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Brief Conclusive Report

Reactive oxygen species-mediated bacterial killing by B lymphocytes

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ABSTRACT

Regulated production of ROS is mainly attributed to Nox family enzymes. In neutrophil granulocytes and macrophages, Nox2 has a crucial role in bacterial killing, and the absence of phagocytic ROS production leads to the development of CGD. Expression of Nox2 was also described in B lymphocytes, where the role of the enzyme is still poorly understood. Here, we show that peritoneal B cells, which were shown recently to possess phagocytic activity, have a high capacity to produce ROS in a Nox2-dependent manner. In phagocytosing B cells, intense intraphagosomal ROS production is detected. Finally, by studying 2 animal models of CGD, we demonstrate that phagocyte oxidase-deficient B cells have a reduced capacity to kill bacteria. Our observations extend the number of immune cell types that produce ROS to kill pathogens. ***J. Leukoc. Biol.*** **97: 1133–1137; 2015.**

Introduction

When exposed to pathogens, phagocytic cells, such as neutrophil granulocytes or macrophages, produce large quantities of microbicidal ROS that have a pivotal role in controlling infection. The source of ROS in phagocytic cells is a multicomponent enzyme complex, phox, which is composed of the membrane-bound cytochrome b-245 and cytosolic components including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTPase Rac2 [1, 2]. Upon stimulation by diverse agonists, the cytosolic components translocate to the membrane-bound cytochrome, initiating the production of superoxide. The cytochrome component of the oxidase itself is a dimer, formed by Nox2 (formerly known as $gp91^{phox}$) and $p22^{phox}$. The fundamental role of phox in host defense is well illustrated by the serious clinical picture of CGD, which develops in the absence of phagocytic ROS production [3]. It has been known for a while that the expression of phox components is not restricted to professional phagocytes, but B cells and many nonleukocytic cells also express components of the oxidase [4–8] The capacity of B cells to produce ROS in a Nox-dependent manner is much less than that of neutrophils. For example, Epstein-Barr-immortalized B lymphocytes were found to produce 100-fold less superoxide than human neutrophils [5]. The function of Nox-generated ROS production in B cells remains poorly understood [9], although a role in MHC class II antigen presentation was recently proposed [10]. As H_2O_2 was described to amplify tyrosine phosphorylation-dependent signaling through the inhibition of protein tyrosine phosphatases [11], a potential role for Nox-derived ROS in signal transduction has been proposed [9, 12]. However, although activation of the BCR initiates ROS generation, early events of signaling through the BCR were found to be essentially unaltered in the absence of an active phox [9].

Importantly, today, even naive B cells cannot be considered as a uniform population. In the mouse, it is widely accepted that at least 2 major B cell lineages exist [13, 14]. Whereas in the spleen, essentially the "conventional" B-2 cells are present, the dominant B cell of the PerC is the "innate-like" B-1 cell. Recently, 3 independent reports described the capacity of mouse B-1 cells to phagocytose and kill bacteria [15–17]. These findings have prompted us to test whether bacterial killing by B cells occurs in a Nox-dependent way. In this report, we show that peritoneal B cells have a higher capacity to produce superoxide than that of Sp B lymphocytes. We also demonstrate that in phagocytosing B cells, Nox2 produces ROS around engulfed bacteria. Finally, we present evidence that B cells lacking Nox2 or $p22^{phox}$ have a restricted ability to kill engulfed pathogens

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Mice

The nmf333 mouse strain carrying a mutation in the *cyba* gene was purchased from The Jackson Laboratory (Ban Harbor, ME, USA). Nox2-deficient animals [18] were a kind gift from Professor Ralf P. Brandes (Goethe University, Frankfurt, Germany). Age- and sex-matched C57Bl/6J animals

Abbreviations: CGD = chronic granulomatous disease, *cyba* = cytochrome b-245 alpha polypeptide, Hv1 = voltage-gated proton channel, KO = knockout, Nox2 = NADPH oxidase 2, PerC = peritoneal cavity, phox = phagocyte oxidase, ROS = reactive oxygen species, Sp = splenic

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were used as controls, and all Nox2-deficient mice were males ($Nox2^{-/0}$). Mice had access to regular chow and water ad libitum.

Antibodies

We used the following antibodies in our experiments: anti-Nox2 (NL7) mouse mAb (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and anti-$p22^{phox}$ 16G7 mouse mAb (generously provided by Françoise Morel, Grenoble University, Grenoble, France). Affinity-purified rabbit polyclonal anti-Hv1 antibody [19], anti-actin mouse mAb (Sigma-Aldrich).

In immunocytochemistry experiments, Nox2 was detected with Alexa Fluor 488-labeled NL7 antibody (AbD Serotec, Oxford, United Kingdom), whereas BCR was detected (intracellular heavy chain) with eFluor 570-labeled anti-mouse IgM (eBioscience, San Diego, CA, USA) or (cell-surface light chain) Alexa Fluor 488-labeled anti-mouse IgG $F(ab')_2$ (Invitrogen, Life Technologies, Carlsbad, CA, USA).

Isolation of PerC and Sp B lymphocytes

Splenocytes and PerC cells were obtained after appropriate euthanasia, as described by Parra et al. [15], with minor modification (details given in Supplemental Methods). To separate B cells, CD19 microbeads were used (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. The purity of B lymphocytes was >95% in all cases tested. Contaminating cells were predominantly macrophages.

Determination of extracellular superoxide generation

Sp or PerC B cells (2×10^6/ml) were resuspended in a 1:1 mixture of HBSS and Diogenes (National Diagnostics, Atlanta, GA, USA) superoxide-detecting medium. B lymphocytes were left unstimulated or stimulated with a 20 μg/ml anti-IgM antibody $F(ab')_2$ fragment (Jackson ImmunoResearch, West Grove, PA, USA). Superoxide release was measured at 37°C by use of a FLUOstar Optima luminofluorimeter (BMG Labtech, Ortenberg, Germany).

Immunoblotting

Cells were lysed in Laemmli buffer containing 5% v/v 2-mercaptoethanol and 2% PMSF. Samples were run on 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. Nonspecific binding of antibodies was attenuated by use of 5% skim-milk powder in PBS + 0.1% Tween 20 for 1 h. The membrane was incubated with the primary antibody for 1 h, and then HRP-conjugated secondary antibody was added, followed by detection of chemiluminescence on Fuji RX films by use of an ECL Western blotting analysis system (GE Healthcare, Little Chalfont, United Kingdom).

Immunocytology

For immunocytological experiments, 10^6/ml cells were resuspended in RPMI-1640 medium containing 10% FBS. Expression of Nox2 was detected in quiescent PerC cells or in purified PerC B cells (for subcellular localization of Nox2). Cells were spun onto slides with cytospin centrifugation and were fixed with 4% formaldehyde in PBS. After washing the cells with PBS, permeabilization was carried out in PBS + 1% BSA + 0.1% Triton X-100 for 20 min. Nonspecific binding sites were blocked by use of a solution containing PBS + 10% FcR blocking reagent (Miltenyi Biotec) + 1% BSA and 1% normal goat serum for 1 h. Slides were incubated overnight at 4°C in a 1:25 dilution of Alexa Fluor 488-labeled anti-Nox2, with or without 1:40 dilution of eFluor 570-labeled anti-IgM and 2.5 μM TO-PRO-3 (Life Technologies, Grand Island, NY, USA). Cells were washed and finally mounted by use of Mowiol 4-88 antifade reagent. Stained cells were subjected to confocal microscopic analysis by use of an LSM 710 laser-scanning confocal unit.

NBT test

PerC cells or magnetically sorted PerC B cells (10^6/ml) were incubated with nonviable Alexa Fluor 594-labeled *Staphylococcus aureus* (Invitrogen, Life Technologies) at 10:1 bacterium:cell ratio in HBSS solution containing 0.5 mg/ml NBT. After 2 h, chilled, nonpermeabilized, live PerC cells were labeled for surface expression of Ig light chain by use of Alexa Fluor 488-labeled anti-mouse IgG in HBSS (1:100), after blocking the FcRs. Cells were washed with HBSS and finally suspended in ice-cold FBS, and smears of the suspension were fast dried onto glass slides. In other experiments (e.g., see Fig. 3B), sorted PerC B cells were subjected to cytospin centrifugation directly after phagocytosis and then fixed with 4% paraformaldehyde. In all cases, cells were mounted by us of Mowiol 4-88 antifade reagent and subjected to transmission and fluorescent microscopy immediately.

Assessment of intracellular killing ability

The bactericidal activity of B cells was examined by use of gentamicin-protection assay, as described previously [17]. In brief, magnetically sorted B cells (10^6/ml cells resuspended in RPMI-1640 medium containing 10% FBS) from wild-type and $p22^{phox}$ or Nox2 KO mice were incubated with viable *S. aureus* (ATCC code: 29213) at a bacterium:cell ratio of 10:1 for 1.5 h. The medium was then supplemented with 5 μg/ml gentamicin. Cells were harvested 0, 1.5, 4.5, and 18 h after gentamicin addition. All incubations were carried out at 37°C in 5% CO_2. Lymphocytes were then washed twice in 500 μl sterile PBS and lysed with 900 μl sterile-distilled water for 10 min, and then osmolarity was normalized with 1.8% NaCl solution. Lysates were serially diluted and spread onto Luria-Bertani agar plates, followed by 12 h incubation at 37°C and colony counting.

Statistical analysis

All data are average ± SEM; Mann-Whitney *U*-test was applied to test statistical significance, unless otherwise specified. Statistical calculations were performed by use of Statistica software (StatSoft, Tulsa, OK, USA)

Study approval

All experimental protocols were approved by the Semmelweis University Animal Experimentation Review Board.

RESULTS AND DISCUSSION

Peritoneal B cells produce high amounts of superoxide in a Nox2-dependent manner

We compared the superoxide-producing capacity of PerC and Sp B lymphocytes isolated from mouse. Both B cell preparations showed some spontaneous superoxide production, and this response was substantially higher in peritoneal B cells (**Fig. 1A** and **B**). When calculated over a 30 min period, superoxide production of peritoneal B cells was ~5 times higher than that of Sp B lymphocytes. BCR activation with anti-IgM $F(ab')_2$ increased the ROS response in both cell populations, but the stimulated superoxide production of peritoneal B cells was much higher (Fig. 1A and B). The higher ROS production of peritoneal B cells was not a result of the presence of a contaminating cell population, as neither neutrophils nor macrophages produced superoxide when they were exposed to anti-IgM $F(ab')_2$ (Supplemental Fig. 1). Furthermore, in pilot studies, unsorted PerC cells displayed a smaller response than purified B cells (data not shown).

As B lymphocytes have been shown to express components of phox [5], we hypothesized that different expression level of oxidase components may explain the observed difference in the ROS response. We used Western blot to detect Nox2 and $p22^{phox}$ proteins in peritoneal and Sp B cells. Indeed, compared with Sp B cells, expression levels of Nox2 and $p22^{phox}$ were strongly elevated in peritoneal B cells (Fig. 1C). Analysis of the Western blot results by densitometry revealed 2-fold higher Nox2 expression and 1.5-fold higher $p22^{phox}$ expression in peritoneal B cells. In granulocytes, phox cooperates with the Hv1 during intense ROS production [20], and the reduction of BCR-dependent ROS production had been reported in Hv1-deficient

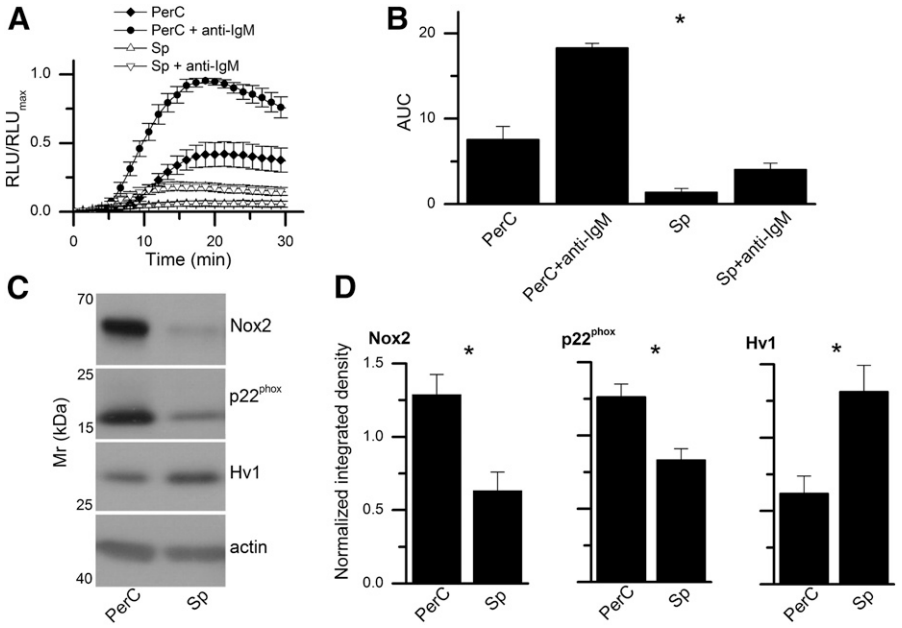

Figure 1. Superoxide release and phox expression of B lymphocytes. (A) Time course of extracellular superoxide release in sorted PerC and Sp B cells, determined with Diogenes reagent. ROS production was measured with or without BCR stimulation by 20 μg/ml anti-IgM F(ab′)$_2$ ($n = 5$). RLU, Relative luminescence unit(s). (B) Area under the curve (AUC) analysis of the data in A. (C) Immunoblot detection of phox subunits in PerC and Sp B cells. The amount of the 2 transmembrane phox subunits (Nox2 and p22^{phox}) and of Hv1 was investigated. Actin labeling serves as a loading control. Mr, Relative molecular mass**.** (D) Densitometric analysis of the Western blot results ($n = 6$; $^*P < 0.05$).

B cells [12]. Interestingly, the amount of Hv1 was found to be higher in Sp B cells (Fig. 1C and D), indicating that the ability of ROS production does not parallel the Hv1 expression level in B cells. We next asked whether phox was responsible for the BCR-induced superoxide production. Peritoneal B lymphocytes were isolated from a Nox2 KO mouse or from animals carrying a point mutation in the *cyba* gene encoding p22^{phox} (nmf333 strain). This latter mutation has been shown to lead to a complete loss of p22^{phox} protein with the consequent absence of phagocytic superoxide production [21]. **Figure 2A** demonstrates that in peritoneal B cells prepared from Nox2-deficient animals, neither components of the cytochrome complex could be detected. Peritoneal B cells prepared from nmf333 animals had no detectable p22^{phox} expression, and their Nox2 content was seriously reduced (Fig. 2A). This result is in agreement with earlier observations, where the presence of Nox2 and p22^{phox} was found to be essential for the formation of a stable cytochrome complex [22]. Superoxide production was completely absent in Nox2- and p22^{phox}-deficient cells (Fig. 2B), thus confirming the essential role of phox in the superoxide release by peritoneal B cells.

Intracellular localization of Nox2 in resting and phagocytosing B cells

Although Nox2 expression had already been described in B lymphocytes, the subcellular distribution of the protein has not yet been examined. We used immunocytochemistry to locate Nox2 in peritoneal B cells. Confocal microscopic analysis revealed that Nox2 was present in IgM-positive cells (i.e., B cells; Fig. 2C), although at a lower level than in IgM-negative cells (macrophages; see below). In purified B cells, Nox2 localized near the cell surface (Fig. 2D). Specificity of the signal was confirmed by staining cells from Nox2 KO animals (Fig. 2C and Supplemental Fig. 2).

B Cells isolated from the PerC or liver were recently shown to phagocytose and kill bacteria. We wanted to know if Nox2 participates in this process. Peritoneal B cells were exposed to fluorescently labeled *S. aureus*, and intracellular localization of the bacteria and Nox2 activity was analyzed by fluorescence microscopy. In agreement with previous reports, only a fraction of B cells (<10%) showed phagocytic activity, whereas the phagocytic activity of wild-type and Nox2 KO cells was essentially the same (as shown in **Fig. 3C**). To follow the ROS formation in the phagosome, B cells were exposed to NBT. In phagocytosing wild-type cells, NBT-formazan deposits colocalized with engulfed bacteria (Fig. 3A and B and Supplemental Fig. 3), whereas no precipitates were observed in p22^{phox}- or Nox2-deficient cells (Fig. 3A and B).

phox Actively participates in bacterial killing by B cells

As the previous experiments suggested the presence of Nox2 activity at the phagosomes, we wanted to know if Nox2-derived ROS contributes to bacterial killing. We studied the microbicidal activity of peritoneal B cells in a gentamicin-protection assay that was successfully used previously to assess the antimicrobial activity of B cells [15–17]. We compared the antimicrobial activity of wild-type, Nox2-deficient, and p22^{phox}-deficient cells, respectively. As shown in Fig. 3C, the intracellular survival of bacteria in Nox2- or p22^{phox}-deficient cells was significantly higher than in wild-type cells. These observations indicate that phox has an active role in killing engulfed bacteria by B cells.

B Cells of fish and amphibians have been described to phagocytose particles and bacteria [23]; however, mammalian primary B cells were originally not considered phagocytic [23]. Recently, 3 groups reported that B-1 cells, isolated from the PerC or from the liver, have the ability to phagocytose and kill bacteria, including *S. aureus* and *Escherichia coli* [15–17]. In this work, we have demonstrated that the microbicidal ability of B cells is dependent on a functional Nox. To our knowledge, our work is the first to assign antimicrobial function for Nox2-derived ROS in B lymphocytes. B-1 cells represent the dominant B cell population in the PerC [13, 14]. These cells are part of the innate immune system and are characterized by the ability to produce natural antibodies. They also share some features with macrophages, including the ability to phagocytose pathogens and to present exogenous antigens. We

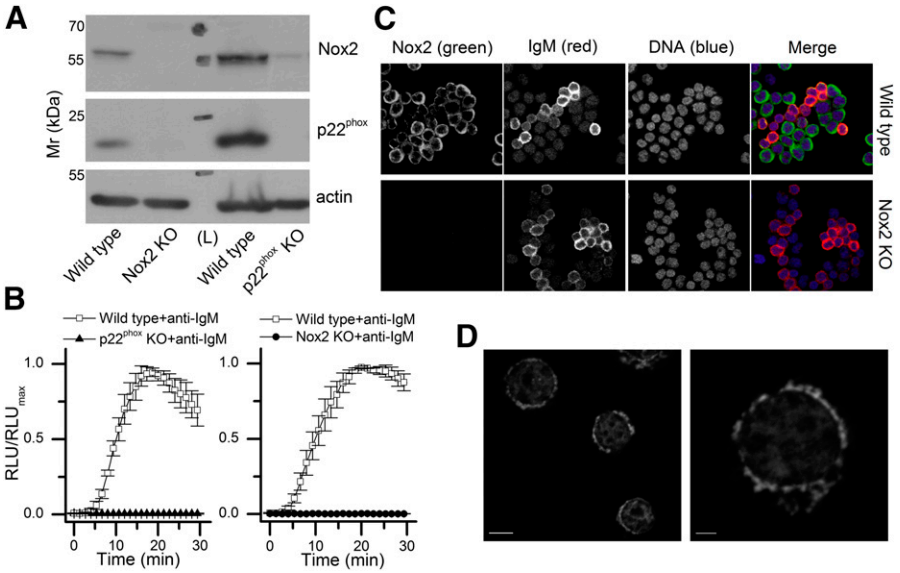

Figure 2. Superoxide release of PerC B cells requires functional phox. (A) Immunoblot analysis of Nox2 and p22^{phox} expression in wild-type, Nox2 KO, and p22^{phox} KO cells. Actin signal serves as loading control. (L), Mr marker. (B) Extracellular superoxide generation of anti-IgM F(ab′)$_2$-stimulated PerC B cells from wild-type, p22^{phox} KO, and Nox2 KO mice (n = 3 for each curve). (C) Expression of Nox2 in IgM+ (i.e., BCR+) and IgM− wild-type and Nox2-deficient PerC cells. Wild-type and KO cells were obtained and labeled in parallel. Image recording and off-line background correction and linear enhancement of the green and red channels were performed the same way. (D) Cellular localization of Nox2 in wild-type, quiescent PerC B cells examined with confocal microscopy. Only the nonnuclear signal is specific for Nox2 (Supplemental Fig. 2). Original scale bars, 5 μM (left); 2 μm (right).

found that peritoneal B cells have much higher capacity to produce ROS than Sp B lymphocytes, and this difference is well explained by their higher Nox2 and p22^{phox} content. The larger capacity of ROS production is a previously unrecognized feature that also supports their similarity to macrophages. Nevertheless, the total ROS (H_2O_2) production of PerC B cells in an Amplex red assay was, on average, 18 times less than that of CD19− PerC cells (mainly macrophages) upon PMA stimulation for 40 min [unpublished results]. Together with their limited phagocytosing and slow killing ability, this observation makes PerC B cells unlikely to play a major role in bacterial clearing of the PerC.

When produced at low amounts, ROS are thought to participate in signaling. Among different ROS, H_2O_2 seems to be a particularly important molecule in signal transduction [24, 25]. Several potential targets of H_2O_2 have been identified over the years; however, the jury is still out on whether their oxidative modification by H_2O_2 is an integral part of signaling pathways. Based on genetic evidence, BCR-induced ROS production has been assigned to the Nox2 system [4]; however, the early events of BCR-induced signaling of Nox-deficient lymphocytes were found to be essentially unaltered [9]. According to the data of Wheeler and Defranco [9], BCR stimulation of primary B cells initiates ROS production in 2 waves, of which Nox2 was shown to mediate only the 1st phase. The 2nd, prolonged phase of the ROS response may originate from mitochondria, and it seems to be required for optimal B cell activation and proliferation. On the other hand, in a different study, Nox2-dependent ROS production, induced by BCR ligation, was described to modulate cell proliferation through the regulation of cyclin-dependent kinase inhibitor 1B expression [26]. The larger ROS-producing capacity of PerC B cells compared with their Sp counterparts might possibly be a result of the higher number of B-1 cells in the peritoneal fluid than in the spleen and may be needed for killing of ROS-sensitive bacteria to enable efficient presentation of their antigens. Additionally, intense phox activity around engulfed bacteria in PerC B cells may play a role in

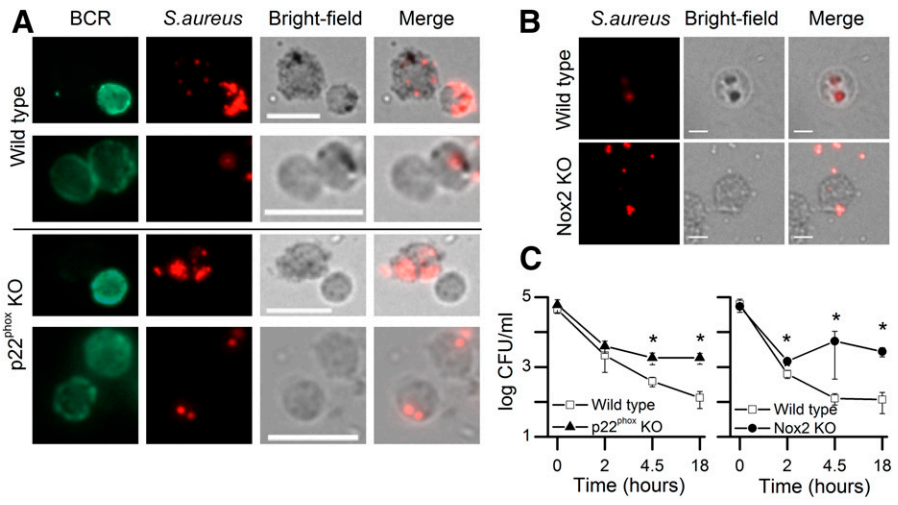

Figure 3. phox is required for phagosomal ROS generation and bacterial killing in PerC B cells. (A) Superoxide release into phagosomes was detected by NBT test. Cells were incubated with Alexa Fluor 594-labeled *S. aureus* (red), followed by labeling the common light chains of cell-surface Igs (i.e., BCR) with Alexa Fluor 488-labeled anti-mouse IgG (green). Formation of massive deposits of dark NBT-formazan around engulfed bacteria was almost always present in phagocytosing wild-type PerC macrophages (larger, BCR− cells) and occasionally in wild-type PerC B cells (smaller, BCR+ cells) but never in any p22phox-deficient cell type. (B) Purified, wild-type PerC B cells, but not their Nox2 KO counterparts, display NBT-formazan deposits. Similar results were obtained by use of bacteria without any surface labeling (Supplemental Fig. 3). Original scale bars, 15 and 5 μm for A and B, respectively. (C) Results of gentamicin-protection assays testing intracellular killing capacity of wild-type and p22^{phox} KO (left) and Nox2 KO (right) cells (n = 3 for all curves; *$P < 0.05$).

optimizing phagosome pH for antigen presentation, as had been shown for dendritic cells [27]. Our results may also have clinical significance, as the finding of defective bacterial killing by oxidase-deficient B cells reveals a previously unrecognized feature of CGD. Interestingly, a previous report has already suggested that B cell physiology might not be normal in CGD, as a serious reduction in the number of $CD27^+$ B cells with the parallel expansion of CD5-positive cells was found in CGD patients [28]. The decreased number of $CD27^+$ B cells in CGD suggests a role of Nox in memory cell formation. Interestingly, human B-1 cells, which were identified as a $CD27^+$, $CD43^+$ cell population, share the CD27 surface antigen with memory B lymphocytes. Griffin and co-workers [29] proposed that some features of memory B cells (such as potent allogenic T cell stimulation), in fact, may be linked to B-1 cells [9].

Future experiments performed on lineage-specific KOs should determine whether the altered antimicrobial activity of B-1 cells contributes to the pathogenesis of CGD.

AUTHORSHIP

I.K. performed experiments, analyzed data, and wrote the manuscript. M.H. performed experiments. A.L. designed experiments. G.L.P. designed and performed experiments, analyzed data, and wrote the manuscript. M.G. designed experiments, analyzed data, and wrote the manuscript.

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DISCLOSURES

The authors declare no conflict of interest.

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