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Letter to the Editor

The PML-nuclear body associated protein Daxx regulates the cellular response to CD40

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Dear Editor,

Daxx has been isolated in several two-hybrid screens and pull-down experiments, and found to interact with numerous proteins involved in apoptosis regulation, such as the promyelocytic leukemia protein (PML),[1] the FAS receptor,[2] transforming growth factor (TGF) β receptor,[3] apoptosis signal-regulating kinase (ASK)1,[4] ZIP kinase,[5] p53 and p73.[6,7]

Several reports demonstrated that Daxx overexpression potentiates apoptosis upon different stimuli, such as FAS treatment,[2,8] UV irradiation[9] and TGFβ stimulation.[3] In particular, Daxx is found to bind the cytosolic domain of the FAS receptor to transduce an apoptotic signal, which is FAS-associated-death-domain(FADD)-independent.[8] We also demonstrated that Daxx overexpression in primary keratinocytes induces programmed cell death.[1] Despite the many reports implicating Daxx as a proapoptotic molecule upon FAS treatment, several reports are in disagreement with this notion.[10–12]

Recent studies suggest that Daxx may play an important role in the lymphoid compartment. First, Daxx is induced by interferon in pre-B cells and is required for interferon-induced apoptosis.[13] Second, Daxx is downregulated in mantle cell lymphomas.[14] Lastly, Daxx and PML colocalize in splenic B cells and Daxx upregulation, and localization into the PML-NB correlates with induction of apoptosis.[1]

However, *in vivo* analysis has not been carried out to date to assess the physiological relevance of these findings in the B cell compartment. Moreover, the function of Daxx in the modulation of the apoptotic response is also extremely controversial, probably because of lack of *in vivo* genetic analysis. In this study, we show in lymphoid-restricted transgenic mice that Daxx is indeed involved in the regulation of B cell function *in vivo*, albeit in an unanticipated manner.

We previously demonstrated that Concavalin A (Con A), which is mainly a T-specific activation agent, causes Daxx induction in B cells.[1] As this effect could be due to a cross talk between T and B cells, we investigated what mechanism could be involved. T helper cells (Th) can activate B cells through the interaction between CD40 Ligand (CD40L) and CD40.[15] We therefore tested whether Daxx induction in B cells was due to this interaction by using an antagonist anti-CD40L antibody. While Daxx was clearly induced upon ConA stimulation in B cells, its induction was substantially reduced in cells treated with blocking antibodies (Figure 1a). The expression of the CD40L-induced molecule[16–19] FAS was blocked by the ConA/anti-CD40L antibody combined treatment (Figure 1a). Moreover, stimulation of purified B220-positive B cells triggered a clear induction of Daxx at the protein level (Figure 1b). Treatment with another B cell-activating stimulus, lipopolysaccaride (LPS), had comparable effects (Figure 1b). The induction of Daxx was concomitant with the one of FAS and BCL-X_L, two transcriptional targets of CD40 stimulation (Figure 1b).[16–21] Daxx was induced at the transcriptional level, and its induction was concomitant with the one of *FAS* (Figure 1c).

We determined the role of Daxx in B-cell activation *in vivo* by generating *Daxx* transgenic animals. A *Daxx* cDNA was cloned under the control of a promoter composed by the proximal *lck* promoter and the $e\mu$ enhancer, which is known to drive expression of the transgene in both thymocytes and B cells (Supplementary Figure 1A).[22] Two transgenic lines were generated and *Daxx* expression was analyzed. *Daxx* transgenic animals were fertile and viable. *Daxx* mRNA was overexpressed at high levels in spleen, bone marrow (BM) and thymus (Supplementary Figure 1B). By contrast, the transgene was not expressed in other organs such as lung, liver and heart (Supplementary Figure 1B). Overexpressed Daxx was predominantly nuclear and accumulates in PML nuclear bodies (PML-NB) (Supplementary Figure 1C). Daxx was clearly overexpressed at the protein level in spleen and thymus (Supplementary Figure 1D). While we found that Daxx was induced upon CD40 stimulation in control cells, constitutively high Daxx levels were observed in both untreated and CD40-treated transgenic cells (Supplementary Figure 1E). We investigated if *Daxx* overexpression would alter B and/or T-cell differentiation. To this end, cells from bone marrow, spleen and thymus were isolated and analyzed for surface markers to identify various subpopulations. pro-B (B220+/CD43+), pre-B (B220+/CD43−) and mature B (B220+/IgM+) cells were equally represented in control and transgenic bone marrows (Supplementary Table S2). Moreover, transgenic spleens contained equal numbers of plasma cells (B220+/CD138+) and surface immunoglobulin-expressing B cells (IgD and IgM, Supplementary Table S2). Although the *Daxx* transgene was highly expressed in thymocytes, thymic development was not affected either (not shown).

Daxx is expressed at very low levels in bone marrow and spleen (Supplementary Figure 1B), but is markedly induced upon CD40 engagement (Figure 1a–c). Upon CD40 stimulation, isolated mature control B cells actively proliferated (Figure 2a). Strikingly, transgenic B cells proliferated at a

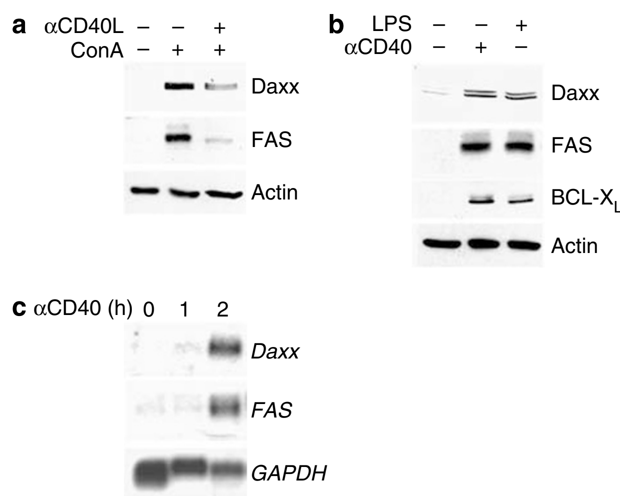

Figure 1 Daxx is induced upon CD40 stimulation. (**a**) Primary spleen cells from 4–8-week-old sv129 animals were isolated and stimulated with concavalin-A (ConA) at 0.5 μg/ml in the presence of either control antibodies or anti-CD40L antibodies at 2 μg/ml. Splenic cells were lysed as previously described.[1] Samples were probed with anti-Daxx and anti-FAS antibodies. Actin levels were measured as loading control. Data shown are representative of three independent experiments. (**b**) Purified B cells were incubated with anti-CD40 antibody at 2 μg/ml or lipopolysaccaride (LPS). At 6 h, cells were lysed and probed with antibodies against Daxx, FAS and BCL-X_L. Actin levels were measured as loading control. Data shown are representative of three independent experiments. (**c**) Purified B cells were incubated with anti-CD40 antibody, and RNA was prepared at 1 and 2 h. Northern blots were probed for *Daxx* and *FAS* expression. *GAPDH* levels were measured as loading control. Data shown are representative of three independent experiments

much lower rate (Figure 2a). However, diminished proliferation was surprisingly not accompanied by reduced viability, demonstrating that Daxx does not trigger cell death in these experimental conditions (not shown). Control cells proliferated more vigorously following IL-4/CD40 treatment, while transgenic cells displayed a much-reduced proliferative rate (Figure 2b). Increasing amounts of anti-CD40 did not rescue the defect observed in transgenic cells (Figure 2a and b). Primary mouse embryo fibroblasts (MEFs) transduced with viruses carrying a *Daxx* mouse cDNA proliferated consistently less at all serum concentrations tested (approximate 35–40% reduction: Figure 2c). Basal levels of apoptosis were once again unaffected upon *Daxx* overexpression (not shown). Thus, Daxx inhibits proliferation in cells derived from different tissues.

Daxx has been proposed to bind the FAS receptor and to positively mediate FAS-induced apoptosis.[2,4,8] Resting mature B cells are resistant to FAS-induced cell death.[21] By contrast, upon CD40 stimulation, B cells become sensitive to FAS-L-induced apoptosis through the upregulation of FAS.[17–19] CD40-stimulated cells were incubated with different concentrations of anti-FAS antibodies for 12 h. While apoptosis was rapidly induced in control cells (over three-fold induction of apoptosis at all concentrations tested: Figure 2d), transgenic cells were not more sensitive to FAS-induced apoptosis (Figure 2d). By contrast, we could observe a moderate, but consistent reduction in cell death. Interestingly, FAS-induced apoptosis was unaffected in *Daxx*-transduced MEFs (not shown). Thus, Daxx does not enhance, but rather suppresses FAS-induced apoptosis in primary B cells.

CD40 signalling results in the concomitant induction of both FAS and the anti-apoptotic molecule BCL-X_L.[16–19,21] FAS induction was comparable in both control and transgenic cells (Supplementary Figure S3). Conversely, BCL-X_L was induced at a higher extent in transgenic cells (Figure 2e), thus suggesting that higher levels of BCL-X_L could counteract FAS-induced apoptosis.

In both B-cell malignancies and autoimmune disorders, B cells are resistant to apoptosis and express high levels of CD40-L, thus indicating the existence of either autocrine or paracrine homotypic B cell interactions to promote survival and proliferation.[15] At the same time, CD40 stimulation of B cells by T helper cells results in FAS upregulation and sensitization to FAS-induced apoptosis.[16–18] Thus, CD40 stimulation can lead either to protection from apoptosis or sensitization to apoptosis depending on the stage of differentiation and microenvironmental conditions. In this study, we demonstrate *in vivo,* in transgenic mice, that Daxx operates downstream CD40 in an unforeseen manner:

(i) we show that induction of *Daxx* in B cells upon activating stimuli (e.g. ConA treatment) is dependent on the CD40 and CD40L interaction and that direct stimulation of B cells with anti-CD40 antibodies results in the rapid induction of Daxx at both transcriptional and protein levels in both mouse and human cells (e.g. Daudi cells; not shown). Moreover, Daxx is induced upon LPS treatment, suggesting that mechanisms for the induction of Daxx may be shared by LPS and CD40.
(ii) We find that when stimulated with anti-CD40 or anti-CD40/IL-4, B cells from *Daxx* transgenic animals proliferate at a much lower rate than control cells, thus revealing the growth-suppressive function of Daxx in this cellular compartment. This in turn unravels the involvement of Daxx in a negative-feedback loop devoted to the control of proliferation upon CD40-mediated B-cell activation.
(iii) Although Daxx overexpression has been reported to potentiate FAS-induced apoptosis, we discover that, *in vivo*, in B cells, Daxx does not potentiate FAS-mediated cell death, but rather inhibits it, which is associated with a stronger induction of the anti-apoptotic molecule BCL-X_L in Daxx transgenic *versus* control B cells. This is in agreement with recent studies, which showed that siRNA-mediated downregulation of Daxx results in higher levels of FAS-induced apoptosis.[20] Based on the evidence presented in this work, FAS-induced apoptosis is largely unaffected by Daxx overexpression *in vivo*, while B-cell activation-induced proliferation is dramatically inhibited (Figure 2f).

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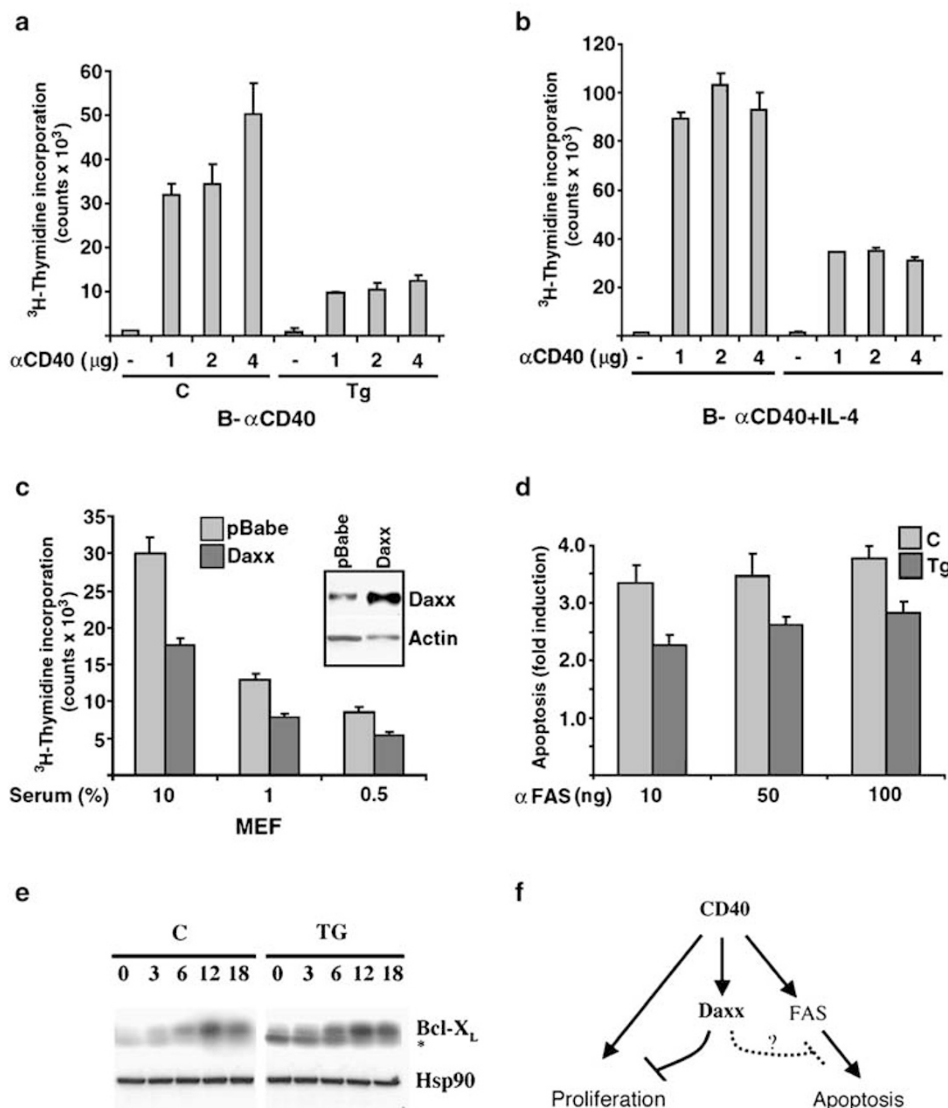

Figure 2 Daxx suppresses CD40-induced proliferation. (**a**) CD40-induced proliferation is reduced in transgenic B cells. Purified B cells from control (**c**) or transgenic (Tg) animals were cultured for 48 h in the presence of increasing concentrations of anti-CD40 antibody (1, 2 and 4 μg/ml). Proliferation was measured by ^{3}H-Thymidine uptake. B cells were plated and incubated with ^{3}H-thymidine for 12 h. Incorporated radioactivity was counted by using a scintillation counter (Pharmacia). Data shown represent one of three independent experiments performed in triplicate. (**b**) CD40/IL-4-induced proliferation is reduced in transgenic B cells. Purified B cells from control (**c**) or transgenic (Tg) animals were stimulated with increasing concentrations of anti-CD40 antibody (1, 2 and 4 μg/ml) and 10 ng/ml of interleukin-4 (IL-4) for 48 h. Proliferation was measured by ^{3}H-Thymidine uptake. Data shown represent one of three independent experiments performed in triplicate. (**c**) Daxx inhibits proliferation in mouse embryonic fibroblasts (MEFs). Phoenix packaging cells were transfected with pBabe and pBabe-Daxx retroviral constructs. MEFs were then infected with pBabe or pBabe-Daxx retroviral supernatants and selected in puromycin (Sigma) for 48 h. Proliferation was measured by ^{3}H-Thymidine uptake in the presence of different serum concentrations (10, 1 and 0.5% fetal bovine serum). Data shown represent one of three independent experiments performed in triplicate. (**d**) FAS-induced apoptosis is reduced in Daxx transgenic B cells. Purified B cells from control (**c**) or transgenic (Tg) animals were cultured in the presence of 2 μg/ml of anti-CD40 antibody for 12 h. Anti-FAS antibody was added at various concentrations and apoptosis was scored at 12 h by propidium iodide (PI) staining and subdiploid peak analysis. Apoptosis is expressed as fold induction over unstimulated cells. (**e**) BCL-X$_L$ expression is augmented in Daxx transgenic B cells. Purified B cells from control (**c**) or transgenic (Tg) animals were stimulated with anti-CD40 antibody at 2 μg/ml. At 0, 3, 6, 12 and 18 h cells were lysed and analyzed for BCL-X$_L$ expression. Heat-shock protein 90 (Hsp90) was measured as loading control. Data shown are representative of three independent experiments. (**f**) Daxx operates downstream CD40. CD40-induced proliferation is reduced upon Daxx overexpression. Surprisingly, FAS-induced apoptosis is also reduced in transgenic B cells

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)