Functional Assays for Macrophages Derived from ESCs/iPSCs

Using this technique, we obtained morphologically typical macrophage-like cells that adhered firmly to the culture dish. To test whether these PS-MPs possessed functional plasticity like primary macrophages, we tried to polarize them into M1 or M2 state by treating them with IFNγ or IL-4, respectively. PS-MPs exhibited typical surface markers that were characteristic of primary M1 or M2 macrophages (**Figure 5A, Figure S5A**). The M1 cytokine pattern is typically IL-12^{high} and IL-10^{low}, whereas the M2 pattern is IL-12^{low} and IL-10^{high}. [5] Pluripotent cell-derived M1 and M2 macrophages (PS-M1/M2) also exhibited cytokine profiles that were comparable to those generated from primary monocytes (**Figure 5B, Figure S5B**).

Discussion

We have established a novel differentiation system for monocytic cells from human ES and iPS cells. Since macrophages and dendritic cells are usually obtained *in vitro* from monocytes, the most important point of the evaluation is to establish whether monocytes differentiated from ESCs/iPSCs are functionally comparable to primary monocytes. In several functional assays, PS-Mo indeed proved to be comparable to primary monocytes, and importantly, PS-DCs and PC-MPs from PS-Mo were also functionally comparable to their primary counterparts.

Although complete M1/M2 macrophage polarization still requires a serum-containing medium, the present results prove that the current method can precisely manipulate macrophages that have the potential to differentiate into M1/M2 macrophages. The cytokine profiles of PS-M1/M2 were also comparable to those of primary M1/M2 macrophages. The expression patterns of surface markers in PS-DCs after LPS stimulation and of PS-MPs after M1/M2 polarization were almost identical to those of DCs/MP derived from primary monocytes. However, the level of IL-10 in PS-DCs after stimulation was higher than that in primary DCs and the expression levels of HLA-DR in PS-DCs/MP were low in comparison with those in DCs/MP derived from primary monocytes. Therefore, further improvement of culture conditions such as the use of a modified medium and cytokine cocktail will be needed.

Several embryonic body methods and feeder cell co-culture methods for PS-DCs/MP differentiation have already been reported. [7,27,29–30] These methods show relatively poor-reproducibility because of the use of xenogeneic feeder cells and/or serum. In an earlier report which describes a protocol that can derive macrophages and dendritic cells from human iPSCs in feeder- and serum-free manner, [7] the authors did not fully characterize the monocytes and noted that PS-DCs/MP were generated only from two of the five iPSC clones tested. The current culture system simply propagated progenitor cells in 2-dimensional cultures without passage or sorting, and floating PS-Mo and PS-DCs/MP could be obtained repetitively from all five ESC/iPSC clones tested (**Figure S2 and S6**). These monocytic cells derived from disease- or patient-specific iPSC would be useful tools for the examination of disease pathologies and for drug discovery in immunological disorders such as autoimmune diseases, immunodeficiencies and autoinflammatory syndromes. However, even in our protocol, there are subtle clonal variations of timing of differentiation such as the day of step 3 to 4 switching which is determined by the emergence of CD43^{+}CD45^{+} cells (day 13–15, data not shown). Fine adjustment of the protocol for each ESC/iPSC clone seemed to further improve the yield of monocytes.

iPSC technology is overcoming immunological and ethical concerns in regenerative medicine using human pluripotent cells. Furthermore, a number of disease-associated iPSCs generated from patients with immunological disorders have been reported. [15,31–34] Because patient- or disease-specific iPS cells will be an important resource for unraveling human immunological disorders, a robust and simple hematopoietic differentiation system that can reliably mimic in vivo hematopoiesis is necessary for this purpose. Our simple and robust protocol to produce monocytic cells is therefore expected to be useful for regenerative medicine and studies of immunological disorders.

Supporting Information

Figure S1 Image of floating hematopoietic cells derived from iPS cells Phase contrast image of floating hematopoietic cells derived from iPS-201B7 at day 21 (step 4).
(PDF)

Figure S2 Phenotype analysis and gene expression pattern of monocytic lineage cells derived from 3 additional pluripotent stem cell lines. (A) The percentage of CD14+ cells within the total floating cells derived from 3 iPSC clones (253G4, CIRA188Ai-W2, and CB-A11) was evaluated from day 13 to day 28. (B) RT-PCR analysis of monocytic lineage cells derived from 253G4, CIRA188Ai-W2, and CB-A11 clones for expression of monocytic lineage marker genes (c-MAF, TLR4, and CCL17). Peripheral blood monocytes and peripheral blood monocyte-derived mature DCs were used as positive controls.
(PDF)

Figure S3 Characteristics of primary monocytes and macrophages. (A) Phase contrast image and (B) flow cytometric analysis of macrophages derived from primary monocytes. (C) The levels of IL-6 and TNF-α in supernatants of primary monocyte culture medium 4 hours after LPS stimulation. (D) The levels of IL-1β were measured 4 hours after LPS stimulation with/without an additional 30-minute ATP stimulation.
(PDF)

Figure S4 Characteristics and functional assays of dendritic cells derived from primary monocytes. (A) Flow cytometric analysis of immature/mature DCs derived from primary monocytes. (B) The levels of IL-10 and TNF-α in supernatants of culture medium with primary-DCs 24 hours after LPS stimulation. (C) The proliferation of allogeneic naïve T cells (1×105 cells per well) co-cultured with 40 Gy-irradiated stimulator cells for 3 days was evaluated. The proliferation of naïve T cells in the last 16 hours was measured by 3H-thymidine uptake.
(PDF)

Figure S5 Characteristics and functional assays of M1/M2 macrophages derived from primary monocytes. (A) Flow cytometric analysis of M1/M2 macrophages derived from primary monocytes. (B) The levels of IL-12p70 and IL-10 in supernatants of culture medium with M1/M2 macrophages derived from primary monocytes 24 hours after LPS stimulation.
(PDF)

Figure S6 Replication assays for 3 additional pluripotent stem cell lines. (A) Phase contrast image (left) and May-Giemsa staining (right) of mature DCs derived from iPSC clones. (B) Phase contrast image of macrophages derived from iPSC clones. (C) Flow cytometric analysis of immature/mature DCs and macrophages derived from iPSC clones.
(PDF)

Table S1 Primers for RT-PCR.
(PDF)

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Author Contributions

iPSC establishment: MDY IA. Conceived and designed the experiments: MDY AN HG TH TN MKS. Performed the experiments: MDY ST SN YM TT JI FHO. Analyzed the data: MDY AN TY KO TN MKS. Wrote the paper: MDY AN TY MKS.

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T-cell receptor ligation causes Wiskott-Aldrich syndrome protein degradation and F-actin assembly downregulation

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Background: Wiskott-Aldrich syndrome protein (WASP) links T-cell receptor (TCR) signaling to the actin cytoskeleton. WASP is normally protected from degradation by the Ca^{++}-dependent protease calpain and by the proteasome because of its interaction with the WASP-interacting protein.
Objective: We investigated whether WASP is degraded after TCR ligation and whether its degradation downregulates F-actin assembly caused by TCR ligation.
Methods: Primary T cells, Jurkat T cells, and transfected 293T cells were used in immunoprecipitation experiments. Intracellular F-actin content was measured in splenic T cells from wild-type, WASP-deficient, and c–Casitas B-lineage lymphoma (Cbl)-b–deficient mice by using flow cytometry. Calpeptin and MG-132 were used to inhibit calpain and the proteasome, respectively.
Results: A fraction of WASP in T cells was degraded by calpain and by the ubiquitin-proteasome pathway after TCR ligation. The Cbl-b and c-Cbl E3 ubiquitin ligases associated with WASP after TCR signaling and caused its ubiquitination. Inhibition of calpain and lack of Cbl-b resulted in a significantly more sustained increase in F-actin content after TCR ligation in wild-type T cells but not in WASP-deficient T cells.
Conclusion: TCR ligation causes WASP to be degraded by calpain and to be ubiquitinated by Cbl family E3 ligases, which targets it for destruction by the proteasome. WASP degradation might provide a mechanism for regulating WASP-dependent TCR-driven assembly of F-actin. (J Allergy Clin Immunol 2013;132:648-55.)

Key words: *Wiskott-Aldrich syndrome, Wiskott-Aldrich syndrome protein, T-cell receptor, calpain, ubiquitination, proteasome, F-actin, Cbl family proteins*

Abbreviations used
Arp: Actin-related protein
Cbl: Casitas B-lineage lymphoma
EVH1: Ena-VASP homology domain 1
IS: Immune synapse
TCR: T-cell receptor
WAS: Wiskott-Aldrich syndrome
WASP: Wiskott-Aldrich syndrome protein
WIP: WASP-interacting protein
WT: Wild-type
ZAP-70: ζ Chain–associated protein kinase of 70 kDa

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by variable immunodeficiency, eczema, and thrombocytopenia.[1] The gene for Wiskott-Aldrich syndrome protein (WASP) is mutated in patients with WAS and X-linked thrombocytopenia. *WAS* is located on Xp11.22-p11.23 and encodes a protein of 502 amino acids and approximately 60 kDa in molecular weight.[2,3] WASP expression is restricted to hematopoietic cells.[3]

WASP has an N-terminal Ena/VASP homology domain 1 (EVH1) domain, a Cdc42/Rac GTPase-binding domain, a proline-rich domain, a G actin–binding verprolin homology (V) domain, a cofilin homology (C) domain, and a C-terminal acidic (A) segment.[1] The last 3 domains are located at the C-terminal end of WASP and are collectively referred to as the VCA domain. WASP interacts with WASP-interacting protein (WIP) through its EVH1 domain; with Cdc42-GTP through its GTPase-binding domain; with multiple SH3 domain–containing proteins, which include Nck, Grb2, and cortactin, through its proline-rich region; and with the actin-related protein (Arp) 2/3 complex that initiates actin polymerization through its VCA domain.[4-6]

WASP plays a critical role in T-cell activation and actin reorganization.[7-9] T cells from patients with WAS and $WASP^{-/-}$ mice are deficient in their ability to increase their F-actin content, secrete IL-2, and proliferate after T-cell receptor (TCR) ligation.[10-12] WASP exists in cells in a closed inactive conformation through intramolecular interactions that prevent the VCA domain from activating the Arp2/3 complex. Binding of Cdc42-GTP or of the SH3 domain of adaptor proteins, as well as phosphorylation of tyrosine (Y) at position 291, is thought to cause a conformational change in WASP, which allows the VCA domain to interact with and activate the Arp2/3 complex.[5,13-15] The WASP-interacting protein (WIP) is expressed at high levels in lymphoid tissues. Most of WASP is associated with WIP in T cells.[16] WIP binds through its C-terminal end to the EVH1 domain of WASP. WIP plays an important role in the recruitment of the WASP-WIP complex to ζ chain–associated protein kinase of 70 kDa (ZAP-70) and

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to the immunologic synapse after TCR ligation.[17] More importantly, WIP stabilizes WASP in T cells. This is evidenced by the finding that WASP levels are significantly reduced in T cells from $WIP^{-/-}$ mice and a WIP-deficient patient.[16,18] Furthermore, most missense mutations in WASP that result in diminished WASP levels are localized to the WIP-binding EVH1 domain of WASP and disrupt the WASP-WIP interaction.[19,20] Expression of the WASP-binding domain of WIP in these cells restores WASP levels close to normal.[21] Treatment with calpain and proteasome inhibitors increases WASP protein levels in T cells from $WIP^{-/-}$ mice and patients with WAS with missense mutations that disrupt WIP binding,[16] indicating that WASP can be subject to degradation by calpain and the ubiquitin-proteasome pathway.

Unregulated activation of WASP is detrimental to many cell types, especially cells of the myeloid lineage. Three different mutations of WASP, namely L270P, S272P, and I294T, destroy the autoinhibitory conformation of WASP, resulting in a constitutively active protein, and cause X-linked neutropenia.[22] The L270P and S272P mutations localize to the GTPase-binding domain,[23] whereas the I294T mutation is located close to the tyrosine residue Y291, which, when phosphorylated, results in the activation of WASP.[24] Knock-in mouse models mimicking the L270P and I294T mutations have been described.[25] T and B cells from these mice show a marked increase in F-actin levels but migrate normally in response to chemokines.

In this study we demonstrate that TCR ligation causes WASP to be degraded by calpain and by Casitas B-lineage lymphoma (Cbl)–mediated ubiquitination and proteasomal destruction. We demonstrate that WASP degradation provides a mechanism for downregulating TCR-driven assembly of F-actin.

METHODS

Cell lines and T cells

Jurkat T cells were obtained from the American Type Culture Collection and maintained in RPMI medium (Gibco, Carlsbad, Calif) supplemented with 10% FBS. 293T cells were a gift from Dr N. Ishii (Tohoku University, Sendai, Japan) and were maintained in Dulbecco modified Eagle medium (Gibco) supplemented with 10% FBS. Spleens from Cbl-b knockout (*Cbl-b*$^{-/-}$) mice and genetically matched wild-type (WT) shipping controls were a generous gift from Dr H. Gu, Columbia University. WASP-deficient mice were obtained from Dr Scott Snapper. Splenic T cells were isolated by using T-cell enrichment columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

Antibodies

Anti-WASP 5A5 mAb, which recognizes the epitope in the region corresponding to amino acids 146 to 265, was developed in our laboratory.[26] Anti-WASP rabbit polyclonal antibody K374 (a gift from Dr Ignacio Molina) is directed to the C-terminal 20 amino acids of WASP.[16] Anti–phospho-WASP antibody, which recognized WASP phosphorylated on Y291, was purchased from Abcam (Cambridge, United Kingdom). Anti-ubiquitin mAb P4D1, anti–c-Cbl mAb A-9, and anti–Cbl-b mAb G-1 were from Santa Cruz Biotechnology (Santa Cruz, Calif). Anti-actin mAb and anti-FLAG mAb were from Sigma (St Louis, Mo). Anti-HA mAb was from Cell signaling (Danvers, Mass). Control rabbit IgG was from Upstate (Billerica, Mass).

TCR stimulation, immunoprecipitation, and Western blotting

TCR ligation was performed, as described previously.[17] Briefly, T cells were incubated with 10 μg/mL anti-CD3 mAb UCHT1 (Calbiochem, San Diego, Calif) on ice for 20 minutes, followed by cross-linking with 15 μg/mL goat anti-mouse IgG (H+L; Caltag, Buckingham, United Kingdom) at 37°C for the indicated period. Cells were lysed in ice-cold lysis buffer containing 1% Triton X-100 and protease inhibitors. Cell lysates were clarified at 14,000*g* for 20 minutes at 4°C. For immunoprecipitation, cell lysates were precleared with protein G–Sepharose (GE Healthcare, Fairfield, Conn) for 2 hours and incubated overnight at 4°C with 4 μg of the indicated antibody preadsorbed onto protein G–Sepharose. Beads were washed 4 times with modified lysis buffer containing 0.2% Triton X-100. Bound proteins were eluted, run on 10% SDS-PAGE gels, and analyzed by means of Western blotting with the indicated antibodies followed by anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase and enhanced chemiluminescent detection (Amersham Life Sciences, Piscataway, NJ). Densitometry was performed with CS Analyzer version 2.08 software (ATTO Corporation, Tokyo, Japan) or ImageJ version 1.45 software.

Calpain and proteasome inhibition

The proteasome inhibitor MG132 and the calpain inhibitor calpeptin were purchased from Calbiochem. Cells were cultured with calpeptin (1 μmol/L) or MG132 (10 μmol/L) for 6 hours before anti-CD3 stimulation.

Expression vectors and transfection

Human pcDNA3.1-EGFP-hWASP-WT was a generous gift from Dr K. A. Siminovitch (University of Toronto, Toronto, Ontario, Canada). Control pAcGFP1-C1 vector was purchased from Clontech (Mountain View, Calif). Human pcDNA3.1-3xFLAG-c-Cbl-WT, pcDNA3.1-3xFLAG-Cbl-b-WT, and pcDNA3.1-HA-Ub were gifts from Drs N. Ishii and Y. Tanaka (Tohoku University).[27] Control p3xFLAG-CMV-14 vector was purchased from Sigma. 293T cells were transiently transfected with lipofectamine, as described previously,[27] and harvested 48 hours after transfection.

Determination of cellular F-actin content

Flow cytometric analysis of F-actin content was performed, as described previously.[16] Briefly, mouse T cells were purified by using negative selection with the Pan T Cell Isolation Kit (Miltenyi Biotec) and then incubated for 6 hours with 1 μmol/L calpeptin. Cells were then washed and incubated with 10 μg/mL rat anti-mouse CD3 mAb (Serotec, Oxford, United Kingdom) for 30 minutes on ice. Cells were stimulated for the indicated times by cross-linking with goat anti-rat IgG (H+L) secondary antibody (Jackson Immunoresearch, West Grove, Pa). Cells were fixed in 4% formaldehyde, washed, and permeabilized with the CytoFix/CytoPerm cell staining kit (BD Biosciences, San Jose, Calif). F-actin was stained with tetramethylrhodamine isothiocyanate–labeled phalloidin (Invitrogen, Carlsbad, Calif). F-actin content was analyzed by measuring the mean fluorescent intensity with FACS LSRFortessa (Becton Dickinson) and FlowJo (TreeStar, Ashland, Ore) software.

Statistical analysis

Statistical analysis was performed with the Student *t* test.

RESULTS

WASP is C-terminally truncated by calpain after TCR ligation

Purified peripheral blood T cells were stimulated with anti-CD3 mAb followed by cross-linking with secondary antibody and cell lysates were immunoblotted for WASP to investigate whether WASP is degraded after TCR ligation. Immunoblotting with mAb 5A5, which recognizes an epitope in the region corresponding to amino acids 146 to 265, revealed a 62- to 64-kDa band in unstimulated T cells (Fig 1, *A*), as previously observed.[28] Stimulation with anti-CD3 resulted in the appearance of a 55-kDa

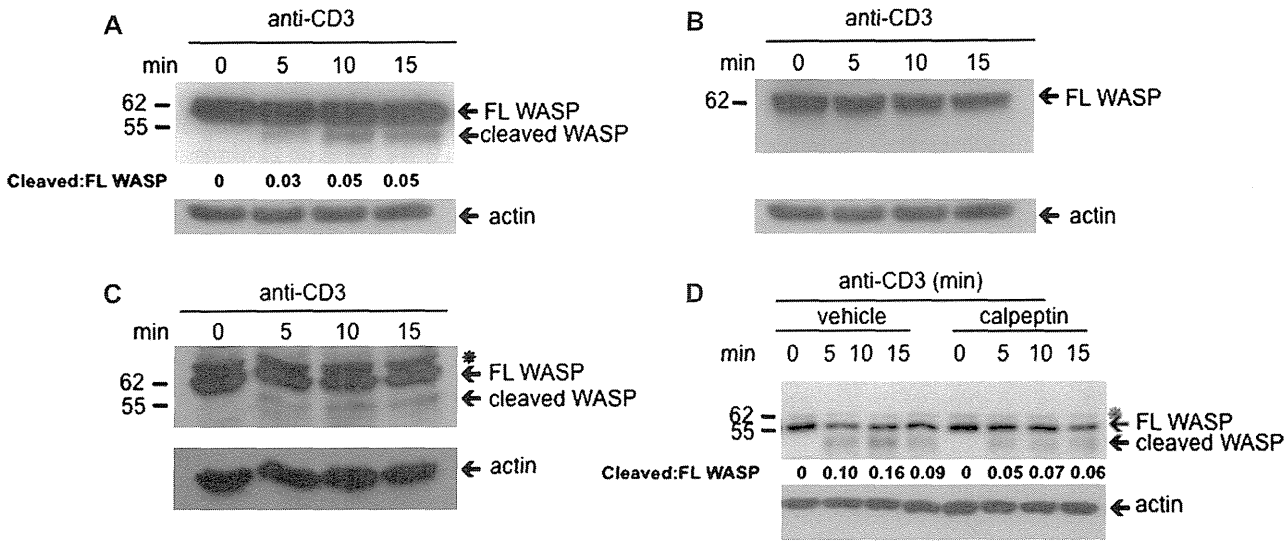

FIG 1. WASP is cleaved by calpain after TCR ligation. **A** and **B,** WASP immunoblot of peripheral blood T cells stimulated for 0 to 15 minutes with anti-CD3 mAb using mAb 5A5 (Fig 1, *A*) or polyclonal antibody K374 (Fig 1, *B*). **C,** WASP immunoblot of Jurkat T cells stimulated with anti-CD3 mAb using mAb 5A5. **D,** Effect of pretreatment for 6 hours with calpeptin on anti-CD3–driven WASP degradation in peripheral blood T cells. Lysates were immunoblotted with mAb 5A5. *Nonspecific band. The positions of molecular weight markers are indicated on the *left* in Fig 1, *A* to *D*. The ratio of cleaved WASP to full-length *(FL)* WASP in Fig 1, *A* and *D*, represents the mean of 5 experiments. Similar results were obtained in Fig 1, *A* to *D*, in 5 independent experiments.

WASP fragment at 5 minutes, which increased at 10 and 15 minutes after stimulation. Scanning densitometric analysis revealed that the intensity of the cleaved WASP band was approximately 5% that of the full-length WASP band at 10 and 15 minutes after stimulation. Similar results were obtained in Jurkat T cells (Fig 1, *C*).

Immunoblotting lysates of T cells with the rabbit polyclonal antibody K374 raised against the C-terminal 20 amino acids of WASP revealed the same 62- to 64-kDa band detected by using mAb 5A5 but did not detect the 55-kDa WASP fragment in anti-CD3–stimulated T cells that was detected by using mAb 5A5 (Fig 1, *B*). Similar results were obtained in Jurkat T cells (data not shown). This result indicates that the 55-kDa WASP fragment lacks the C-terminal VCA domains of WASP (amino acids 421-502) responsible for its actin-polymerizing activity.

Calpain cleaves WASP *in vitro*[29] and contributes to WASP degradation in WIP-deficient T cells.[16,30] To examine whether calpain was responsible for the cleavage of WASP after TCR ligation, T cells were pretreated with the calpain inhibitor calpeptin for 6 hours, washed, and stimulated with anti-CD3 mAb for 5 minutes. Preincubation with calpeptin attenuated by approximately 50% the generation of the 55-kDa WASP fragment in response to anti-CD3 stimulation (Fig 1, *D*), strongly suggesting that calpain mediates the C-terminal truncation of WASP after TCR/CD3 ligation, at least in part.

WASP is ubiquitinated and degraded by the proteasome in T cells after TCR ligation

In the absence of WIP, WASP is degraded by the ubiquitin-proteasome pathway.[16,30] To investigate whether WASP is a substrate for ubiquitination, we incubated *in vitro* transcribed and translated WASP with purified ubiquitin and ubiquitin-conjugating enzymes (mixture of E1, E2, and E3 enzymes), and the reaction mixture was immunoblotted with anti-ubiquitin mAb. WASP was polyubiquitinated in the presence of ubiquitin and ubiquitin-conjugating enzymes, as indicated by an intense high-molecular-weight smear (Fig 2, *A*). Addition of the 26S proteasome fraction to the ubiquitination mixture resulted in marked attenuation of the ubiquitinated WASP smear. These results indicate that after TCR/CD3 ligation, WASP is subject to ubiquitination, which targets it for destruction by the proteasome.

We next examined whether WASP is ubiquitinated in T cells after TCR ligation. Fig 2, *B*, shows the appearance of polyubiquitinated WASP after anti-CD3 mAb stimulation of Jurkat T cells. To examine whether WASP ubiquitinated after TCR ligation is targeted for destruction by the proteasome, Jurkat T cells were pretreated with the proteasome inhibitor MG132 for 6 hours and then stimulated with anti-CD3 mAb for 10 minutes, and WASP immunoprecipitates were prepared from their lysates and probed for ubiquitin. Fig 2, *C*, shows that ubiquitinated WASP was weakly detectable in unstimulated Jurkat cells, but its levels increased after TCR/CD3 stimulation. Pretreatment with MG132 modestly increased the amounts of ubiquitinated WASP in unstimulated Jurkat cells and strongly increased the amounts of ubiquitinated WASP detected after TCR/CD3 ligation. These results indicate that WASP is ubiquitinated and degraded by the proteasome after TCR ligation.

The Cbl family proteins c-Cbl and Cbl-b associate with WASP after TCR ligation and act as E3 ubiquitin ligases for WASP

Members of the Cbl family of E3 ubiquitin ligases are negative regulators in TCR signaling.[31,32] We tested the hypothesis that Cbl proteins might be involved in WASP ubiquitination. We first

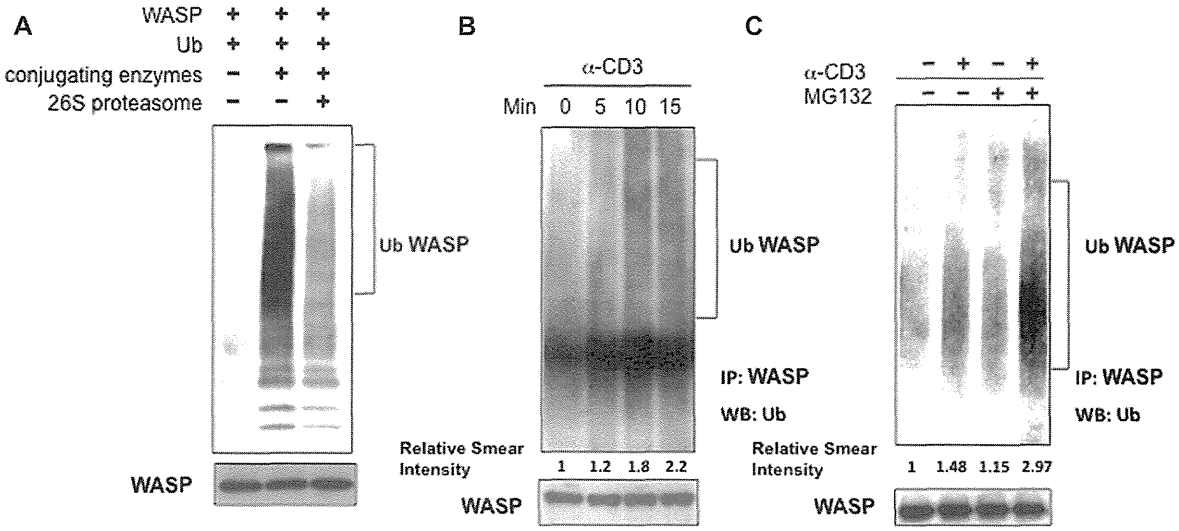

FIG 2. WASP is ubiquitinated and degraded by the 26S proteasome *in vitro* and in anti-CD3–stimulated Jurkat cells. **A,** Ubiquitination of *in vitro* translated purified WASP by ubiquitin-conjugating enzymes and its degradation by the 26S proteasome. Reaction mixtures were probed with anti-ubiquitin. **B,** Generation of ubiquitinated WASP in Jurkat T cells after stimulation with anti-CD3 mAb. WASP immunoprecipitates were probed with anti-ubiquitin mAb. Polyubiquitinated WASP appears as a smear. **C,** Protection of ubiquitinated WASP from degradation by the proteasome inhibitor MG132 in anti-CD3–stimulated Jurkat T cells. Similar results were obtained in Fig 2, *A* to *C*, in 4 independent experiments. The relative smear intensity in Fig 2, *B* and *C*, represents the mean of 4 experiments. *IP*, Immunoprecipitate; *Ub*, ubiquitin; *WB*, Western blot.

investigated whether Cbl proteins and WASP form a complex. WASP immunoprecipitates from Jurkat cell lysates were probed for c-Cbl and Cbl-b. c-Cbl, but not Cbl-b, coprecipitated weakly with WASP in unstimulated Jurkat T cells. TCR ligation increased the association of c-Cbl with WASP. It also induced the association of Cbl-b with WASP at 10 and 15 minutes after stimulation (Fig 3, *A*).

To investigate whether Cbl proteins act as E3 ubiquitin ligases for WASP, we transiently transfected 293T cells with plasmids coding for WT WASP, HA-tagged ubiquitin, FLAG-tagged c-Cbl, or FLAG-tagged Cbl-b. WASP coprecipitated with both c-Cbl and Cbl-b and was polyubiquitinated significantly more when co-transfected with ubiquitin, c-Cbl, and Cbl-b than with ubiquitin and empty vector (Fig 3, *B*).

To examine the role of Cbl-b in WASP ubiquitination after TCR ligation, we used purified T cells from spleens of *Cbl-b*$^{-/-}$ mice. Ubiquitination of WASP after TCR ligation was reduced, although not completely abrogated, in T cells from *Cbl-b*$^{-/-}$ mice (Fig 3, *C*), suggesting that WASP is a substrate for Cbl-b in antigen-stimulated T cells. We could not examine the role of c-Cbl on WASP ubiquitination after TCR ligation because we had no access to T cells from *c-Cbl*$^{-/-}$ mice.

WASP degradation after TCR/CD3 ligation limits TCR/CD3-driven F-actin assembly in T cells

WASP is important for F-actin assembly in T cells.[10] We examined whether WASP degradation after TCR/CD3 ligation regulates TCR/CD3-driven F-actin assembly. Purified T cells from WT and WASP-deficient mice were incubated for 6 hours with calpeptin or left untreated, washed and stimulated with anti-CD3 mAb, and cross-linked with a secondary antibody. The cells were then fixed, permeabilized, stained for F-actin with fluorescein isothiocyanate–conjugated phalloidin, and analyzed by means of flow cytometry. As previously reported, WASP-deficient T cells had a lower F-actin content than WT T cells.[16] TCR/CD3 ligation caused a parallel increase in F-actin levels in both WT and WASP-deficient T cells, which peaked at 5 minutes after stimulation and returned almost to baseline 10 minutes after stimulation. Pretreatment with calpeptin had no effect on F-actin content of the T cells at baseline or at 2 and 5 minutes after stimulation; however, it significantly increased the F-actin content of WT T cells at 10 minutes after anti-CD3 stimulation, maintaining it at almost the peak level achieved at 5 minutes after stimulation. In contrast, pretreatment with calpeptin had no effect on the F-actin content of WASP-deficient T cells 10 minutes after anti-CD3 stimulation. These results suggest that calpain-mediated WASP degradation limits the duration of F-actin assembly after TCR/CD3 ligation.

We next examined whether ubiquitination, which targets WASP for proteosomal degradation, regulates F-actin assembly after TCR/CD3 ligation. Because Cbl-b participates in WASP ubiquitination, we examined F-actin assembly in T cells deficient in Cbl-b. Baseline F-actin content and TCR-driven F-actin assembly were both significantly increased in T cells from c-Cbl-deficient mice compared with T cells from WT control animals (Fig 4, *B*). These results suggest that WASP degradation by ubiquitination regulates baseline and TCR-driven F-actin assembly.

DISCUSSION

Our results demonstrate that TCR ligation triggers the degradation of WASP by calpain-mediated cleavage and Cbl-mediated ubiquitination and subsequent proteasomal degradation. We present evidence that WASP degradation provides a mechanism for limiting the duration of TCR-driven assembly of F-actin.

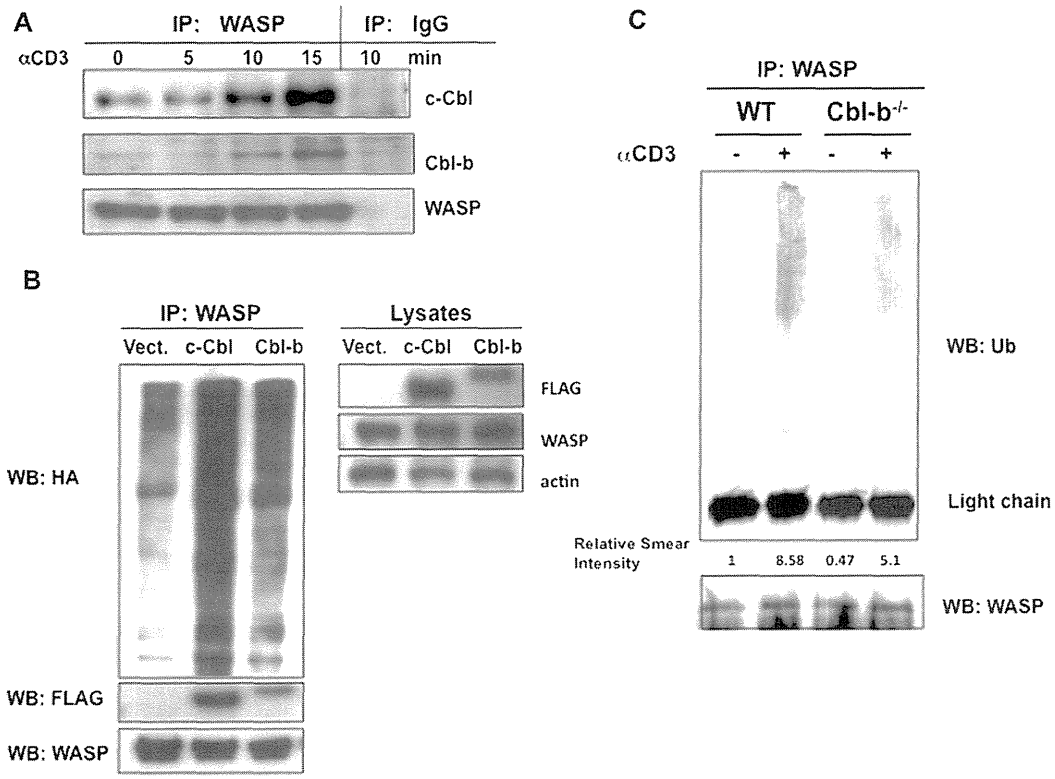

FIG 3. Cbl family E3 ubiquitin ligases associate with and ubiquitinate WASP after TCR ligation. **A,** Western blot analysis of WASP immunoprecipitates from anti-CD3–stimulated Jurkat T cells for c-Cbl and Cbl-b. IgG control antibody precipitates were prepared 10 minutes after anti-CD3 stimulation and used as controls. **B,** Ubiquitination of WASP in 293T cells transfected with WT WASP and HA-tagged ubiquitin plus either FLAG vector alone *(Vect.)*, FLAG-tagged c-Cbl, or FLAG-tagged Cbl-b. In the *left panel* WASP immunoprecipitates were probed for HA, FLAG, and WASP. In the *right panel* total lysates were probed for FLAG–c-Cbl or FLAG–Cbl-b, WASP, and actin. **C,** WASP ubiquitination after TCR ligation in T cells from *Cbl-b*$^{-/-}$ mice and WT control animals. WASP immunoprecipitates were probed for ubiquitin. Similar results were obtained in Fig 3, *A* to *C*, in 4 independent experiments. The relative smear intensity in Fig 3, *C*, represents the mean of 4 experiments. *IP*, Immunoprecipitate; *Ub*, ubiquitin; *WB*, Western blot.

TCR/CD3 ligation resulted in the degradation of a small fraction of WASP through calpain-mediated cleavage and the ubiquitin proteasome pathway. We estimated that approximately 5% of WASP is degraded after TCR/CD3 ligation. This is possibly an underestimate because the truncated 55-kDa WASP might be less stable than intact WASP. We could not detect a decrease in the levels of intact WASP in anti-CD3–activated T cells, probably because Western blotting is not sensitive enough to detect a small decrease in protein levels. We were unable to detect the cleaved, C-terminal, approximately 10-kDa fragment using an antibody to the C-terminus of WASP. This is most likely because such a small cleaved fragment would be rapidly degraded in the cell. Normally, WASP is protected from degradation by its partner, WIP.[16] The conformational changes in WASP induced by TCR signaling, which involve a change from an inactive to an active form capable of activating the Arp2/3 complex and F-actin polymerization, possibly increases the susceptibility of WASP to calpain cleavage and to ubiquitination and proteasomal degradation. The observation that WASP is degraded by calpain after TCR ligation is consistent with previous observations that WASP can be degraded in platelet lysates by calpain[29] and that *in vitro* translated WASP is a substrate for calpain I and II.[16] The increase in intracellular Ca^{++} concentration that follows TCR ligation could be the trigger for the Ca^{++}-dependent activation of calpain in anti-CD3–stimulated T cells.

Both c-Cbl and Cbl-b associated with WASP when overexpressed in 293T cells and acted as E3 ubiquitin ligases for WASP ubiquitination *in vitro*. More importantly, WASP ubiquitination after TCR ligation was impaired in Cbl-b-deficient T cells, implicating at least Cbl-b in WASP ubiquitination in T cells. Cbl family proteins act as negative regulators of TCR signaling by virtue of their ability to ubiquitinate LCK and ZAP-70,[33] which are upstream of WASP. Thus Cbl family members might regulate WASP activity indirectly by dampening TCR signaling upstream of WASP, as well as directly by ubiquitinating WASP and targeting it for degradation. Evidence has been presented that the activated WASP phosphorylated at Y291 is a target for ubiquitination.[34] We have also found that inhibition of the proteasome by MG132 increases the amount of tyrosine-phosphorylated WASP in anti-CD3–stimulated cells (see Fig E1 in this article's Online Repository at www.jacionline.org). This observation lends further support to the notion that activated WASP molecules are targets for degradation after TCR ligation.

It is not clear whether the interaction between WASP and c-Cbl and Cbl-b is direct or mediated by other proteins. It has been reported that c-Cbl associates with multiple proteins, which

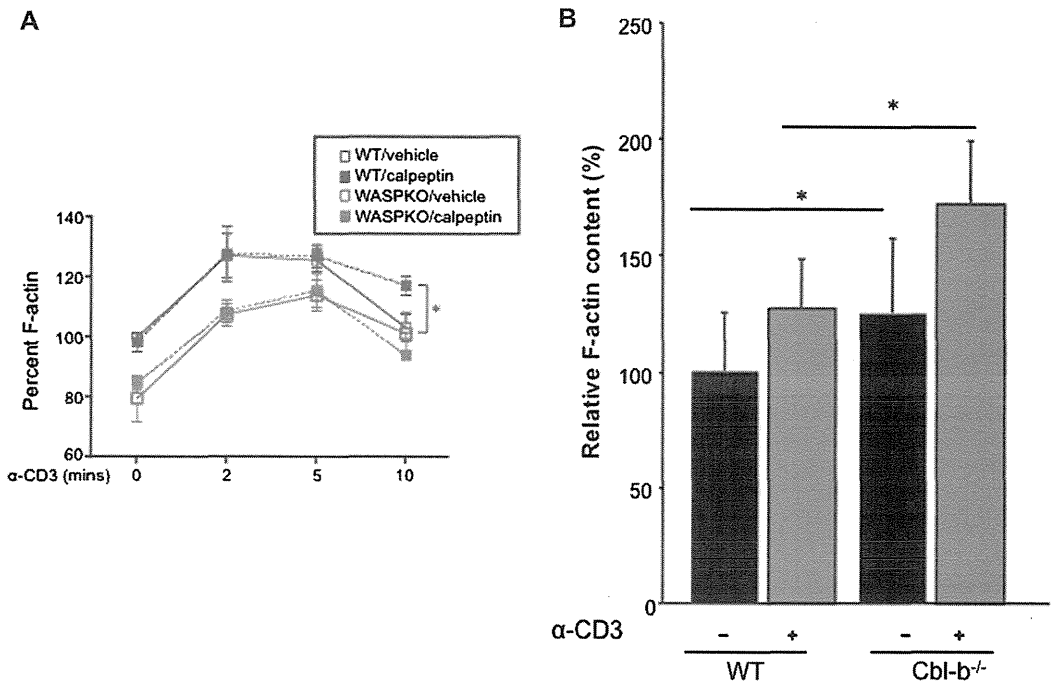

FIG 4. Effect of calpain inhibition and loss of Cbl on F-actin assembly in T cells. **A,** Effect of pretreatment of T cells from WT and WASP knockout mice with calpeptin on TCR-driven assembly of F-actin. Pretreatment with the vehicle dimethyl sulfoxide was used as a control. **B,** TCR-driven assembly of F-actin in T cells from *Cbl-b*$^{-/-}$ mice and WT control animals. T cells were stimulated with anti-CD3 for 10 minutes. Results are expressed as a percentage of the baseline F-actin content in unstimulated WT T cells and represent the means ± SDs of 3 independent experiments. *$P < .01$.

include tyrosine phosphorylated ZAP-70,[35] the adaptor proteins Nck[36] and Grb2[37] through their SH3 domains, CrkL through its SH2 domain,[38] Src tyrosine kinases through their SH3 domains,[39,40] and Vav and the p85 regulatory subunit of phosphatidylinositol-3-OH kinase through their SH2 domains.[41,42] Because these proteins are also reported to associate with WASP, its partner WIP, or both,[17,43] an indirect association of Cbl with WASP cannot be ruled out. Alternatively, c-Cbl and Cbl-b could directly interact with an activated form of WASP, such as tyrosine-phosphorylated WASP. Indeed, while this manuscript was in preparation, it was shown that WASP phosphorylation at tyrosine 291 after TCR activation results in recruitment of Cbl-b.[34]

Our data suggest that the degraded fraction of WASP includes activated WASP. This is supported by the observation that calpain inhibition and lack of the WASP-ubiquitinating E3 ligase Cbl-b resulted in more sustained F-actin assembly in WT T cells after TCR/CD3 ligation. The small fraction of WASP that is cleaved after TCR ligation could be important for F-actin polymerization because of its location close to the TCR. Indeed, we have shown previously that a fraction of WASP translocates together with a fraction of the TCR/CD3 complex to lipid rafts.[17] It is also well known that a fraction of WASP colocalizes with TCR molecules in the immune synapse (IS).[17,44,45] Cbl family molecules, which are also recruited to the IS, where they are activated by LCK and ZAP-70,[46,47] could ubiquitinate WASP molecules recruited to the IS, targeting them for degradation. The IS is a dynamic structure that constantly undergoes protein kinase Cθ–dependent dissolution and WASP/F-actin–dependent reformation of its peripheral supramolecular activation complex.[45] Protein kinase Cθ–dependent dissolution breaks the symmetry of the IS and allows T-cell motility. WASP/F-actin–dependent reformation of the IS is important for the sustained signaling that is necessary for IL-2 production. We speculate that cycles of TCR-triggered recruitment and activation of WASP in the IS followed by local degradation of the activated WASP might be important for IS dynamics and T-cell function.

The observation that baseline F-actin content was increased in *Cbl-b*$^{-/-}$ T cells, but not in calpeptin-treated T cells, suggests that under steady-state conditions, Cbl ubiquitination and proteasome degradation, but not calpain, degrade WASP molecules in activated in T cells. The observation that calpain inhibition had no effect on F-actin assembly in WASP-deficient T cells indicates that calpain regulates F-actin assembly by targeting WASP for degradation. These results strongly suggest that degradation of activated WASP by calpain and by the ubiquitin/proteasome pathway provide an important homeostatic mechanism for terminating signaling to the cytoskeleton after TCR ligation. Furthermore, WASP mutants that are resistant to ubiquitination are associated with enhanced T-cell activation, supporting the notion that WASP degradation limits TCR activation.[34]

Protein cleavage is used by prokaryotes and eukaryotes to activate or terminate signaling. Well-documented examples include the coagulation cascade, the complement activation cascade, degradation of the nuclear factor κB inhibitor IκBα, TNF receptor–associated factor 3, Argonaute, and voltage-gated calcium-channel proteins.[48-53] Degradation of activated WASP might regulate receptor signaling to the cytoskeleton not only in T cells but also in other hematopoietic cells. Such a control mechanism would avoid the potential pathology observed in patients with mutations that cause sustained WASP activation and manifest as X-linked neutropenia.

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Key message

- TCR signaling causes WASP to be degraded by calpain and by Cbl-family members through ubiquitination and destruction by the proteasome, limiting TCR-driven assembly of F-actin.

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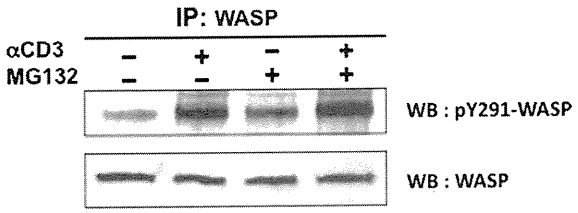

FIG E1. Tyrosine-phosphorylated WASP generated after TCR ligation is a target for proteasomal degradation. Effect of pretreatment of MG132 on the amount of tyrosine-phosphorylated WASP in anti-CD3–stimulated Jurkat T cells is shown. WASP immunoprecipitates were probed with anti-pY291-WASP antibody (Abcam). Similar results were obtained in 3 experiments. *IP*, Immunoprecipitate; *WB*, Western blot.

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Current Opinion in Immunology

Molecular determinants of sterile inflammation

Hajime Kono, Akiko Onda and Tamiko Yanagida

Necrotic cell death alerts the acquired immune system to activate naïve T cells even in the absence of non-self derived molecules (e.g. pathogens). In addition, sterile necrosis leads to innate immune-mediated acute inflammation. The dying cells still represent a threat to the body that should be eliminated by the host immune response. Although the inflammatory response plays important roles in protecting the host and repairing tissues, it can also cause the collateral damage to normal tissues that underlies disease pathogenesis. Tissue resident macrophages recognize the danger signals released from necrotic cells via the pattern recognition receptors and secrete IL-1 that results in acute neutrophilic inflammation. This article will review our current knowledge especially focusing on the role of IL-1 in the sterile necrotic cell death induced inflammation.

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Introduction

The immune system can differentiate among forms of cell death and respond to a perceived threat accordingly. Unlike apoptosis, necrotic cell death induces an acute inflammatory response, even in the absence of pathogens. The innate immune system can sense changes in the integrity of the body. For example, sterile physical damage, such as a burn or bruise, evokes acute inflammation, which consists of the four canonical signs of inflammation: rubor (redness), dolor (pain), calor (heat), and tumor (swelling). These signs were described as early as 2700 years ago. By contrast to the longstanding recognition of the role of inflammation in sterile cell death, our understanding of the mechanism underlying this inflammatory response is just emerging [1–3]. One of the most important steps in this has been the identification of the key mediator interleukin 1 (IL-1) [4••]. Of the 11 members of the IL-1 family, IL-1α and IL-1β has been shown to be responsible for mediating the inflammatory responses to sterile cell death, whereas the IL-1R antagonist (IL-1Ra) is a physiological inhibitor of IL-1. All of IL-1α, IL-1β, and the IL-1Ra bind to IL-1RI signaling receptor which recruits adaptor protein Myd88 and culminated in NF-κB-induced transcription of inflammatory genes. IL-1α is preformed as a active molecule without further modification in cells mainly works as a membrane bound molecule [5]. IL-1β is not expressed under normal homeostatic conditions by bone marrow-derived myeloid cells and is induced as an inactive form by various inflammatory stimuli. Generation and secretion of active IL-1β as well as secretion of IL-1α are mediated by caspase-1 activation on the inflammasome [6••,7].

The mechanism leading to IL-1 production, inflammasome formation, is shared by a variety of stimuli, ranging from sterile stimuli such as dead cells and particulates to microbial stimuli such as bacteria, fungi, or viruses [7]. Although the final 4 canonical signs of inflammation is identical for infectious and sterile causes, the processes of responses to the various stimuli are diverse. Regarding the relationship between cell death and inflammation, apoptosis is a non-inflammatory programmed cell death which is a necessary part of development and tissue homeostasis to remove unwanted cells. Necrosis (oncosis) is an inflammatory non-programmed cell death, which is caused by passive disruption of the plasma membrane that results in releasing of the cytosolic contents to the extracellular space. Other than necrosis, macrophages and dendritic cells undergo an inflammatory programmed cell death, named pyroptosis, that is distinct from apoptosis and necrosis [8]. Pyroptosis is a lytic cell death and mediated by caspase-1 and inflammasome, while apoptosis is non-lytic and mediated by caspase 3/6/7 and apoptosome. Pyroptosis is inflammatory by releasing IL-1 family molecules and other cytosolic contents. More importantly, inflammation caused by necrosis is mediated and/or amplified by macrophages which sometimes undergo pyroptosis. This review focuses on inflammation triggered by sterile dead cells, and specifically on the molecular and cellular mechanisms of both IL-1α and IL-1β.

Danger theory and its extension to acute inflammation

Janeway proposed the principle of discriminating self from non-self by utilizing the recognition of conserved molecular patterns of pathogens, called pathogen-associated molecular patterns or PAMPs (Figure 1) [9]. In this theory, antigen-presenting cells can present the appropriate pathogen-derived peptide to and activate naïve T cells in the presence of PAMPs. This theory was confirmed by identifying Toll-like receptors (TLRs) as the PAMP receptors. TLR engagement on antigen

presenting cells indirectly enhances activated T cell proliferation, differentiation, and survival by promoting the up-regulation of costimulatory molecules and the secretion of proinflammatory cytokines. Nevertheless, this 'Stranger theory' cannot explain some of the well-known immune responses, including the sterile organ transplantation response. Matzinger proposed a theoretical answer to this question, named the 'Danger model' [10]. She explained how the immune system can recognize damage to self by sensing a 'Danger signal'. Danger signals are also capable of activating dendritic cells, which results in the activation of naïve T cells in the same manner as with PAMPs. This theory was confirmed by several studies in which dead cells promoted CD4+ and CD8+ T cell responses [11,12]. The original notion of danger theory was expanded to acute inflammation. TLRs and Nod-like receptors were characterized as pattern recognition receptors, which sense PAMPs and induce acute inflammatory responses [13]. When cells die *in vivo*, neutrophils and monocytes infiltrate the site [14]. This phenomena is also observed in the absence of pathogens; that is, under sterile conditions [15]. The precise mechanism that senses own cell death and induces inflammation was not clear. Advances in the last decade have revealed the molecular identity of the danger signal and the receptors and amplifiers that play a role in this sterile cell death-induced inflammation.

Players in the recognition and amplification of cell-death-induced inflammation

Danger signals

A variety of danger signals have been identified and are classified into two categories on the basis of their actions: (1) molecules that are usually sequestered inside cells and released on necrotic cell death and (2) extracellular matrix, which exposes hidden molecular patterns when fragmented [16]. The hidden intracellular danger signals include uric acid, HMGB1, the myosin heavy chain, SAP130, S100 proteins, ATP, and nucleic acids including mitochondrial DNA [17] (Table 1). Cytokines such as IL-1α and IL-33 have also been identified as danger signals and are released passively from necrotic cells [18–20]. Fragments generated from hyaluronic acid, collagen, elastin, and laminin all stimulate inflammation [16].

Uric acid was the first endogenous molecule identified as functioning as a danger signal via the activation of dendritic cells and enhances the CD8+ T cell responses *in vivo* [21•]. Later uric acid is shown to mediate acute neutrophilic inflammation to necrotic cells in the acute inflammatory responses to the injection of necrotic thymocyte cells or in acetaminophen-induced liver toxicity [22]. Uric acid also stimulates sterile-injury-derived inflammation in the lung [23] or kidney [24]. Uric acid is the end product of the cellular catabolism of purines, and present at near-saturating levels in body fluids and at much higher concentrations in the cytoplasm of cells. In addition, uric acid is produced after cell death by xanthine oxidase [21•,22]. It is speculated that production of uric acid from necrotic cells leads to the phase transition from soluble uric acid to urate crystals (i.e. monosodium urate crystal) around the dying cells with the high sodium extracellular milieu, although the formation of the crystals has not been proven *in vivo*. Monosodium urate (MSU) crystal is formed in human joints which causes acute gouty arthritis [25]. MSU crystals also have been shown to both activate dendritic cells and augment immune responses [21•]. MSU crystals, but not uric acid, induces chemokines and cytokines when added to eosinophils [26]. Collectively, MSU crystals rather than uric acid are the danger signals that can mount both of innate and adaptive immune responses in response to cell death.

Another prototype inflammatory danger signal is HMGB1 protein [27•]. In the normal setting, HMGB1 functions as a chromatin-binding nuclear factor, but it can also be actively secreted by activated immune cells and initiate inflammatory responses [28,29]. HMGB1 is passively released from necrotic cells; by contrast, apoptotic cells modify their chromatin so that HMGB1 binds tightly and thus is not released [27•]. Initially HMGB1 was reported to exert a cytokine like activity including release of TNF-α, IL-1α and IL-β, IL-1Ra, IL-6, IL-8, and MIP-1α and MIP-β when added to mononuclear cells [30]. Later it was shown that recombinant HMGB1 itself induce little or no cytokine secretion [31–33]. At this point, it is shown that HMGB1 forms complex with other molecules including ssDNA, LPS, IL-1β and nucleosomes and exerts inflammatory properties [29]. HMGB1 contributes to evoking inflammation in response to cell death in the sterile liver toxicity model [27•].

Mitochondria is a rich source of danger signals, including mitochondrial DNA, formyl peptides, cytochrome C, and ATP. The highly concentrated danger signals make mitochondria potent stimulators of inflammation [34].

IL-1α also functions as a primary danger signal in certain settings, including the death of dendritic cells or vascular smooth muscle cell [18,35,36]. We confirmed that the necrotic dendritic cells from IL-1α-deficient mice induced reduced inflammation when injected into the peritoneum, compared with cells from wild-type mice [37]. However, necrotic tissue from the liver, brain, and heart of IL-1α-deficient mice had a similar neutrophilic response [37]. Zheng *et al.* also showed the necrosis-induced IL-1α inflammatory activity is highly cell type dependent, and further revealed the molecular mechanism which regulates the activity of IL-1α when released from necrotic tissue [38•]. A decoy IL-1R type II (IL-1R2) which constitutively associates with cytosolic IL-1α and prevents its interaction with IL-1R1 upon release from necrotic cells. Moreover the active caspase-1 cleaves IL-1R2 to make IL-1α accessible to IL-1R1 in

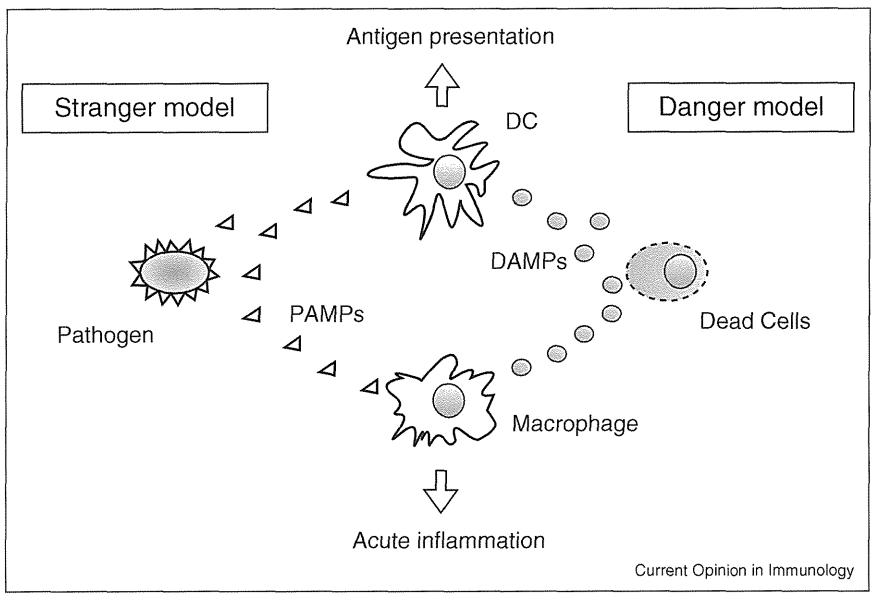
Table 1

Intracellular danger signals, receptors and their biological effects *in vitro* and *in vivo*.

Danger signal	Putative receptor	Biological effect *in vitro*	Biological effect *in vivo*	Related pathologies in human
HMGB1	RAGE [89,90]	Chemotaxis [91] Cytokine induction [92] Macrophage activation [92] DC activation [93,94] Endothelial cell activation [95]	Antitumor immunity promotion [93,96] Liver injury [27•,97] Arthritis [98] Heart ischemia [90] Brain ischemia [99,100] Aortic aneurysm [101]	Sepsis [102] Brain ischemia [99] Aortic aneurysm [101]
Uric acid (MSU)	Cholesterol of plasma membrane [70] TLR2&4 [103] NOT TLR2&4 [73] CD14 [104]	Cytokine production [26,72] DC/MP activation [21•,72] Th17 differentiation [105] Chemotaxis & degranulation of eosinophil [26]	Liver injury [22] Adjuvant activity [21•] Neutrophil recruitment [22] Bleomycin induced lung injury [23]	Gout [106,107] Tumor lysis syndrome [108] Osteoarthritis [109]
Histone/nucleosome/ chromatin/DNA/RNA	TLR2&4 [110] TLR9 [86,111,112] RAGE [113] RIG-I family members [114] TLR3 [115]	Cytokine induction [115,116] DC and MP activation [117,118] B cell and DC activation in forms of chromatin-IgG complex [111,112] Neutrophil activation [119] Endothelial cell death [120] Complement factor B production [121] NF-κB activation [113]	DC maturation [117,118] Liver injury [86,110] Sepsis [120,122] Neutrophil recruitment [113] Sunburn [115]	Systemic lupus erythematosus Sepsis [120]
ATP	P2X and P2Y receptors [123]	Inflammasome activation [124] Chemotaxis [125] Cytokine production [26]	Neutrophil recruitment [43]	
Adenosine	P1 receptors (A2A) [47•,48,61,123]	Suppression of cytokine induction [61]	Protection of liver injury [47•,48]	
SAP130	Clec4e (Mincle) [65•]	Cytokein and chmokine production [65•]	Nerutrophil recruitment [65•]	
Actin filaments	Clac9a (DNGR-1) [68,69]	Cross presentation [66•] Divert necrotic cell cargo into a recycling endosomal compartment [67]	Cross presentation [66•,63,67]	
Non-muscle myosin heavy chains (type IIA and C)	Natural IgM [126]	Activation of complements [127]	Ischemia of heart, muscle and intestine [128,129]	
Mitochondria *N*-formyl peptides	FPR1 [130]	Chemotaxis [131] Cytokine production [43,132]	Neutrophil recruitment [43,133] SIRS [134•] Liver injury [135]	
Mitochondria DNA/ mitochondrial transcription factor A	TLR9 [134•,136,137] RAGE [137]	Type I IFN production [137] Cytokine production [134•]	Neutrophil recruitment [134•] SIRS [134•] Dilated cardiomyopathy [136]	
Peroxideroxin	TLR2&4 [100]	IL-23 production [100]	Brain ischemia [100]	
IL-1α	IL-1R1 [7]	Cytokine production [35,38•] Chemikine production [18,35]	Neutrophil recruitment [18,37,38•]	
IL-33	IL-1RL1(ST2) [39]	Cytokine production [19,20]	Neutrophil rectuitment [19,39] Allergic airway inflammation & Colitis [138]	

addition to its known role in secreting IL-1α. Therefore, IL-1α from necrotic cells contributes to neutrophilic inflammation as a primary danger signal; although this is not always true and appears to be cell type dependent. IL-1α also serves as an indispensable second messenger actively secreted from macrophages which receives danger signals (see below section [Sensor cells for danger signals that generate IL-1]). IL-33 is also released from dead fibroblasts and provokes an inflammatory response [20,39].

Figure 1



Stranger and danger models. To discriminate self and non-self, dendritic cells require an appropriate signal to be activated. Dendritic cells recognize pathogen associated molecular patterns (PAMPs) released from pathogens and get activated to present pathogen derived peptide to naïve T cells (Stranger model). Dendritic cells also recognizes danger signal or damage associated molecular patterns (DAMPs) released from necrotic cells and initiate the antigen presentation (Danger model). These models are applied to the acute inflammatory response in macrophages in response to PAMPs and DAMPs.

Extracellular ATP is a prototype inflammasome activator; it engages the P2X7 ATP-gated ion channel, triggering a K^+ efflux and inducing the gradual recruitment of pannexin-1 membrane pores [40,41]. ATP is released upon necrotic cell death and induces inflammation *in vivo* [42,43]. ATP is also released upon apoptotic cell death and works as a 'find-me' signals that recruit motile phagocytes, leading to the prompt clearance of the dying cells [44•]. They indicated that early apoptotic cells release very small amounts of ATP (<2% of intracellular ATP), which should be much less and could be distinguished from necrotic cells that may release much of their ATP content [45]. Intriguingly, adenosine is also released from necrotic cells but identified as an immunoregulatory danger signal that limit the excessive collateral tissue damage [46,47•,48].

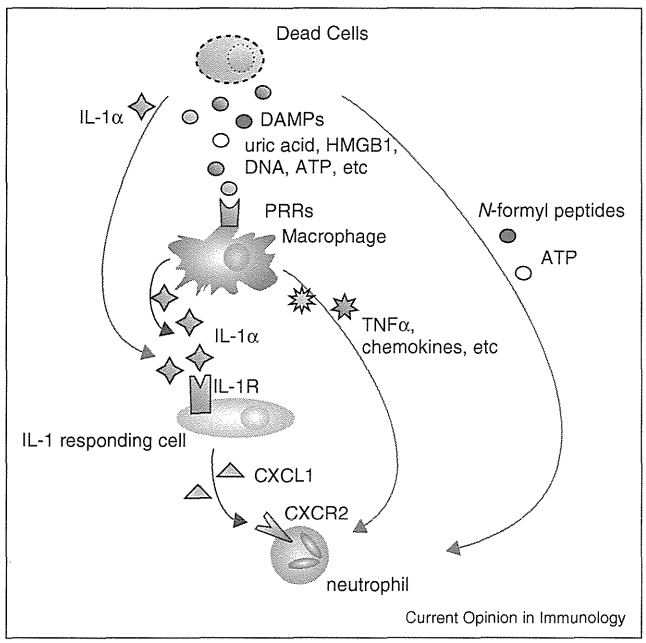
Ligands for C-type lectin receptors including SAP130 or filamentous actin were identified as danger signals. These are described in section [Danger signal receptors] below.

As stated above, neutralization of HMGB1 reduces cell death induced liver toxicity. On the other hand, necrotic mutant fibroblasts lacking HMGB1 are as proinflammatory as wild-type fibroblasts when injected into murine peritoneum [4,27•]. Therefore, there is redundancy in danger signals *in vivo*, which makes it difficult to evaluate the roles of each. As such, the dominant danger signal varies in the context of the sterile injury, including the location (organ, tissue or region), magnitude, manner of cell death, and time point after the insult. Matzinger also suggested that the tissue influences the outcome of the immune responses to a large extent [49].

Receptors for danger signals

It was speculated that the two major stimuli of inflammation, infection and sterile injury, also share recognition pathways. In infections, TLRs recognize microbial components and stimulate inflammation via the activation of transcription factors NF-κB or interferon regulatory factors (IRFs), and the expression of proinflammatory cytokines [50,51]. Chen *et al.* examined the role of each TLR using the *in vivo* model of peritoneal inflammation in response to necrotic cells. The inflammation was reduced modestly in TLR2 and TLR4 double-deficient mice, but not in mice deficient only in each individual TLR (TLR1, 2, 3, 4, 6, 7, 9, and 11) [4••]. TLRs signal via one or more different intracellular TIR adaptor molecules: MyD88, TIRAP/Mal, TRIF, and TRAM. The inflammatory response to dead cells was reduced greatly in mice lacking MyD88, but not TIRAP/Mal or TRIF. The IL-1 receptor, which also utilizes MyD88, was identified as playing a major role in sterile inflammation in response to dead cells [4••]. The IL-1 receptor recognizes both IL-1α and IL-1β. IL-1α serves as a primary danger signal from dead cells in certain settings, as described above. IL-33 also

Fig. 2



The role of IL-1 in necrotic cell death induced inflammation. Necrotic cells release intracellular contents, some of which act as danger signals (DAMPs). Macrophages at the site of tissue injury recognize DAMPs via Pattern recognition receptors (PRRs) and release IL-1α. IL-1α acts as one of the primary DAMPs when dendritic cells become necrotic. IL-1α then binds the IL-1 receptor (IL-1R) on a non-bone marrow-derived responding cell, resulting in the attraction and activation of neutrophils. IL-1α stimulates the chemoattractant chemokines, that is, CXCL1 and CXCL2, and the receptor CXCR2 expression is required for the neutrophil recruitment. In addition to IL-1 pathway, macrophages also respond to DAMPs to generate chemokines and cytokines including TNFα that leads to neutrophil recruitment. ATP and *N*-formyl peptides directly guides neutrophils to injured cells.

acts as a primary danger signal via the ST2 receptor [20,39]. In other experiments, TLR9 was shown to be responsible for inducing liver toxicity in response to excess amounts of acetaminophen or pancreatitis in response to cerulein [52,53].

Recently, a growing number of receptors that recognize innate cytosolic patterns have been identified; these sense either DNA or RNA, including AIM2, RIG-I, and DAI [54,55]. The setting of compromised DNA hydrolysis, such as DNase deficiency, leads to the development of DNA-driven, IFN-dependent autoimmune diseases [56,57]. Although there is no direct evidence of a role for DNA sensors in cell-death-induced acute inflammation, there is emerging evidence that these cytosolic receptors play a major role in autoimmunity-related inflammation [58] or have adjuvant properties [59,60].

ATP is recognized by the P2X7 and P2Y2 receptors in cell-death-induced inflammation [26,43]. The anti-inflammatory properties of adenosine are media by A2A receptor [47•,48,61].

HMGB1 binds to RAGE and TLRs and exerts its fu tion in inflammation [27•,32]. Later it is shown t HMGB1 binds to other molecules including ssDI LPS, IL-1β and nucleosomes, which are recognized TLR9, TLR4, IL-1R, and TLR2 receptors, respectiv [62•].

Recent advances in the research in C-type lectin rec tors revealed their functions in recognizing danger sigı in addition to apoptotic cells or microbial compone [63,64]. C type lectin, Lox-1, Mgl1 and DEC205 w known for sensing apoptotic cells. Mincle (Clec4e expressed on macrophages and neutrophils that ser SAP130 released from necrotic cells to stimulate infl mation including cytokine production and neutro recruitment [65•]. DNGR-1 (Clec9a) is expressed CD8α+ dendritic cells and promotes cross-presentat of dead cell-associated antigens to activate cytotoxi lymphocytes [63,66•,67]. Subsequently actin filament cytoskeleton were identified as ligand for DNGR-1. A filaments which are usually sequestered inside of cells exposed to extracellular space when cells are dama [68,69].

To date, no specific receptor for uric acid or MSU crys working as a danger signal has been identified. Us atomic force microscopy, cholesterol within the plas membrane was identified as the receptor for MSU crys [70]. Martinon *et al.* found that the NLRP3 protei NOD-like receptor, forms a multi-molecule comp with ASC and caspase-1, named the inflammasome analogy to the apoptosome [71•]. The inflammasome as a platform for activating caspase-1, which results in maturation of inactive pro IL-1β to active IL-1β. T NLRP3 inflammasome is shown to be required secretion of IL-1β and neutrophil recruitment *in v* [72].

It was initially proposed that pore formation allows ex cellular NLRP3 agonists to access the cytosol and activ NLRP3 directly [41,73]. However, the structural diver of growing number of NLRP3 activators argues again direct interaction between NLRP3 and all of its act tors. Subsequently, Hornung *et al.* presented evide that NLRP3 senses vesicular rupture, which led to a r concept: NLRP3 monitors cell health and respond internal cell damage [74]. This is another example of concept of danger signals fitting inside cells whicl analogous to the concept, 'ontogeny recapitulates ph geny' of Ernst Haeckel. At the level of an organism, danger signals, usually hidden molecules from immune system, are released to extracellular milieu wl tissues are broken down to evoke the responses. At cellular level, NLRP3 inflammasome responds to

ːllular changes that indicate cellular damage, for ıle, pore formation of plasma membrane, vesicular e or cellular stress. It was shown that mitochondrial rane permeabilization represents the point of no of programmed cell death pathways that leads to ɔsis or programmed necrosis [75,76]. It is recently that mitochondrial dysfunction and ROS pro- n activates NLRP3 inflammasome that is enhanced ɔphagy/autophagy does not kick in appropriately *,79,80]. In addition, mitochondrial DNA or cardi- released to cytosol also induces NLRP3 inflamma- activation [78*,81,82]. These recently added ıces along with the previous ones shed the light central role of mitochondria as the central regulator ular stress management linking to inflammation. In ɔn, NLRP3 inflammasome senses internal damage cell including the damage of plasma membrane via lic potassium concentration [83] or cell volume

r cells for danger signals that generate IL-1

ted above, one of the major recent discoveries in ːath-induced inflammation is that cytokine IL-1 an important role in this response. Since IL-1 is ıted and released by cells, studies investigated cell lineage produces IL-1. Since many cell types oduce this cytokine, we generated chimeric mice bone marrow lacked functional IL-1 genes or radioresistant parenchyma cells and challenged with necrotic cells intraperitoneally [37]. Utilizing D11b-DTR system and reconstitution, we ident- ːD11b+ macrophages as the key player sensing cell and producing IL-1α *in vivo* (Figure 2). Of note, ɔ+ cells also have the ability to reduce inflammation 11b-DTR mice. However, in another set of exper- ; using CD11c-DTR mice in which DT treatment ːntly deleted most dendritic cell subsets and the ɔ+ subset of CD8+ T cells, we found that CD11c+ tic cells do not play major role in the acute inflam- / response to cell death. Interestingly, macrophages ː major source of IL-1α needed for the cell-death- ːd inflammatory response in our models. In ɔn, IL-1β was required for the cell-death-induced matory response. The dominant source of IL-1β in sponse was bone marrow-derived cells. The IL-1α -1β release from macrophages in response to dead epends on MAP3K8 (Cot/tpl2) [85]. Macrophages uppfer cells were shown to be NLRP3 inflamma- ıctivated and secrete IL-1β in response to necrotic 42,86]. Although IL-1α secretion and IL-1β proces- dependent on inflammasome *in vitro*, caspase-1 is quired *in vivo* for much of the IL-1β-dependent inflammatory response [87]. IL-1β is processed by ːyte serine proteases which are controlled by cath- C in the caspase-1 independent pathway [87]. dendritic cells recognize necrotic cells via R-1 (Clac9a) to cross present the dead cell associated antigens *in vitro* and *in vivo* [66*]. Monocytes respond to necrotic cells to produce TNF-α [27*]. Another innate immune cell type resident in tissues is mast cells. Mast cells mount an inflammatory response to dead fibroblasts by recognizing the dead-cell-spilling IL-33 as a primary danger signal [20,39]. Eosinophils also participate in the inflammatory responses to necrotic cell-derived danger signals including MSU crystal to secrete cytokines and chemokines, although shown only *in vitro* [26,88].

Effector cells that recruit neutrophils

IL-1 and the IL-1 receptor pathway are essential for neutrophil recruitment to the site of cell-death-induced inflammation, as described above. CD11b+ macrophages are required to produce IL-1α and bone-marrow-derived cells are required to produce IL-1β. Bone marrow chimeric experiments revealed that the IL-1 receptor is required on parenchymal, non-bone-marrow-derived cells, but not on bone-marrow-derived cells [4**]. These results indicate that neutrophils are recruited to the site of inflammation in a manner other than by recognizing IL-1 on their receptors directly. The neutrophil recruitment is initiated by the activation of adhesion molecules (e.g. ICAM-1) on the endothelium neighboring the injury. In response to necrotic dendritic cells which releases abundant IL-1α that is sensed by the IL-1R on mesothelial cells to induce the production of neutrophil-attracting chemokines (e.g. CXCL1) [18]. Neutrophils transmigrate to the vicinity of the necrotic focus according to the gradient of CXCR2 ligands, such as CXCL1 [18,43].

Conclusions

A significant progress has been made in revealing the mechanisms underlying the generation of the neutrophilic inflammatory response in response to sterile necrosis. The identification of IL-1 as a key molecule for this process was an important step; and is shared by inflammation to sterile particulates. In addition to the role of NLRP3 inflammasome for IL-1α and IL-1β processing and secretion, new potential players that function in this process have been identified. These findings open the possibility of developing new therapies and therapeutics to treat diseases that are caused by necrotic cell death- or sterile particulates-induced inflammation including myocardial infarction, stroke, drug induced liver injury, SIRS, and NLRP3 inflammasome related diseases including gout, diabetes mellitus and atherosclerosis.

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