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Brief report

Deficiency of the oxidative damage-specific DNA glycosylase NEIL1 leads to reduced germinal center B cell expansion

Hiromi Mori[a], Rika Ouchida[a], Atsushi Hijikata[b], Hiroshi Kitamura[b], Osamu Ohara[b], Yingqian Li[c,a], Xiang Gao[c], Akira Yasui[d], R. Stephen Lloyd[e], Ji-Yang Wang[a,*]

[a] Laboratory for Immune Diversity, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi, Yokohama 230-0045, Japan
[b] Immunogenomics Group, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, Yokohama 230-0045, Japan
[c] Model Animal Research Center, MOE Key Laboratory of Model Animal for Disease Research, Medical School of Nanjing University, Nanjing 210061, China
[d] Department of Molecular Genetics, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan
[e] Center for Research on Occupational and Environmental Toxicology, Oregon Health & Science University, Portland, OR 97239-3098, USA

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ABSTRACT

Mammalian cells possess multiple DNA glycosylases, including OGG1, NTH1, NEIL1, NEIL2 and NEIL3, for the repair of oxidative DNA damage. Among these, NEIL1 and NEIL2 are able to excise oxidized bases on single stranded or bubble-structured DNA and has been implicated in repair of oxidative damage associated with DNA replication or transcription. We found that *Neil1* was highly constitutively expressed in the germinal center (GC) B cells, a rapidly dividing cell population that is undergoing immunoglobulin (Ig) gene hypermutation and isotype switching. While *Neil1*$^{-/-}$ mice exhibited normal B and T cell development and maturation, these mice contained a significantly lower frequency of GC B cells than did WT mice after immunization with a T-dependent antigen. Consistent with the reduced expansion of GC B cells, *Neil1*$^{-/-}$ mice had a decreased frequency of Ig gene hypermutation and produced less antibody against a T-dependent antigen during both primary and secondary immune responses. These results suggest that repair of endogenous oxidative DNA damage by NEIL1 is important for the rapid expansion of GC B cells and efficient induction of humoral immune responses.

1. Introduction

Reactive oxygen species (ROS) are continuously generated as by-products of cellular respiration and are one of the major endogenous causes of DNA damage [1,2]. ROS react with DNA and generate a variety of base lesions, such as 8-oxoguanine (8-oxoG), formamidopyrimidines (Fapys) and thymine glycol (Tg) [1–3]. 8-OxoG pairs with adenine and is thus highly mutagenic whereas Fapys and Tg lesions may block DNA replication and thereby affect cell division and survival. The oxidized lesions are primarily repaired by the base excision repair pathway, which is initiated with excision of the damaged bases by DNA glycosylases. In mammals, five oxidized damage-specific DNA glycosylases, OGG1, NTH1, NEIL1, NEIL2 and NEIL3, have thus far been identified [4,5]. These enzymes have overlapping but distinct substrate specificities presumably to allow for efficient excision of different types of oxidized bases [2]. Among these enzymes, the expression of NEIL1 is elevated during S phase of the cell cycle [6] and is active in excision of oxidized bases on single stranded or bubble-structured DNA [7]. These observations suggest that NEIL1 is involved in repair of oxidative damage associated with DNA replication or transcription. Inhibition of NEIL1 expression by RNAi in ES cells resulted in increased sensitivity to low doses of ionizing irradiation [8]. NEIL1-deficient mice appeared normal up to 4–6 months of age but developed severe obesity, dyslipidemia and fatty liver diseases as they aged although the phenotypes appear to be variable and may also be dependent on the genetic background [9]. Recently, it has been shown that *Neil1*$^{-/-}$ mice are cancer-prone and that double knockouts of *Neil1*$^{-/-}$ *Nth1*$^{-/-}$ had very high frequencies of lung and liver cancers [10]. Additionally, it was shown that downmodulation of NEIL1 by antisense oligonucleotides resulted in elevated oxidative damage in the genome and enhanced spontaneous mutation in the *Hprt* locus both in human and Chinese hamster cells [11].

During the course of identifying genes that are involved in B cell activation and terminal differentiation, we found that *Neil1* was abundantly expressed in the splenic germinal center (GC) B cells and in Peyer's patches, which are rich in GC B cells. GC B cells represent a unique cell population that arise in the sec-

Abbreviations: GC, germinal center; Ig, immunoglobulin; SHM, somatic hypermutation; AID, activation-induced cytidine deaminase; NP-CGG, 4-hydroxy-3-nitrophenyl-acetyl coupled to chicken gamma-globulin; LPS, lipopolysaccharide; Tg, thymine glycol; 8-oxoG, 8-oxoguanine; Fapys, formamidopyrimidines.

* Corresponding author. Tel.: +81 45 503 7041; fax: +81 45 503 7040.
E-mail address: oh@rcai.riken.jp (J.-Y. Wang).

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ondary lymphoid organs such as spleen, lymph node, and Peyer's patch during an immune response against foreign antigens [12]. These rapidly dividing cells undergo dynamic genetic alterations including somatic hypermutation (SHM) of the immunoglobulin (Ig) genes. SHM is initiated by activation-induced cytidine deaminase (AID) [13], which is thought to catalyze the deamination of cytosine (C) to uracil (U) and generate a U:G DNA lesion [14]. Mutations are introduced during replication and repair of the AID-triggered U:G lesion and accumulate as GC B cells undergo cell division [15–17]. SHM results in altered affinity of antibodies and those with increased affinity for antigen are selected to differentiate into antibody-producing plasma cells or memory B cells. Upon re-stimulation by the same antigen, the memory B cells are promptly activated and differentiate to secrete large amounts of antigen-specific antibodies.

The dramatic upregulation of *Neil1* expression in GC B cells suggests a role for NEIL1 in B cell activation and terminal differentiation. It is interesting that the preferred substrates of NEIL1 are the oxidized bases on single stranded or bubble-structured DNA. AID, the enzyme responsible for SHM has a similar preference for single stranded regions [14]. In the present study, we have analyzed B cell development, maturation, activation and terminal differentiation in *Neil1*$^{-/-}$ mice. Our results suggest that repair of endogenous oxidative damage by NEIL1 is important for the rapid expansion of GC B cells and efficient SHM and antibody production.

2. Materials and methods

2.1. Isolation of B-lineage cell subpopulations and RT-PCR analyses

Spleen B cells were isolated by using negative sorting with the IMag B cell purification kit (BD Biosciences, Mountain View, CA). To isolate follicular and marginal zone B cells, spleen cells were stained with APC-B220, FITC-anti-CD21 and PE-anti-CD23. B220^{+}CD23^{high}CD21^{dull} (follicular) and B220^{+}CD23^{dull}CD21^{high} (marginal zone) B cells were then sorted with a FACSVantage^{TM} (BD Biosciences). For GC B cells, mice were injected *i.p.* with 100 μg of NP-CGG (4-hydroxy-3-nitrophenyl-acetyl coupled to chicken gamma-globulin) precipitated with alum. Two weeks later, spleen cells were stained with PE-B220 and FITC-PNA and the B220^{+}PNA^{high} GC B cells were sorted using a FACSVantage-turbo cell sorter. RNA was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA) and first-strand cDNA was synthesized with *Superscript III* reverse transcriptase and random primers. The following primers were used in RT-PCR analyses: *Neil1*/s212, 5′-AGCCACTGTCCCTTGTCTTC-3′; *Neil1*/as949, 5′-CTGGAAACGGACTTGCTTGA-3′; *Neil2*/s336, 5′-AGGGAATGTGGCAGAAAGAG-3′; *Neil2*/as610, 5′-GGAAGCCACCACCACTAAAA-3′; *Neil3*/s627, 5′-TGCTGTGTGATGTGTTGCTG-3′; *Neil3*/as1013, 5′-TCCGTAAAGCAATCCTCTCC-3′; *Nth1*/s225, 5′-TGAGGAAGGCGAAGATGCTG-3′; *Nth1*/as764, 5′-TGGGGTCTTGGTCATCTTCT-3′; *Ogg1*/s630, 5′-TCCAAGGTGTGAGACTGCTG-3′; *Ogg1*/as1083, 5′-CTTAGGATGCCAGCCGTAGT-3′; *β-actin*/s80,5′-ATGGATGACGATATCGCT-3′; *β-actin*/as630, 5′-ATGAGGTAGTCTGTCAGGT-3′. RT-PCR was performed using Taq polymerase (TOYOBO, Japan) under the following conditions: *Neil1*, *Neil3*, *Nth1* and *Ogg1*, 95 °C for 2 min followed by 95 °C for 10 s, 60 °C for 20 s and 72 °C for 1 min for 30 (for *Neil1* and *Ogg1*) or 35 (for *Neil3* and *Nth1*) cycles; *Neil2*, 95 °C for 2 min and then 95 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s for 35 cycles; *β-actin*, 95 °C for 2 min followed by 95 °C for 5 s, 54 °C for 10 s and 72 °C for 1 min for 25 cycles.

2.2. NEIL1-deficient mice and FACS analysis

Neil1$^{-/-}$ mice were generated in a 129/C57BL/6 mixed background [9] and have been backcrossed with C57BL/6 mice for five generations. Mice were kept in specific pathogen-free conditions and all experiments were approved by the Animal Facility Committee of RIKEN Yokohama Institute (permission number 20-025). FACS analysis was performed essentially as described [18,19].

2.3. Somatic hypermutation assays

Two WT and three *Neil1*$^{-/-}$ mice (10-week old) were immunized with 100 μg of NP-CGG (Biosearch Technologies, Novato, CA) with alum. Two weeks later, B220^{+}PNA^{high} GC B cells were sorted from spleens of each mouse and analyzed for SHM as described [20].

2.4. Immune response

Eight pairs of WT and *Neil1*$^{-/-}$ mice (9-week old) derived from breeding of *Neil1*$^{+/-}$ mice were injected *i.p.* with 100 μg of NP-CGG precipitated with alum and boosted 5 weeks later. Mice were bled weekly and serum titers of NP-specific IgG1 were analyzed by ELISA, using NP-specific monoclonal high (clone C6) and low (clone N1G9) affinity antibodies as a standard [21].

2.5. Immunofluorescence

Spleen B cells purified from mice immunized with NP-CGG were first attached to the slide glass by cytospin and then fixed and permeabilized as described previously [18]. The cells were then stained with FITC-GL7 (BD Biosciences) and mouse IgG1 anti-thymine glycol (MTG-100P, Japan Institute for the Control of Aging, Shizuoka, Japan), followed by Texas Red-goat anti-mouse IgG1 (Santa Cruz).

2.6. Sensitivity to hydrogen peroxide (H_2O_2)

Spleen B cells (5×10^5/ml) were cultured in the presence of 10 μg/ml of lipopolysaccharide (LPS) for 1 day. The cells were then washed once with serum-free medium and resuspended in serum-free medium containing different concentrations of H_2O_2. After incubation on ice for 10 min, the cells were washed three times with culture medium containing serum and cultured for 1 additional day in the presence of 10 μg/ml of LPS. The cells were then collected and stained with FITC-Annexin V and propidium iodide (PI) to detect apoptotic and necrotic cells, respectively (BioVision, Mountain View, CA). Annexin V^{-}PI^{-} live cells were determined by FACS.

3. Results

3.1. Neil1 is highly expressed in GC B cells

We first examined the expression of DNA glycosylases involved in repair of oxidative damage during B cell differentiation and activation *in vivo*. *Neil1* was expressed at low levels in freshly isolated mature spleen B (Fig. 1, lane 1), follicular B (lane 2) and marginal zone B (lane 3) cell subpopulation, but abundantly expressed in GC B cells (Fig. 1, lane 4) and in Peyer's patches, which contain a high proportion of GC B cells (not shown). In contrast, *Neil2* and *Nth1* transcripts were undetectable in GC B cells whereas *Neil3* and *Ogg1* were only weakly expressed (Fig. 1, lane *4*). These results demonstrate that only *Neil1* is highly expressed in GC B cells, which are proliferating cells that have been activated by antigen and helper T cells *in vivo*.

3.2. Reduced GC B cell expansion in Neil1$^{-/-}$ mice

B cell development and maturation, T cell differentiation and B cell Ig class switching appeared normal in *Neil1*$^{-/-}$ mice (Supplemental Fig. S1). The high levels of *Neil1* expression in GC