Brca2 Deficiency Leads to T Cell Loss and Immune Dysfunction

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Germline mutations in the breast cancer type 2 susceptibility gene (BRCA2) are linked to familial breast cancer and the progressive bone marrow failure syndrome Fanconi anaemia. Established Brca2 mouse knockout models show embryonic lethality, but those with a truncating mutation at the C-terminus survive to birth and develop thymic lymphoma at an early age. To overcome early lethality and investigate the function of BRCA2, we used T cell-specific conditional Brca2 knockout mice, which were previously shown to develop thymic lymphoma at a low penetrance. In the current study we showed that the number of peripheral T cells, particularly naive pools, drastically declined with age. This decline was primarily ascribed to improper peripheral maintenance. Furthermore, heterozygous mice with one wild-type Brca2 allele manifested reduced T cell numbers, suggesting that Brca2 haploinsufficiency might also result in T cell loss. Our study reveals molecular events occurring in Brca2-deficient T cells and suggests that both heterozygous and homozygous Brca2 mutation may lead to dysfunction in T cell populations.

INTRODUCTION

Germline mutations in the breast cancer type 2 susceptibility gene (BRCA2) are linked to familial breast cancer and the progressive bone marrow failure syndrome Fanconi anaemia (FA) (Ford et al., 1998; Howlett et al., 2002). Deleterious mutation in one BRCA2 allele predisposes carriers to breast and ovarian cancer with a 30-60% and 2-19% cumulative risk respectively (King et al., 2003; Risch et al., 2006). The incidence of other tumours (Alter et al., 2003) and previously reported that the [Brca2⁻/⁺]; Lck-Cre] mice developed thymic lymphoma at a low penetrance (Park and Lee, 2008). Except for the small number of mice that developed thymic lymphoma, the mice remained normal and healthy. In this study, we closely examined the Brca2-deficient T cells and found a gradual loss of T cell populations, particularly the naive pools. Brca2-deficient T cells showed activation of the p53 pathway, supporting the notion that activation of p53 induces apoptosis of Brca2-deficient cells (Cheung et al., 2002; Jonkers et al., 2001). Importantly, heterozygous mice also exhibited a gradual loss of T cells. Taken together, our findings suggest that a heterozygous germline BRCA2 mutation may confer immune dysfunction and that mature naïve T cell populations are highly susceptible to death triggered by Brca2 deficiency.

MATERIALS AND METHODS

Keywords: breast cancer type 2 susceptibility gene (BRCA2), knockout mouse, T cell

Mice and preparation of tissues

Brca2⁻/⁺ and Lck-Cre mice were kind gifts from Dr. Anton...
Berms (The Netherland Cancer Institute, The Netherlands). These mice were backcrossed to the FVB/N background for more than 10 generations to generate Brca2 conditional knockout mice. All experiments were approved by the Institutional Animal Care and Use Committees of Seoul National University, and followed the guidelines of Policy and Regulation for the Care and Use of Laboratory Animals.

The thymus and spleen of mice were placed in ice-cold PBS and ground with frosted slides to give a single cell suspension. The suspension was centrifuged at 400 × g for 10 min and red blood cells were lysed with ACK lysis buffer (155 mM NH4Cl, 10 mM KHCO3 and 0.1 mM EDTA). Cells were washed with PBS and resuspended in RPMI-1640 medium (HyClone, USA) supplemented with 10% FCS (HyClone), penicillin/streptomycin, L-glutamine, HEPES, sodium pyruvate, NEAA, and β-mercaptoethanol. Supplements and chemicals were obtained from Sigma (USA).

Flow cytometry analysis
The lymphocyte suspensions were washed in PBS containing 1% BSA and 0.01% sodium azide and incubated with various antibodies for 45 min at 4°C. Stained cells were analysed using the FACS Canto (BD Biosciences, USA). The following antibodies were used for staining: FITC-anti-B220, PE-anti-CD3, FITC-anti-CD44, PE-anti-CD62L from Biolegend (USA); and APC-anti-CD8α from BD Pharmingen (USA).

Western blot analysis
Mouse tissues or cell pellets were homogenised in NETN buffer (150 mM NaCl, 20 mM Tris-Cl pH 8.0, 0.5% v/v Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml aprotonin, 1 μg/ml pepstatinA, 2 μg/ml Na3VO4, and 1 μg/ml leupeptin). Lysates (100-200 μg) were heated at 55°C for 15 min and separated by SDS-PAGE for Western blotting. The lymphocyte suspensions were washed in PBS containing 1% BSA and 0.01% sodium azide and incubated with various antibodies for 45 min at 4°C. Stained cells were analysed using the FACS Canto (BD Biosciences, USA). The following antibodies were used for staining: FITC-anti-B220, PE-anti-CD3, FITC-anti-CD44, PE-anti-CD62L from Biolegend (USA); and PerCP-anti-CD4 and APC-anti-CD8α from BD Pharmingen (USA).

Purification and culture of CD4+ T cells
CD4+ T cells were purified from spleen using anti-CD4 (L3T4) antibody-magnetic beads (Miltenyi Biotech, Germany), according to the manufacturer’s instructions. Purified CD4+ T cells (2 × 10⁶ cells/ml) were stimulated by plate-bound anti-CD3 (2C11, eBioscience) and anti-CD4 and APC-anti-CD8 (Biolegend, USA) at a 1:2 ratio. The lymphocytes suspensions were washed in PBS containing 1% BSA and 0.01% sodium azide and incubated with various antibodies for 45 min at 4°C. Stained cells were analysed using the FACS Canto (BD Biosciences, USA). The following antibodies were used for staining: FITC-anti-B220, PE-anti-CD3, FITC-anti-CD44, PE-anti-CD62L from Biolegend (USA); and APC-anti-CD8α from BD Pharmingen (USA).

RESULTS

Mutant protein expression in [Brca2fl/fl; Lck-Cre] mice
We used Brca2fl/fl (Brca2 conditional knockout mice harbouring loxp sites flanking exon 11 (Jonkers et al., 2001) to monitor the molecular events occurring in Brca2-deficient T cells. In previous studies, Brca2 knockout mice targeting different regions of the gene showed variable degrees of lethality and tumour susceptibility, suggesting a partial function of truncated or mutant Brca2 proteins (Evers and Jonkers, 2006). To clarify the status of Brca2 expression in [Brca2fl/fl; Lck-Cre] mice, we generated a sheep polyclonal antibody using the Brca2 C-terminal fragment (Choi et al., 2012). This antibody detected the Brca2 protein at highest levels in the thymus, spleen, and testis of young mice (Fig. 1A). This expression profile recapitulated the Brca2 expression pattern previously assessed at the mRNA level (Callens et al., 2002; Flores et al., 2002; Rajan et al., 1997). Interestingly, we detected a band similar in size to the exon 11-deleted Brca2 protein in the [Brca2fl/fl; Lck-Cre] thymus (Fig. 1B). The truncated Brca2 is likely to span all Brca2 exons except for the floxed-out exon 11, as indicated by the RT-PCR results (Fig. 1C). These findings demonstrate that [Brca2fl/fl; Lck-Cre] mice express a mutant Brca2 protein. Notably, many BRCA2 mutations found in patients with breast cancer generate truncated or mutated proteins (Couch et al., 1996; Goggins et al., 1996; Spain et al., 1999).

Loss of splenic T cells in [Brca2fl/fl; Lck-Cre] mice
In our mouse model Brca2 deletion should occur from the thymocytes because [Brca2fl/fl; Lck-Cre] mice express Cre recombinase under the proximal lck (p56) promoter (Gu et al., 1994). Therefore, to define the effects of Brca2 deletion, we first examined thymocytes and found that the number of thymocytes was slightly diminished in [Brca2fl/fl; Lck-Cre] mice (Fig. 2A). The diminution was more apparent in the double positive T cells compared to the single CD4+ or CD8+ T cells. Nevertheless, the reduction was not statistically significant and thymic T cell profiles remained unaltered (Fig. 2B). It was previously reported that Brca2 is not required for T cell development (Cheung et al., 2002; Patel et al., 1998). The non-involvement of Brca2 in the thymic developmental process was also evident from the identical T cell receptor (TCR) repertoire in splenic T cells from [Brca2fl/fl; Lck-Cre] mice compared with that in wild-
Type (WT) mice (Fig. 2D). With age, the thymus degenerated, and the number of thymocytes declined drastically in both WT and [Brca2]\(^{F11/+/F11/+}\); Lck-Cre] mice, resulting in the same low cell numbers by week 12. Next, we examined mature T cells in the spleen. Although thymic T cells were not much affected by Brca2 deficiency, splenic T cell numbers were significantly reduced in the [Brca2]\(^{F11/F11}\); Lck-Cre] mice (Fig. 2C). This decrease was observed for both CD4\(^+\) and CD8\(^+\) T cell populations, but not for B cells (Fig. 3A). Interestingly, the reduction in splenic T cell number was also evident in the heterozygous [Brca2]\(^{F11/F11}\); Lck-Cre] mice, suggesting haploinsufficiency of Brca2.

**Loss of naïve T cells in [Brca2]\(^{F11/F11}\); Lck-Cre] mice**

To further characterize the T cell populations susceptible to Brca2 deficiency, we examined surface markers that distinguish naïve or activated/memory T cells, i.e. CD44 and CD62L (Dutt et al., 2007; Dutton et al., 1998). As shown in Figs. 3B and 3C, we found that the decrease in cell number was most dramatic for T cell populations with CD44\(^{hi}\)/CD62L\(^{lo}\) naïve surface marker expression. The reduction in total T cell numbers and disappearance of naïve T cell populations suggest that Brca2 is required for the proliferation/survival of slowly proliferating peripheral T cells. Disappearance of the naïve T cell pool also suggests that immune function might be compromised in the [Brca2]\(^{F11/F11}\); Lck-Cre] mice.

**Reduced immune response in [Brca2]\(^{F11/F11}\); Lck-Cre] mice**

In the next experiment, we examined whether Brca2-deficient cells manifest functional impairment. First, we stimulated CD4\(^+\) T cells with plate-bound anti-CD3 and anti-CD28 antibodies to examine cytokine production. Because Brca2-deficient mice have a lower percentage of T cells in the spleen, we purified CD4\(^+\) T cells and used a constant number of cells in the assay. As shown in Fig. 4A, Brca2-deficient T cells produced similar levels of IL-2 to wild-type cells, indicating that Brca2-deficient T cells were competent for IL-2 production. However, these cells produced less IFN-\(\gamma\) and IL-4, suggesting a functional defect of Brca2-deficient T cells. Notably, there was more profound reduction in the IL-4 production.

Second, we determined the effect of Brca2 deficiency on T cell function in vivo by assessing T-cell-dependent antibody responses. We immunized Brca2 WT [Brca2]\(^{+/+}\); Lck-Cre] or Brca2-deficient [Brca2]\(^{F11/F11}\); Lck-Cre] mice with chicken ovalbumin in complete Freund’s adjuvant twice at a two-week interval. After 4 weeks, we collected the immune serum and measured ovalbumin-specific IgM, IgG1, and IgG2a antibody levels. As shown in Fig. 4B, Brca2-deficient mice produced lower levels of ovalbumin-specific antibodies than WT mice. Notably, Brca2 deficiency had a more profound effect on production of IgM (9-fold reduction) compared with IgG1 (3-fold reduction) or IgG2a (less than 3-fold reduction) isotypes. Overall, these data demonstrate that Brca2 deficiency compromised T cell function.

**Activation of the p53 pathway in Brca2-deficient T cells**

Brca2 is an essential regulator of homologous recombination, a vital pathway for error-free repair of DNA double strand breaks (Thorslund and West, 2007). Therefore, in the absence of functional Brca2 proliferating cells accumulate DNA double strand breaks, which activate the p53 checkpoint (Connor et al., 1997; Sharan et al., 2007). The activation of p53 might trigger cell cycle arrest or cell death and could explain the T cell deficit in Brca2-deficient mice. To investigate whether p53 was activated in the Brca2-deficient T cells, we assessed the expression and phosphorylation of p53. In response to DNA damages, p53 is known to undergo extensive post-translational modifications and to become stabilized and activated (Dai and Gu, 2010). Phosphorylation at Ser15 in human p53 has been shown to relieve the inhibition or degradation of p53 by MDM2 (Shieh et
al., 1997), whereas the mouse equivalent pSer18 has been implicated in the pro-apoptotic function of p53 (Sluss et al., 2004). As shown in Fig. 5, p53 protein expression was increased to a moderate extent in thymic and splenic T cells of the Brca2<sup>F11/F11</sup>; Lck-Cre mice (Figs. 5A and 5B). Upon T cell activation, up-regulation and phosphorylation (pSer18) of p53 was apparent in the Brca2-deficient T cells (Fig. 5C). We then examined several p53 downstream targets, such as p21 and Puma. The induction of p21 and Puma has been linked to cell cycle arrest and apoptosis respectively (Jung et al., 2010; Yu et al., 2003). Moderate induction of both p21 and Puma was observed in thymic and splenic T cells in the Brca2<sup>F11/F11</sup>; Lck-Cre mice (Figs. 5A and 5B). Upon T cell activation, up-regulation of p21 and Puma was clearly observed in Brca2-deficient T cells (Fig. 5C). Together, these results demonstrate the activation of p53 pathway in Brca2-deficient T cells and suggest that the p53
A checkpoint might be responsible for the loss of Brca2-deficient T cells.

**DISCUSSION**

In this study we used conditional Brca2 knockout mice to investigate the role of BRCA2 in T cells. The [Brca2<sup>F11F1</sup>; Lck-Cre] mice exhibited gradual loss of splenic T cells and impaired T cell-dependent immune function. These findings suggest that BRCA2 is required to maintain adequate T cell numbers as well as functional capacity. As heterozygous [Brca2<sup>F11+/</sup>; Lck-Cre] mice also manifested T cell loss, albeit to a lower extent, our findings suggest that individuals with a single allelic BRCA2 mutation may suffer from T cell deficiency.

Previous reports that mice with a Brca2 deletion had no overt T cell phenotype suggested that Brca2 might be dispensable for T cells (Cheung et al., 2002; Patel et al., 1998). We demonstrated that this is not the case, as our mice developed T cell loss and dysfunction. One difference between our mice and others is that the conditional allele targeted a different region. The targeted allele for our mice was selected based on the frequent mutations found in BRCA2 exon 11 (Jonkers et al., 2001; Tavtigian et al., 1996). The 6174delT mutation found in the Ashkenazi Jewish population (Neuhausen et al., 1996) is also present within exon 11 and generates a truncated BRCA2 protein (Goggins et al., 1996; Spain et al., 1999). Our mice expressed a mutant form of Brca2 that spanned all exons except for the floxed-out exon 11. Because exon 11 contains most of the BRC repeats, a series of unique BRCA2 domains that associate with Rad51 (Pellegrini et al., 2002; Wong et al., 2013).
Fig. 4. T cells from [Brca2<sup>F11/F11</sup>;Lck-Cre] mice show reduced immune responses. (A) CD4<sup>+</sup> T cells were isolated from WT or [Brca2<sup>F11/F11</sup>;Lck-Cre] mice and stimulated with plate-bound anti-CD3 and anti-CD28 antibodies. Culture supernatants were collected after 48 h and subjected to cytokine ELISA. Experiments were repeated twice. The p values < 0.05 are marked as asterisks. (B) Sera were collected from WT and [Brca2<sup>F11/F11</sup>;Lck-Cre] mice 4 weeks after 2nd immunization with chicken ovalbumin, and the level of ovalbumin-specific antibodies were determined. Experiments were repeated twice and data points show average +/- SEM from 6 mice. Statistical significance was determined at dilution points giving ~50% maximum O.D. readings, and the p-values < 0.05 are marked with asterisks for IgM and IgG1.

Fig. 5. The p53 pathway is activated in T cells of [Brca2<sup>F11/F11</sup>;Lck-Cre] mice. Western blot analysis was performed on the thymic (A) and CD4<sup>+</sup> splenic T cells as fresh (B) or 48 h after anti-CD3 and anti-CD28 antibody stimulation (C), from the WT and [Brca2<sup>F11/F11</sup>;Lck-Cre] mice. Actin was used as a loading control. Signals for p53 phosphorylation (mouse pSer18) were detectable only in activated T cells. Western blot data were quantified by densitometry and normalized by Actin signals (bar graphs on the right). On the bar graphs, Y axis is an arbitrary unit and each bar represents average +/- SD from 3 mice. The p values < 0.05 are marked as asterisks.
1997), the mutant Brca2 would not be fully functional. The variable phenotypes among mouse models suggest that each truncated or mutant Brca2 protein retains BRCA2 function to a different degree.

The best characterized molecular function of BRCA2 is the control of homologous recombination through recruitment of the Rad51 recombinase to damaged DNA sites (Holmman, 2011). BRCA2 deficiency therefore results in the failure of Rad51-mediated DNA double strand break repair, leading to accumulation of DNA damage (Marx, 1997). In Brca2<sup>−/−</sup>; Lck-Cre mice, the T cell loss initiated in the thymus but only became apparent in the periphery. Our interpretation of this observation is that T cell loss is a cumulative phenotype associated with accumulation of DNA damage during cell division. Alternatively, but not exclusively, mature naïve T cells could be more susceptible to cell death or cell cycle arrest inflicted by Brca2 deficiency. The differential effect of Brca2 deficiency in cytokines or antibody isotypes may also indicate differential susceptibility of the cell types involved. Further studies are required to address whether T cell loss has been caused by the intrinsic property of particular cell types.

The Brca2-deficient T cells of our mouse model showed prominent activation of the p53 pathway. Activation of p53 has been suggested in many other Brca2-deficient mouse models and is thought to determine the cell fate between survival and apoptosis. This dual hypothesis is well corroborated by the pathology findings and is thought to determine the cell fate between survival and apoptosis. Alternatively, accumulation of DNA damage during cell division. Alternatively, accumulation of DNA damage during cell division. Alternatively, accumulation of DNA damage during cell division. Alternatively, accumulation of DNA damage during cell division. Alternatively, accumulation of DNA damage during cell division. Alternatively, accumulation of DNA damage during cell division. Alternatively, accumulation of DNA damage during cell division. Alternatively, accumulation of DNA damage during cell division.

ACKNOWLEDGMENTS

Brca2<sup>−/−</sup> and Lck-Cre mice were a gift from A. Berns (NCI, The Netherlands). This study was supported by the Basic Science Research Program Grant 2012-R1A1A3010579 awarded to Hae-Ock Lee and 2011-0018630 awarded to Hyunsook Lee, through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

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