RAE1 Ligands for the NKG2D Receptor Are Regulated by STING-Dependent DNA Sensor Pathways in Lymphoma

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Abstract

The immunoreceptor NKG2D originally identified in natural killer (NK) cells recognizes ligands that are upregulated on tumor cells. Expression of NKG2D ligands (NKG2DL) is induced by the DNA damage response (DDR), which is often activated constitutively in cancer cells, revealing them to NK cells as a mechanism of immunosurveillance. Here, we report that the induction of retinoic acid early transient 1 (RAE1) ligands for NKG2D by the DDR relies on a STING-dependent DNA sensor pathway involving the effector molecules TBK1 and IRF3. Cytosolic DNA was detected in lymphoma cell lines that express RAE1 and its occurrence required activation of the DDR. Transfection of DNA into ligand-negative cells was sufficient to induce RAE1 expression. If3–/–Eμ-Myc mice expressed lower levels of RAЕ1 on tumor cells and showed a reduced survival rate compared with If3+/–Eμ-Myc mice. Taken together, our results suggest that genomic damage in tumor cells leads to activation of STING-dependent DNA sensor pathways, thereby activating RAE1 and enabling tumor immunosurveillance. Cancer Res; 74(8); 2193–203. ©2014 AACR.

Introduction

The NKG2D system is an arm of innate immune recognition, which is important in the context of both cancer and infection (1–3). Transformed and infected cells increase their expression of NKG2D ligands (NKG2DL). Engagement of the NKG2D receptor on natural killer (NK) cells and certain T cells stimulates their effector functions, which aid in tumor control (4, 5). Recently, we elucidated a principle mechanism that induces NKG2DLs in cancer cells: the DNA damage response (DDR) (ref. 6). DNA damage upregulates the expression of numerous NKG2DLs, including different retinoic acid early transcript (RAE1) isoforms and mouse UL16-binding protein-like transcript 1 (MULT1) in mouse cells. The DDR molecules ataxia telangiectasia and Rad3 related (ATR) ataxia telangiectasia mutated homolog (ATM), and checkpoint kinase 1 homolog (CHK1) are required for expression of NKG2DLs in response to DNA damage and the constitutive expression of NKG2DLs in some tumor cell lines (6). Additional effector molecules of the DDR required for mouse NKG2DL expression have not been identified.

Optimal immune responses to autologous cells often require the presence of pathogen- and damage-associated molecular patterns. Pattern recognition receptors (PRR) that recognize self-molecules, such as DNA, have been suggested to play a role in cancer (7). Recently identified candidate cytosolic DNA sensors include Z-DNA binding protein 1 (ZBP1/DAI) and retinoic acid inducible gene I (Rig-I; ref. 8). Upon recognizing DNA, these sensors activate stimulator of interferon (IFN) genes (STING), TANK-binding kinase 1 (TBK1), and/or the related IKK-related kinase epsilon (IKKε; ref. 9). Activated TBK1 and IKKε directly phosphorylate IFN regulatory factor-3 (IRF3), which subsequently undergoes dimerization and translocation into the nucleus (10). Nuclear IRF3 induces the expression of target genes, including Ifnb and chemokine C-C motif ligand-5 (Ccl5; ref. 11).

Expression of the proto-oncogene c-MYC is deregulated in 70% of human cancers (12, 13). Overexpression of c-MYC induces DNA damage and the DDR, which was suggested to act as a barrier against tumor development in premalignant cells. In Eμ-Myc transgenic mice, c-Myc expression is driven by the immunoglobulin heavy chain Eμ enhancer, leading to precursor B-cell malignancies similar to human Burkitt’s lymphoma (14, 15). The tumor suppressors that prevent the development of c-Myc–expressing premalignant cells have not been well characterized.

In the present study, we show that the DDR leads to the presence of cytosolic DNA and activation of IRF3 in lymphoma cell lines. The induction of RAЕ1 ligands by the DDR depended on IRF3. Transfection of cells with cytosolic DNA induced the...
expression of RAE1 molecules. Tumors in If3β+/−;Eμ-Myc mice expressed lower levels of RAE1 and developed lymphoma earlier, resulting in a shortened life span when compared with If3β+/−;Eμ-Myc mice. Taken together, these findings link genotoxic stress to cytosolic DNA sensor signaling pathways and the induction of RAE1 in lymphoma cell lines.

Materials and Methods

Cells

BC2 (a kind gift by Dr. L. Corcoran, Walter + Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia) and EμM1 cells were derived from Eμ-Myc mice (16). Yac-1 cells were purchased from American Type Culture Collection. Cells were cultured in RPMI-1640 medium (Invitrogen) with 10% FCS (Hyclone), 50 μmol/L 2-mercaptoethanol, 100 μmol/L asparagine, 2 mmol/L glutamine (Sigma), 1% penicillin-streptomycin (Invitrogen), and 1/1,000 plasmocin (Invivogen).

EμM1, mouse embryonic fibroblasts (MEF), and tumor cells in Eμ-Myc mice (C57BL/6) express RAE1β and/or RAE1ε. BC2 (C57BL/6;129) and Yac-1 (A/Sn) cells express RAE1α, RAE1β, RAE1γ, and RAE1ε.

Reagents

Aphidicolin, caffeine, CGK733, cytosine β-D-arabinofuranoside hydrochloride (Ara-C), Dimethyl sulfoxide (DMSO), Poly G; C, Poly A;U, and Poly I:C were purchased from Sigma. TransFectin was purchased from BioRad. KU55933 and VE-821 were obtained from Tocris Bioscience or Axon Medchem. ODN1585, OND1G55, and lipopolysaccharides (LPS) were purchased from Invivogen. DNA was conjugated to Alexa-488 using the Ulysis labeling kit according to the manufacturer’s instructions (Invitrogen).

Constructs and transduction

If3β, Tbk1, Ikke, Sting-HA, IRF3-Egfp, and IRF3Δ7-Egfp were subcloned into the pMCSV2.2-RES-Gfp vector (gift of Dr. Sha, University of California, Berkeley, CA). Wild-type (WT) and mutant Sting fibroblasts were kindly provided by Dr. Vance (University of California, Berkeley, CA). Retroviral supernatants were generated as described in (17). Short hairpin RNA (shRNA) constructs were cloned into the MSCV/LTRmiR30-PIG vector (Open Biosystems; See Supplementary data).

Quantitative real-time reverse transcription PCR

Quantitative real-time reverse transcription (RT)-PCR was performed as described previously (6).

Western blotting

Whole cell extracts were electrophoresed in 10% or 4% to 20% SDS-PAGE gels and blotted onto nitrocellulose membranes (BioRad). Antibodies against IRF3, IRF3pS388, TBK1, Tbk1pS172, ATM, ATMpS1981 (Cell Signaling Technology), BCL2L12 (clone E-13; Santa Cruz), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma) and horseradish peroxidase–coupled second stage reagents were used (Thermo). Blots were exposed on X-ray film (Fuji); densitometry analysis was performed using ImageJ-1.46r.

Flow cytometry

The following antibodies were used: pan-RAE1, RAE1βγ, RAE1βε, RAE1ε (R&D Systems), B220-PerCP, IgM-APC, CD16/CD32, MHC class II (eBioscience), rabbit-anti-phospho-IRF3-Ser396 or rabbit-anti-phospho-TRK1-Ser172 (Cell Signaling Technology), and rat IgG-APC (eBioscience) or rabbit IgG-Alexa-488 (Invitrogen). Propidium iodide (PI, 1 μg/mL) was added to all stainings, and PI-negative cells are shown. For intracellular staining, cells were fixed according to the manufacturer’s protocol. Some cells were treated with 2 U/μL λ-phosphatase (New England Biolabs) at 37°C for 90 minutes before staining. Stained cells were analyzed using FACSCalibur and FlowJo 8.8.7 (Treestar). Bromodeoxyuridine incorporation analysis was performed as described (18).

Microscopy

Cells were fixed and stained for DNA according to the manufacturer’s instructions (Millipore). A detailed protocol is provided in the Supplementary data.

CD107a degragation assay and in vitro NK cell stimulation

Performed as described in refs. 4 and 19.

Statistical analyses

Groups were compared using two-tailed unpaired t tests (Prism; 5.0c; GraphPad). Survival was represented by Kaplan–Meier curves, and statistical analysis was performed with the log-rank Mantel–Cox test.

Results

IRF3 is necessary for RAE1 expression in response to DNA damage

IRF3 has previously been shown to be activated in response to DNA damage (20, 21). We therefore investigated the role of IRF3 in the expression of NKG2DLs in cells exposed to DNA damaging agents. Phosphorylation of serine 396 (S388 of mouse IRF3) has been shown to be critical for the activation of IRF3 (22). Phosphorylation of IRF3S388 increased after treatment with DDR-inducing agents Ara-C or aphidicolin, although not to the same degree as LPS, a known inducer of IRF3 (Fig. 1A and B; Supplementary Fig. S1A; ref. 23). The late kinetics of IRF3S388 phosphorylation were similar to kinetics previously observed for DNA damage–induced upregulation of NKG2DLs (6). Notably, treatment of BC2 cells with Ara-C also induced activated IRF3 characterized by nuclear translocation of IRF3 (22). Phosphorylation of IRF3S388 increased after treatment with DDR-inducing agents Ara-C or aphidicolin, although not to the same degree as LPS, a known inducer of IRF3 (Fig. 1A and B; Supplementary Fig. S1A; ref. 23). The late kinetics of IRF3S388 phosphorylation were similar to kinetics previously observed for DNA damage–induced upregulation of NKG2DLs (6). Notably, treatment of BC2 cells with Ara-C also induced activated IRF3 characterized by nuclear translocation of endogenous IRF3 (Fig. 1C) and overexpressed chimeric IRF3-GFP (Fig. 1D and E), consistent with previous reports (20, 21). No nuclear translocation was observed with a mutant form of IRF3 (IRF3Δ7-GFP) that is unable to be activated (Fig. 1D and E). Treatment of BC2 cells with Ara-C or aphidicolin induced expression of several IRF3 target genes to a similar or greater extent than Poly I.C, an established IRF3 activator, suggesting that IRF3 is transcriptionally active in response to DNA damage (Fig. 1F and G).

To test whether IRF3 is required for DDR-mediated upregulation of NKG2DLs, we transduced BC2 cells with an If3β-specific shRNA (Supplementary Fig. S1B). Compared with
Figure 1. IRF3 is activated and necessary for optimal induction of RAE1 ligands for NKG2D in response to DNA damage. A, phosphorylation of IRF3S388 in BC2 cells treated with 10 μmol/L Ara-C, 4 μmol/L aphidicolin, 1 μg/mL ODN1585 (red line), or DMSO (blue line) was analyzed by intracellular flow cytometry at indicated time points. Filled histograms, isotype staining of Ara-C–treated cells. Mean fluorescence intensity (MFI) ± SD are shown. B, phosphorylation of IRF3S388 after 16 hours of 10 μmol/L Ara-C or DMSO treatment was analyzed by Western blotting. RAW 264.7 cells were treated with LPS for 4 hours. C, nuclear translocalization of endogenous IRF3 (red) in BC2 cells treated with 10 μmol/L Ara-C for 16 hours and stained for IRF3 in the presence of DAPI (blue). D and E, BC2 cells expressing IRF3-Gfp or IRF3A7-Gfp were treated with 10 μmol/L Ara-C, 10 μg/mL Poly I:C, or DMSO for 16 hours. Some cells were pretreated with 10 μmol/L of the ATM/ATR-specific inhibitor CGK733. Localization of IRF3 in DAPI-stained cells was analyzed by fluorescent microscopy (D). Quantification of BC2 cells with nuclear (white bar; >90% nuclear), partial nuclear (gray bar; 10%–90% nuclear) and cytosolic (black bar; <10% nuclear) localization of IRF3 (E). F, BC2 cells were treated with DMSO (white bar), 10 μmol/L Ara-C (light-gray bar), 4 μmol/L aphidicolin (gray bar), 1 μg/mL Poly I:C for 20 hours (black bar), or 1 μg/mL Poly I:C for 4 hours (dark-gray bar). Relative mRNA levels of IRF3 target genes were measured by qRT-PCR. Means ± SD of three independent experiments normalized to DMSO-treated cells are shown. G, levels of IFN-β in supernatants of 0.75 × 10^6 BC2 cells/mL treated with DMSO, 10 μmol/L Ara-C, or 1 μg/mL Poly I:C for 24 hours were determined by ELISA. *P < 0.05. H, RAE1 expression in Irf3–specific (red line) or control (blue line) shRNA-transduced BC2 cells, which were cultured for 5 days in puromycin before treatment with 10 μmol/L Ara-C for 16 hours. DMSO-treated Irf3 (dashed line) or control (dotted line) shRNA-transduced cells are also shown. Filled histograms, isotype staining of Ara-C–treated cells. MFI ± SD are indicated. I, IL-2–activated NK cells were cocultured with Irf3–specific (circles) or control shRNA-transduced (squares) BC2 cells in the presence of NKG2D-blocking antibodies (open symbols) or IgG2a isotype control antibodies (filled symbols) and treated with 10 μmol/L Ara-C (red) or DMSO (black) for 16 hours. The effector (E) to target (T) ratio is indicated. Results ± SD of three independent experiments are shown.
control shRNA, \textit{If}β3-specific shRNA significantly inhibited the upregulation of RAЕ1 ligands of NKG2D, but not MHC class II, in response to Ara-C (Fig. 1H). Bimodal expression of RAЕ1 is likely to reflect specific activation of the DDR in S-phase of the cell cycle. No consistent effects were observed for the NKG2D ligands on untreated cell lines (24). In agreement, phosphorylation of IRF3S388 was detected in EμM1 cells and Yac-1 lymphoma cells (Fig. 2A). Endogenous IRF3 (Fig. 2B) and exogenous chimeric IRF3-GFP (Fig. 2C) were partially localized to the nucleus, indicating that a subset of IRF3 is activated in EμM1 and Yac-1 cells. In contrast, IRF3A7 exhibited exclusively cytosolic localization in Yac-1 cells. Inhibition of IRF3 expression in EμM1 and Yac-1 cells by shRNA decreased the expression of IRF3 target genes (Fig. 2D and E), RAE1 transcription (Supplementary Fig. S1C), and RAЕ1 cell surface levels (Fig. 2F). Mouse strains were found to express different RAЕ1 isoforms and the cell lines used in this study vary in their genetic background (see Supplementary Materials and Methods; ref. 25). The incomplete reduction of cell surface RAЕ1 expression by \textit{If}β3-specific shRNAs may reflect incomplete turnover of preformed RAЕ1 or incomplete knockdown of IRF3 (Supplementary Fig. S1B).

TBK1 is necessary for RAЕ1 expression in response to DNA damage

IRF3 is activated by the IKK-related serine/threonine kinases TBK1 and IKKɛ (10). We therefore tested whether TBK1 was phosphorylated in response to DNA damage. Similar to results with IRF3, substantial phosphorylation of TBK1 on serine 172 was detected after 15 hours of treatment with Ara-C or aphidicolin, although the activation was weaker than TBK1 phosphorylation in response to LPS (Fig. 3A and B; Supplementary Fig. S2A). No Ikke expression was detected in BC2 cells (data not shown).

Transduction of BC2 cells with the TBK1 inhibitor Sike caused substantial reduction in RAЕ1 expression in response to DNA damage (Fig. 3C). Similarly, BC2 cells transduced with \textit{Tk}bk1-specific shRNA expressed less transcripts and RAЕ1 at the cell surface in response to Ara-C (Fig. 3D; Supplementary Fig. S2B). \textit{Tbk}1- and \textit{Ikke}-deficient MEFs failed to upregulate RAЕ1 in response to Ara-C (Fig. 3E). Genetic reconstitution of \textit{Tbk}1+/−;\textit{Ikke}−/− MEFs with \textit{Tbk}1 or \textit{Ikke} was sufficient to induce RAЕ1 expression on a fraction of cells and to restore the capacity of cells to upregulate RAЕ1 in response to Ara-C (Fig. 3E). The induction of RAЕ1 in untreated cells is likely a consequence of overexpression of \textit{Tbk}1 or \textit{Ikke}, which has been shown to result in unregulated activation of the pathway (26). In summary, the data suggest that TBK1 is required for induction of RAЕ1 in response to DNA damage in BC2 cells.

TBK1 is required for constitutive RAЕ1 expression

Similarly to IRF3, constitutive phosphorylation of \textit{Tbk}1S172 was detected in Yac-1 and EμM1 cells (Fig. 3F). Transduction of Sike into Yac-1 cells caused reductions in RAЕ1 levels (Fig. 3G). Expression of a \textit{Tbk}1-specific shRNA in Yac-1 cells decreased the amount of RAЕ1 transcripts, and a significant but less complete reduction in RAЕ1 at the cell

![Figure 2. IRF3 is necessary for constitutive RAЕ1 expression in Yac-1 and EμM1 cells. A, expression of IRF3S388 in Yac-1 and EμM1 cells was detected by intracellular flow cytometry (blue line). Some cells were pretreated with λ-phosphatase before staining (red line). Filled histogram, isotype staining of Yac-1 cells. MFI ±SD are indicated. B, Yac-1 and EμM1 cells were stained by intracellular flow cytometry (blue line). Some cells were pretreated with λ-phosphatase before staining (red line). Filled histogram, isotype staining of Yac-1 cells. MFI ±SD are indicated. C, IRF3S388 expression was detected in Yac-1 and EμM1 cells treated with λ-phosphatase and control shRNA (white bar)-transduced Yac-1 and EμM1 cells were cultured with interleukin 10 µm and the cell lines used in this study vary in their genetic background (see Supplementary Materials and Methods; ref. 25). The incomplete reduction of cell surface RAЕ1 expression by \textit{If}β3-specific shRNAs may reflect incomplete turnover of preformed RAЕ1 or incomplete knockdown of IRF3 (Supplementary Fig. S1B).](image-url)
Transduction with retroviral vectors encoding activation of ATR and ATM (27). To address the role of ATM, phosphorylation of IRF3 and TBK1 depends on the DDR, surface (Fig. 3H and I). No Ikke transcripts were detected in Yac-1 cells (data not shown).

**Phosphorylation of IRF3 and TBK1 depends on the DDR**

Treatment of cells with Ara-C results in DNA breaks and the activation of ATR and ATM (27). To address the role of ATM and ATR in the activation of TBK1 and IRF3, we blocked ATM and ATR function with different chemical inhibitors. Attempts to efficiently block both ATM and ATR by shRNA were not successful. Induction of RAE1 expression in BC2 cells by Ara-C was inhibited by the combined treatment of cells with ATM and ATR inhibitors (Fig. 4A; Supplementary Fig. S3A).
Inhibition of ATM and ATR also impaired TBK1 and IRF3 phosphorylation (Fig. 4B and C; Supplementary Fig. S3B) and nuclear localization of chimeric IRF3-GFP (Fig. 1D and E) in Ara-C–treated BC2 cells. The effects of ATM or ATR inhibition on RAEl expression and phosphorylation of TBK1 and IRF3 were less pronounced, suggesting that ATM and ATR act redundantly (Fig. 4A–C).

Inhibition of ATR impaired constitutive RAEl expression and phosphorylation of TBK1 and IRF3 in Yac-1 cells (Fig. 4D and E). Nuclear localization of IRF3-GFP in Yac-1 cells was also suppressed by inhibition of ATR and ATM (Fig. 4F). Thus, activation of TBK1 and IRF3 in Yac-1 cells depends on ATR, suggesting that ATR activates DNA damage, which preferentially activates ATR, is present in Yac-1 cells (28).

**Accumulation of cytosolic DNA depends on the DDR**

Recognition of cytosolic DNA by DNA sensors activates TBK1 and IRF3 (8). To test whether DNA damage leads to appearance of cytosolic DNA, we stained cells with antibodies specific for single-stranded (ss) DNA or double-stranded (ds) DNA (Supplementary Fig. S4A). The specificity of the DNA staining was verified by pretreating cells with 1 mM nuclease to degrade ssDNA or DNase I to digest dsDNA before staining (Supplementary Figs. S4B and S4C). All cells were treated with RNase before staining. Strikingly, we found that ssDNA and dsDNA was present in the cytosol of BC2 cells in response to Ara-C treatment and cytosolic DNA was constitutively present in Yac-1 cells (Fig. 5A and B). To substantiate the presence of DNA in the cytosol of cells, we stained BC2 cells with Picogreen, a vital dsDNA-specific dye (Supplementary Fig. S4D). In agreement with the dsDNA-specific antibody stainings, Picogreen staining showed the presence of cytosolic dsDNA in Yac-1 and Ara-C–treated BC2 cells (Fig. 5C; Supplementary Figs. S4E–S6). As Ara-C disrupted Mitotracker staining, we treated BC2 cells with aphidicolin, an inhibitor of DNA synthesis, and found that Picogreen staining was abolished in the presence of aphidicolin.
inhibitor of nuclear DNA synthesis that activates the DDR but does not affect replication of mitochondrial DNA (Fig. 5C; Supplementary Fig. S4D; ref. 29). Three-dimensional rendering of confocal microscopy data showed that most cytosolic DNA is present outside of mitochondria in Yac-1 and Ara-C–treated BC2 cells (Fig. 5C; Supplementary Figs. S4D and S5).

To test whether the DDR influences the occurrence of cytosolic DNA, we pretreated BC2 cells with ATM and/or ATR inhibitors before treatment with Ara-C. Blocking of ATM and ATR prevented appearance of cytosolic DNA in response to Ara-C (Fig. 5A; Supplementary Fig. S4D and S5). The presence of DNA in the cytosol activates STING-dependent DNA sensors, leading to the activation of TBK1 and IRF3.

**Cytosolic DNA induces RAE1 expression**

To test whether cytosolic DNA induces RAE1 expression in BC2 cells, we transfected cells with Alexa-488–labeled plasmid DNA, genomic DNA, or ssDNA. We were unable to purify sufficient quantities of cytosolic DNA to determine whether cytosolic DNA present in Ara-C–treated BC2 cells directly induces RAE1 expression. Alexa-488–positive BC2 cells upregulated expression of RAE1, although to a lesser degree than Ara-C–treated cells (Fig. 6A). The presence of DNA in the cytosol activates STING-dependent DNA sensors, leading to the activation of TBK1 and IRF3.

Figure 5. DNA damage leads to the appearance of cytosolic DNA. A, staining of BC2 cells for the presence of ssDNA (left columns) or dsDNA (right columns) in the presence of DAPI (first and third column). BC2 cells were pretreated with 10 µmol/L of the ATM inhibitor KU55933, the ATR inhibitor VE-821, KU55933 + VE-821, or DMSO followed by treatment with DMSO or 10 µmol/L Ara-C for 14 hours. BC2 cells were treated with RNase and stained with ssDNA- or dsDNA-specific antibodies (red) and DAPI (blue). B, Yac-1 cells were treated with 10 µmol/L KU55933, VE-821, KU55933 + VE-821, or DMSO for 14 hours and stained as outlined in A. C, DMSO (left) or 4 µmol/L aphidicolin-treated (middle; 14 hours) BC2 cells and Yac-1 cells (right) were incubated with the dsDNA-specific dye PicoGreen (green) for 1 hour and Mitotracker dye (red) for 15 minutes. Z-stack images were acquired by confocal microscopy and analyzed using Imaris software to generate iso-surface plots. White arrows, presence of cytosolic DNA.
Zbp1/Dai modestly inhibited RAE1 but had little effect on RAE1 expression in Yac-1 cells (Fig. 6D). Inhibition of RIG-I, a RNA sensor that may indirectly mediate responses to cytosolic DNA, had no effect on RAE1 expression in BC2 or Yac-1 cells (Fig. 6C and D). Hence, DNA sensors other than ZBP1/DAI are likely to participate in inducing RAE1 expression in response to DNA damage, in line with other evidence suggesting the existence of DNA sensors that act redundantly (30). Taken together, these data suggest that cytosolic DNA sensor pathways regulate RAE1 expression in cells exposed to DNA damage.

IRF3 regulates RAE1 expression in B-cell lymphomas of Eμ-Myc mice

To address whether IRF3 regulates RAE1 expression in lymphomas, Irf3-deficient mice were bred to mice overexpressing c-Myc under the control of immunoglobulin heavy-chain enhancer region (Eμ), analogous to human Burkitt lymphoma (31). Spontaneous B220<sup>low</sup> B-cell lymphomas develop by 15 to 20 weeks of age, and the progression of lymphomas is accelerated in Nkg2d<sup>−/−</sup> : Eμ-Myc mice (4, 32). Tumor cells in Eμ-Myc mice express phosphorylated ATM (ATM pS1981; Fig. 7A; ref. 19). Staining of tumor cells with a dsDNA-specific antibody revealed the presence of cytosolic dsDNA in B220<sup>low</sup> tumor cells, but not normal B220<sup>+</sup> B cells (Fig. 7B). The accumulation of cytosolic DNA was strictly dependent on the DDR as administration of the ATM inhibitor KU55933 resulted in reduced levels of cytosolic dsDNA (Fig. 7C). Irf3<sup>−/−</sup> : Eμ-Myc mice (median survival = 62 days) experienced a significantly reduced survival rate compared with Irf3<sup>+/−</sup> : Eμ-Myc mice (median survival = 116 days; Fig. 7D). We were not able to generate Irf3<sup>−/−</sup> : Eμ-Myc mice because Irf3<sup>−/−</sup> : Eμ-Myc mice failed to breed. Heterozygosity of Irf3 in Eμ-Myc mice resulted in 2.5-fold decrease of IRF3 levels and reduced expression of IRF3 target genes in splenic B-cell lymphomas when compared with Irf3<sup>+/−</sup> : Eμ-Myc mice, suggesting that cytosolic DNA in...
Linkage of RAE1 Expression and PRR Pathways

Figure 7. RAE1 expression in c-Myc-driven lymphomas depends on IRF3. A, immunoblot analysis of splenic B-cell lymphomas (>78% purity) from Irf3−/−; Eμ-Myc, Irf3+/−;Eμ-Myc, Irf3+/−;WT, Irf3+/−;WT, and Irf3−/−;WT probed with antibodies for IRF3, BCL2L12, ATM, ATMpS1981, and GAPDH (left). Densitometry analysis of immunoblots showing mean ± SD from three mice normalized to GAPDH levels; **P < 0.01. B, B220 green) cells of WT and Eμ-Myc mice were stained for dsDNA (red) in the presence of DAPI (blue) at 54 days of age. C, Eμ-Myc mice were injected intraperitoneally with 5 mg/kg KU55933 (n = 3) or vehicle (n = 3) at 34 and 36 days of age and stained for dsDNA (red), B220 (green), and DAPI (blue) at 38 days of age. D, Irf3−/−;Eμ-Myc mice (thick) exhibit decreased survival compared with Irf3−/−;Eμ-Myc (thin line), nontransgenic (dashed line), or Irf3−/− (dotted line) mice. The Kaplan–Meier analysis of survival of Irf3−/−;Eμ-Myc mice (n = 25; median survival, 116 days), Irf3−/−;Eμ-Myc mice (n = 17; median survival, 62 days), and Irf3+/− or Irf3−/− mice (n = 25; median survival, >250 days). P < 0.0001 by log-rank test or by the Gehan-Breslow-Wilcoxon test. E, relative mRNA levels of indicated IRF3 target genes in purified tumor cells of Irf3−/−;Eμ-myc and Irf3+/−;Eμ-Myc mice were measured by qRT-PCR. F, EAE1 expression in tumor cells of three Irf3−/−;Eμ-Myc mice. B220+ cells in blood of moribund Irf3−/−;Eμ-Myc, Irf3+/−;Eμ-Myc, and C57BL/6 mice (bold line) were stained for the indicated NKG2D ligand expression. Filled histogram, isotype staining of B220+ tumors. G, Bc2/212-IRF3-Gfp (red line) or IRF3-Gfp-transduced (blue line) BC2 (left) or EμM1 (right) cells were stained for the indicated NKG2D-L3 3 days posttransduction. Dashed line, isotype staining of Bc2/212-IRF3-Gfp–transduced cells. Fine line, isotype staining of IRF3-Gfp–transduced cells. Filled histograms, isotype staining of untransduced cells. H, IL-2–activated NK cells derived from Irf3−/−;Eμ-Myc (white squares, n = 8) or Irf3+/−;Eμ-Myc (black circles, n = 7) mice were cocultured with Yac-1 cells at an effector to target ratio of 3:1. After 4 hours, the percentage of CD107a and IFN-γ-expressing NK1.1+CD3+ cells was determined by flow cytometry (left and middle). Freshly isolated splenocytes of Irf3−/−;Eμ-Myc (white squares, n = 4) and Irf3+/−;Eμ-Myc (black circles, n = 4) mice were stimulated in vitro for 5 hours on plates coated with NKG2D-specific antibodies (60 µg/mL) or isotype control (10 µg/mL) before staining and analysis. Intracellular IFN-γ was detected by flow cytometry gated on NK1.1+CD3+ cells (right). Error bar denotes SE of mean.
lymphomas activates IRF3 (Fig. 7A and E). Importantly, reduced levels of IRF3 in lymphomas impaired RAEl1 expression, the only RAEl family member detected in Eμ-Myc tumor cells (Fig. 7F; ref. 33).

The null mutation introduced into the If3 allele also resulted in functional inactivation of the neighboring Bcl2l12 gene, which promotes or suppresses tumorigenesis depending on the cellular context (34, 35). However, heterozygosity of the gene-targeted locus did not result in reduced BCL2L12 levels or on the cellular context (34, 35). However, heterozygosity of the gene, which promotes or suppresses tumorigenesis depending resulted in functional inactivation of the neighboring gene, which promotes or suppresses tumorigenesis depending.

expression in lymphoma cells in response to DNA damage for showed that cytosolic DNA contributes to the induction of RAE1.

Discussion

Our previous results provided evidence that the DDR activates immune responses by inducing NKG2DLs (6). Here, we show that cytosolic DNA contributes to the induction of RAEl expression in lymphoma cells in response to DNA damage for the following reasons: (i) inhibition of the DDR impaired the induction of cytosolic DNA and RAEl molecules (ii) transfection of DNA into cells upregulated RAEl expression; (iii) inhibition of STING, TBK1, or IRF3 impaired RAEl expression; (iv) TBK1 and IRF3 were activated in response to DNA damage in a DDR-dependent manner; and (v) overexpression of TBK1 or IKCe induced RAEl expression.

Linking the DDR to STING-initiated pathways is of interest immunologically, because STING is a critical component of a major pathway common to receptors that detect cytosolic DNA and RNA of pathogens (8). Previous studies provided indications that the DDR induces phosphorylation of IRF3 and that certain Toll-like receptor agonists induce Rael1 gene expression in peritoneal macrophages (37), but the linkage of these pathways had not been explored. Much remains to be determined about the relation of the DDR and STING pathways. We observed less phosphorylation of IRF3 in response to DNA damage when compared with LPS, suggesting that IRF3 translocation and transcriptional activity is differentially regulated in response to DNA damage. Consistent with this possibility, Noyce and colleagues reported that no minimal posttranslational modification of IRF3 correlated with its transcriptional activity (38). Of interest was that DNA damage consistently led to lower induction of IFN than Poly IC. The reduced induction likely reflects the fact that the DDR failed to induce IRF7 activation, which is necessary for efficient transcription of IFN genes (data not shown).

Cytosolic DNA has been shown to be present in cells upon infection or the uptake of apoptotic cells (8). Our data show the presence of cytosolic DNA in uninfected lymphoma cell lines. An intriguing question is where cytosolic DNA originates from and the mechanism leading to cytosolic DNA in tumor cells. DNA damage is known to induce transcription of retroelements, including transposases, derived from functional endogenous retrovirus present in the genome (39). Alternatively, cytosolic DNA could be generated during DDR-dependent DNA repair that can result in deletion of genomic DNA.

An important question is the nature of the DNA sensor recognizing the cytosolic DNA. The induction of RAEl by Ara-C partially relied on ZBP1/DAI. ZBP1/DAI is a candidate sensor that is reported to activate TBK1/IRF3 (40). However, additional TBK1-activating DNA sensors exist as MEFs from Zbp1−/− deficient mice mount a normal type I IFN response to DNA (18, 30). These sensors may be required for constitutive RAEl expression in Yac-1 cells. Hence, unidentified DNA sensors may play a predominant role in YAC-1 cells, or may function redundantly with ZBP1/DAI, in the induction of RAEl.

NKG2D plays an important role in immunosurveillance of tumors in Eμ-Myc mice (4, 5). The accelerated development of lymphoma in If3/Bcl2l12−/−/Eμ-Myc mice when compared with NKG2D-deficient mice suggests that IRF3 induces the expression of molecules other than RAEl ligands important for immunosurveillance or suppression of tumorigenesis. IRF3 and Bcl2L12 are known to induce genes implicated in apoptosis (11). However, we observed no differences in the rates of apoptosis or proliferation comparing WT and heterozygous tumor cells, suggesting that accelerated tumorigenesis of If3/Bcl2l12−/−/Eμ-Myc mice is not due to effects of IRF3 or Bcl2L12 on apoptosis or proliferation. In summary, our data suggest that tumorigenesis leads to accumulation of cytosolic DNA and subsequent activation of an antitumor immune response that may partially depend on NKG2D.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Linkage of RAE1 Expression and PRR Pathways

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RAE1 Ligands for the NKG2D Receptor Are Regulated by STING-Dependent DNA Sensor Pathways in Lymphoma

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