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Selenoprotein K Knockout Mice Exhibit Deficient Calcium Flux in Immune Cells and Impaired Immune Responses

Saguna Verma,*† FuKun W. Hoffmann,†,‡ Mukesh Kumar,* Zhi Huang,†
Kelsey Roe,* Elizabeth Nguyen-Wu,† Ann S. Hashimoto,† and Peter R. Hoffmann†

Selenoprotein K (Sel K) is a selenium-containing protein for which no function has been identified. We found that Sel K is an endoplasmic reticulum transmembrane protein expressed at relatively high levels in immune cells and is regulated by dietary selenium. Sel K−/− mice were generated and found to be similar to wild-type controls regarding growth and fertility. Immune system development was not affected by Sel K deletion, but specific immune cell defects were found in Sel K−/− mice. Receptor-mediated Ca2+ flux was decreased in T cells, neutrophils, and macrophages from Sel K−/− mice compared with controls. Ca2+-dependent functions including T cell proliferation, T cell and neutrophil migration, and Fcy receptor-mediated oxidative burst in macrophages were decreased in cells from Sel K−/− mice compared with that in cells from controls. West Nile virus infections were performed, and Sel K−/− mice exhibited decreased viral clearance in the periphery and increased viral titers in brain. Furthermore, West Nile virus-infected Sel K−/− mice demonstrated significantly lower survival (2 of 23; 8.7%) compared with that of wild-type controls (10 of 26; 38.5%). These results establish Sel K as an endoplasmic reticulum-membrane protein important for promoting effective Ca2+ flux during immune cell activation and provide insight into molecular mechanisms by which dietary selenium enhances immune responses. The Journal of Immunology, 2011, 186: 2127–2137.

Selenium is an essential micronutrient important for many aspects of human health, including optimal immune responses (1). Both innate and adaptive immunity are impaired in Se-deficient individuals, and changes in Se intake affect a range of immune responses including antiviral immunity, vaccine responses, and allergic asthma (2). Our recent study demonstrated that increased Se intake boosted Ca2+ flux and downstream cell signaling in CD4+ T cells, which dramatically influenced their activation, proliferation, and differentiation (3). The biological effects of Se are exerted mainly through its incorporation into selenoproteins as the amino acid selenocysteine. Twenty-five selenoproteins have been identified in humans, all but one of which also exist as selenocysteine-containing proteins in mice and rats (4). Some selenoproteins such as the glutathione peroxidases and thioredoxin reductases have been functionally characterized as antioxidant enzymes, serving to mitigate damage caused by reactive oxygen species and to regulate redox tone within or outside of cells (5). However, not all selenoproteins act as antioxidant enzymes, and functions for several selenoproteins have yet to be determined.

One selenoprotein for which no function has been identified is selenoprotein K (Sel K), which is a small (~12 kDa) protein first identified through a bioinformatics analysis of the human genome (4). Both human and mouse Sel K contain one selenocysteine residue and share 91% amino acid sequence identity. One investigation into potential functions of Sel K showed that its overexpression in cardiomyocytes decreased sensitivity to treatment with hydrogen peroxide (6). However, it was not demonstrated whether endogenous Sel K serves in an antioxidant capacity or what are the potential mechanisms by which this may occur. The Drosophila melanogaster orthologue of Sel K was not found to contribute to antioxidant potential in this organism (7). Moreover, Sel K lacks defined redox motifs found in antioxidant selenoproteins like the glutathione peroxidase (GPx) and thioredoxin reductase enzymes (8). A recent study suggested that Sel K expression in the HepG2 cell line is regulated by endoplasmic reticulum (ER) stress, and decreasing its expression with small interfering RNA augmented cell death induced with ER stress-inducing agents (9). Whether Sel K is directly or indirectly linked to ER stress in vivo remains unclear.

The Sel K amino acid sequence contains a predicted transmembrane domain, a feature found in only four other selenoproteins: deiodinase 2 and selenoproteins I, N, and S (5). In one study, overexpression of GFP-tagged Sel K resulted in its localization to the endoplasmic reticulum (ER) (6), whereas other data have suggested that Sel K may localize to the plasma membrane (4). These two different findings may be reconciled by the fact that regions of ER come in close proximity to the plasma membrane (within 10–25 nm), regions commonly referred to as puncta (10). Puncta provide microenvironments in which key signaling events occur between the ER and the extracellular matrix.
and the plasma membrane, particularly during Ca\(^{2+}\) influx and activation of T cells (11). Overall, important questions remain regarding subcellular localization and biological functions of Sel K, and more information is needed regarding its in vivo role. Clues to potential biological roles for Sel K may be obtained by analyzing its tissue distribution. Based on Northern blot analysis, expression of Sel K was suggested to be relatively high in the heart (6). However, real-time RT-PCR data subsequently published by our laboratory demonstrated that Sel K mRNA expression levels are widely distributed throughout most tissues, with particularly high levels detected in spleen and testes (12). An immunohistochemical survey of human tissues suggested relatively high expression in the salivary gland and lymphoid tissues (13). We report in this study that Sel K is an ER-membrane protein expressed at relatively high levels in lymphoid tissues and a variety of immune cells, which raises the question of the role it plays in leukocytes during immune system development and immune responses. To address this issue, Sel K

\(^{-/-}\) mice were generated and found to be similar to wild-type (WT) controls in terms of immune responses when infected with West Nile virus (WNV) as demonstrated by decreased viral clearance in periphery, increased viral titers in brain, and increased mortality, suggesting an important role for Sel K in maintaining effective immunity.

**Materials and Methods**

**Construction of targeting vector and embryonic stem cell clones**

A targeting vector was designed and constructed in conjunction with the Targeting Laboratory (Stony Brook, NY) and included an 11.31-kb region of the WT sel K locus subcloned from a positively identified C57BL/6 (RPCL23: 227C3) BAC clone. The region was designed such that the short homology arm extended ∼1.53 kb 5’ to exon 2. The long homology arm terminated 3’ to exon 3 and was 7.52 kb in length. The loxp-flanked Neo cassette was inserted on the 3’ side of exon 2, and a single loxP site was inserted at the 3’ side of exon 3. The target region was 2.27 kb and included exons 2–3. The total size of the targeting construct, including vector backbone (pSP72; Promega), and Neo cassette was 15.52 kb. Ten micrograms of the targeting vector was linearized by NotI and then transfected into iTIL C1 C57BL/6 embryonic stem cells by electroporation. After selection with G418 antibiotic, surviving clones were expanded and analyzed by PCR to identify recombinant embryonic stem cell (ES) clones. Secondary confirmation of positive clones identified by PCR was performed by Southern blot analysis. DNA was digested with PstI and electrophoretically separated on 0.8% agarose gel. After transfer to a nylon membrane, the digested DNA was hybridized with a 5’ external probe. DNA from the C57BL/6 mouse strain was used as a WT positive control.

**Generation of Sel K-deficient mice**

ES clones were injected into C57BL/6 blastocysts to produce chimeras with one WT and one floxed Sel K allele, Sel K

\(^{+/+}\) mice, which were then mated to generate Sel K

\(^{+/\text{lox}}\) mice on a C57BL/6 background. FLPL1 and CMV-Cre transgenic mice were purchased from The Jackson Laboratory. FLPL1 transgenic mice were mated with Sel K

\(^{+/\text{lox}}\) mice to generate offspring with the Neo cassette excised, which were mated to regenerate Sel K

\(^{+/+}\) mice. These mice were then mated with CMV-Cre transgenic mice to generate offspring in which Sel K was deleted in all tissues (Sel K

\(^{-/-}\) mice). Exclusion of Sel K was confirmed in all offspring using PCR that amplified a 408-bp product in the targeted region present in the WT allele (forward, 5’-CTG CCC TAG TGT AGT TCT TCT-3’; reverse, 5’-TGT ATG CCA TTC TTA GTA CAG TCT-3’), and a 1.6 kb product in the excised allele (forward, 5’-GCA GCC CGA GAA TTA CAT ACT GA-3’; reverse, 5’-CGT GG ACC AAG GT-3’). C57BL/6J mice were purchased from The Jackson Laboratory to generate a colony of WT mice. In some cases, diets with low (0.08 ppm), medium (0.25 ppm), and high (1.0 ppm) Se were fed to mice for 8 wk as previously described (3). In all other experiments, standard chow (∼0.25 ppm Se) was used. All animal experimental protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee.

**Abs and reagents**

Abs for flow cytometry included PE-anti–CD3, PE-anti–CD4, allophycocyanin-anti–CD4, PE-anti–CD11b, allophycocyanin-anti–CD16/32 (all purchased from eBioscience); allophycocyanin-Cy7-anti–CD11b, PerCP-Cy5.5-anti–Gr-1, and PE/texas red-anti–B220 (all purchased from BD Pharmingen); PE-anti–F4/80, and Alexa 647-anti–CXCR2 (BioLegend). Rabbit polyclonal anti-Sel K purchased from Sigma or custom rabbit polyclonal Ab purchased from ProSci were used for Western blots and immunohistochemistry. Mouse monoclonal anti–α-tubulin was purchased from Novus Biologicals, anti–β-actin from Sigma, anti-stromal interaction molecule 1 from Abgent, and both anti-KDEL and anti-Tata binding protein from Miltenyi. Rabbit polyclonal anti-Akt was purchased from Cell Signaling. HRP- or fluorochrome-conjugated secondary Abs were purchased from Jackson ImmunoResearch or Invitrogen, respectively. Thapsigargin, ionomycin, tunicamycin, and polyinositot/cytosine [poly(iC)] were purchased from Sigma. Recombinant KC, RANTES, and stromal cell-derived factor 1 (SDF-1) were purchased from R&D Systems.

**Preparation of ex vivo cells and flow cytometry**

Tissues were homogenized using a Miltenyi gentleMACS cell dissociator. For lymph nodes, two inguinal and two axillary lymph nodes were pooled from each mouse, and cell numbers were calculated per lymph node. A Miltenyi cell separation system with appropriate magnetic beads was then used to negatively purify pan T cells, CD4+ T cells, or CD8+ T cells from spleens or to positively purify Gr-1+ neutrophils from bone marrows. After washing with RPMI 1640 medium, cells were resuspended in complete RPMI 1640 medium and purification confirmed using flow cytometry. For bone marrow-derived macrophages (BMDMs), bone marrow was flushed from femurs and tibiae with HBSS using a 25-gauge needle. Cells were released from clumps using a syringe with an 18-gauge needle under continuous washing with a 40-μm-pore cell strainer to remove tissue debris. The cells were then plated in DMEM containing 10% FCS, 1% penicillin/streptomycin/glutamine (Invitrogen), and 5 ng/ml recombinant M-CSF (R&D Systems) and used on day 7 of culture.

**Western blotting and immunohistochemistry**

Western blots were performed as previously described (12), with slight modifications. To maximize yield of Sel K, tissues and cell pellets were lysed in a low-salt buffer containing 10 mM Tris pH 7.5, 1% Triton X-100, 5 mM EDTA, proteinase and phosphatase inhibitors, and 5 mM NaCl. Densitometry was performed using the Li-Cor Odyssey imaging system. A compartment protein extraction kit (Millipore) was used to prepare membrane, nuclear, and cytoplasmic fractions from whole-cell lysates. Immunohistochemistry was performed as previously described (14), with modifications including the use of rabbit polyclonal anti-Sel K (ProSci) as a primary Ab followed by HRP–anti–rabbit IgG.

**Oxidative burst assay and head uptake**

IgG-opsonized BSA for Fcγ receptor-mediated Ca\(^{2+}\) flux assays was generated by adding 20 μg BSA (Sigma) with 200 μg rabbit polyclonal anti–BSA (Upstate/Millipore) and PBS in a final volume of 500 μl. Oxidative burst was determined in a time-course manner using Fc OxyBURST fluorescent assay reagent (Invitrogen) and measured on a FACSCaliber (BD Biosciences). For phagocytosis assays, yellow-green fluorescent, 1 μm carbohydrate-modified FluoroSpheres were purchased from Invitrogen and incubated with BMDMs for 1 h at 37°C. After washing nonphagocytosed beads, BMDMs were fixed with 2% paraformaldehyde and stained with PE-anti–F4/80 as a plasma membrane fluorescent stain. Confocal images were captured using a Zeiss Axiovert 200M attached to a Zeiss LSM 5 Pascal imaging system and three-dimensional rendering performed using National Institutes of Health ImageJ software.

**Poly(i:C)-induced peritonitis and cytokine measurement**

Each mouse was injected i.p. with 150 μg poly(i:C) in 150 μl PBS and maintained in cages for 48 h to allow infiltration of several different types of innate immune cells. Mice were then sacrificed, and a syringe with an 18-gauge needle was used to inject and withdraw cold complete RPMI 1640 medium. Cells were centrifuged, washed twice with cold RPMI 1640.
containing no phenol red, and resuspended in cold FACS buffer (PBS with 2% FBS). Four-color flow cytometry was then performed on cell samples from each peritoneal lavage as previously described (15). Also, serum was collected from each mouse and analyzed by Cytometric Bead Array and Flexbead kits (BD Biosciences) per the manufacturer’s instructions.

**ER-stress measurement**

To determine levels of ER stress, an established protocol (16) was followed using conventional RT-PCR and Pst digestion to determine relative levels of spliced and unspliced X-box–binding protein-1. Primers used to amplify X-box–binding protein-1 included forward, 5'-A AAC AGA GTA GCA GCG CAG ACT GC-3' and reverse, 5'-TC TTG CTG AGA CCT CTG GGA G-3', and PCR conditions included 94°C for 4 min, 35 cycles of 94°C for 30 s, 63°C for 60 s, and 72°C for 30 s, followed by 72°C for 10 min final extension. Tunicamycin was added as a control to induce ER stress at 5 μg/ml for 7 h. ER stress was also determined by Western blot analysis of BiP (Grp78) using whole-cell lysates from spleen or BMDMs untreated or treated with tunicamycin for 24 h.

**Calcium flux and migration assays**

Purified T cells or neutrophils were loaded with fluo 3-AM and fura red (3 μM and 3.3 μM, respectively; Invitrogen) for 30 min at room temperature in RPMI 1640 with no FBS. After two washes with complete RPMI 1640 media, cells were resuspended in complete RPMI 1640 media with no phenol red and Ca2+2–flux assays performed by measuring the ratio of fluo 3 (FL-1) to fura red (FL-3) fluorescence on a FACSCalibur as previously described (17). Migration assays were performed using CytoSelect 24-well Cell Migration Assay kits (Cell Biolabs). For neutrophils, 1.5 × 105 cells in 300 μl serum-free medium were added to the upper chamber of the Transwell plates with 3-μm-pore filters. Lower chambers contained 500 μl RPMI 1640 medium and varying concentrations of KC, and migration was carried out for 2.5 h at 37°C and 10% CO2. For T cells, 105 cells in 200 μl serum-free medium were added to the upper chamber of Transwell plates with 5-μm-pore filters. Lower chambers contained 500 μl RPMI 1640 medium with varying concentrations of either RANTES or SDF-1, and migration was carried out for 3.5 h at 37°C and 10% CO2. The number of cells migrating to lower chambers was determined by fluorescence on a Victor 2 plate reader (PerkinElmer) per the manufacturer’s instructions. For macrophages, BMDMs were grown on glass coverslips and loaded with fluorescent Ca2+ indicator dye fluo 4-AM (Invitrogen) at 5 μM for 30 min followed by washes and covered with complete DMEM with no phenol red. Cells were mounted in a rapid-exchange Warner perfusion system for confocal imaging using a Zeiss Axiovert 200M attached to a Zeiss LSM 5 Pascal imaging system. Fluo 4 signal was quantitatively analyzed using ImageJ software.

**Proliferation assays**

Proliferation of T cells was measured using [3H]thymidine techniques by plating 5 × 103 cells in 200 μl per well in a 96-well plate precoated with anti-CD3 (1 μg/ml) plus anti-CD28 (10 μg/ml). Cells were incubated for 72 h in RPMI 1640 media containing 10% FBS. For the last 18 h, a master mix containing 0.5 μl [3H]thymidine/well (spec. act. 25 Ci/mol, radioactive concentration 1.0 μCi/ml; Amersham [methyl-3H]) was added to each well. Cells were harvested using a Skatrom Instruments SemiAutomatic Cell Harvester (Lier, Norway) and levels of isotope incorporated into DNA measured using a Packard Bioscience/PerkinElmer (Waltham, MA) Tri-Carb 2900TR liquid scintillation counter.

**WNV infections, plaque assays, and RT-PCR**

All infection experiments were performed using the lineage I WNV strain (NY99) and in accordance with the guidelines of the University of Hawaii’s Animal Care and Use Committee. For survival studies, male and female mice between 10 and 12 wk of age maintained on normal mouse chow (<0.25 ppm Se) were inoculated via the footpad route with 102 PFU WNV, and the disease symptoms and mortality was observed for 18 d. On days 3 and 6, 100–200 μl blood was collected from tail veins, from which sera were separated and frozen for subsequent analyses. WNV replication in the sera was analyzed by plaque assay using Vero cells as previously described (18, 19). For quantitation of viral burden in the brain at day 7 postinfection, separate groups of mice were perfused with 10 to 15 ml PBS, whole brains harvested, tissue homogenized, and total RNA was extracted using RNaseasy kit (Qiagen). WNV copy number was quantitated using quantitative real-time RT-PCR analyses using a WNV-specific probe as previously described (18, 19).

**Statistical analyses**

Comparison of means was carried out using an unpaired Student t test (parametric test) or Mann–Whitney test (nonparametric test) using GraphPad Prism version 4.0. For survival analyses, Prism 4.0 was used to perform a Kaplan–Meier log-rank test to compare curves. All comparisons were considered significant at p < 0.05.

**Results**

Sel K is an ER-localized membrane protein expressed in immune tissues and cells that is regulated by dietary Se levels

Data describing the relative levels of Sel K mRNA in different tissues have been conflicting (6, 12), and limited information is available regarding Sel K protein expression. To assess Sel K tissue distribution in mice, Western blot analysis of eight tissues was performed, and results demonstrated relatively high expression of Sel K in spleen and, to a lesser extent, in intestine (Fig. 1A). Western blot analyses also detected Sel K protein in several different types of human and mouse immune cells (Fig. 1B). Some selenoproteins are more sensitive to changes in dietary Se in terms of expression levels (20). So, we examined spleens of mice fed diets with low (0.08 ppm), medium (0.25 ppm, which is equivalent to levels in standard chow), and high (1.0 ppm) Se levels and found that Sel K expression was significantly decreased in the mice fed low Se (Fig. 1C). The effects of increased Se intake on Sel K expression was similar to that observed for Western blot analysis of GPx1 (Supplemental Fig. 1) and both GPx and thioredoxin reductase activities (3). Also, higher dietary Se (1.0 ppm) increased Sel K expression to detectable levels in most tissues examined except for heart (Supplemental Fig. 2). Although the Sel K amino acid sequence contains a predicted transmembrane domain, no data are available confirming membrane localization. Subcellular fractionation of Jurkat T cells was performed followed by Western blotting, and Sel K was detected only in the membrane fraction (Fig. 1D). Fluorescent microscopy revealed that endogenous Sel K localized to the ER in human lymphocytes (Fig. 1E), which is consistent with previous data showing that overexpressed, GFP-tagged Sel K localized to the ER in HEK293 cells (6). Moreover, Sel K was detected by immunohistochemistry in mouse lymph nodes and exhibited a perinuclear distribution consistent with ER localization (Fig. 1F, Supplemental Fig. 3). Taken together, these results suggest that Sel K is an ER-membrane protein expressed at relatively high levels in immune cells and is regulated by dietary Se levels.

Sel K-deficient mice are healthy with normal immune system development

Sel K-deficient mice were generated using a conditional knockout approach due to the possibility that conventional knockout of Sel K may be embryonic lethal. Mice were generated with loxP sites flanking exons 2 and 3 of the Sel K gene on a C57BL/6 genetic background and crossed with CMV-Cre mice to produce offspring with Sel K deleted in all tissues (Fig. 2A). Excision of the targeted region of the Sel K gene was confirmed by PCR (Fig. 2B) and both GPx and thioredoxin repression was similar to that observed for Western blot analysis of GPx1 (Supplemental Fig. 1) and both GPx and thioredoxin reductase activities (3). Also, higher dietary Se (1.0 ppm) increased Sel K expression to detectable levels in most tissues examined except for heart (Supplemental Fig. 2). Although the Sel K amino acid sequence contains a predicted transmembrane domain, no data are available confirming membrane localization. Subcellular fractionation of Jurkat T cells was performed followed by Western blotting, and Sel K was detected only in the membrane fraction (Fig. 1D). Fluorescent microscopy revealed that endogenous Sel K localized to the ER in human lymphocytes (Fig. 1E), which is consistent with previous data showing that overexpressed, GFP-tagged Sel K localized to the ER in HEK293 cells (6). Moreover, Sel K was detected by immunohistochemistry in mouse lymph nodes and exhibited a perinuclear distribution consistent with ER localization (Fig. 1F, Supplemental Fig. 3). Taken together, these results suggest that Sel K is an ER-membrane protein expressed at relatively high levels in immune cells and is regulated by dietary Se levels.

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Sel K−/− mice were born at the expected Mendelian ratio and developed normally to adulthood. Sel K−/− mice were fertile and showed no differences from WT mice in terms of weight gain, although no detailed analyses have yet been performed to characterize metabolism in these mice fully. Because of the relatively high levels of Sel K expressed in lymphoid tissues such as thymus, lymph nodes, and spleen (Fig. 1, Supplemental Fig. 4), immune system development was examined in the knockout mice. Com-
pared with WT controls, Sel K−/− mice exhibited no differences in cell numbers in lymphoid tissues (Table I), suggesting that immune system development was not affected by Sel K deletion. Also, percentages of myeloid and lymphoid cells in liver and lung were similar between WT and Sel K−/− mice (data not shown). Overall, Sel K deletion appeared to have no overt effect.

FIGURE 1. Characterization of Sel K expression in mouse and human tissues. A, Western blot analysis of lysates from eight different mouse tissues revealed relatively high levels of Sel K in spleen. Note that α-tubulin was used as a loading control because β-actin is not expressed in the heart. B, Western blot demonstrated that Sel K is detectable in different primary immune cells (left panel) and cell lines (right panel). C, Western blots were performed on spleen cells from mice fed low (0.08 ppm), medium (0.25 ppm), and high (1.0 ppm) Se diets, and densitometry was performed comparing signal for Sel K/β-actin. Results are expressed as means ± SEM with n = 3 per dietary group. *p < 0.05 (Student t test). D, Fractionation of Jurkat T cell lysates showed that Sel K is detected in the membrane fraction. Markers for each fraction included stromal interaction molecule 1 (STIM1) for membrane, Tata binding protein (TBP) for nucleus, and Akt for cytoplasm. E, Primary human lymphocytes were isolated from PBMCs and analyzed by immunofluorescence microscopy to detect Sel K, which colocalized with the ER marker KDEL. Scale bar, 5 μm. F, Immunohistochemistry performed on frozen mouse lymph node tissue revealed perinuclear Sel K staining consistent with ER localization (arrows). Scale bars, 100 μm for ×20 image and 10 μm for ×100 image. Note that the stain used for detection was horseradish peroxidase and the darker stain around the edges was an artifact of tissue folding. The images for preimmune negative controls are shown in Supplemental Fig. 3.

FIGURE 2. Generation of Sel K-deficient mice. A, The mouse Sel K locus on chromosome 14 contains five exons consisting of translated (blue) and untranslated (green) regions. A neomycin-encoding cassette (Neo) was inserted between exons 1 and 2. The Neo cassette was excised through recombination of the two FRT sites (yellow block arrows) by breeding with an FLP-recombinase transgenic mouse. Presence of the loxP sites (black block arrows) flanking exons 2 and 3 allowed excision of the 2.27-kb target region by expression of Cre recombinase under control of the CMV promoter. B, PCR genotyping of mice was performed to detect presence of targeting region in WT allele (408 bp) or excision of targeting region in KO allele (1.6 kb) using primers as described in Materials and Methods. C, Western blot analysis of spleens from Sel K+/+, Sel K+/−, and Sel K−/− mice indicated complete deletion of Sel K in homozygous KO mice.
on the phenotype of the mice including immune system development and immune cell populations in peripheral tissues.

Sel K-deficient T cells, neutrophils, and macrophages exhibit decreased receptor-mediated calcium flux

Our finding that Sel K is a transmembrane protein localized to the ER of immune cells was of interest because the ER is the key site for initiation of store-operated Ca\(^{2+}\) entry (SOCE) during immune cell activation (21). Thus, we analyzed SOCE by measuring intracellular Ca\(^{2+}\) levels during activation of T cells, neutrophils, and macrophages from WT and Sel K\(^{-/-}\) mice. Activation through the TCR induced lower Ca\(^{2+}\) flux in T cells from Sel K\(^{-/-}\) mice compared with WT controls (Fig. 3A). Also, Ca\(^{2+}\) flux was reduced in response to two chemokines, RANTES and SDF-1, in

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\(^{a}\)Values represent mean ± SE (n = 5).

FIGURE 3. Receptor-induced Ca\(^{2+}\) flux is reduced in T cells, neutrophils, and macrophages from Sel K\(^{-/-}\) mice. T cells loaded with fluo 3 and fura red were analyzed for the ratio of fluo 3 to fura red fluorescence (FL-1/FL-3) for 1 min and then were stimulated with anti-CD3 (A), RANTES (B), SDF-1 (C), or thapsigargin (D). Ionomycin was added for the last 30 s to confirm equivalent response to this Ca\(^{2+}\) ionophore. Neutrophils were analyzed for Ca\(^{2+}\) flux in a similar manner as described above for T cells, with stimulation including KC treatment (E) and thapsigargin treatment (F). For A–F, results are representative of three independent experiments. Ca\(^{2+}\) flux was measured in BMDMs using live-cell, confocal fluorescent microscopy to analyze the ratio of fluo 4 fluorescence (F1) to baseline fluo 4 fluorescence (F0) with stimulation by addition of IgG-opsonized BSA (G) or thapsigargin (H). For G and H, fluorescence was measured for a minimum of 25 cells per group for each experiment with results expressed as means ± SEM for three independent experiments.
Ca2+ flux induced by engagement of Fc receptors using IgG-opsonized BSA, and results demonstrated that Ca2+ flux was significantly decreased in Sel K−/− mice compared with WT controls (Fig. 3B). BMDMs were analyzed for Ca2+ flux induced by engagement of Fcγ receptors using IgG-opsonized BSA, and results demonstrated that Ca2+ flux was significantly decreased in Sel K−/− BMDMs compared with WT controls (Fig. 3G). For all three cell types examined, Sel K deletion did not affect Ca2+ flux induced by thapsigargin, which inhibits sarcoplasmic:endoplasmic reticulum Ca2+ ATPases and nonspecifically releases Ca2+ from ER stores causing Ca2+ to enter through calcium release-activated Ca2+ (CRAC) channels in the plasma membrane (Fig. 3D, 3F, 3H). This suggests that Sel K deletion does not affect levels of Ca2+ stored in the ER or Ca2+ influx at the level of plasma membrane CRAC channels. The Ca2+ ionophore ionomycin was added as a control at the end of these experiments to open Ca2+ channels and intracellular stores on top of that already mobilized by the previous stimuli. Knockout (KO) cells did not differ from WT in fluorescence by an amount more than the difference from the first stimuli for all three cell types, suggesting the KO cells were healthy and able to flux Ca2+ in response to this ionophore. We also examined the possibility that Sel K deletion may cause ER stress in these cells, which may lead to generalized ER dysfunction. However, there were no differences in ER-stress markers between WT and Sel K−/− spleen cells or BMDMs (Supplemental Fig. 5). Overall, Sel K deletion does not affect the ability of the ER to induce SOCE in a general manner but specifically impairs receptor-mediated ER Ca2+ flux.

Sel K-deficient T cells exhibit decreased proliferation and migration

Because Ca2+ flux was impaired in T cells from Sel K−/− mice and Ca2+ flux is important for T cell proliferation, we analyzed the effect of Sel K deficiency on the proliferative capacity of T cells. Proliferation was decreased in both CD4+ and CD8+ T cells from Sel K−/− mice compared with WT controls (Fig. 4A). Proliferation was only partially diminished (15 and 20% decrease for CD4+ and CD8+ T cells, respectively), but the decreases were statistically significant. T cell migration induced through chemokine receptors also requires efficient Ca2+ flux (22), and this function was measured in response to two T cell chemokines, SDF-1 and RANTES. Migration in response to both chemokines was significantly decreased in Sel K−/− T cells compared with WT controls (Fig. 4B, 4C). Notably, migration from upper to lower chambers in absence of either chemokine was also decreased in Sel K−/− T cells, suggesting an effect of Sel K deletion on passive migration through the Transwell pores.

Sel K-deficient neutrophils exhibit reduced migration

Similar to T cells, chemotaxis of neutrophils depends on efficient Ca2+ release from ER upon chemokine receptor engagement (23, 24). To determine if neutrophils from Sel K−/− or WT mice demonstrated differences in chemotactic activity, migration assays were performed using neutrophils purified from bone marrow of these mice. The frequency of neutrophils in bone marrow and expression of the chemokine receptor for KC, CXCR2, were similar between WT and Sel K−/− mice (Fig. 5A). However, when these cells were purified and tested for their ability to migrate in response to KC, migration was significantly decreased in neutrophils from Sel K−/− mice compared with WT controls (Fig. 5B).

To test the in vivo effect of Sel K deletion on neutrophil migration, peritonitis was induced in WT and Sel K−/− mice using i.p. injection of a viral mimetic, poly(i:c), and cellular infiltration was analyzed. Although no differences were found in total cellular infiltration, Gr-1+ neutrophil influx was slightly decreased in Sel K−/− mice (Fig. 6A, 6B). Other innate cell types did not differ significantly, including monocytes/macrophages (CD11b+), dendritic cells (CD11c+), and NK cells (NK1.1+). Importantly, no differences were found between KO and WT in terms of leukocytes circulating in blood, including Gr-1+, CD11b+, CD3+, and B220+ cells (Supplemental Fig. 6). This supports the notion that lower neutrophil populations in the inflamed peritoneal tissue of KO were not due to lower frequency of circulating cells but to impaired migration of these cells. Sera from these mice was analyzed for levels of TNF-α, MCP-1, IL-6, IL10, IL-12p70, IFN-γ, and KC. Of these cytokines, three were detectable: TNF-α, MCP-1, and KC (Fig. 6C). Decreased levels of MCP-1 and KC were detected in Sel K−/− mice compared with those in WT controls, whereas TNF-α levels did not differ. Thus, the infiltration of neutrophils as well as the production of chemokines important for inducing their infiltration were impaired in Sel K−/− mice during peritonitis.

Sel K-deficient macrophages exhibit reduced Fcγ receptor-mediated oxidative burst

An important function of macrophages during immune responses is the efficient phagocytosis and elimination of IgG-opsonized pathogens through Fcγ receptor-mediated oxidative burst, which
controls (Fig. 7A) the percentage of neutrophils (Gr-1+) macrophages (CD11b+), dendritic cells (CD11c+), and NK cells (NK1.1+) was enumerated by four-color flow cytometry to determine lower Fcγ receptor-mediated oxidative burst and TLR-induced secretion of select cytokines.

Viral infection of Sel K-deficient mice leads to inadequate viral clearance and increased mortality

Proper activation and migration of host immune cells is required for effective immune responses to pathogens, and the importance of these immune functions is particularly evident in experiments involving the mouse model of WNV infection. Inadequate WNV clearance in the periphery due to diminished or defective immune responses leads to increased WNV invasion into CNS tissue and higher mortality due to higher numbers of infected neurons (27). Sel K−/− mice were infected with 10^2 PFU WNV in the footpad and analyzed for survival and viral clearance. As shown in Fig. 8A, survival was significantly reduced in Sel K−/− mice (2 of 23; 8.7%) compared with that in WT controls (10 of 26; 38.5%). To understand how Sel K deletion increased susceptibility to WNV infection, levels of WNV titers were determined by plaque assay and quantitative real-time RT-PCR in sera and brain, respectively. At day 1 postinfection, WNV titers in sera were below detectable levels (data not shown) and then peaked at day 3 in both WT and Sel K−/− mice (Fig. 8B). At day 6 postinfection, WNV titers were lower than those at day 3 for both WT and Sel K−/−, but significantly higher levels of WNV were detected in Sel K−/− sera compared with those in WT controls. Also, WNV titers were higher (by 1.5 log_{10} PFU) in brains from Sel K−/− at day 7 compared with WT controls (Fig. 8C). Immune responses to WNV depend on antiviral cytokines produced by immune cells in the periphery to limit neuroinflammation that may lead to death (27). Compared with WT mice, KO mice exhibited decreased levels of key cytokines in sera suggesting impaired anti-WNV immunity while neuroinflammation was increased as determined by brain tissue cytokines and histol-

FIGURE 5. Chemotaxis is reduced in neutrophils from Sel K−/− mice. A. Percentage of bone marrow cells expressing neutrophil markers (Gr-1+ CD11b+) were similar for WT and Sel K−/− mice, and expression of the chemokine receptor that binds KC, CXCR2, was similar for neutrophils from WT and Sel K−/− mice. B. Migration assays were performed using neutrophils purified from bone marrow and added to Transwell plates with 0, 1, or 10 ng/ml KC added to the lower chamber. Results are expressed as means ±SEM for triplicates and representative of two independent experiments. *p < 0.05 (Student t test).

FIGURE 6. Decreased neutrophil infiltration and chemokine secretion are exhibited during peritonitis in Sel K−/− mice. A. Peritonitis was induced in WT and Sel K−/− mice by i.p. injection of the viral mimetic poly(i,c). After 48 h, total cells in peritoneal lavages were enumerated. B. Peritoneal lavages were analyzed by four-color flow cytometry to determine the percentage of neutrophils (Gr-1+), macrophages (CD11b+), dendritic cells (CD11c+), and NK cells (NK1.1+). C. Three cytokines were detectable in sera from mice at 48 h after induction of peritonitis, including TNF-α, KC, and MCP-1. Results are expressed as means ±SEM for six mice per group pooled from two independent experiments. *p < 0.05 (Student t test).

is dependent on effective Ca^{2+} flux (25). To determine the effect of Sel K deficiency on Fcγ receptor-mediated oxidative burst, we performed a time-course experiment using BMDMs from Sel K−/− and WT mice. Despite similar levels of Fcγ receptors (CD16/32) on their cell surfaces, Sel K−/− BMDMs produced a significantly lower Fcγ receptor-mediated oxidative burst compared with WT controls (Fig. 7A, 7B). Importantly, phagocytosis in general was not impaired in BMDMs from Sel K−/− mice as determined by polystyrene bead uptake (Fig. 7C). TLR-induced cytokine production is another function that has been suggested to depend on Ca^{2+} flux, although the data are limited (26). BMDMs from Sel K−/− and WT mice were analyzed for production of proinflammatory cytokines TNF-α, MCP-1, IL-6, IL-12p70, and IFN-γ in response to two TLR agonists: LPS and poly(i,c). Results demonstrated that of the three cytokines that were secreted by BMDMs (MCP-1, IL-6, and TNF-α), only IL-6 and TNF-α were significantly diminished in Sel K−/− BMDMs compared with WT controls (Supplemental Fig. 7). Overall, Sel K deletion selectively affected the functions of BMDMs that require Ca^{2+} flux including Fcγ receptor-mediated oxidative burst and TLR-induced secretion of select cytokines.
gestion of beads (green) between plasma membrane (red) was similar to dimensional rendering of Z-stack images confirming that complete in-polystyrene beads was similar between WT and KO. Insets display three-

cantly reduced in macrophages from Sel K receptor-mediated phagocytosis of IgG-opsonized complexes was significant.

Values.

between WT and Sel K macrophages was significantly reduced in macrophages from Sel K mice compared with WT controls. Results are expressed as means ± SEM for triplicates representative of two independent experiments. Note that SEM bars are present in B but do not appear due to their small size in comparison with y-axis values. *p < 0.05 (Student t test).

Phagocytosis of 1-μm-diameter polystyrene beads was similar between WT and KO. Insets display three-dimensional rendering of Z-stack images confirming that complete ingestion of beads (green) between plasma membrane (red) was similar between WT and KO BMDMs.

Discussion

No in vivo roles have been revealed for Sel K since the identification of the Sel K gene in 2003 (28). An initial clue to potential functions for Sel K was our finding that this protein is expressed at relatively high levels in lymphoid tissues and immune cells. To our knowledge, this is the first evidence of a selenoprotein exhibiting enriched expression in immune cells or tissues. Higher expression in lymphoid tissues combined with its localization to the ER membrane in immune cells suggests Sel K may serve a particularly important role in immune cell activation. Using a novel Sel K mouse model, Sel K was clearly demonstrated to be involved in promoting efficient Ca\textsuperscript{2+} flux during activation of immune cells such as T cells, neutrophils, and macrophages. Ca\textsuperscript{2+} flux induced during activation of other immune cells such as B cells is possible given that we detected Sel K expression in primary B cells. Future studies will determine if B cells, mast cells, and other immune cells required Sel K for activation dependent on efficient Ca\textsuperscript{2+} flux.

Although Ca\textsuperscript{2+}-dependent functions were impaired in immune cells from Sel K-deficient mice, many functions were only partially impaired. For example, T cell proliferation was only partially decreased (15–20%), whereas T cell migratory capacity was effectively eliminated. Ex vivo neutrophil migration in response to high doses of KC (10 ng/ml) or in vivo migration during peritonitis were only partially decreased. Finally, the peak Fcγ receptor-mediated oxidative burst was significantly reduced in Sel K macrophages, but only by 33%. Thus, these functions do not entirely depend on the expression of Sel K and suggest a supportive role for this selenoprotein in promoting Ca\textsuperscript{2+}-dependent immune cell functions. In this sense, Sel K should not be considered a limiting factor during Ca\textsuperscript{2+}-dependent signaling and more likely serves to coordinate or facilitate Ca\textsuperscript{2+} flux during immune cell activation.

The role of Sel K in Ca\textsuperscript{2+}-dependent activation of immune cells may provide important insight into the mechanisms by which dietary Se enhances immunity. Our recent study demonstrated that increased dietary Se led to increased TCR-induced Ca\textsuperscript{2+} flux in CD4\textsuperscript{+} T cells (3). Increased cellular Ca\textsuperscript{2+} has been shown to be an indispensable step in T cell proliferation (29, 30), and the ER is the main Ca\textsuperscript{2+} store in T cells and other immune cells (31). Activation of T cells through the TCR initiates release of Ca\textsuperscript{2+} from this organelle, which subsequently activates CRAC channels in the plasma membrane (32–34). Although our data suggest a role for Sel K in promoting Ca\textsuperscript{2+} flux and the subsequent signaling events in immune cells that rely on this Ca\textsuperscript{2+} flux, the exact mechanism by which Sel K may carry out this function remains unknown. The Sel K amino acid sequence does not contain motifs for canonical Ca\textsuperscript{2+}-binding domains such as EF-hands, epidermal growth factor-like repeats, cadherin repeats, or thrombospondin repeats (35, 36). Thus, it is unlikely that Sel K is directly involved in fluxing Ca\textsuperscript{2+} or sensing loss of Ca\textsuperscript{2+} from ER stores in a manner similar to another ER transmembrane protein, stromal interaction molecule 1 (37). Sel K may act in a more indirect manner to coordinate or facilitate Ca\textsuperscript{2+} flux through protein–protein interactions with signaling molecules, other ER-membrane proteins, or cytoskeleton proteins. For example, the cytoplasmic region of Sel K has a predicted rigid structure that includes several proline residues, with two canonical SH3-binding sequences (R/K-X-X-P-X-X-P-) (38). SH3 domains are present in a variety of cytoplasmic proteins that serve a scaffolding function to promote protein–protein interactions. This suggests that some or all of the functions of Sel K may involve interactions with SH3 domain-containing proteins and formation of protein complexes. Structure/function studies are currently under way to determine how the SH3-binding domain of Sel K may affect Ca\textsuperscript{2+} flux and potential binding partners for Sel K. Sel K is not the only selenoprotein localized to the ER. In fact, there are five other ER-localized selenoproteins, two of which are located in the ER membrane (Sel N and Sel S). Sel N has been demonstrated to participate in protein–protein interactions with the ryanodine receptor (RyR), which is a major component of the RyR intracellular Ca\textsuperscript{2+} release pathway (39). However, the RyR system is not used by immune cells for SOCE, and Sel N appears to regulate RyR-mediated Ca\textsuperscript{2+} mobilization required for normal muscle development and differentiation (5). Sel S is widely expressed in a variety of tissues and has been suggested to participate in the removal of misfolded proteins from the ER lumen for degradation and to regulate ER stress-induced apoptosis (40). Sel K has been suggested to play a similar role (9). Our data
suggest that ER stress is not the mechanism by which Sel K deletion leads to impaired immune cell activation. However, Sel K may play a role similar to Sel S in regulating ER stress in immune and nonimmune cells, and knocking out Sel K may not produce detectable ER stress due to redundancy with Sel S. In this sense, Sel K may serve a common role to alleviate ER stress in most cells that require low abundance but play an additional Ca²⁺ flux-related role in immune cells. A better understanding of the roles Sel K, Sel N, and Sel S play in the ER membrane in different cells and tissues warrants further investigation.

Several different types of immune cells are affected by Sel K deficiency, and the cumulative effect of deleting Sel K is an immunocompromised host. In both humans and mice, increased susceptibility to WNV neuroinvasive disease is correlated with depressed immunity (41). Humoral and cell-mediated responses are important in restricting WNV infection in the periphery, as both B cell- and T cell-deficient mice showed significantly higher viral burden and lethality (42–44). Our data reflect a similar pattern, with Sel K⁻/⁻ mice exhibiting higher viral burden in the periphery and brain accompanied by increased mortality, thereby suggesting inadequate viral clearance by the immune system. However, it must also be considered that Sel K deficiency may increase WNV replication or WNV-induced mortality through mechanisms that do not involve impaired immunity. For example, it is possible that Sel K deletion leads to increased viral replication in infected cells. However, given the decreased ex vivo functions found in both innate and adaptive immune cells, the more likely explanation is that Sel K⁻/⁻ mice are immunocompromised. Less robust immune responses, both innate and adaptive immunity, have been demonstrated in Se-deficient animals (2). A particularly important role for sufficient dietary Se intake has been demonstrated for protecting against viral infection, including H3N2 and H1N1 influenza, HIV-1, and coxsackievirus (45–48). It would be of interest to test different infectious disease models on various selenoprotein KO mice to determine which selenoproteins are important for protecting against different pathogens.

Our results demonstrated a specific effect of Sel K deficiency on macrophage secretion of cytokines using ex vivo BMDMs. Both IL-6 and TNF-α were decreased in Sel K⁻/⁻ BMDMs, but not MCP-1. These results differ from those obtained with in vivo injection of poly(I:C), which did not produce detectable levels of serum IL-6 and showed similar TNF-α levels in WT and KO mice. These differences may be due to the times at which cytokines were measured, and time-course experiments for different cell types responding to different TLR agonists would better reveal differences between WT and KO cells. Also, receptor uptake of secreted...
cytokine may dramatically differ between in vivo and ex vivo conditions, contributing to differences measured in each scenario. Another important issue involves the finding with T cell migration wherein Sel K−/− T cell migration was decreased compared with that in WT even in the absence of chemokines. This suggests that other factors in addition to chemokine-induced Ca2+ flux may be affected by Sel K deficiency, including adhesion molecule expression or cellular cytoskeleton organization required for cellular movement.

Also, in our in vivo experiments involving the TLR3 agonist, poly(i:c), revealed both decreased migration and decreased production of chemokines that attract these cells from the circulating blood in KO compared with WT mice. Activation of cells induced by TLR agonists has been shown to depend on effective Ca2+ flux (26), and this results in decreased chemokine production. Further investigation into the role that Sel K and Ca2+ flux in general play in chemokine production is warranted. Also, it is interesting that TNF was not affected by Sel K deletion in the in vivo or ex vivo experiments, whereas other cytokines or chemokines were decreased. This suggests that TNF secretion does not depend on the effect of Ca2+ flux to the same degree as other cytokines or chemokines.

Overall, our results involving a novel Sel K-deficient mouse model establish this selenoprotein, for which no function has yet been described, as important for immune system function. Sel K is required for effective Ca2+ flux during immune cell activation, and Sel K deletion in mice leads to insufficient immune responses. Our finding that Sel K is important for Ca2+-dependent immune cell functions opens a new field of biological inquiry into molecular mechanisms of Se in enhancing immune responses. Sel K may also prove an effective target of therapeutic modulation of immunity. It is important to consider that Sel K may play other roles not associated with Ca2+ signaling, and these roles may be important in a wide variety of cells and tissues. This seems likely given that Sel K is slightly detectable, but consistently expressed in nonimmune cells and tissues that do not use SOCE for activation. Further studies involving Sel K structure and function are currently under way and will allow a better understanding of the role this selenoprotein plays in immune responses and other biological roles.

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Disclosures

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References


