLIP_L is pivotal to the regulation of caspase-8 activation and hence proliferation of primary T cells. Consistent with this idea is the finding that increased expression of c-FLIP_L in T cells leads to both enhanced caspase activity as well as increased proliferation (15, 16). T cell proliferation and survival are also severely reduced in the absence of c-FLIP (58, 61). Given the relatively ubiquitous expression of caspase-8 and c-FLIP_L, a similar molecular complex may be critical for the growth or development of a variety of cell types, as demonstrated in neurons (62), endothelial cells (63), monocytes (63, 64), and bone marrow stromal stem cells (65).

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REFERENCES

1. Degterev A, Boyce M, Yuan J. *Oncogene*. 2003; 22:8543–8567. [PubMed: 14634618]
2. Alam A, Cohen LY, Aouad S, Sekaly RP. *J. Exp. Med.* 1999; 190:1879–1890. [PubMed: 10601362]
3. Kennedy NJ, Kataoka T, Tschopp J, Budd RC. *J. Exp. Med.* 1999; 190:1891–1896. [PubMed: 10601363]
4. Misra RS, Jelley-Gibbs DM, Russell JQ, Huston G, Swain SL, Budd RC. *J. Immunol.* 2005; 174:3999–4009. [PubMed: 15778357]
5. Chun HJ, Zheng L, Ahmad M, Wang J, Speirs CK, Siegel RM, Dale JK, Puck J, Davis J, Hall CG, Skoda-Smith S, Atkinson T, Straus SE, Lenardo MJ. *Nature*. 2002; 419:395–399. [PubMed: 12353035]
6. Su H, Bidere N, Zheng L, Cubre A, Sakai K, Dale J, Salmena L, Hakem R, Straus S, Lenardo M. *Science*. 2005; 307:1465–1468. [PubMed: 15746428]

7. Salmena L, Lemmers B, Hakem A, Matsysiak-Zablocki E, Murakami K, Au PY, Berry DM, Tamblyn L, Shehabeldin A, Migon E, Wakeham A, Bouchard D, Yeh WC, McGlade JC, Ohashi PS, Hakem R. *Genes Dev.* 2003; 17:883–895. [PubMed: 12654726]
8. Irmeler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer J-L, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. *Nature.* 1997; 388:190–195. [PubMed: 9217161]
9. Chang DW, Xing Z, Pan Y, Algeciras-Schimmich A, Barnhart BC, Yaish-Ohad S, Peter ME, Yang X. *EMBO J.* 2002; 21:3704–3714. [PubMed: 12110583]
10. Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C, Grutter MG. *J. Biol. Chem.* 2002; 277:45162–45171. [PubMed: 12215447]
11. Boatright KM, Deis C, Denault JB, Sutherland DP, Salvesen GS. *Biochem. J.* 2004; 382:651–657. [PubMed: 15209560]
12. Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. *J. Biol. Chem.* 2001; 276:20633–20640. [PubMed: 11279218]
13. Scaffidi C, Schmitz I, Zha J, Korsmeyer SJ, Krammer PH, Peter ME. *J. Biol. Chem.* 1999; 274:22532–22538. [PubMed: 10428830]
14. Kataoka T, Budd RC, Holler N, Thome M, Martinon F, Irmeler M, Burns K, Hahne M, Kennedy N, Kovacsovic M, Tschopp J. *Curr. Biol.* 2000; 10:640–648. [PubMed: 10837247]
15. Dohrman A, Kataoka T, Cuenin S, Russell JQ, Tschopp J, Budd RC. *J. Immunol.* 2005; 174:5270–5278. [PubMed: 15843523]
16. Lens SM, Kataoka T, Fortner KA, Tinel A, Ferrero I, MacDonald RH, Hahne M, Beermann F, Attinger A, Orbea HA, Budd RC, Tschopp J. *Mol. Cell. Biol.* 2002; 22:5419–5433. [PubMed: 12101236]
17. Egawa T, Albrecht B, Favier B, Sunshine MJ, Mirchandani K, O'Brien W, Thome M, Littman DR. *Curr. Biol.* 2003; 13:1252–1258. [PubMed: 12867038]
18. Gaide O, Favier B, Legler DF, Bonnet D, Brissoni B, Valitutti S, Bron C, Tschopp J, Thome M. *Nat. Immunol.* 2002; 3:836–843. [PubMed: 12154360]
19. Hara H, Wada T, Bakal C, Kozieradzki I, Suzuki S, Suzuki N, Nghiem M, Griffiths EK, Krawczyk C, Bauer B, D'Acquisto F, Ghosh S, Yeh WC, Baier G, Rottapel R, Penninger JM. *Immunity.* 2003; 18:763–775. [PubMed: 12818158]
20. Ruefli-Brasse AA, French DM, Dixit VM. *Science.* 2003; 302:1581–1584. [PubMed: 14576442]
21. Ruland J, Duncan GS, Elia A, del Barco Barrantes I, Nguyen L, Plyte S, Millar DG, Bouchard D, Wakeham A, Ohashi PS, Mak TW. *Cell.* 2001; 104:33–42. [PubMed: 11163238]
22. Ruland J, Duncan GS, Wakeham A, Mak TW. *Immunity.* 2003; 19:749–758. [PubMed: 14614861]
23. Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, Dixit VM. *Mol. Cell.* 2000; 6:961–967. [PubMed: 11090634]
24. Millet I, Phillips RJ, Sherwin RS, Ghosh S, Voll RE, Flavell RA, Vignery A, Rincon M. *J. Biol. Chem.* 2000; 275:15114–15121. [PubMed: 10809748]
25. Voll RE, Jimi E, Phillips RJ, Barber DF, Rincon M, Hayday AC, Flavell RA, Ghosh S. *Immunity.* 2000; 13:677–689. [PubMed: 11114380]
26. Muppidi JR, Siegel RM. *Nat. Immunol.* 2004; 5:182–189. [PubMed: 14745445]
27. Balamuth F, Leitenberg D, Unternaehrer J, Mellman I, Bottomly K. *Immunity.* 2001; 15:729–738. [PubMed: 11728335]
28. Blank N, Gabler C, Schiller M, Kriegel M, Kalden JR, Lorenz HM. *J. Immunol. Methods.* 2002; 271:25–35. [PubMed: 12445726]
29. Dohrman A, Russell JQ, Cuenin S, Fortner K, Tschopp J, Budd RC. *J. Immunol.* 2005; 175:311–318. [PubMed: 15972663]
30. Kataoka T, Tschopp J. *Mol. Cell. Biol.* 2004; 24:2627–2636. [PubMed: 15024054]
31. Thome M. *Nat. Rev. Immunol.* 2004; 4:348–359. [PubMed: 15122200]
32. Habelhah H, Takahashi S, Cho SG, Kadoya T, Watanabe T, Ronai Z. *EMBO J.* 2004; 23:322–332. [PubMed: 14713952]
33. Yang K, Zhu J, Sun S, Tang Y, Zhang B, Diao L, Wang C. *Biochem. Biophys. Res. Commun.* 2004; 324:432–439. [PubMed: 15465037]

34. Chen ZJ. *Nat. Cell Biol.* 2005; 7:758–765. [PubMed: 16056267]
35. Dykstra M, Cherukuri A, Sohn HW, Tzeng SJ, Pierce SK. *Annu. Rev. Immunol.* 2003; 21:457–481. [PubMed: 12615889]
36. Cherukuri A, Dykstra M, Pierce SK. *Immunity.* 2001; 14:657–660. [PubMed: 11420035]
37. Snipas SJ, Wildfang E, Nazif T, Christensen L, Boatright KM, Bogoy M, Stennicke HR, Salvesen GS. *Biol. Chem.* 2004; 385:1093–1098. [PubMed: 15576331]
38. Lucas PC, Yonezumi M, Inohara N, McAllister-Lucas LM, Abazeed ME, Chen FF, Yamaoka S, Seto M, Nunez G. *J. Biol. Chem.* 2001; 276:19012–19019. [PubMed: 11262391]
39. Fortner KA, Budd RC. *J. Immunol.* 2005; 175:4374–4382. [PubMed: 16177078]
40. Aouad SM, Cohen LY, Sharif-Askari E, Haddad EK, Alam A, Sekaly RP. *J. Immunol.* 2004; 172:2316–2323. [PubMed: 14764700]
41. Scaffidi C, Schmitz I, Krammer PH, Peter ME. *J. Biol. Chem.* 1999; 274:1541–1548. [PubMed: 9880531]
42. Golks A, Brenner D, Fritsch C, Krammer PH, Lavrik IN. *J. Biol. Chem.* 2005; 280:14507–14513. [PubMed: 15701649]
43. Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, Annes J, Petrzilka D, Kupfer A, Schwartzberg PL, Littman DR. *Nature.* 2000; 404:402–407. [PubMed: 10746729]
44. Bidere N, Snow AL, Sakai K, Zheng L, Lenardo MJ. *Curr. Biol.* 2006; 16:1666–1671. [PubMed: 16920630]
45. Sun L, Deng L, Ea CK, Xia ZP, Chen ZJ. *Mol. Cell.* 2004; 14:289–301. [PubMed: 15125833]
46. Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ. *Cell.* 2000; 103:351–361. [PubMed: 11057907]
47. Zhou H, Du MQ, Dixit VM. *Cancer Cell.* 2005; 7:425–431. [PubMed: 15894263]
48. Zhou H, Wertz I, O'Rourke K, Ultsch M, Seshagiri S, Eby M, Xiao W, Dixit VM. *Nature.* 2004; 427:167–171. [PubMed: 14695475]
49. Sebald A, Mattioli I, Schmitz ML. *Eur. J. Immunol.* 2005; 35:318–325. [PubMed: 15597322]
50. O'Reilly LA, Divisekera U, Newton K, Scalzo K, Kataoka T, Puthalakath H, Ito M, Huang DC, Strasser A. *Cell Death Differ.* 2004; 11:724–736. [PubMed: 15017386]
51. Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW. *Genes Dev.* 1998; 12:806–819. [PubMed: 9512515]
52. Tuosto L, Parolini I, Schroder S, Sargiacomo M, Lanzavecchia A, Viola A. *Eur. J. Immunol.* 2001; 31:345–349. [PubMed: 11180097]
53. Viola A. *Trends Immunol.* 2001; 22:322–327. [PubMed: 11377292]
54. Viola A, Schroeder S, Sakakibara Y, Lanzavecchia A. *Science.* 1999; 283:680–682. [PubMed: 9924026]
55. Tavano R, Gri G, Molon B, Marinari B, Rudd CE, Tuosto L, Viola A. *J. Immunol.* 2004; 173:5392–5397. [PubMed: 15494485]
56. Bachmann MF, Gallimore A, Linkert S, Cerundolo V, Lanzavecchia A, Kopf M, Viola A. *J. Exp. Med.* 1999; 189:1521–1530. [PubMed: 10330431]
57. Micheau O, Tschopp J. *Cell.* 2003; 114:181–190. [PubMed: 12887920]
58. Zhang N, He YW. *J. Exp. Med.* 2005; 202:395–404. [PubMed: 16043517]
59. Kreuz S, Siegmund D, Rumpf JJ, Samel D, Leverkus M, Janssen O, Hacker G, Dittrich-Breiholz O, Kracht M, Scheurich P, Wajant H. *J. Cell Biol.* 2004; 166:369–380. [PubMed: 15289496]
60. Hu WH, Johnson H, Shu HB. *J. Biol. Chem.* 2000; 275:10838–10844. [PubMed: 10753878]
61. Chau H, Wong V, Chen NJ, Huang HL, Lin WJ, Mirtsos C, Elford AR, Bonnard M, Wakeham A, You-Ten AI, Lemmers B, Salmena L, Pellegrini M, Hakem R, Mak TW, Ohashi P, Yeh WC. *J. Exp. Med.* 2005; 202:405–413. [PubMed: 16043518]
62. McLaughlin B. *Apoptosis.* 2004; 9:111–121. [PubMed: 15004508]
63. Kang TB, Ben-Moshe T, Varfolomeev EE, Pewzner-Jung Y, Yorgev N, Jurewicz A, Waisman A, Brenner O, Haffner R, Gustafsson E, Ramakrishnan P, Lapidot T, Wallach D. *J. Immunol.* 2004; 173:2976–2984. [PubMed: 15322156]

64. Sordet O, Rebe C, Plenchette S, Zermati Y, Hermine O, Vainchenker W, Garrido C, Solary E, Dubrez-Daloz L. *Blood*. 2002; 100:4446–4453. [PubMed: 12393560]
65. Miura M, Chen XD, Allen MR, Bi Y, Gronthos S, Seo BM, Lakhani S, Flavell RA, Feng XH, Robey PG, Young M, Shi S. *J. Clin. Investig.* 2004; 114:1704–1713. [PubMed: 15599395]

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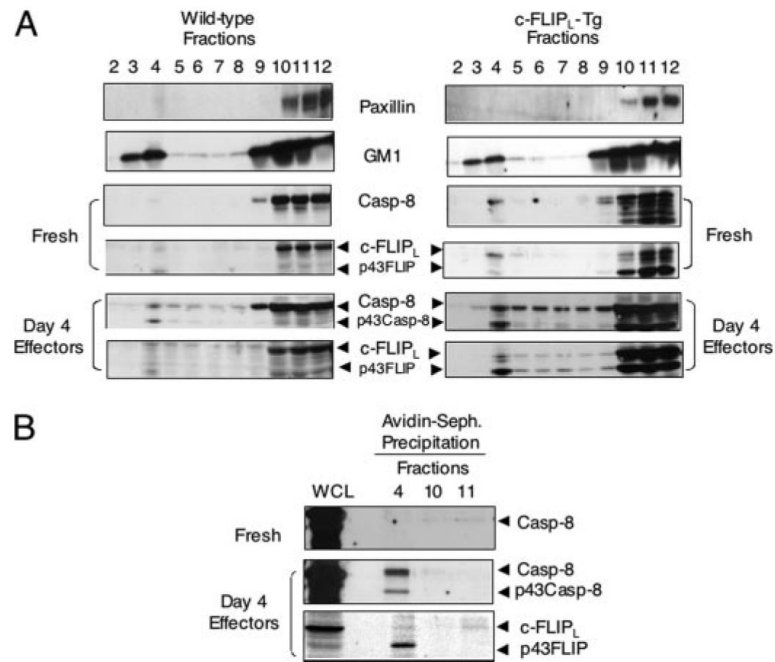


FIGURE 1. Active full-length caspase-8 and c-FLIP are present in lipid rafts of effector, but not fresh T cells

Wild-type and c-FLIP_L-Tg fresh and day 4 effector T cells were treated with biotin-VAD-fmk prior to lysis. Lipid raft and cytoplasmic compartments were isolated in 12 1-ml fractions as described under “Experimental Procedures.” A, cytosolic and lipid raft fractions were identified by immunoblots for control proteins paxillin (cytosolic) and GM1 (raft). Immunoblots were also performed for caspase-8 and c-FLIP to determine cellular localization in wild-type (*left*) and c-FLIP_L-Tg (*right*) T cells. B, 800 μ l of lipid raft fraction 4, or cytosolic fractions 10 and 11, of fresh or day 4 wild-type effector T cells were incubated with streptavidin-Sepharose beads and immunoblot analysis performed on precipitates to identify active full-length caspase-8 and cleaved p43 caspase-8, as well as full-length c-FLIP_L and processed p43 FLIP. Lipid raft fractions from fresh and day 4 effector populations contained comparable amounts of protein. Whole cell lysate (WCL) from day 4 effector T cells was included as a positive control. Data are representative of three experiments.

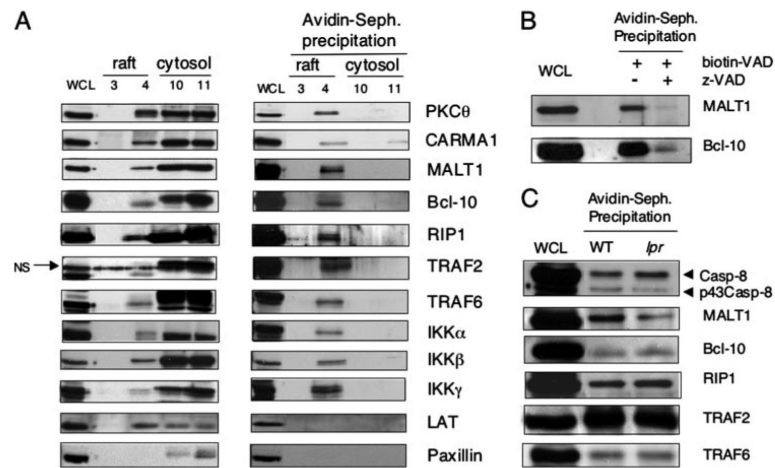


FIGURE 2. NF- κ B signaling molecules associate with active caspases in lipid rafts of effector T cells

A, wild-type day 4 effector T cells were labeled with biotin-VAD-fmk, lysed, and separated into lipid raft (3 and 4) and cytosolic (10 and 11) 1-ml fractions. 50 μ l of each fraction were separated by SDS-PAGE and immunoblotted for the indicated molecules (*left side panels*). 800 μ l of the same fractions were then treated with streptavidin-Sepharose and active caspase precipitates were separated and immunoblotted for the same molecules (*right side panels*). LAT was included to demonstrate that not every raft-associated molecule was associated with active caspases. NS, non-specific band. *B*, effector T cells were treated with biotin-VAD-fmk with or without 10-fold excess of non-biotinylated Z-VAD-fmk prior to lysis. Active caspase precipitates were immunoblotted for MALT1 and Bcl-10. *C*, active caspases were precipitated from wild-type (WT) and Fas-deficient *lpr* day 4 effector T cells and immunoblotted for the indicated molecules. Results shown are from two independent experiments. WCL, whole cell lysate.

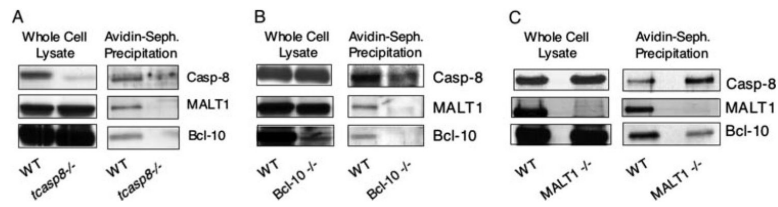


FIGURE 3. Bcl-10 is necessary for recruitment of MALT1 to active caspase complexes

Effector T cells were generated from *tcasp8*^{-/-} mice and wild-type controls (*WT*) (A) and *Bcl-10*^{-/-} mice and their normal littermate wild-type controls (B), or *MALT1*^{-/-} mice and their normal littermate wild-type controls (C), and labeled with biotin-VAD-fmk, lysed, and active caspases precipitated. Immunoblot analysis was performed for caspase-8, Bcl-10, and MALT1 on whole cell lysates (*left*) and streptavidin-Sepharose precipitates of active caspases (*right*). Results are representative of two independent experiments.

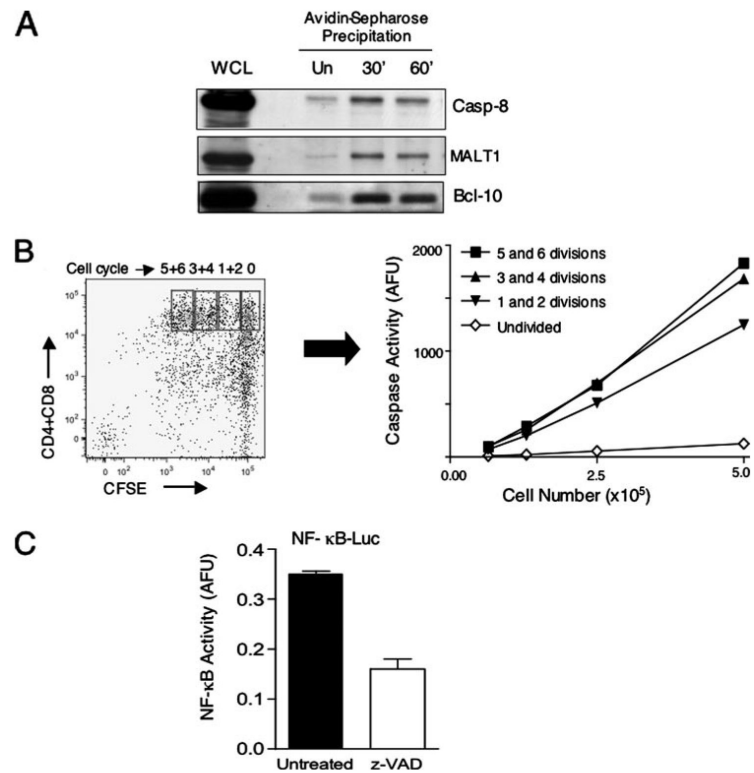


FIGURE 4. Rapid formation of active caspase complex following TCR ligation and requirement of active caspases for NF- κ B activation

A, wild-type resting T cells were either unstimulated (*Un*) or stimulated with soluble anti-CD3 cross-linked with goat anti-hamster IgG for 30 and 60 min and then treated with biotin-VAD-fmk for an additional 15 min before lysis. Active caspases were precipitated from 600 μ g of whole cell lysates and immunoblots performed for caspase-8, MALT1, and Bcl-10. Results shown are from one of three independent experiments. *B*, wild-type lymph node cells were labeled with the membrane dye CFSE followed by stimulation with anti-CD3/anti-CD28 for 2 days. Cells were then transferred to new wells containing fresh medium plus IL-2 for an additional 24 h. T cells were identified by surface staining CD4 plus CD8 and sorted based on the number of cell cycles completed. A caspase activity assay using DEVD-rhodamine was then performed on each population. *C*, T cells from NF- κ B-luciferase reporter mice were activated with anti-CD3/anti-CD28 plus IL-2 in the presence of Me₂SO vehicle control (untreated) or Z-VAD (100 μ M). After 48 h cells were analyzed for luciferase activity. Results shown are from one of three experiments. Differences were statistically significant by paired *t* test ($p < 0.05$). *WCL*, whole cell lysate.

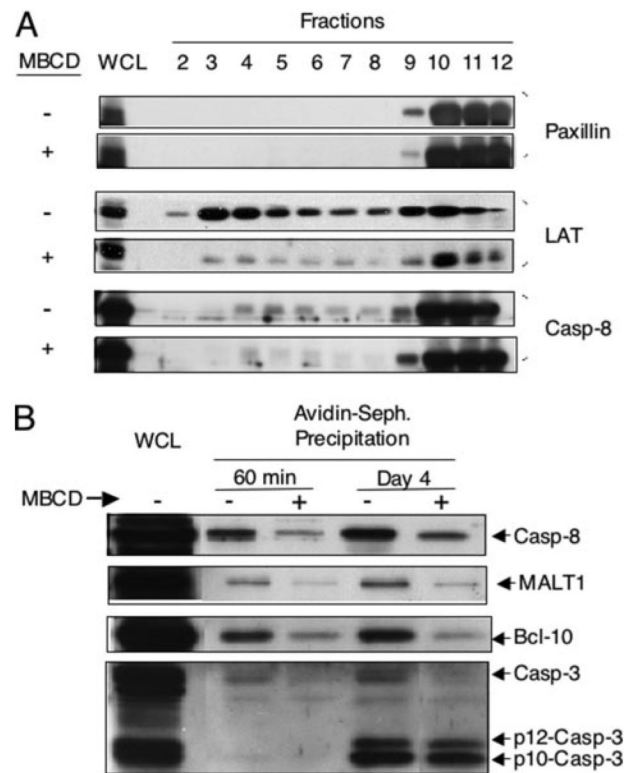


FIGURE 5. Cholesterol depletion displaces caspase-8 from lipid rafts and disrupts the activation of full-length caspase-8 and association of MALT1 and Bcl-10 with active caspases

A, day 4 wild-type effector T cells were treated with 20 mM (+) or not treated (–) for 10 min prior to lysis. Lipid raft (fraction 4) and cytosolic (fractions 10 and 11) compartments were isolated and immunoblots performed for paxillin, LAT, and caspase-8. *B*, fresh and effector T cells were either treated or not treated for 10 min with 20 mM MBCD. Fresh cells were then stimulated with soluble anti-CD3 cross-linked with goat anti-hamster for 60 min. Effector and short term-stimulated T cells were subsequently treated with biotin-VAD-fmk for 15 min, and active caspases were precipitated from 600 μ g of total cellular protein and immunoblotted for caspase-8, MALT1, Bcl-10, and caspase-3. Empty lanes within the same gel and exposure were electronically removed to allow approximation of the samples of interest. *WCL*, whole cell lysate.