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Peroxisiredoxin II Regulates Effector and Secondary Memory CD8+ T Cell Responses

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Reactive oxygen intermediates (ROI) generated in response to receptor stimulation play an important role in cellular responses. However, the effect of increased H2O2 on an antigen-specific CD8+ T cell response was unknown. Following T cell receptor (TCR) stimulation, the expression and oxidation of peroxiredoxin II (PrdxII), a critical antioxidant enzyme, increased in CD8+ T cells. Deletion of PrdxII increased ROI, S phase entry, division, and death during in vitro division. During primary acute viral and bacterial infection, the number of effector CD8+ T cells in PrdxII-deficient mice was increased, while the number of memory cells were similar to those of the wild-type cells. Adoptive transfer of P14 TCR transgenic cells demonstrated that the increased expansion of effector cells was T cell autonomous. After rechallenge, effector CD8+ T cells in mutant animals were more skewed to memory phenotype than cells from wild-type mice, resulting in a larger secondary memory CD8+ T cell pool. During chronic viral infection, increased antigen-specific CD8+ T cells accumulated in the spleens of PrdxII mutant mice, causing mortality. These results demonstrate that PrdxII controls effector CD8+ T cell expansion, secondary memory generation, and immunopathology.

CD8+ T cells are critical for the immune system’s response to infectious agents and tumors (3, 53). During an infection, naive T cell activation commences upon T cell receptor (TCR) recognition of cognate antigen presented by major histocompatibility complex (MHC) proteins on the surface of antigen-presenting cells (APCs). Antigenic recognition triggers a signaling cascade marked by protein tyrosine phosphorylation, increased generation of reactive oxygen intermediates (ROI), and a rise in intracellular calcium (20, 33, 37). Together, these signals initiate effector cell differentiation that is accompanied by increased protein synthesis, cell growth, and effector functions such as cytokine production and cytolytic activity (12). Activation also results in massive clonal expansion beginning 24 h after stimulation and continuing with one division every 6 to 8 h (35). Upon pathway clearance, the immune system returns to homeostasis through apoptosis of the majority of effector cells, while surviving cells further differentiate into long-lived memory cells (12). During a chronic infection, CD8+ T cells still expand but gradually lose effector function through a process termed exhaustion due to persistent antigen exposure, and they fail to differentiate to memory cells (45). Understanding the mechanisms regulating the function and differentiation of antigen-specific T cells is essential for designing new vaccine and immunotherapy strategies.

Multiple studies have established a critical role for ROI in regulating signaling in a variety of cell types. As early as 1979, May and de Haen demonstrated that H2O2 acts as a “second messenger” during insulin stimulation (31), and in another study it was found that H2O2 subsequently induces oxidative inactivation of the tyrosine phosphatase PTP1B to promote signal transduction (32). In addition, ROI have also been implicated in the regulation of transcription factors, such as AP-1 (1) and NF-κB (30, 38), and kinases such as protein kinase C (15). To prevent oxidative stress, cells must possess the means to regulate ROI in order to modulate signal transduction. Peroxisiredoxins have been proposed to act as a cellular switch that determines whether hydrogen peroxide acts as a deleterious oxidant or a beneficial second messenger through the use of reducing equivalents provided by thioredoxin (46). Because of its high affinity for H2O2 (Michaelis constant [Km] for H2O2, <10 μM) and abundance in the cytoplasm, peroxiredoxin II (PrdxII) can modulate receptor signaling pathways while protecting cells from nonspecific oxidation of cellular components (8). PrdxII expression affects cellular functions such as proliferation, differentiation, heme metabolism, intracellular signaling, and chemotherapeutic resistance of cancer cells (10, 11, 28). In the case of the immune system, deletion of PrdxII has been shown to promote differentiation of bone marrow cells into CD11c-positive dendritic cells and to enhance concanavalin A-induced splenocyte proliferation in vitro (34). However, the contribution of PrdxII during an adaptive immune response was not determined.

T cell activation and proliferation are highly energetic processes in which cells adapt their metabolism (39) and electron transport (51) to become fully activated. During this phase, the ROI generated are required for activation and function of CD8+ T cells (14, 33). Previous studies demonstrated that antioxidants inhibit proliferation and interleukin-2 (IL-2) production when administered during the early stages of T cell activation (9). In both infectious (27) and autoimmune models (41), antioxidant treatment has been shown to decrease the proliferation and cytokine production of antigen-specific T cells. Together, these findings suggest that ROI generated in response to receptor stimula-
tion are positive mediators of lymphocyte activation. In contrast, high levels of ROI have been implicated with T cell dysfunction in diseases such as cancer, HIV, and systemic lupus erythematosus and in aging (7, 13, 25, 40). Therefore, maintaining optimal levels of ROI is critical to T cell proliferation and function.

We hypothesized that PrdxII, through modulation of ROI, was a critical regulator of T cell proliferation, function, and differentiation. During the initial stages of CD8+ T cell activation, we observed an increase in both PrdxII expression and oxidation. By utilizing mice deficient in PrdxII, we present the first report, to our knowledge, in which the contribution of increased H2O2 to the antiviral immune response is determined. Along with elevated ROI levels, we measured increased CD8+ T cell division in vitro and effector cell expansion in vivo in lymphocytic choriomeningitis virus (LCMV)-infected PrdxII−/− mice. Surprisingly, the generation of secondary but not primary memory CD8+ T cells was increased in mutant mice. Lack of PrdxII during chronic LCMV infection resulted in mortality that was mediated by an increased ROI. These results demonstrate that PrdxII is a regulator of ROI levels and the expansion of antigen-specific CD8+ T cells.

MATERIALS AND METHODS

Mice, virus, bacteria, and infections. PrdxII−/− mice, described previously (28), were backcrossed onto the C57BL/6 background for 10 generations. Wild-type and knockout littermates were separated and used to generate lines that were maintained by homozygous mating. Genotypes were confirmed by PCR amplification of tail DNA. P14 PrdxII−/− mice were generated by crossing PrdxII+/− mice with P14 mice that were maintained on a C57BL/6 (CD45.2) background. CD45.1 mice were purchased from the National Cancer Institute (Frederick, MD). All studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Wake Forest University School of Medicine. Six- to eight-week-old mice were infected with 2 × 10^7 PFU of lymphocytic choriomeningitis virus strain Armstrong (LCMV-Armstrong) intraperitoneally (i.p.) or 2 × 10^5 PFU of LCMV strain Clone 13 (LCMV-Clone 13) intravenously (i.v.). Virus was grown and quantitated as described (2). For bacterial infection, 6- to 8-week-old mice were infected i.v. with 5 × 10^5 CFU of Listeria monocytogenes expressing the LCMV epitope GP33-41, which was prepared as described previously (52).

Cell isolation. The spleen was removed from mice after cervical dislocation. Following mechanical disruption of splenocytes on a wire mesh screen, red blood cells were removed by osmotic lysis in ACK buffer (NH4Cl, KHCO3, and EDTA). Splenocytes were then resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1-glutamine, penicillin-streptomycin, and β-mercaptoethanol (complete medium). For isolation of liver lymphocytes, mice were euthanized, the abdomen was opened, the hepatic vein was cut, and 5 ml of ice-cold phosphate-buffered saline (PBS) was injected directly into the hepatic artery to peritonealize the liver. The liver tissue was homogenized with a wire screen and incubated in 0.25 mg of collagenase B/ml (Boehringer Mannheim) and 1U/ml ofDNase I (Boehringer Mannheim) for 45 min. Digested liver was centrifuged, and the pellet was resuspended in 5 to 10 ml of 44% Percoll (Sigma) solution and incubated with 0.25 mg of collagenase B/ml (Boehringer Mannheim) and 1U/ml ofDNase I (Boehringer Mannheim) for 45 min. Digested liver was centrifuged, and the pellet was resuspended in 5 to 10 ml of 44% Percoll (Sigma). This solution was underlaid with 56% Percoll and spun at 2,000 rpm for 20 min at 20°C. The intrahepatic lymphocyte populations were harvested from the interface, and red blood cells were lysed with 0.83% ammonium chloride, washed, and counted.

CD8+ T cell purification. CD8+ T cells were negatively selected by magnetic bead enrichment from the spleens of naive 6- to 8-week-old mice using the Miltenyi MicroBead system according to the manufacturer’s protocol. Purity was >95% as determined by flow cytometry.

CD8+ T cell stimulation. For anti-CDS/CD28 stimulations, 48-well flat-bottom plates were coated with 10 μg/ml of anti-CD3 and anti-CD28 antibodies or 20 μg/ml control IgG in PBS overnight at 4°C. All antibodies were purchased from BD Pharmingen. After removal of PBS, purified T cells were added at 3 × 10^5 cells per ml. Phorbol myristate acetate (PMA) and ionomycin (ION) stimulation was induced at 50 ng/ml and 500 ng/ml, respectively.

Real-time RT-PCR. Purified CD8+ T cells (3 × 10^5) were stimulated for the times indicated with anti-CD3 and anti-CD28 antibodies. RNA was isolated using the RNasy Minikit (Qiagen), and cDNA was generated by reverse transcription (RT) (Superscript II; Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed on an ABI Prism 7000 Sequencer and system using SDS V1.2 software with TaqMan Gene Expression kits for murine PrdxII (Mm00489896_m1), c-myc (Mm00487804_m1), and GAPDH (Mm03968990_s1) and signal normalized to GAPDH, and the fold increase relative to ex vivo was determined.

Western blotting. To measure PrdxII and Prdx-SO3 levels, 2 × 10^6 purified CD8+ T cells were lysed in 50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 20 mM β-mercaptoethanol, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% Igepal, 0.5% Triton X-100, 1 mM Na3VO4, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin (pH 8.0). Lysates were immediately sonicated and frozen at −70°C. To measure dimer formation in PrdxII, cells were pretreated for 10 min with 100 mM N-ethylmaleimide (NEM) and subsequently lysed in the presence of NEM. Samples were then separated on a 12% SDS denaturing gel, transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane, and blocked for 1 h at room temperature in 5% skim milk. PrdxII and Prdx-SO3 antibodies (Abs) were purchased from Lab Frontier and were used at a dilution of 1:10,000 in 5% milk and incubated with membranes overnight at 4°C. After three washes, the blots were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary Ab (Southern Biotechnology) at a 1:10,000 dilution in 5% milk for 2 h at 4°C. The blot was then visualized using the SuperSignal Dura West Chemiluminescent Substrate from Pierce according to the manufacturer’s protocol. The blot was then stripped with Restore Western blot stripping buffer (Pierce) for 10 min at room temperature, blocked, probed with anti-actin (Santa Cruz) at a 1:1,000 dilution, and subsequently developed as described above. For quantitation of protein expression, actin and PrdxII levels were normalized using a Kodak Image Station 2000RT and Kodak Molecular Imaging software. The fold increase in PrdxII was calculated by multiplying the fold difference in the normalized actin value by the PrdxII signal.

DCFDA oxidation. DCFDA ([5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate-acetyl ester] was purchased from Invitrogen and resuspended in dimethyl sulfoxide (DMSO) as a 2 mM stock. Cells were stimulated in a 96-well flat-bottom plate, transferred to a 96-well round-bottom plate after stimulation, and incubated with DCFDA for 30 min at 37°C before being washed in fluorescence-activated cell sorter (FACS) buffer (2% FCS and PBS), stained with anti-CD8α Ab, and acquired immediately on a FACS Calibur instrument. Data are presented as changes in mean fluorescent intensities compared with unstained wild-type cells.

Surface and intracellular staining. In this study, the following Abs were used: rat anti-mouse CD8α-phycocerythrin (CD8α-PE), rat anti-mouse CD8α-peridinin chlorophyll protein (CD8α-PerCP), rat anti-mouse CD8α-APC, rat anti-mouse CD127-fluorescein isothiocyanate (CD127-FITC), rat anti-mouse CD44-FITC, rat anti-mouse CD4-PE, rat anti-mouse gamma interferon (IFN-γ)-FITC, rat anti-mouse tumor necrosis factor alpha (TNF-α)-PE, rat anti-mouse IL-2-APC, rat anti-mouse killer cell lectin-like receptor G1 (KLRG1)-PE, rat anti-mouse CD27-PE, rat anti-mouse CD62L-FITC, rat anti-mouse LAG3-PE, and hamster anti-mouse PD-1. KLRG1 antibody was purchased from Abcam. CD127 and PD-1 antibodies were purchased from eBioscience. All other antibodies...
were purchased from BD Pharmingen. D6GP33-41, D6NP39-404, and D6GP276-286 MHC class I tetramers were generated as previously described (36). Surface staining was performed by incubation of Abs at a 1:100 dilution in FACs buffer for 30 min at 4°C. KL2RG1 staining was performed at a 1:25 dilution. To measure intracellular cysteine levels, cells were treated with BD Biosciences Cytofix/Cytoperm kit according to the manufacturer’s instructions.

Cell cycle analysis. BrdU (Sigma-Aldrich) labeling was performed as described previously by Tebo et al. (44). Briefly, purified CD8+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies. During the final hour of stimulation, samples were pulsed with 10 μM BrdU for 60 min, resuspended in 1% paraformaldehyde with 0.05% Igepal (Sigma-Aldrich), shaken, and incubated overnight at 4°C. Cells were then washed two times in room temperature PBS at 290 g for 6 min, resuspended in 1 ml of PBS and 4.2 mM MgCl2 containing 50 Kunitz U/ml DNase I (Sigma-Aldrich), and incubated for 30 min at 37°C. After two washes with wash buffer (5% FCS with 0.5% Igepal in PBS) at 290 g and 4°C for 6 min, cells were resuspended in the same buffer containing 2% mouse serum and a 1:5 dilution of anti-BrdU-FITC (BD Pharmingen) and incubated on ice for 45 min. Samples were washed two times in wash buffer at 290 g and 4°C for 6 min. For 7-aminocytidine D (7-AAD) staining, cells were resuspended in 20 μl of 7-AAD (Pharmince) and FACS buffer for 10 min on ice. Samples were acquired immediately using a FACScalibur instrument.

CFSE labeling. CFSE (5-6-carboxyfluorescein diacetate, succinimidyl ester) was purchased from Invitrogen Life Technologies and dissolved in DMSO as a 5 mM stock. After purification, cells were washed three times in PBS and suspended at a concentration of 2 × 10^5 cells/ml in PBS. The CFSE stock was diluted to 6.67 μM in PBS and mixed with cells 1:1 (vol/vol), resulting in a final concentration of 3.33 μM. After 3 min, samples were vortexed and then incubated for an additional 2 min. After this time, 1/10 volume of FCS was added for 1 min followed by vortexing. The cells were then washed three times with complete medium and used in experiments. The percentage of cells in the final division was determined with FlowJo software (Treestar).

In vivo CD8 depletion. To deplete CD8+ T cells in vivo, mice were administered isotype control or 300 μg of rat anti-mouse CD8 antibody clone 2.43 (BioXcell Corp.) at days −2, 0, and 2 postinfection with LCMV-Clone 13. Control testing demonstrated greater than 98% depletion of CD8+ T cells from naive mice.

Statistical analysis. Data from all experiments were analyzed by the Student’s t test using Microsoft Excel, and a P value of <0.05 was considered significant.

RESULTS

PrdxII expression increases following naive CD8+ T cell activation. Previous studies have demonstrated that H2O2 generated in response to naïve T cell activation is an important contributor to cytokine oxidation and protein signaling (26, 33). Therefore, we reasoned that CD8+ T cells possess a mechanism that regulates ROI levels to promote signaling, while preventing oxidation of nonspecific targets. To address this hypothesis, we focused on the contribution of the antioxidant peroxiredoxin II (PrdxII), a regulator of H2O2, to T cell activation and differentiation. Since H2O2 is produced following T cell stimulation, we wanted to determine if PrdxII levels increase. Figure 1A demonstrates that following T cell receptor (TCR) stimulation, naïve CD8+ T cells increased PrdxII steady-state mRNA levels almost 8-fold by 18 h. This increase was not transient and was maintained throughout the stimulation. The kinetics of PrdxII expression were distinct from those of the oncogene c-Myc, which was rapidly induced at 6 h, peaked at 18, and had declined by 30 h. Since we observed that mRNA levels were increased, it was critical to determine changes on a protein level. Figure 1B and C demonstrate that following TCR stimulation, naïve CD8+ T cells increased PrdxII protein expression 2-fold by 18 h and 5-fold by 30 h. This increase in PrdxII levels correlated with S-phase entry and T cell proliferation (33).

In the presence of H2O2, PrdxII becomes oxidized to sulfenic acid on the peroxidatic cysteine. This residue then undergoes nucleophilic attack by the resolving cysteine on a neighboring PrdxII monomer and generates a disulfide bond that can subsequently be reduced in the presence of thioredoxin and thioredoxin reductase (47). To determine if PrdxII dimerization is altered during T cell activation, we measured disulfide bond formation by polyacrylamide gel electrophoresis under nonreducing conditions following TCR stimulation (Fig. 1D). The greatest increase in PrdxII dimerization was detected by 18, 24, and 30 h and indicates an important role for PrdxII in eliminating H2O2 during activation and proliferation.

When ROI are produced in a concentrated manner, the flood-gate hypothesis predicts that PrdxII becomes irreversibly oxidized to sulfenic (SO2H) or sulfonic (SO3) acid (46). This prevents disulfide bond formation and allows the accumulation of H2O2 within the cell to promote signaling. To determine if PrdxII undergoes overoxidation in response to T cell activation, we measured the levels of PrdxII, utilizing a PrdxII antibody that recognizes the sulfenic and sulfonic acid forms of PrdxII, I, II, III, and IV. Within 6 h of activation, there was an increase in PrdxII levels (Fig. 1E) that continued to increase for the duration of the stimulation. We also observed that peroxiredoxins I, III, and IV were overoxidized following stimulation. Together, these data demonstrate that both PrdxII expression and oxidation increase during naïve CD8+ T cell activation and proliferation.

PrdxII−/− CD8+ T cells generate increased ROI and undergo more proliferation than wild-type cells. Since increased PrdxII expression correlated with T cell activation, we hypothesized that deletion of PrdxII would increase ROI levels. To address this question, we used mice that were deficient in PrdxII. To measure ROI production following T cell activation, we used the oxidant-sensitive dye DCFDA, which is cell permeant and nonfluorescent until oxidized by peroxides, peroxynitrite, and/or hydroxyl radicals. Within 15 min of stimulation, there was a 20% increase in DCFDA oxidation in wild-type CD8+ T cells. As predicted, the PrdxII−/− T cells possessed even higher levels of DCFDA oxidation both before and following stimulation (Fig. 2A). These results demonstrate that PrdxII is an important antioxidant protein in CD8+ T cells and regulates intracellular ROI levels.

Prior studies have documented that antioxidants can suppress lymphocyte proliferation in vitro and in vivo (9, 27, 41). To determine if increasing ROI promoted S-phase entry, purified CD8+ T cells were activated with anti-CD3 and anti-CD28 antibodies and were pulsed with BrdU. After intracellular staining, cells were incubated with 7-AAD to determine cell cycle progression. Following 18 h of stimulation, 8.9% of wild-type CD8+ T cells (Fig. 2B and C) were in S phase. By 24 h, 70.5% of cells had progressed to S phase. This contrasts with mutant CD8+ T cells, of which 25.9% had entered S phase by 18 h. When results were quantitated from multiple experiments (Fig. 2C), they indicated that mutant cells entered S phase earlier but progressed through at a similar rate. To determine how loss of PrdxII affects division, CD8+ T cells were purified and CFSE labeled. T cells were then stimulated with anti-CD3 and anti-CD28, and proliferation was assessed by the loss of CFSE fluorescence in comparison to undivided cells (Fig. 2D). Although cells from both genotypes underwent extensive division,
there were always more PrdxII−/− cells in the final division (Fig. 2D and E). Because division was increased, it was critical to determine how this affected cell numbers. To verify that PrdxII−/− cells were not more susceptible to cell death in vitro, the number of viable cells following isotype stimulation was determined (Fig. 2F), and no difference between wild-type and mutant cells was observed. This contrasted with the results following anti-CD3 and anti-CD28 antibody stimulation. Initially, cell numbers were similar for wild-type and mutant cells. But as time progressed, wild-type cells preferentially accumulated compared to PrdxII−/− cells (Fig. 2G). Thus, loss of PrdxII promotes S-phase entry and increased division but results in decreased survival in vitro.

Increased expansion of primary effector CD8+ T cells in PrdxII−/− mice. To determine the importance of increased ROI production during a primary immune response, wild-type or PrdxII−/− mice were infected with LCMV-Armstrong. Prior to infection, the number and development of T and B cells in the bone marrow and thymus were not significantly different for wild-type and for PrdxII−/− mice (data not shown). Figure 3A shows that the overall number of CD8+ T cells in the spleen was slightly elevated, but this difference was not significant. When naïve (CD44low) and activated/memory phenotype (CD44high) CD8+ T cells were examined, there was a slight increase (~20%) in the number of activated/memory phenotype cells in PrdxII−/− mice. LCMV-Armstrong is a natural mouse pathogen that replicates in multiple tissues. When the initial viral replication on days 1 and 3 postinfection was examined (Fig. 3B), similar levels were observed in the two strains of mice. On day 8 postinfection, at the peak of T cell expansion, we observed a 2-fold increase in the number of LCMV-specific CD8+ T cells in the spleens (Fig. 3C) and livers (Fig. 3D) of PrdxII−/− mice. Studies by Joshi and colleagues have demonstrated that the effector pool can be subdivided based on the expression of IL-7 receptor α chain (CD127) and killer cell lectin-like receptor G1 (KLRG1) (24). Short-lived effector cells (SLEC) are CD127lowKLRG1high and are not maintained following infection. Double-positive effector cells (DPEC) are CD127highKLRG1high, while long-lived memory precursor effector (MPEC) cells are CD127highKLRG1low. The effector and memory CD8+ T cell pools can also be subdivided based on the expres-
sion of CD62L and CD27. Expression of both sets of surface markers (Fig. 3E and F) was similar on LCMV-specific CD8 T cells for wild-type and PrdxII−/− mice. When results were quantitated from multiple experiments (Fig. 3G and H), a small difference in SLEC and DPEC was observed only in the GP33-41-specific CD8 T cells in PrdxII−/− mice. No differences were observed in cytokine production (data not shown). Thus, during primary infection PrdxII−/− mice have increased numbers of effector CD8 T cells that differentiate normally.

Following the peak of the antigen-specific CD8 T cell response on day 8, a contraction phase ensues and reaches a memory set point by 35 days postinfection. Although effector CD8 T cells were modestly increased, the number of antigen-specific CD8 T cells declined over time to a similar level in PrdxII−/− mice. Once the memory set point was reached, stable numbers were maintained in both genotypes for up to 1 year (Fig. 4A). When the liver was examined at day 35 postinfection, similar numbers of LCMV-specific CD8 T cells were observed in the two strains of mice (data not shown).

Because we observed these increases during an acute viral infection, it was important to verify whether these effects could be observed in other infectious models. To address this question, mice were infected with *Listeria monocytogenes* expressing the GP33-41 epitope from LCMV. At the peak of the response on day 7, the number of effector CD8 T cells was increased 2-fold (Fig. 4B) in PrdxII−/− mice, but the set point following
the contraction phase was similar. To determine if these changes were T cell autonomous, P14 TCR transgenic mice that lacked PrdxII were generated. Naïve wild-type or PrdxII−/− P14 cells (n = 6,000) were transferred to naïve CD45.1 congenic recipients, which were then infected with LCMV-Armstrong. During early infection, responses were modestly increased in mice that had received PrdxII−/−. P14 cells, but by day 7 responses were ~2-fold greater than those found in mice that received wild-type P14 cells. From day 7 to day 8 postinfection, PrdxII−/− P14 cells began to contract, while the number of wild-type P14 cells plateaued. Following day 8, both populations declined, reaching a similar memory set point by day 35 (Fig. 4C). Taken together, these results suggest that loss of PrdxII increases primary effector CD8+ T cell responses in an autonomous fashion.

Loss of PrdxII increases secondary effector CD8+ T cell responses and alters surface marker phenotype. To determine if the remaining antigen-specific CD8+ T cells following acute infection were "bona fide" memory CD8+ T cells, mice that were previously infected with LCMV-Armstrong 97 days earlier were challenged with LCMV-Clone 13. After infection, unimmunized mice of both genotypes had viral titers of ~10^8 PFU/g (Fig. 5A) in their spleens 5 days postinfection, while mice that had been previously infected with strain Armstrong cleared the infection. When the number of antigen-specific CD8+ T cells was determined, secondary effector responses were increased 2-fold in PrdxII−/− mice for all three epitopes examined (Fig. 5B). Upon secondary infection, memory CD8+ T cells redifferentiate into secondary effector cells. Regardless of antigen specificity, CD8+ T cells were skewed in their differentiation toward a more "memory-like" phenotype with fewer SLECs and more MPECs in PrdxII−/− mice (Fig. 5C). This phenomenon was not restricted solely to KLRG1 and CD127, as similar results were observed when the markers CD62L and CD27 were analyzed (Fig. 5D). Thus, loss of

FIG 3 Increased expansion of PrdxII−/− effector CD8+ T cells during primary viral infection. (A) Naïve 6- to 8-week-old wild-type or PrdxII−/− mice were sacrificed, and the numbers of total, naïve (CD44lo), and activated/memory phenotype (CD44hi) CD8+ T cells in the spleen were determined; the averages and standard deviations were plotted. (B) Wild-type or PrdxII−/− mice were infected with LCMV-Armstrong and the viral titer in the indicated organs was determined on days 1 and 3 postinfection by plaque assay. The bar indicates the mean. (C and D) Mice were sacrificed on day 8 postinfection, and splenocytes (C) or liver lymphocytes (D) were stained with anti-CD8α and the MHC class I tetramers D^bGP33-41, D^bNP396-404, or D^bGP276-286, and the averages and standard deviations were plotted. On day 8 postinfection, splenocytes were stained with anti-CD8α, MHC class I tetramers D^bGP33-41, D^bNP396-404, or D^bGP276-286, and either anti-KLRG1 and CD127 (E) or anti-CD62L and CD27 (F). The dot plots are gated on CD8+ Tetramer+ cells, and the number in each quadrant indicates the percentage of each population. (G and H) The averages and standard deviations were plotted for each population. Five to 10 mice were analyzed for each genotype in two to four independent experiments. *, significant difference between PrdxII−/− and wild-type samples; P ≤ 0.05.

FIG 4 Increased expansion of effector CD8+ T cells but similar memory set point in PrdxII−/− mice following primary viral and bacterial infection. Naïve 6- to 8-week-old wild-type or PrdxII−/− mice were infected with LCMV-Armstrong. (A) Mice were sacrificed at various time points following infection, and the number of CD8+ Tetramer+ T cells was determined in the spleen. (B) To assess effects during acute bacterial infection, mice were inoculated with Listeria monocytogenes expressing the GP33-41 epitope and sacrificed at the indicated time points. The number of CD8+ IFN-γ+ T cells in the spleen following GP3-41 peptide stimulation was determined. (C) Naïve P14 wild-type or PrdxII−/− cells (n = 6,000) were adoptively transferred into congenic (CD45.1-) hosts and infected with LCMV-Armstrong. At the time points indicated, the spleen was examined, and the numbers of CD45.2+ CD8+ D^bGP33-41+ T cells (averages and standard deviations) were plotted. Five to 10 mice were analyzed for each genotype in two to four independent experiments. *, significant difference between PrdxII−/− and wild-type samples; P ≤ 0.05.
PrdxII increases the magnitude of secondary effector CD8<sup>+</sup> T cell responses and alters their differentiation to a more memory-like phenotype.

**Loss of PrdxII increases the secondary memory CD8<sup>+</sup> T cell pool and alters their phenotype.** Because the number and phenotype of secondary effector CD8<sup>+</sup> T cells were altered, we determined whether the secondary memory pool was affected. Thirty-five days following LCMV-Clone 13 rechallenge, the spleen was harvested and the number of virus-specific CD8<sup>+</sup> T cells was determined (Fig. 6A). During secondary responses, D<sup>9</sup>NP396-404- and D<sup>9</sup>GP276-286-specific CD8<sup>+</sup> T cells undergo greater expansion than D<sup>9</sup>GP33-41<sup>+</sup> T cells (16, 44). By day 60, we observed a 3-fold increase in NP396-404-specific cells, while GP276-286 cells were only increased 1.5-fold. Surprisingly, there was no difference in GP33-41-specific CD8<sup>+</sup> T cells. In addition to increased numbers, secondary memory cells have been shown to be phenotypi-
cally different from primary memory cells (22). To measure functional differences, we performed intracellular cytokine staining for IFN-γ, TNF-α, and IL-2 following peptide restimulation. Similar to results obtained with MHC class I tetramer staining, the number of IFN-γ-producing CD8+ T cells was increased in PrdxII−/− mice (Fig. 6B). When secondary memory CD8+ T cells were restimulated from wild-type mice, the vast majority produced both IFN-γ and TNF-α (Fig. 6C and D). This contrasted with PrdxII−/− secondary memory CD8+ T cells, in which the production of TNF-α was significantly decreased. In addition to TNF-α, memory CD8+ T cells also produce IL-2. Following peptide restimulation, more IL-2 production was observed in PrdxII−/− secondary memory cells (Fig. 6E and F). When the surface phenotype of the secondary memory cells was interro-

FIG 6 Loss of PrdxII increases the size and alters the quality of the secondary memory CD8+ T cell pool. Naïve 6- to 8-week-old wild-type or PrdxII−/− mice were infected with LCMV-Armstrong. On day 91 postinfection, LCMV-Armstrong immune mice were rechallenged with LCMV Clone 13. Mice were sacrificed on day 35 postchallenge. (A) Splenocytes were stained with anti-CD8 and MHC class I tetramers, and the averages and standard deviations were plotted. (B) Splenocytes were stimulated with the indicated peptide, and the number of IFN-γ-producing CD8+ T cells was determined. The averages and standard deviations were plotted. (C and E) In addition to IFN-γ, the production of TNF-α (C) or IL-2 (E) was determined. Dot plots are gated on CD8+ T cells, and the number indicates the percentage in each quadrant. (D and F) The percentage of IFN-γ+TNF-α+ (D) or IFN-γ+IL-2+ (F) cells was quantitated for each epitope, and the averages and standard deviations were plotted. (G) To examine surface marker phenotype, splenocytes were stained with anti-CD8α, MHC class I tetramer, anti-CD127, and anti-KLRG1. The averages and standard deviations were plotted. Five mice were analyzed for each genotype in two independent experiments. *, significant difference between PrdxII−/− and wild-type samples; P ≤ 0.05.
gated, there were more CD127highKLRG1low virus-specific CD8+ T cells in PrdxII−/− mice regardless of antigen specificity (Fig. 6G). Taken together, these results demonstrate that loss of PrdxII alters the size and differentiation of the secondary memory CD8+ T cell pool.

Increased expansion of antigen-specific CD8+ T cells causes lethality during chronic infection. Because T cell stimulation is accompanied by increased ROI production, we hypothesized that PrdxII would play a critical role during repeated TCR stimulation. Wild-type and PrdxII−/− mice were infected with LCMV-Clone 13, which induces a chronic infection that persists in the brain and kidneys of mice for extended periods. Infection of wild-type mice with Clone 13 was not associated with mortality. However, in PrdxII−/− mice mortality was observed by day 9, and within 13 days postinfection almost all of the mice were dead (Fig. 7A).

When viral loads were examined in the spleen and liver, no differences were observed between wild-type and PrdxII−/− mice (Fig. 7B). Enumeration of the effector CD8+ T cell response on day 10 postinfection revealed a 2-fold increase across multiple antigen specificities in PrdxII−/− mice (Fig. 7C). During chronic infection, virus-specific CD8+ T cells undergo a process termed exhaustion (45). Although functional effector cells are initially generated, they progressively lose the ability to produce IL-2, TNF-α, and then IFN-γ. When cytokine production was examined (Fig. 7D and E), CD8+ T cells from both wild-type and PrdxII−/− T cells had manifested loss of function. Since multiple reports have documented a role for the inhibitory receptors PD-1 (4) and LAG-3 (5) in regulating T cell function during chronic infection,
we measured the surface expression of these molecules on CD8+ D6GP33-41+ T cells (Fig. 7F). For both markers the level of inhibitory receptors was higher on mutant than on wild-type T cells. Because we observed increased numbers of effecter CD8+ T cells in PrdxII−/− mice, we reasoned that these cells were causing immunopathology. To address this hypothesis, CD8+ T cells were depleted by anti-CD8α antibody injection on days −2, 0, and +2 relative to LCMV-Clone 13 infection. This resulted in >98% depletion of CD8+ T cells. Antibody depletion completely rescued PrdxII−/− mice from death, while all of the isotype-treated animals succumbed from days 9 to 11 postinfection (Fig. 7G). Thus, loss of PrdxII increases effecter CD8+ T cell expansion during chronic infection, causing lethal immunopathology.

DISCUSSION

In this study, we examined the role of the antioxidant PrdxII in promoting CD8+ T cell activation, proliferation, and differentiation during viral infection. Here we report five novel observations. First, PrdxII mRNA, protein levels, and oxidation increased in response to naïve CD8+ T cell activation, and this modulated ROI levels in cells. Second, elevated ROI in PrdxII−/− mice were accompanied by increased CD8+ T cell proliferation both in vitro and in vivo. Third, increased effecter expansion in vivo was T cell autonomous. Fourth, during secondary infection, lack of PrdxII skewed the differentiation of effecter CD8+ T cells to a more “memory-like” phenotype, resulting in a larger and altered secondary memory pool. Fifth, during chronic infection, lack of PrdxII increased the expansion of antigen-specific CD8+ T cells, resulting in lethal immunopathology. Taken together, these results demonstrate that PrdxII and its regulation of ROI are critical in controlling CD8+ T cell responses.

Prior studies from our laboratory and others have demonstrated that naïve T cell activation is accompanied by ROI production (14, 33). These changes are critical because antioxidant treatment, which lowers ROI, decreases activation and proliferation (9). However, T cells must possess mechanisms to allow signaling but limit oxidative damage. Here we report that in response to naïve CD8+ T cell activation, and this modulated ROI levels in cells. Second, elevated ROI in PrdxII−/− mice were accompanied by increased CD8+ T cell proliferation both in vitro and in vivo. Third, increased effecter expansion in vivo was T cell autonomous. Fourth, during secondary infection, lack of PrdxII skewed the differentiation of effecter CD8+ T cells to a more “memory-like” phenotype, resulting in a larger and altered secondary memory pool. Fifth, during chronic infection, lack of PrdxII increased the expansion of antigen-specific CD8+ T cells, resulting in lethal immunopathology. Taken together, these results demonstrate that PrdxII and its regulation of ROI are critical in controlling CD8+ T cell responses.

Prior studies from our laboratory and others have demonstrated that naïve T cell activation is accompanied by ROI production (14, 33). These changes are critical because antioxidant treatment, which lowers ROI, decreases activation and proliferation (9). However, T cells must possess mechanisms to allow signaling but limit oxidative damage. Here we report that in response to naïve CD8+ T cell activation, PrdxII mRNA and protein increase. The changes in mRNA occur relatively late compared to the oncogene c-myc and suggest that there are two waves of PrdxII function: an initial buffering of signaling by protein present at activation and then a later increase that blocks oxidative damage as cells begin to divide. By generating a disulfide bond with another monomer, PrdxII can efficiently catalyze the elimination of intracellular H2O2. Indeed, loss of PrdxII increased the resting and stimulated levels of H2O2. The presence of dimer that we observe in naïve CD8+ T cells stands in contrast to prior studies of immortalized Jurkat T cells (29). In those experiments, dimerization was weakly detected in untreated cells and lost as exogenous H2O2 was increased. Therefore, during a physiologic response, the regulation of PrdxII dimerization is more important to T cell physiology than previously estimated and illustrates an important difference between redox regulation in primary versus transformed cells. Levels of overoxidation of multiple Prdx proteins also rose, further indicating that these proteins are undergoing sustained enzymatic turnover in the presence of high levels of H2O2. In addition to dimerization and overoxidation, we also observed the presence of higher-order-molecular-weight complexes between PrdxII and other cellular proteins in activated T cells (Fig. 1D). This suggests that PrdxII could influence T cell activation and proliferation through heterologous interactions such as those observed with platelet-derived growth factor (PDGF) (10) and sulfiredoxin (23). Taken together, these results indicate that PrdxII is critical for regulation of H2O2, following T cell activation.

Aside from increased ROI production in PrdxII−/− mice, we also observed a greater expansion of CD8+ T cells both in vitro and in vivo. Prior studies of PrdxII−/− mice demonstrated increased proliferation of splenocytes in response to concanavalin A stimulation or of smooth muscle cells during vascular remodeling (10, 34). Here we extend these studies, demonstrating that PrdxII−/− CD8+ T cells undergo more division due to earlier entry into S phase. In agreement, we observe a greater expansion of CD8+ T cells in response in vivo to physiologically relevant infections. We observed 2-fold-greater numbers during acute and chronic infections of PrdxII−/− mice. Given that LCMV infection induces one of the largest expansions of CD8+ T cells, PrdxII’s importance is illustrated by the further amplification of the response. These results contrast with earlier studies utilizing antioxidants. By pharmacologically lowering ROI levels in vivo, decreased CD8+ T cell expansion, contraction, and cytokine production during an acute LCMV infection (27) or inducible diabetes model (41) were observed.

Our results have important implications for antiviral immune responses. Taken as a whole, they argue that redox regulation of CD8+ T cell activation and differentiation is complex. Although the elevation in primary effecter CD8+ T cell responses in PrdxII−/− mice was not sustained, immunological memory was maintained for extended periods, suggesting that multiple mechanisms help to prevent oxidative stress from driving cells to senescence or apoptosis. In addition to its antiapoptotic properties, Bcl-2 can function in an antioxidant pathway to prevent cell death (18). In the absence of PrdxII, high levels of Bcl-2 in memory cells (17) could prevent both apoptosis and oxidative stress. Additionally, while we did not observe a major alteration in cell phenotype during primary infection, secondary effecter CD8+ T cells displayed surface marker expression that was skewed to a memory phenotype, resulting in an expanded secondary memory pool that displayed altered cytokine production and surface phenotype. Effectors and memory cell fate is controlled by multiple signals (12), including antigen stimulation, costimulation, and inflammatory cytokines that converge on the transcription factors T-bet, Eomesodermin (Eomes), Blimp-1, Id2, Id3, and Bcl-6 (19, 21, 24, 42, 50). Our observations of increased “memory-like” phenotype effecter CD8+ T cells during secondary LCMV infection are similar to those observed during infection of Blimp1−/− mice (42). This suggests that under conditions where infection is limited, such as a secondary challenge, increased ROI are beneficial for memory differentiation, perhaps through increased oxidation of Blimp-1. Since the DNA-binding activity of multiple transcription factors, including AP-1 and NF-κB, has been shown to be controlled through oxidation in the cytoplasm and reduction in the nucleus (43, 48), loss of PrdxII could lead to Blimp-1 overoxidation and inactivation of its DNA binding. When the secondary memory set point was reached, the pool was increased in PrdxII−/− mice. Prior studies have demonstrated that secondary memory cells display decreased CD62L and IL-2 production compared to memory cells generated following primary infection (22). Loss of PrdxII actually increased the production of IL-2, and no difference was observed in CD62L levels (data not shown). In contrast to the positive effect on IL-2 production, loss of PrdxII negatively regu-
lated TNF-α production in secondary memory cells. This result contrasts with previous studies demonstrating that loss of PrdxII can enhance TNF-α production in macrophages following lipopolysaccharide (LPS) stimulation (49). The role of PrdxII in regulating CD8+ T cell responses was not restricted solely to acute infection, as we observed increased lethality during chronic LCMV infection of mutant mice that was mediated by CD8+ T cells. Although we observed increased numbers of effector CD8+ T cells in PrdxII−/− mice, these cells were not more functional than their wild-type counterparts and actually had increased inhibitory receptor expression. Further confirmation that cytokine differences may not be the critical defect was provided by etanercept treatment, which failed to rescue PrdxII−/− mice from lethality (data not shown). Our results with chronic LCMV contrast with the increased lethality observed during acute influenza virus infection of mice that possess a T cell-specific deletion of manganese superoxide dismutase. In this study, Case and colleagues (6) demonstrated that increased ROI, specifically superoxide, decreased the number of antigen-specific effector CD8+ T cells during infection, causing death. Taken together with our data demonstrating increased H2O2 augmenting responses, these results argue that the effects of ROI on CD8+ T cell activation and differentiation are complex and intermediates may have distinct biological effects.

In conclusion, we demonstrate that PrdxII is required for regulating the immune response and mediating survival during chronic infection. These results have important implications for regulating immune responses. During chronic viral infection, manipulation of PrdxII, once the virus and immune system have reached equilibrium, could lead to increased T cell number and viral clearance. In addition, modulation of PrdxII could increase recruitment and expansion of CD8+ T cells following vaccination. Together, our results suggest that PrdxII may make an attractive therapeutic target for modulating the proliferation and expansion of CD8+ T cells.

ACKNOWLEDGMENTS

This work was supported by NIAID grant RO1-A1068952 to I.M.G. and NCI and NIGMS grants CA126659 and GM050389 to L.B.P. R.D.M. and K.E.C. were supported by NIAID grant 5T32AI007401-20.

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