AID-expressing epithelium is protected from oncogenic transformation by an NKG2D surveillance pathway

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Abstract

Activation-induced deaminase (AID) initiates secondary antibody diversification in germinal center B cells, giving rise to higher affinity antibodies through somatic hypermutation (SHM) or to isotype-switched antibodies through class switch recombination (CSR). SHM and CSR are triggered by AID-mediated deamination of cytosines in immunoglobulin genes. Importantly, AID activity in B cells is not restricted to Ig loci and can promote mutations and pro-lymphomagenic translocations, establishing a direct oncogenic mechanism for germinal center-derived neoplasias. AID is also expressed in response to inflammatory cues in epithelial cells, raising the possibility that AID mutagenic activity might drive carcinoma development. We directly tested this hypothesis by generating conditional knock-in mouse models for AID overexpression in colon and pancreas epithelium. AID overexpression alone was not sufficient to promote epithelial cell neoplasia in these tissues, in spite of displaying mutagenic and genotoxic activity. Instead, we found that heterologous AID expression in pancreas tissues, in spite of displaying mutagenic and genotoxic activity, was not sufficient to promote epithelial cell neoplasia in these tissues, in spite of displaying mutagenic and genotoxic activity. Instead, we found that heterologous AID expression in pancreas tissues, in spite of displaying mutagenic and genotoxic activity, was not sufficient to promote epithelial cell neoplasia in these tissues, in spite of displaying mutagenic and genotoxic activity.

Keywords: activation-induced deaminase; cancer; epithelium; NKG2D; pancreas

Subject Categories: Cancer; Immunology

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Introduction

Activation-induced deaminase (AID) is the enzyme that initiates the reactions of secondary antibody diversification: somatic hypermutation (SHM) and class switch recombination (CSR) (Muramatsu et al., 2000). These reactions enable the generation of antibodies with increased affinity for antigen (SHM) and with diversified, specialized functions for antigen removal (CSR), and are therefore critical for a competent immune response (Di Noia & Neuberger, 2007; Stavnezer et al., 2008; Alt et al., 2013; Robbiani & Nussenzweig, 2013). Accordingly, defective AID activity promotes a Hyper-IgM immunodeficiency syndrome in humans (Revy et al., 2000). AID triggers SHM and CSR by direct deamination of cytosine nucleosides in the DNA of immunoglobulin genes, resulting in the generation of U:G mismatches (Petersen-Mahrt et al., 2002; Alt et al., 2013; Robbiani & Nussenzweig, 2013). These U:G mismatches are in turn processed by alternative repair pathways that ultimately lead in SHM to the fixation of a mutation, and in CSR to a DNA double-strand break (DSB) and a recombination reaction (Di Noia & Neuberger, 2007; Stavnezer et al., 2008; Alt et al., 2013; Robbiani & Nussenzweig, 2013).

Activation-induced deaminase activity is not confined to immunoglobulin genes and can promote mutations and DSB followed by illegitimate chromosomal translocations in other regions of the genome (Ramiro et al., 2004, 2006; Liu et al., 2008; Robbiani et al., 2008, 2009). Importantly, chromosomal translocations are the hallmark of mature B-cell lymphomas, the most frequent of all human lymphomas. Indeed, AID deficiency delays the onset of lymphomagenesis in the mouse (Ramiro et al., 2004; Kovalchuk et al., 2007; Pasqualucci et al., 2008), establishing a direct link between AID collateral genotoxic activity and neoplastic transformation in B lymphocytes.

In the last few years, it has become clear that AID expression is not, as originally thought, exclusively restricted to activated B cells. AID expression has been reported in several tissues, including...
gastric, hepatic, and gut epithelia (Endo et al., 2007, 2008; Matsumoto et al., 2007; reviewed in Marusawa et al. (2011)). AID expression in these tissues is most frequently associated with inflammatory conditions and the activation of the NF-κB pathway (Endo et al., 2007; Matsumoto et al., 2007) and has been claimed to promote the accumulation of mutations in epithelial cells (Matsumoto et al., 2007, 2010; Takai et al., 2009; reviewed in Marusawa et al. (2011)). Given that chronic inflammation in epithelial tissues predisposes to cancer development (Mantovani et al., 2008), the finding that the mutagenic activity of AID can be induced in an inflammatory context has fostered the idea that AID might contribute to or even constitute the link between inflammation and cancer (Takai et al., 2012; reviewed in Marusawa et al. (2011)).

Several gain-of-function mouse models have been generated to address the contribution of AID to neoplastic transformation. Ubiquitous AID overexpression led mostly to early T cell neoplasia (Okazaki et al., 2003), hampering a thorough analysis of other malignancies. In contrast, B-cell-specific AID overexpression did not result in lymphomagenesis (Muto et al., 2006; Robbiani et al., 2009) unless the tumor suppressor p53 was removed (Robbiani et al., 2009). However, to date, the impact of specific AID expression in epithelial tissues, classically subject to inflammation-induced neoplastic transformation, has not been addressed. Here, we aimed to test this possibility directly by generating conditional knock-in models of AID overexpression. AID expressed in colon and pancreas epithelia was not sufficient to promote carcinogenesis, in spite of being expressed at high levels and displaying genotoxic activity. Instead, AID triggered the expression of NKG2D ligands and the recruitment of immune cells and promoted a cytotoxic response and cell death. Our data indicate that the oncogenic potential of AID in epithelial cells is neutralized by an immunosurveillance pathway that prevents the expansion of pre-tumoral cells.

Results

Inflammation-induced AID does not contribute to carcinogenesis

Inflammation is known to play a critical role in the etiology of colorectal and pancreatic ductal adenocarcinoma (reviewed in Feagins et al. (2009); Vonderheide & Bayne (2013)). To investigate whether inflammatory conditions promote AID expression in these tissues, we treated human epithelial cell lines derived from colorectal adenocarcinoma (LoVo and SW480) and pancreatic adenocarcinoma (AsPC and PaTu) with the pro-inflammatory cytokine TNF-α and measured AID expression by qRT-PCR. TNF-α stimulation increased AID mRNA expression in all cell lines analyzed (Fig 1A and B). To assess whether primary, non-transformed cells were also able to express AID in response to inflammatory stimuli, we generated explants from mouse pancreatic acinar cells and treated them with TNF-α. As with the human tumor cells, mouse primary epithelial cells expressed AID upon exposure to TNF-α (Fig 1C). TNF-α treatment typically induced 4–30-fold increases in AID mRNA levels in the different cell types tested, consistent with previous findings in liver, gastric and colorectal cell lines (Endo et al., 2007, 2008; Matsumoto et al., 2007). Together, these data confirm previous results showing that inflammatory stimuli can trigger AID expression in cell lines originated from human colorectal adenocarcinoma (Endo et al., 2008), and show that pancreatic adenocarcinoma cells and primary pancreatic cells are also responsive to TNF-α treatment.

Inflammation-induced AID expression has been proposed to contribute to or even be the leading cause of some epithelium-derived tumors, such as colorectal adenocarcinoma (Marusawa et al., 2011). To address whether endogenous AID expressed in epithelium under inflammatory conditions could contribute to carcinogenesis, we made use of the well-established model of dextran sulfate sodium (DSS)-induced colitis-associated cancer (CAC) (Cooper et al., 2000). AID−/− mice or AID+/− littermates were treated for 10 cycles with 3% DSS for 10 cycles, and colonic sections were analyzed by histologic inspection after H/E staining. Graphs represent mean frequency values of adenoma and adenocarcinoma lesions of five independent experiments. n = 28 (AID−/− males); 35 (females); 23 (AID+/− males); 25 (females). P-value: male: 0.8; female: 0.246.

Conditional AID expression in epithelial cells does not promote adenocarcinoma development

The absence of a significant contribution of endogenous AID to carcinogenesis in DSS-treated mice could be explained by an insufficient amount of AID in this model. Indeed, AID expression is known to be limiting for its activity in B cells (Sernandez et al., 2008), and AID levels in B cells are typically 100–1,000 fold higher than those detected in epithelial cells under inflammatory conditions (unpublished observations). To directly evaluate whether AID expression...
can contribute to carcinogenesis, we generated two mouse models for conditional AID expression in epithelial cells of colonic and pancreatic origin (Fig 2A). We introduced an AID-GFP-encoding cassette in the endogenous Rosa26 locus preceded by a transcriptional stop flanked by two loxP sites (R26AID+/KI mice). To achieve specific expression of AID in epithelial cells, we bred R26AID+/KI mice with mice expressing the Cre-recombinase under a villin promoter, which specifically drives expression in colon (el Marjou et al, 2004) (R26AID+/KI/villin-CRE+/TG mice), or the pancreas-specific Ptf1 (p48) gene (Kawaguchi et al, 2002) (R26AID+/KI p48-CRE+/KI mice). R26AID+/+ villin-CRE+/TG and R26AID+/+ p48-CRE+/KI mice were used as controls. To confirm that the Rosa26 AID-GFP cassette was functional, we first evaluated the expression of the reporter protein GFP by immunofluorescence in colon of R26AID+/+villin-CRE+/TG mice and pancreas of R26AID+/+ p48-CRE+/KI mice (Fig 2B). GFP was expressed in R26AID+/+ villin-CRE+/TG colon and R26AID+/+ p48-CRE+/KI pancreas but not in control mice (Fig 2B) or in other tissues (not shown). We next measured AID transcript levels by qRT-PCR. In R26AID+/+ villin-CRE+/TG and R26AID+/+ p48-CRE+/KI mice, the amount of AID in the targeted epithelial tissues was similar to that found in B cells activated in vitro with LPS + IL4, whereas AID expression in control mice remained at background level (Fig 2C). AID is thus expressed in the epithelium of R26AID+/+ villin-CRE+/TG colon and R26AID+/+ p48-CRE+/KI pancreas at levels known to be functional in B cells.

To assess the contribution of AID to adenocarcinoma development, we monitored tumor incidence in R26AID+/+ villin-CRE+/TG and R26AID+/+ p48-CRE+/KI mice. The onset of pancreatic and colorectal adenocarcinoma in a variety of mouse models ranges from 5–6 months to 1–1.5 years (Fodde & Smits, 2001; Aguilar et al, 2004; Martinelli et al, 2015). Therefore, to avoid confounding results arising from spontaneous tumorigenesis in very old mice, we set analysis end points at 75–100 weeks. Survival of R26AID+/+ villin-CRE+/TG mice was indistinguishable from that of R26AID+/+ villin-CRE+/TG littermate controls (Fig 2D, left). Likewise, survival of R26AID+/+ p48-CRE+/KI did not differ from that of R26AID+/+ p48-CRE+/KI controls (Fig 2D, right). To rule out the presence of early malignancies in aged animals, we performed thorough pathological analysis of colon and pancreas sections of all animals, but could not detect any tumor development in R26AID+/+ villin-CRE+/TG and R26AID+/+ p48-CRE+/KI animals at 75–100 weeks (Fig EV1). Expression of AID in colon or pancreatic epithelial cells is thus not sufficient to promote tumor development.

**AID generates mutations and DNA double-strand breaks in pancreatic epithelium**

The failure of AID expression to trigger tumorigenesis prompted us to evaluate its activity in epithelial cells. We first analyzed the in vivo mutagenic activity of ectopically expressed AID. The primary target sequences for AID mutagenic activity are immunoglobulin genes; although other genes are known to be susceptible to AID-induced mutagenesis, this occurs at much lower rates (~10−4 mutations.bp) and the mechanisms responsible for this susceptibility are poorly understood. One of the best-characterized requirements for AID activity is that the target sequence be transcriptionally active (Chaudhuri et al, 2003; Ramiro et al, 2003; Pavri & Nussenzweig, 2011). To simplify the mutagenesis analysis, we made use of the p48 pancreatic AID expression model to take advantage of the known low complexity transcriptome of acinar cells (MacDonald...
AID preferentially targets the consensus hotspots WRCY/RYGW and particularly AGCT motifs (Rogozin & Kolchanov, 1992; Pham et al., 2003; Perez-Duran et al., 2012). Based on this, we analyzed the presence of mutations in 800–900 bp downstream of the transcriptional start site of two highly transcribed genes in pancreas, Elastase1 (Ela1) and Elastase2 (Ela2a), by next-generation sequencing, which allows large number of mutations to be analyzed (Perez-Duran et al., 2012). Graphs show cysteine mutation frequency overall (total) or at AGCT hotspots. Results of two independent experiments are shown. ****P < 0.0001.

Activation of the DNA damage response (DDR) pathway induces the expression of NKG2D ligands in epithelial cells, which are in turn recognized by NKG2D receptors expressed by NK cells and subsets of T cells (Diefenbach et al., 2001; Gasser et al., 2005; Champsaur & Lanier, 2010; Raulet et al., 2013). This cross talk promotes the elimination of precancerous cells and is therefore a mechanism to prevent tumor development (Guerra et al., 2008). Given that AID expression in pancreas promotes mutations and DNA damage without leading to tumor development, we sought for the evidence of precancerous cells and found that pancreas from aged R26AID+/+p48-CRE+/KI mice contained more proliferating cells, as assessed by Ki67 staining, than control pancreas (Fig 4A), indicating that pancreatic AID expression leads to an abnormal rate of cell division. The epithelial identity of Ki67+ cells was confirmed both by morphology (Fig 4A, magnified micrograph on the right) and by staining with the epithelium-specific anti-cytokeratin 8 antibody (Figs 4B and EV2). We next asked whether the NKG2D immune surveillance pathway could be in play in R26AID+/+p48-CRE+/KI mice. To test this hypothesis, we first analyzed the expression of the Raen NKG2D ligand in epithelial cells from pancreatic explants of R26AID+/+p48-CRE+/KI and control mice by flow cytometry. Acinar cells from R26AID+/+p48-CRE+/KI mice expressed higher levels of Raen than their control littermates, although the difference was not statistically significant (Fig 4C). To assess whether RAe ligands were expressed by pancreatic cells in vivo, we prepared pancreas extracts from aged (75-week-old) R26AID+/+p48-CRE+/KI mice and controls and measured the amount of five RAe isoforms by droplet digital PCR (ddPCR). With this technique, each sample is fractioned into thousands of droplets, in which PCR amplification reactions occur independently, thereby increasing the sensitivity and quantitative potential of the amplification. Amplification of RAe isoforms was detected in more drops from R26AID+/+p48-CRE+/KI mice than from controls, indicating that AID generates DSBs in this cellular context.

**Table 1. Analysis of AID mutagenic activity by Sanger sequencing.**

| Genotype      | Total clones analyzed | Mutations | Total bp sequenced | Frequency (×10⁻⁶) |
|---------------|-----------------------|-----------|--------------------|-------------------|
| Elastase1     | R26AID+/+p48-CRE+/KI  | 84        | 4                  | 70,018            |
|               | R26AID+/+p48-CRE+/KI  | 82        | 13                 | 69,355            | 1.87              |
| Trp53         | R26AID+/+p48-CRE+/KI  | 66        | 0                  | 59,472            | 0.185             |
|               | R26AID+/+p48-CRE+/KI  | 59        | 1                  | 53,936            |
samples than from controls (Fig 4D), indicating that AID promotes the expression of NKG2D ligands in pancreas, most likely as a result of DSB and DDR. We found that primary explants from R26AID+/+p48CRE+/KI pancreas contained cells undergoing apoptotic cell death, detected by caspase-3 immunohistochemistry (Fig 5E), indicating that NKG2D ligand expression in AID-expressing pancreas is functional.

We next asked whether the expression of RAE ligands promoted the recruitment of immune cells to R26AID+/+p48CRE+/KI pancreas in vivo. Hematoxylin–eosin staining of pancreas sections from aged mice clearly revealed the presence of immune infiltrates in AID-expressing pancreas of R26AID+/+p48CRE+/KI mice (Fig 5A). The composition of these immune infiltrates was analyzed by antibody staining to detect macrophages (F4/80), B cells (Pax5) and T cells (CD3). The vast majority of cells in the immune infiltrates of R26AID+/+p48CRE+/KI mice were CD3+ T cells (Fig 5B), with only a negligible contribution from B cells and macrophages (not shown). To discount age-related effects, we analyzed 20-week-old mice, finding that the accumulation of T cell infiltrates is detectable in these young animals (Fig 5C). The main NKG2D-expressing T cell subset is the CD8+ population (Raulet et al., 2013), and immunofluorescence analysis of immune infiltrates revealed that a high proportion of the CD8+ infiltrate is composed of CD8+ T cells (Fig 5C), a finding consistent with the reported recruitment of CTL cells to pancreatic islets transgenically expressing Rae1 (Markiewicz et al., 2012). Finally, we found that aged R26AID+/+p48CRE+/KI mice had significantly higher levels of pancreatic TNF-α mRNA than control littermates (Fig 5D), indicating that AID promotes the expression of effector cytotoxicity. Consistently, R26AID+/+p48CRE+/KI pancreas contained cells undergoing apoptotic cell death, detected by caspase-3 immunohistochemistry (Fig 5E, P = 0.054).

Together, these results indicate that heterologous AID expression in pancreas promotes a cytotoxic response, most likely arising from the generation of genotoxic activity and NKG2D ligand expression and the recruitment of NKG2D-expressing CTL cells.

Discussion

In recent years, the finding that inflammatory cues induce AID expression in epithelial cells has boosted interest in the notion that AID might promote carcinogenesis and even be the causative link between inflammation and neoplastic transformation (Marusawa et al., 2011; Takai et al., 2012). In this regard, our analyses of AID expression in response to TNF-α have confirmed previous data in colonic cell lines and have expanded these observations to pancreatic cell lines. In addition, we found that primary pancreatic epithelium is also sensitive to TNF-α, suggesting that AID
expression can indeed take place in vivo in pro-inflammatory contexts.

We wanted to further explore the physiological relevance of AID expression in promoting epithelial malignant transformation. We found that AID deficiency does not reduce the incidence of oncogenic lesions in an inflammation-induced carcinoma model. Our results contrast with the finding that AID deficiency reduces colon neoplasia in IL10−/− mice (Takai et al., 2012). It may be that the effect reported by Takai et al is not specifically driven by epithelial cells, but rather by B cells in IL10−/− mice, a possibility that could be tested using conditionally rather than constitutively AID-deficient animals.

Previous reports have claimed that AID heterologous expression leads to epithelial cell neoplasia in various tissues (Endo et al., 2008; Takai et al., 2009, 2012; Marusawa et al., 2011). In those studies, overexpression was always achieved with transgenes, none of which was epithelial specific, and the incidence of neoplasias was extremely low, and varied with the insertion sites and with ongoing mouse generations (Okazaki et al., 2003). In contrast, here we have developed epithelium-specific conditional knock-in models, thus avoiding both widespread expression and transgene-derived artifacts. Remarkably, our models allowed B-cell-like AID expression levels, a basic prerequisite for AID to be competent, given that its levels are rate limiting in its native context (Sernandez et al., 2008; Takizawa et al., 2008). We found that AID expressed ectopically in pancreatic cells is able to mutate non-immunoglobulin genes and to generate genotoxic DSBs, suggesting that in this context, there are no obvious mechanisms for negative regulation of AID activity, as they were previously proposed for transgenic AID expression in B cells (Muto et al., 2006). AID mutagenic activity was detected in two highly expressed pancreatic genes (Ela1 and Ela2), which is expected from the well-established link between AID activity and transcription of its target genes (Chaudhuri et al., 2003; Ramiro et al., 2003; MacDonald et al., 2010; Pavri & Nussenzweig, 2011).

Our data indicate that AID activity in pancreas does not promote pancreatic carcinogenesis; instead, it triggers an NKG2D-mediated cytotoxic response that would eliminate preterminal cells and prevent carcinoma development, in line with the published finding

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**Figure S5. AID expression in pancreas promotes immune infiltration and cell death.**

A Hematoxylin–eosin (HE) staining of pancreas from aged (75-week-old) R26AID+/-p48CRE+/- and R26AID+/-p48CRE+/- mice. Left: Representative HE staining showing an immune infiltrate in a R26AID+/-p48CRE+/- mouse. Scale bar: 200 μm. Right: Quantification of number of foci per mm² of tissue. n = 13 (R26AID+/-p48CRE+/-); 21 (R26AID+/-p48CRE+/-). *P = 0.0098. B CD3 immunohistochemistry of pancreas from 75-week-old R26AID+/-p48CRE+/- and R26AID+/-p48CRE+/- mice. Left: Representative image of a CD3 infiltrate in a R26AID+/-p48CRE+/- mouse. Scale bar: 100 μm. Right: Quantification of the number of CD3-positive cells per mm² of tissue. n = 12 (R26AID+/-p48CRE+/-); 13 (R26AID+/-p48CRE+/-). *P = 0.0149. C Representative immunofluorescence staining of CD3 and CD8 in pancreas from 20-week-old R26AID+/-p48CRE+/- and R26AID+/-p48CRE+/- mice. Scale bar: 20 μm. D TNF-α expression. Total RNA was isolated from pancreas of aged (75-week-old) R26AID+/-p48CRE+/- and control mice, and TNF-α expression was quantified by qRT-PCR. Each dot represents an individual mouse. n = 8 (R26AID+/-p48CRE+/-); 15 (R26AID+/-p48CRE+/-). *P = 0.0992. E Cell death detection. Pancreas from 75-week-old R26AID+/-p48CRE+/- and R26AID+/-p48CRE+/- mice were stained with anti-caspase-3. Left: Representative staining from an R26AID+/-p48CRE+/- mouse (top) and an R26AID+/-p48CRE+/- mouse (bottom). Scale bar: 50 μm. Right: Quantification of number of cells per mm² of tissue. Each dot represents an individual mouse. n = 9 (R26AID+/-p48CRE+/-); 11 (R26AID+/-p48CRE+/-). P = 0.0545.

Data information: Statistical differences were analyzed by two-tailed unpaired Student’s t-test.
that AID promotes an NKG2D immune response in B cells infected with the Abelson murine leukemia virus (Gourzi et al., 2006). The lymphomagenic potential of AID was previously shown to be dampened in B cells (Muto et al., 2006; Robbiani et al., 2009), where p53 exerts a cell-intrinsic tumor suppressor function (Robbiani et al., 2009). Here, we provide evidence of a further protective mechanism triggered by AID activity and carried out through an extrinsic immunosurveillance pathway in epithelial tissues. These data highlight the diversity of safeguarding events in AID-expressing cells and encourage a refined view of the previously acknowledged contribution of endogenous AID to epithelial-derived tumors.

Materials and Methods

Mice

R26AID mice were generated by insertion of a construct encoding mouse AID cDNA into the Rosa26 IRES-GFP targeting vector (Nyabi et al., 2009). AID cDNA was PCR-amplified from C57BL/6 mice (primers: forward 5'-TTCTGGAGACGCAAGCT-3' ; reverse 5'-CCCTTCCAGGCTTTGAAA-3'), cloned into the pENTR/D-TOPO Gateway vector (Invitrogen), and subsequently recombined into the Rosa 26 targeting vector, in which the cloned construct is preceded by a loxP-flanked transcriptional stop cassette and followed by an internal ribosomal entry site and GFP. The construct was linearized with PvuI before electroporation into hybrid 129/C57BL/6 ES cells. Clones positive for homologous recombination in the Rosa26 locus were identified by Southern blot of EcoRV-digested genomic DNA hybridized with a 5’-arm Rosa26 probe. R26AID mice were backcrossed to C57BL/6 background for 5 generations. R26AID mice were crossed with Villin-CRE+/− mice (el Marjou et al., 2004) and p48-CRE+/− mice (Kawaguchi et al., 2002) to promote expression of AID in colonic and pancreatic epithelial cells, respectively. Both Villin-CRE and p48-CRE mice were backcrossed to C57BL/6 for 5 generations. Balb/c AID+/− mice were generated by backcrossing AID−/− mice (Muramatsu et al., 2000) for 6 generations by speed congenics (Ramiro et al., 2004). Mice of both genders were used unless specified otherwise. All animals were housed in the Centro Nacional de Investigaciones Cardiovasculares animal facility under a 12-h light/dark cycle with food ad libitum.

Number of animals per group to detect biologically significant effect sizes was calculated using appropriate statistical sample size formula and indicated in the biometrical planning section of the experimental and other scientific purposes, enforced in Spanish law under RD 53/2013.

Cell lines and primary acinar cell culture

Primary pancreatic acinar cells were isolated and cultured as described in Gout et al. (2013). Briefly, complete pancreas from 8-week-old mice was mechanically and enzymatically digested with collagenase to obtain isolated acinar structures. Acini were grown in Waymouth’s medium supplemented with 2.5% FBS, 10 mM HEPES, 0.25 mg/ml trypsin inhibitor (Sigma) and 25 ng/ml of recombinant human epidermal growth factor (Sigma). PaTu-8988S, AsPC-1, LoVo and SW480 cells were grown in DMEM supplemented with 10% FCS and 10 mM HEPES. PaTu-8988S cell line was kindly provided by Dr Thomas Gress (University of Marburg). AsPC-1, LoVo and SW480 cell lines were obtained from the ATCC. All of them were mycoplasma negative. TNF-α (50 ng/ml) was added when indicated.

DSS-induced colitis-associated cancer (CAC) experiments

8–10-week-old Balb/c AID+/− and AID−/− (Ramiro et al., 2004) mice were given 3% dextran sulfate sodium salt (DSS, Sigma) in their drinking water for 5 days followed by regular drinking water for 10 days. Colon samples were obtained from these mice after 10 cycles of DSS treatment, and Swiss roll preparations were fixed, embedded in paraffin for section, and stained with hematoxylin/eosin (H/E).

Immunofluorescence

Pancreas and colon specimens were fixed with 4% paraformaldehyde, incubated with 30% sucrose, embedded in OCT compound (Olympus), and frozen in dry ice. 10-μM sections were permeabilized and blocked with Image-IT FX signal enhancer (Invitrogen, Molecular Probes). The following antibodies were used: rabbit anti-AID (Abcam, 1/100), rat anti-mouse CD8α (BD Pharmingen, 1/100), mouse anti-Cytokeratin 8 (TROMA I, 1/50), goat anti-rabbit Alexa Fluor 488 (Molecular Probes, 1/500), goat anti-mouse Alexa Fluor 488 (Molecular Probes, 1/500), and goat anti-rat Cy3 (Jackson ImmunoResearch, 1/500). Slides were mounted with Vectashield mount medium containing DAPI (Vector Laboratories).

Immunohistochemistry

Pancreases were fixed in neutral-buffered 10% formalin solution (Sigma), embedded in paraffin blocks, and cut in 5-μM sections. H/E staining was performed using standard protocols. For immunohistochemistry, sodium citrate buffer was used for antigen retrieval. The following antibodies were used: polyclonal rabbit anti-human CD3 (Dako, 1/200), rabbit anti-Ki67 (Abcam, 1/100), mouse anti-Cytokeratin 8 (TROMA I, 1/50), goat anti-rabbit Alexa Fluor 488 (Molecular Probes, 1/500), goat anti-mouse Alexa Fluor 488 (Molecular Probes, 1/500), and goat anti-rat Cy3 (Jackson ImmunoResearch, 1/500). Slides were mounted with Vectashield mount medium containing DAPI (Vector Laboratories). Results are shown as the number of reactive cells per mm² of tissue.

qRT–PCR

RNA was extracted from colon samples with TRizol (Sigma). RNA from pancreas samples was extracted with GTC solution, following a standard phenol–chloroform protocol (reagents from Sigma), followed by DNase treatment (Qiagen). CDNA was synthesized using Random Hexamers (Roche) and SuperScript II reverse transcriptase. mRNA was quantified by SYBR green assay (Applied Biosystems), with normalization to GAPDH. The following primers were used: human-AID (forward) 5'-AAAGTCTTCCTCAGGCTTTC-3', (reverse) 5'-GGGAGGATGAGAAGATGCAGCCT-3';
human-GAPDH (forward) 5'-GAA GGT GAA GGT CGG AGT C-3', (reverse) 5'-GAA GAT GAT CAT GGG ATT TC-3'; mouse AID (forward) 5'-ACC TTC GCA ACA AGT CTG GCT-3', (reverse) 5'-AGC TTG GTC TCC ACA GAA-3'; mouse-GAPDH (forward) 5'-TGA AGC AGG CAT CTG AGG G-3', (reverse) 5'-CGA AGG TGG AAG AGT GGC AG-3'; mouse-TNF-α (forward) 5'-AGC CCA CTG GTG AGC AAA CCA-3', (reverse) 5'-ACA ACC CA CGG CTG GCA CC-3'.

**Next-generation sequencing for detection of mutations**

DNA from three R26AID+/p48-CRE+/KII and three R26AID+/p48-CRE+/KII p48-CRE+/KII mice was extracted and amplified using the following oligonucleotides: Elastase1 (forward) 5'-GCA CAG CAT CTG TTG TTT GGC TAA-3', (reverse) 5'-GGG GAC AGT GGT CTA CTC TCT-3'; Elastase2a (forward) 5'-AGC AGT CCA GGA CAA TCA GAG A-3', (reverse) 5'-TGA TAA GGC CAC TCA TAA AAA GGA-3'. Amplification reactions were carried out with 2.5 U of Pfu Ultra (Stratagene) in a 50 μl reaction with the following profile: 94°C for 5 min followed by 25 cycles at 94°C for 10 s, 60°C for 30 s, and 72°C for 1 min. For NGS sequencing, the PCR product from two reactions per mouse were pooled, and an equimolar amount of DNA from each of the two mouse genotypes was pooled and mixed.

**γ-H2AX staining and Opera acquisition**

Primary pancreatic acinar cells were grown on 96-well plates (Perkin Elmer), and γ-H2AX (Millipore, 1/500) immunofluorescence was performed using standard procedures. Images were automatically acquired from each well with the Opera High-Content Screening System (Perkin Elmer). A 40× magnification lens was used, and pictures were taken at nonsaturating conditions. Images were segmented using DAPI staining to generate masks matching cell nuclei, and the mean per-cell γ-H2AX signal was calculated.

**Droplet digital PCR**

Each 20 μl ddPCR contained 10 μl of 2x ddPCR Supermix (Bio-Rad), 1 μl of cDNA generated from 1.4 μg of RNA, and 500 nM of each primer: pan-RAE (forward) 5'-TGG ACA CTC ACA AGACCA ATG-3', (reverse) 5'-CCC AGG TGG CAC TAG GAG T-3'; and 250 nM Taqman probe (panRAE-FAM 5'-CCA TGA TTT ATC GGC CAA AAG GGC AGG GCC-3'). After droplet generation, a mean of 12,000 droplets were obtained per sample. Samples were transferred into a 96-well plate (Eppendorf) and cycled in a thermal cycler (Bio-Rad) under the following conditions: 95°C for 10 min followed by 38 cycles of 94°C for 30 s, 58°C for 1 min, and a final step at 98°C for 10 min. After amplification, samples were transferred to a droplet reader (QX100 Droplet Digital PCR, Bio-Rad) from which positive-drop data were extracted with QuantaSoft software. Results are represented as the proportion of positive drops in duplicates of each sample.

**NK killing assay in vitro**

Primary pancreatic acinar cells from R26AID+/p48-CRE+/KII and R26AID+/p48-CRE+/KII mice were isolated as described above. After 6 days of culture, acinar cells were trypsinized and stained with CFSE. NK cells were isolated by cell sorting from wild-type C57BL/6 mice. Both male and female aged 6–8 weeks were used as NK cells donors. NK cells and acinar pancreatic cells were co-cultured for 4 h in the presence of IL2 (2,000 U/ml, Peprotech) at a 1:10 (target cell:NK effector cell) ratio. Killing was analyzed by staining with DAPI by flow cytometry. Data are presented as the proportion of CFSE+DAPI+ cells normalized to the same population in cultures lacking NK cells.

**Statistics**

Statistical analyses were performed with GraphPad Prism (version 6.01 for Windows, GraphPad Software, San Diego, CA, USA) using two-tailed Student’s t-test. P ≤ 0.05 was considered statistically significant. Error bars in figures represent standard error of the mean (SEM). Normal distribution of data was assessed by applying a D’Agostino & Pearson omnibus normality test. F-test was used to compare variances between groups. For the survival analyses, GraphPad Prism was used and the Mantel-Cox test was applied. Differences were considered statistically significant at P ≤ 0.05.

**Expanded View**

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**Author contributions**

AP-G and ARR designed the research, analyzed data, and wrote the manuscript. AP-G, PP-D, TW, IVS, and SMM performed experiments. MC performed histopathological analyses. FXR designed the research and provided expertise and reagents on pancreatic adenocarcinoma.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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