Supplementary Fig. S1: Flow cytometric detection of apoptosis in HNSCC cells.

HNSCC cells were treated with 50 μg/ml poly(I):(C) for 48h followed by staining with Annexin V/Propidium Iodide and Flow cytometric analysis as described in materials and methods. Fraction of cells in the green boxes indicate the apoptotic cell population.
Supplementary Fig. S2: Cell viability (A) and TLR expression profiles (B) of HNSCC cells.

(A) HNSCC cells were treated with poly(I):poly(C) as indicated for 24 h followed by MTT assay. Cellular viability were expressed as fold changes compared to untreated cells.

(B) Expression profiling of HNSCC cells for TLR4, TLR7 and TLR8. Total mRNA from HNSCC and positive/negative control HEK293 cells were subjected to quantitative RTPCR with respective primer pairs. Following normalization with internal RPL32 controls, fold change in mRNA expressions with respect to HEK293 were plotted.
Supplementary Fig. S3: TLR3 protein Expression in HNSCC cells.

TLR3 protein levels in PCI-6 and UMSCC22 cells were measured by flow cytometry as described in materials and methods.
**Supplementary Fig. S4: Enhanced apoptosis in PCI-15B is mediated via TRIF dependent signaling.**

(A) Upon poly(I):poly(C) treatments PCI-15 cells induce comparable levels of CCL5 cytokine. HEK293-TLR3 and PCI-15 cells were treated with poly(I):poly(C) (50 μg/ml) for 24 h as indicated. Following treatments culture supernatants were collected and CCL5 protein levels were measured by ELISA.

(B) Type I IFN production is not responsible for enhanced apoptosis in PCI-15B cells. PCI-15B cells were either treated with poly(I):poly(C) (50 μg/ml) or IFNα (500 U/ml) for 24 h, in presence or absence of 10 μg/ml anti-IFNAR2 antibody (6h pretreatment) as indicated. Gene induction (ISG56) and apoptosis (c-PARP) were analyzed by immunoblotting.

(C) IFNAR1 expression levels in PCI-15 cells following IFNAR1 knockdown. Quantitative RTPCR of IFNAR1 mRNA in PCI-15 cells following transduction with IFNAR1- or Ctrl-shRNA-pLKO.1 lentivirus.

(D) TLR3-TRIF signaling pathway is essential for PCI-15B cell apoptosis. PCI-15B cells were treated with poly(I):poly(C) in presence of 60 μM of TRIF-neutralizing peptide (Lane 3) or control peptide (Lane 4) Apoptosis (c-PARP) and gene induction (ISG56) were assayed as before.
Supplementary Fig. S5: Defective NF-κB signaling in PCI-15B.

(A) Induction of IL-8 mRNA in PCI-15 cells. Cells were treated as indicated for 24 h followed by the detection of IL-8 mRNA by quantitative RTPCR.

(B) Defective IL-8 protein induction in PCI-15B. HNSCC cells were treated with 50 μg/ml poly(I):poly(C), 1 μg/ml LPS, 80 HAU/ml Sendai virus and 5 μg/ml R848 for 24 h. Culture supernatants were collected after treatment and subjected to ELISA as described in Materials and Methods.

(C) and (D) Z-VAD-FMK inhibits poly(I):poly(C) mediated apoptosis in both PCI-15 cell lines. PCI-15 cells were pretreated with indicated amounts of Z-VAD-FMK for 1 h before poly(I):poly(C) (50 μg/ml) treatment. Cells were harvested after 24 h of poly(I):poly(C) treatment and whole cell lysates were either immunoblotted with anti-cleaved PARP and actin antibodies (C) or subjected to Caspase 3/7 cleavage assay (D).
Supplementary Fig. S6: Enhanced apoptosis in PCI-15B is due to defective NF-κB activation.

PCI-15A and B cells were pretreated with 2 μM Bay11-7082 (A) or with 10 ng/ml IL-1β for 30 min (B), followed by 24h poly(I):poly(C) treatment as indicated. Apoptosis inductions were measured by Caspase-Glo 3/7 assay as before.
Supplementary Materials and Methods

Antibodies against the cleaved PARP and IκBα were purchased from Cell Signaling Technology (Beverly, MA). Anti-NF-κB p50 (NLS), anti-actin and anti-tubulin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NF-κB p65 was from Abcam (Cambridge, MA). Anti-ISG56, anti-ISG60, anti-IRF3, anti-DRBP76 antibodies have been described before (33). Human interferon alpha/beta receptor antibody (anti-IFNAR) and recombinant human interferon α (IFNα) were from PBL interferon source (Piscataway, NJ). LPS and NF-κB peptide inhibitor BAY11-7082 was obtained from Sigma-Aldrich (St. Louis, MO). Resiquimod (R848) was from Alexis Biochemicals (Plymouth Meeting, PA), and Sendai virus (SeV) from Charles River (Wilmington, MA). Z-VAD-FMK was from Promega (Madison, WI) and human IL-1β and TNFα were from Peprotech (Rocky Hill, NJ).

Nuclear fractionation and Western blotting analysis

To prepare nuclear extracts, cells were washed in ice-cold PBS, scraped, collected in hypotonic buffer (10 mM Tris pH 8.0, 2 mM MgCl2, 3 mM CaCl2, 0.5 mM DTT, 300 mM sucrose, 0.1 % NP-40), and kept on ice for 5 minutes before dounce homogenization. Nuclei were then pelleted at 1,000g for 5 minutes at 4°C, washed with PBS and lysed in lysis buffer (20 mM HEPES pH 7.4, 1 % Triton-X 100, 150 mM NaCl, 1.5 mM MgCl2, 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na3VO4, 1 mM PMSF plus 1x protease inhibitors). The whole cell lysates were also prepared in above cell lysis buffer. Equal amounts of protein extracts were subjected to SDS-PAGE and western blotting with appropriate antibodies.
**Immunofluorescence microscopy**

HNSCC cells (1×10^5 cells) were grown on glass cover slips and incubated with poly(I):poly(C) (50 μg/ml) for 24 hours at 37 °C. Cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After washing 3 times with PBS, cells were permeabilized in 0.2% TritonX-100 in PBS for 15 min at room temperature. The permeabilized cells were blocked in PBS containing 0.02% Tween 20, 3% BSA for two hours and incubated with primary antibody: anti-NF-κB p65 or p50 at a dilution of 1:100 overnight at 4°C. After washing with PBS, the cells were stained with Alexa Fluor® 488 conjugated anti-rabbit or mouse IgG (H+L) F(ab')2 Fragment (Cell Signaling) for 1 h. The cell nuclei were counter stained with DRAQ5 (Cell Signaling) at a dilution of 1:5000 for 5min in dark. After washing with PBS, the coverslips were mounted on microscope slides with ProLong® Gold antifade reagent (Invitrogen), and visualized using a Leica TCSSL confocal Microscope.

**Flow cytometry**

For intracellular flow cytometry, cells were washed with PBS, the cell pellet resuspended in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS and incubated for 10 min at room temperature. The cells were then permeabilized using 100% methanol overnight at -20°C. The cells were then washed in 2% fetal bovine serum (Cellgro, Manassas, VA) in PBS and stained for 1h at 4°C with either a FITC-conjugated anti-human TLR3 antibody (Imgenex, San Diego, CA) or an isotype-matched FITC-IgG control antibody (BD Biosciences, San Jose, CA). For apoptosis detection, following treatment cells were washed with PBS, resuspended in 1x Annexin binding buffer (BD Pharmingen, San Jose, CA) and stained with 5μl Annexin V-FITC and 5μl Propidium Iodide in 100μl binding buffer for 15 min at room temperature in the
dark. Flow cytometry was performed on a CyAn ADP Analyzer (Beckman Coulter, Miami, FL) and analyzed using Summit v4.3 software as described before (1).

**IL-8 and CCL5 ELISA**

The cell culture supernatants of HEK293, HEK293-TLR3, PCI-15A and PCI-15B were collected 24 hours post stimulation with different ligands and subjected to IL-8 and CCL5 measurement as described before (2, 3).

**Quantitative PCR analysis of gene expression**

Total RNA was prepared using Trizol reagent (Invitrogen) and treated with DNase I (DNA Free kit, Ambion, Foster City, CA). Total RNA (600 ng) was used for reverse transcription using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and subjected to real-time PCR using a CFX96 real time system (Bio-Rad) according to manufacturer’s instructions. Primers for TLR3, TLR4, TLR7, TLR8 and TRIF are given in the Supplementary Material; primers for IFNα, IFNβ, IL-8 and RPL32 have been reported before (3). Each sample was normalized to RPL32 and expressed as fold change of the untreated control.

**Sequence of the Primer sets used:**

**TLR3:**
- forward 5’-CCTGGTTTGTTAATTGGATTAACGA-3’
- reverse 5’-TGAGGTGGAGTGTTGCAAAGG-3’

**TLR4**
- forward 5’-CAGAGTTTCCTGCAATGGATCA-3’
- reverse 5’-GCTTATCTGAAGGTGTTGCACAT-3’

**TLR7**
- forward 5’-TCCTTGGGGCTAGATGGTTTC-3’
- reverse 5’-TCCACGATCACATGGTTTTT-3’
TLR8  forward 5'- TGTGAGTTATGCGCCGAAGAA-3'  
reverse 5' - GTTTGGGAACTTCCTGTAGTC-3'  

TRIF  forward 5'- CAGGAGCCTGAGGAGATGAG-3'  
reverse 5'-CTGGGTAGTTGGTGCTGGTT-3'  

IκBα  forward 5'- CTCCGAGACTTTCGAGGAAATAC-3'  
reverse 5'- GCCATTGTAGTTGGTACCTTCA-3'  

References:

