Regulation of NF-κB–Dependent Lymphocyte Activation and Development by Paracaspase

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Supporting Online Material

Materials and Methods

Generation of Paracaspase-Deficient Mice

A genomic paracaspase clone was isolated from a 129/R1 library and used for constructing a targeting vector (Fig. S1A) containing exons 11 and 12 flanked by loxP sites that was electroporated into ES cells. Two homologous recombinants were identified by Southern blotting probes 5' of genomic sequence present in the targeting vector. These ES cell clones were then transfected with a plasmid carrying cre-recombinase. Subsequent ES cell clones were screened by PCR and Southern blot for the loss of exons 11 and 12. Four ES cell clones, two from each parental line, homologous for the deletion of exons 11 and 12, were injected into blastocysts and chimeric offspring were backcrossed onto C57BL/6N mice. Germline transmission was confirmed by PCR and Southern blot analysis of genomic tail DNA. Immunoprecipitation followed by Western blot using a monoclonal antibody raised against amino acids 352-824 of paracaspase confirmed deletion of paracaspase protein from *paracaspase*$^{-/-}$ spleens. This antibody recongnizes a region 127 amino acids N-termial to exons 11 and 12. All *paracaspase*$^{-/-}$ mice used for this study were 6-14 weeks old and were generated from F1 *paracaspase*$^{-/+}$ intercrosses.

Cell isolation, Immunofluorescence Staining, and Flow Cytometry

Purified splenic T and B cells were isolated using either a Pan-T cell kit or CD19 microbeads and AutoMACs separation columns (Miltenyi Biotech). Cell purity was typically >95% for purified B and T cells as assessed by FACs analysis. Single cell suspensions prepared from thymus, spleen, bone marrow, and lymph nodes were stained with monoclonal antibodies conjugated to FITC, PE, or APC. For detection of apoptosis,

activated T cells were stained with annexin V and propidium iodide (PI) using the Apoptosis detection kit (R&D Systems) according to the manufacturer's instructions. All samples were analyzed by flow cytometry using a FACScan (Becton Dickinson).

Immunohistochemistry

Frozen spleen sections from 14-week-old wild-type and *paracaspase*$^{-/-}$ mice were stained with Texas red-conjugated anti-murine B220 (Pharmingen) and FITC-conjugated PNA (Vector Laboratories), or Alexa Fluor 546-conjugated goat anti-mouse IgM (Molecular Probes) and FITC-conjugated rat anti-mouse metallophilic macrophages (MOMA-1) (Serotec). Sections were examined by fluorescence microscopy.

Lymphocyte Activation and IL-2 Production

Purified splenic T and B cells were cultured in high glucose-containing Dulbecco's modified Eagle's medium supplemented with 250 μM L-asparagine, 50 μM 2-mercaptoethanol, and 10% fetal calf serum. T cells (10^5 cells/ml) were stimulated with plate-bound 145-2C11 anti-CD3ε and 37.51 anti-CD28 antibodies (BD Biosciences; 1.25-10 μg/ml of each antibody in PBS) or phorbol myristate acetate (PMA) (0.25-2 ng/ml) (Sigma) and ionomycin (12.5-100 μg/ml) (Sigma), in the presence or absence of 40 ng/ml recombinant mouse interleukin-2 (R&D Systems). B cells ($3x10^5$ cells/ml) were stimulated with 20 μg/ml $F(ab')_2$ goat anti-mouse IgM (Jackson ImmunoResearch), 20 μg/ml LPS (Sigma) or 20 μg/ml FGK-45 anti-CD40 (ATCC) in the presence or absence of 40 ng/ml recombinant mouse interleukin-4 (R&D Systems). Cultures were pulsed for 8 hours with [^{3}H]-thymidine (1 μCi/well) and incorporation of [^{3}H]-thymidine was measured using a Matrix 96 direct β counter system (Hewlett Packard). Data represent triplicate samples and are representative of at least three separate experiments. Endogenous IL-2 production of purified T cells treated as above was measured by ELISA (R&D Systems).

***In Vivo* Immunity**

Eight-week-old wild-type and *paracaspse*$^{-/-}$ mice were immunized i.p. with 100 μg DNP-OVA absorbed to alum (Sigma). DNP-specific immunoglobulin concentrations were

determined by ELISA on TNP-BSA-coated plates. Basal levels of Ig isotypes were analyzed by ELISA on serially diluted serum samples using anti-mouse IgG1, IgG2a, IgG2b, IgG2, IgA, or IgM antibodies (BD Biosciences) according to the manufacturer's instructions.

Immunoprecipitations and Western Blotting

Purified T cells (2×10^6) were stimulated with 10 μg/ml each of plate-bound anti-CD3 and anti-CD28 or 50 ng/ml each of PMA and ionomycin or 10 ng/ml TNFα for 0-30 minutes. Purified B cells were stimulated with 50 ng/ml each of PMA and ionomycin or 10 μg/ml $F(ab')_2$ goat anti-mouse IgM for 0-40 minutes. Cell lysates were prepared in ice cold lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1.0% Triton X-100, 20 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, complete protease inhibitors (Roche), and phosphatase inhibitors I and II (Sigma). Western blots were performed using phospho-specific antibodies for IκBα, ERK1/2, and JNK (Cell Signaling Technology). Blots were stripped and re-probed with antibodies to total IκBα (Cell Signaling Technology) and phospho-tyrosine (Santa Cruz).

NF-κB Luciferase Assays

Mouse embryonic fibroblasts (MEFs) were transfected using Lipofectamine 2000 (Invitrogen) with a total of 2 μg expression vectors (V5-Bcl10, 3Xflag-paracaspase, or HA-pcDNA3), 0.25 μg pLam3 (NF-κB responsive luciferase), and 0.025 μg pR-TK (control luciferase). Luciferase activity was measured 48 hours post-transfection using the Dual-Luciferase Reporter Assay System (Promega). Data represent triplicate samples and are representative of at least two separate experiments.

NF-κB ELISA

Purified T cells ($2x10^7$) were treated for 12 hours with either media alone, 50 ng/ml each PMA and ionomycin, or 10 μg/ml each of plate-bound anti-CD3 and anti-CD28. Purified B cells ($2x10^7$) were treated for 12 hours with 10 μg/ml $F(ab')_2$ goat anti-mouse IgM (Jackson ImmunoResearch) or 20 μg/ml LPS (Sigma). Nuclear lysates were prepared

using the NE-PUR extract system (Pierce). ELISAs were performed using 15 μg of nuclear lysate with the BD Mercury Transfactor family kit for NF-κB according to the manufacturer's instructions (BD Biosciences).

Supplementary Figure Legends

Fig. S1. Gene targeting of paracaspase. (A) Genomic paracaspase sequences and construction of the neomycin resistance (neo) insertion vector. *Paracaspase* exons are shown as boxes and loxP sites are shown as triangles. The *paracaspase* flanking probe used for Southern blotting and the expected fragment sizes after digests of wild-type and mutant genomic DNA are indicated. (B) Genomic DNA was isolated from *paracaspase*$^{+/+}$, *paracaspase*$^{+/-}$, *and paracaspase*$^{-/-}$ mice, digested with BglII, and analyzed by Southern blotting using the 5' flanking probe shown in (A). (C) Immunoprecipitation followed by Western blot analysis of paracaspase protein expression in *paracaspase*$^{+/+}$, *paracaspase*$^{+/-}$, *and paracaspase*$^{-/-}$ splenocytes using a monoclonal antibody reactive against mouse paracaspase (* denotes heavy chain band and ** denotes light chain band).

Fig. S2. Lack of marginal zone B cells in paracaspase$^{-/-}$ spleens. Wild-type and *paracaspase*$^{-/-}$ mice were stained with antibodies to MOMA-1 (green) to detect metallophilic macrophages and anti-IgM (red) to visualize B cells. The IgM^{+} MZ B cell layer (arrow) external to the ring of metallophilic macrophages is nearly absent from *paracaspase*$^{-/-}$ spleens.

Fig. S3. Normal total tyrosine phosphorylation in paracaspase$^{-/-}$ T and B cells and impaired NF-κB binding activity. (A) Purified T cells and (B) B cells were treated for the indicated times with antibodies to CD3 and CD28 or anti-IgM. Whole cell lysates were analyzed by western blot for total tyrosine phosphorylation levels. (C) Purified T cells and (D) B cells were treated with the indicated mitogens for 12 hours and nuclear extracts were analyzed by ELISA for DNA binding activity to oligos specific for NF-κB subunits p50 and p65.

Fig. S1

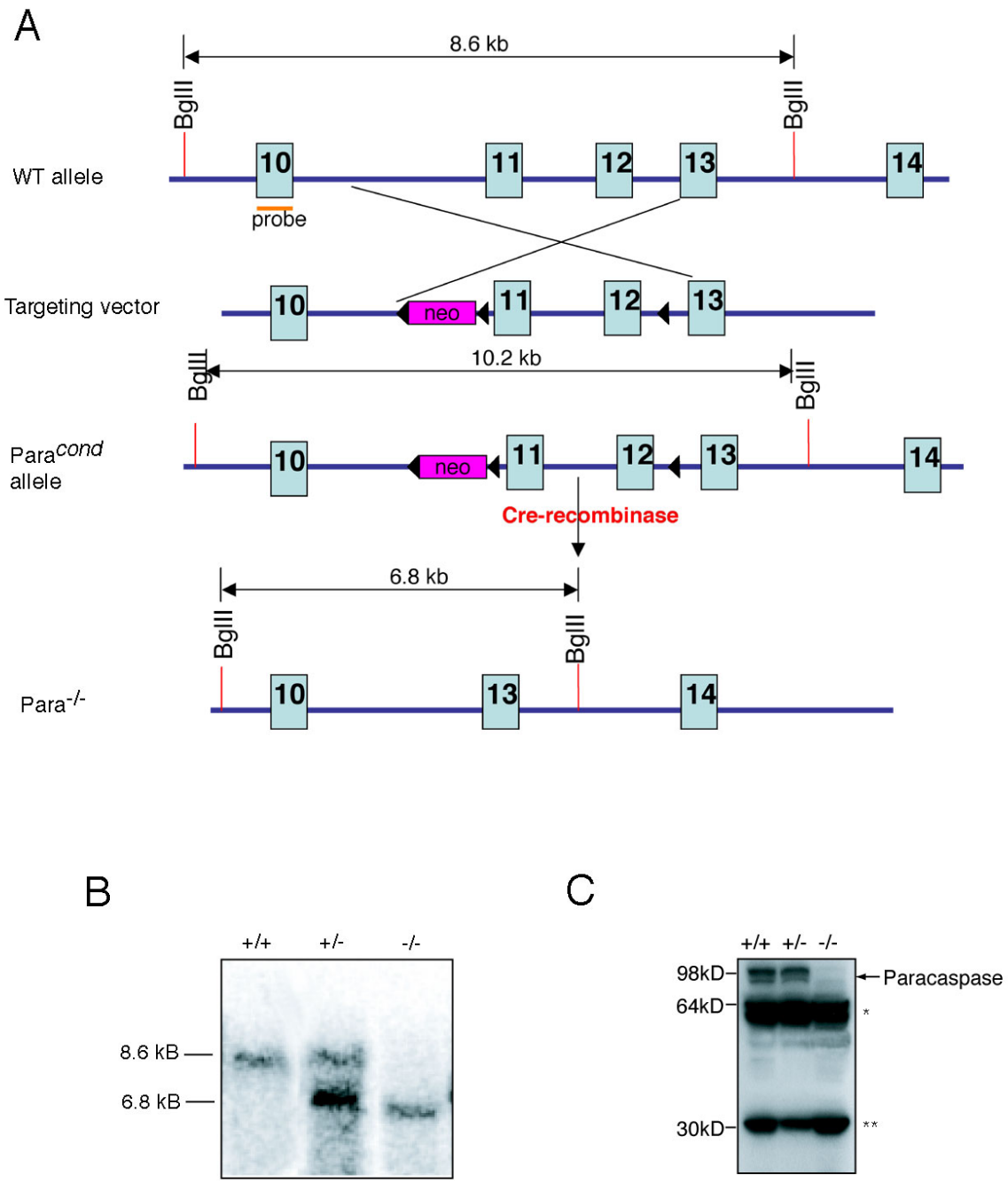
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Fig. S2

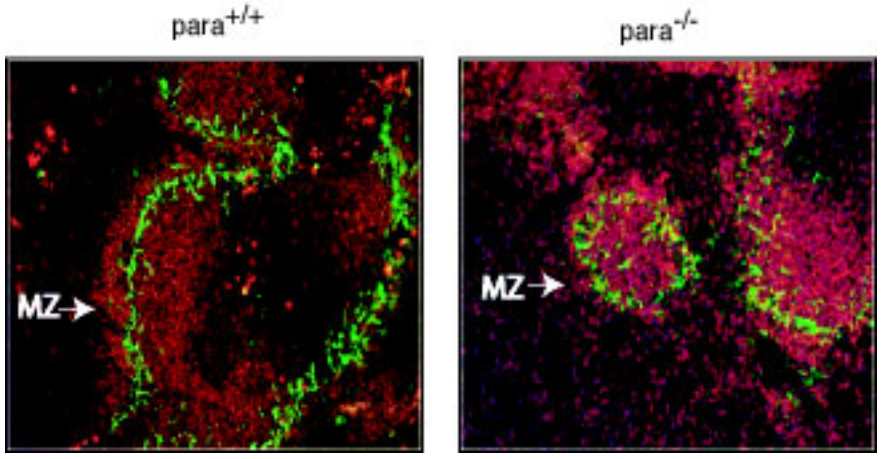
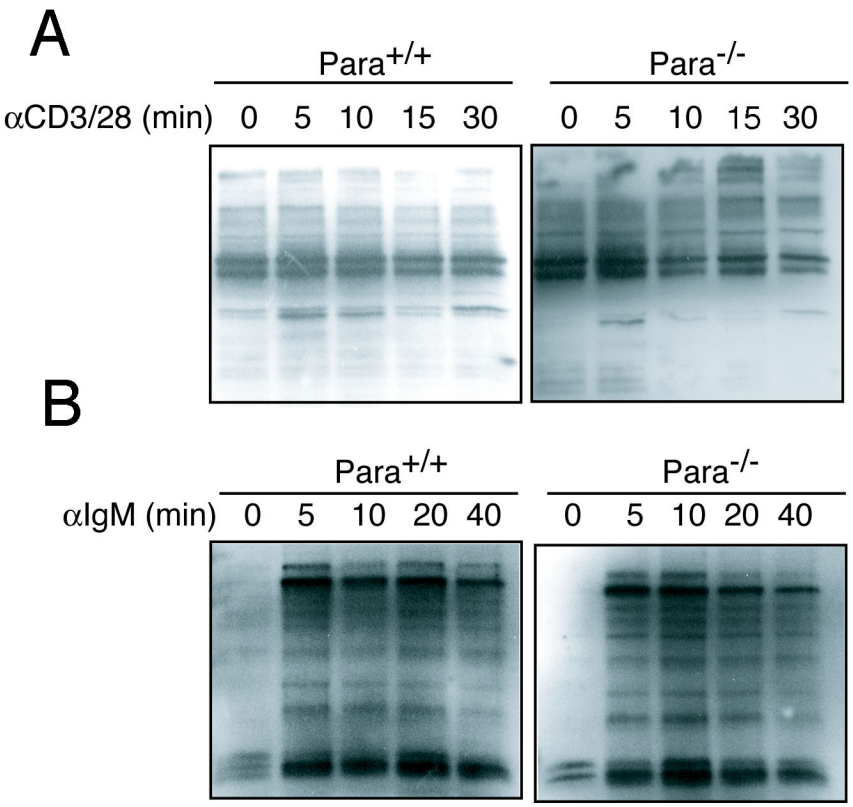


Fig. S3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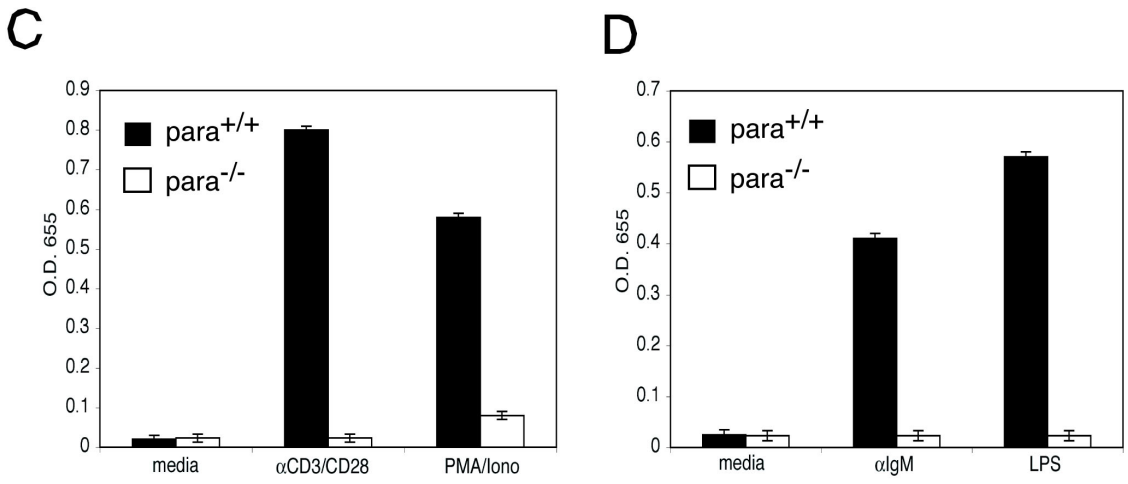
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