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Protection from RNA and DNA Viruses by IL-32

Jarod A. Zepp,* Claudia A. Nold-Petry,*[†] Charles A. Dinarello,* and Marcel F. Nold*[†]

Several studies have documented a proinflammatory role for IL-32, which induces IL-1 α , IL-1 β , IL-6, TNF, and chemokines via NF- κ B, p38MAPK, and AP-1. However, IL-32 also participates in the responses to infection with viruses such as HIV-1 and influenza. In this study, we explored these antiviral properties of IL-32. Vital staining assays demonstrated that low concentrations (5–10 ng/ml) of rIL-32 γ protected epithelial WISH cells from vesicular stomatitis virus-induced cell death. By lactate dehydrogenase assays, treatment with IL-32 γ resulted in a 3- to 4-fold decrease in viral load. Specific silencing of IL-32 revealed that the antiviral responses triggered by the synthetic analogs of ssRNA viruses (polyuridine) and dsRNA viruses (polyinosinic-polycytidylic acid) were significantly weaker (2- to 3-fold more virus) in WISH cells in the absence of IL-32. Importantly, we discovered that the polyinosinic-polycytidylic acid-induced increase in production of IFN- α in human PBMC was nearly completely abolished when IL-32 was silenced. Moreover, we observed that IL-32 antagonizes the DNA virus HSV-2 in epithelial Vero cells as well as in human umbilical cord endothelial cells, as production of HSV-2 increased 8-fold upon silencing of IL-32 ($p < 0.001$). Mechanistically, we found that IL-32 used the PKR-eIF-2 α as well as the MxA antiviral pathways. Unexpectedly, a considerable part of the antiviral properties of IL-32 was not dependent on IFNs; specific blockade of IFN activity reduced the antiviral properties of IL-32 only moderately. In conclusion, these data suggest a central role for IL-32 in the immune response to RNA and DNA viruses, which may be exploitable for clinical use in the future. *The Journal of Immunology*, 2011, 186: 4110–4118.

Interleukin-32 has been implicated in several aspects of inflammatory responses through its induction of IL-1 β , IL-6, IL-8, and TNF through the p38-MAPK, NF- κ B (1), and AP-1 (2) signaling pathways. Also, IL-32 is involved in diseases characterized by inflammation such as rheumatoid arthritis (3) and inflammatory bowel disease (1, 4). Expression and regulation of IL-32 has been described in several cell types including T cells, monocytes, NK cells, endothelial cells, and epithelial cells. In endothelial cells, IL-32 is a major mediator of IL-1 β -induced inflammatory responses (5). In the A549 and WISH epithelial cell lines, IL-32 protein levels are markedly increased by treatment with IFN- γ (1, 6). Furthermore, in primary human macrophages, *Mycobacterium tuberculosis* stimulates IL-32 production via caspase-1, IL-18, and IFN- γ (7).

To investigate the host's response to virus invasion in vitro, the chemical compounds polyinosinic-polycytidylic acid (poly-IC), polyuridine [poly(U)], and polydeoxyadenylic-polydeoxythymidylic acid [poly(dA-dT)] are commonly used to mimic viral pathogen-associated molecular patterns (PAMPs). The compound poly-IC is structurally similar to viral dsRNA (as occurs in the rotavirus) and is recognized by TLR3 and MDA-5. To mimic viral ssRNA (as

occurs in the influenza and vesicular stomatitis viruses [VSV]), which is recognized by TLR7/8, poly(U) oligonucleotides can be used. The recognition of DNA viruses (e.g., HSV1 and -2, cowpox virus), mimicked by poly(dA-dT), has been elusive; however, the adaptor molecule AIM2 (8) and RNA polymerase-III (9) appear to be involved in such recognition. Once detected by the cell, the aforementioned compounds initiate the antiviral response.

Aside from its proinflammatory properties, recent reports have highlighted that IL-32 is regulated during viral infection. Elevated levels of IL-32 were found in serum from patients infected with HIV-1 (10) or influenza A (11), suggesting that this cytokine is relevant in viral infection in humans. Positive and negative feedback interactions between IL-32 and cyclooxygenase-2, PGE₂, and inducible NO synthase (iNOS) (12) during influenza A infections have been described. Furthermore, it has been demonstrated that epigenetic modifications modulate the efficacy of the antiviral properties of IL-32 (13). We reported that IL-32 interferes with viral function: silencing of IL-32 in PBMCs acutely infected with HIV-1 resulted in 4-fold increases in the production of HIV-1 (2). We observed similar increases in the latently infected U1 macrophage cell line. Moreover, treatment of U1 cells with recombinant human IL-32 γ resulted in 72% decreases in the HIV-1 p24 protein. That study provided the first evidence that IL-32 regulates the production of a virus. Furthermore, small interfering RNA (siRNA) to IL-32 resulted in a 51% reduction of IFN- γ protein levels (2). Encouraged by the advances described above, we searched the promoter of the *il-32* gene for sites sensitive to regulation by IFN regulatory factors (IRFs). IRFs are key inducers of antiviral effectors and have been reported to bind to tandem repeats of the core motif GAAA (14, 15). Indeed, the region up to 2000 bp upstream of the transcriptional start site of *il-32* contains several occurrences of the IRF consensus sequences.

Given the mounting evidence for a role of IL-32 in the immune response to viral infection and its regulation by and induction of IFNs, we set out to investigate whether and how antiviral responses depend on IL-32. We employed a WISH cell model to study the RNA-based VSV as well as an endothelial and an epithelial cell assay for infections with the DNA-based HSV-2.

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Abbreviations used in this article: C_T, threshold cycle; eIF-2 α , eukaryotic translation initiation factor 2 α ; iNOS, inducible NO synthase; IRF, IFN regulatory factor; LDH, lactate dehydrogenase; PAMP, pathogen-associated molecular pattern; PKR, protein kinase R; poly(dA-dT), polydeoxyadenylic-polydeoxythymidylic acid; poly-IC, polyinosinic-polycytidylic acid; poly(U), polyuridine; scr, scrambled small interfering RNA; siIL-32, small interfering RNA to IL-32; siRNA, small interfering RNA; soluble IFN- α / β , soluble IFN- α / β receptor; VSV, vesicular stomatitis virus.

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Materials and Methods

Reagents

MEM, Isocove's DMEM, FCS, penicillin/streptomycin, and trypsin were purchased from Cellgro (Herndon, VA). rIL-1 β , rIL-32 γ (different lots were tested as well as another rIL-32 γ from another company, all of which had similar results), and the human IFN- α multisubtype ELISA were obtained from R&D Systems (Minneapolis, MN). rIFN- γ and rIFN- β were from PeproTech (Rocky Hill, NJ). TLR agonists, poly-IC, poly(U), and poly(dA-dT) were purchased from Invivogen (San Diego, CA). We purchased the detection kit for lactate dehydrogenase (LDH) from BioVision (Mountain View, CA). L-NAME was purchased from Enzo Life Sciences (Plymouth Meeting, PA). The Nucleofector II electroporation device and reagents were from Lonza/Amata (Cologne, Germany). The soluble IFN- α/β receptor (sol-IFN- α/β R) was kindly provided by Dr. Daniela Novick, Weizmann Institute, Rehovot, Israel. The affinity-purified goat anti-human polyclonal IL-32 Ab used for Western blotting was described previously (1). The rabbit anti-human protein kinase R (PKR) and phospho-eukaryotic translation initiation factor 2 α (eIF-2 α) (Ser⁵¹) were obtained from Cell Signaling Technology (Danvers, MA). The mouse mAb anti-MxA was a gift from Dr. Georg Kochs, Department of Virology, University of Freiburg, Freiburg, Germany, and was described previously in Ref. 16.

Cell culture

Human amnion-derived WISH cells and the monkey kidney epithelial Vero cells were obtained from American Tissue Culture Collection and cultured in MEM with 10% FCS and 50 U/ml and 50 μ g/ml penicillin/streptomycin. HUVEC were obtained and propagated as described previously (5). For each cell type in each experiment that required stimulation with exogenous protein (e.g., IL-32 γ), growth medium was removed and replaced with reduced serum media before stimulation. For WISH and Vero cells, MEM with 1% pooled human serum and 1% penicillin/streptomycin was used; for HUVEC, stimulation medium was the same as growth medium, except FCS was reduced to 2%. For HUVEC experiments, all cell-culture plates were coated with 1% gelatin.

Human PBMC were obtained from healthy volunteers that abstained from taking any medications for at least 1 wk before blood draw. Heparinized (20 U/ml) blood was collected from the antecubital vein, and PBMC were separated using the Ficoll-Hypaque density gradient. After removal of the mononuclear cell layer, the PBMC were washed two times with cold PBS and resuspended in pure RPMI 1640 without additives. All experiments involving PBMC were approved by the University of Colorado Multiple Institutional Review Board.

Viruses used in this study were the Indiana strain of VSV and a clinically isolated type 2 HSV. Virus was propagated in WISH (VSV) and VERO (HSV-2), and aliquots of virus-rich supernatant were frozen at -80°C . Only one freeze-thaw cycle was allowed.

Antiviral bioassays

To investigate modulation of virally induced cytopathic effects, we employed the VSV/WISH cell model to study RNA-based viruses and the HSV-2/Vero or HUVEC model to explore DNA-based viruses. Both models were described previously (17). WISH or Vero cells (passages 1–10) were trypsinized, and live cells were counted using trypan blue. A total of 5×10^4 cells were seeded into each well of a 96-well flat-bottom polystyrene plate in 0.1 ml growth medium and grown until a confluent monolayer was obtained (20–24 h). Unless indicated otherwise, the growth medium was then removed and replaced with stimulation medium, immediately followed by addition of the stimuli. The cells were challenged with VSV or HSV-2 24 h poststimulation. Twenty-four (VSV) or 48 (HSV-2) h postinfection, small aliquots of the supernatants were removed for LDH determinations where indicated. For characterization of the relation between viral load and LDH values in the VSV/WISH and HSV-2/Vero models, serial dilutions of VSV or HSV-2 were added to the cells, which had been plated as described above. The indicated dilutions of virus were prepared and added to the cells. Twenty-four (VSV) or 48 (HSV-2) h later, LDH release was measured. sol-IFN- α/β R was added 30 min before stimulation with exogenous IL-32 γ .

Transfections

For experiments involving transfection, WISH cells or HUVEC were counted and subjected to electroporation using the Amata Cell Line Nucleofector kit V (Amata) and program T-020 for WISH cells and the HUVEC Nucleofector kit and program U-001 for HUVEC. Per transfection condition, one cuvette contained 1.6×10^6 WISH cells or 0.8×10^6 HUVEC plus the indicated concentration of scrambled siRNA (scr) or

siRNA to IL-32 (Thermo Fisher Scientific, Lafayette, CO). For sequences, please see Ref. 2. Cells were recovered from the cuvette with 0.5 ml pure MEM for WISH or Opti-MEM 1 \times (Cellgro) for HUVEC warmed to 37°C followed by 5 min of recovery at 37°C . Cells were then reseeded in 24-well flat-bottom plates with 1 ml growth medium at a concentration of 0.1×10^6 cells/ml. For cytokine analysis, transfected cells were grown overnight, then medium was replaced with stimulation media, and stimuli were added for 24 h. Thereafter, cells were challenged with VSV or HSV-2.

PBMC were transfected using Amata Nucleofector kit T cell as described previously (2). In brief, 10×10^6 cells were used per transfection condition per cuvette. Transfected cells were recovered from the cuvette with 0.5 ml pure, prewarmed RPMI 1640. Cells rested overnight at 37°C in polypropylene tubes in human T-Cell Media (Lonza) plus 1% human serum plus 2 mM L-glutamine (Cellgro). Cells were then resuspended and viable cells were counted via trypan blue exclusion. The cells were spun at $200 \times g$ for 10 min, then resuspended in PBMC stimulation medium: RPMI 1640 plus 1% human serum plus 10 mM HEPES buffer (Cellgro) plus 100 μ g/ml primocin (Lonza) at a concentration of 2×10^6 cells/ml.

HSV quantitative PCR

After 48 h of HSV infection, HUVEC cell cultures were frozen and thawed. The freeze-thaw suspension was analyzed for LDH and cytokines, and DNA was extracted from the remaining cell-supernatant mixture. Viral DNA was extracted, and real-time PCR was performed using reagents and devices by Roche (Boulder, CO). The primer/probe sets targeted HSV-1 and -2; HSV-1 was not detected in any of the samples. Total DNA content was determined on a NanoDrop (Thermo Fisher Scientific); each sample was measured in triplicate. After acquisition of threshold cycles ($C_{T\text{S}}$) by real-time PCR, $\Delta C_{T\text{S}}$ values were calculated by subtracting the $C_{T\text{S}}$ of the scr-transfected controls from the $C_{T\text{S}}$ of the small interfering RNA to IL-32 (siIL-32)-transfected conditions. Fold changes in HSV content were obtained by normalization to DNA content.

Electrochemiluminescence assays and ELISAs

Electrochemiluminescence measurements of protein levels of the human cytokines IL-1 α , IL-6, IL-8, IL-32, and TNF were performed with specific Ab pairs and standards made with recombinant proteins, all provided by R&D Systems (Minneapolis, MN), and assayed on the Origen Analyzer from Wellstat Diagnostics (Gaithersburg, MD) as described previously (2). The Ab pairs for human IFN- γ were purchased from Fitzgerald Industries International. ELISAs for IFN- α and IFN- β were from PBL InterferonSource (Piscataway, NJ) and performed according to the manufacturer's instructions.

LDH assay

After 24 (VSV) or 48 (HSV) h of infection, 10 μ l cell supernatant was removed and immediately assayed for LDH according to the manufacturer's instructions. All samples were run in duplicate. For percent decrease calculations, wells that received virus alone were defined as uninhibited virus-induced cell death. Therefore, percent decrease was calculated by using LDH values in the following equation: $[(\text{stimulated}/\text{positive cell death control}) - 1] \times 100$.

Crystal violet staining

Staining involved a crystal violet solution in 100% ethanol performed when virus-induced cell death in wells with virus alone was nearly complete. This was determined by light microscopy. Supernatants were removed, and 50 μ l crystal violet solution was added to all wells. Residual (nonabsorbed) crystal violet was washed out by repeated rinses with distilled water.

Immunoblotting

The total protein content of each sample was determined using the Bradford method. Twenty-five micrograms total protein were then solubilized in SDS buffer with 2-ME. Samples were separated by electrophoresis on 4–15% gradient SDS-polyacrylamide gels (Bio-Rad). Proteins were transferred to nitrocellulose membranes (0.2 μ m), which were assessed for equal loading by Ponceau staining. Equal loading was also ascertained by β -actin (Santa Cruz Technologies) staining. Densitometry on immunoblots was performed using Adobe Photoshop software (Adobe Systems).

Measurement of nitrate/nitrite

Supernatants from WISH cells treated with vehicle or L-NAME with or without IL-32 γ for 24 h were collected and assayed for total nitrate/nitrite concentration using a kit purchased from Cayman Chemical (Ann Arbor, MI).

Statistical analysis

Raw datasets were tested for normality by the Kolmogorov-Smirnov method and for equal variance (p value to reject = 0.05). Afterwards, data were analyzed using the unpaired t test (α set at 0.05 in all cases) or the Mann-Whitney rank-sum test.

Results

Production and regulation of IL-32 in WISH cells

To determine whether WISH cells produce the IL-32 protein, cell-culture lysates were assayed by ELISA, electrochemiluminescence assay, and immunoblotting. Fig. 1B shows that IL-1 β and IFN- γ increase the production of IL-32 protein 4.2- and 5.5-fold, respectively. IFN- β moderately induced IL-32. Classical inducers of type I IFNs, the synthetic ligands of TLR3 and 7/8 [poly-IC and poly(U)], exhibited a significant 3.8- and 2-fold increase in IL-32 abundance (Fig. 1C). These observations were also confirmed by immunoblot as demonstrated in Fig. 1A. Blocking experiments using IL-1R antagonist revealed that the contribution of TLR ligand-stimulated IL-1 α to the induction of IL-32 was minimal for poly-IC and absent for poly(U) (data not shown). Neither IL-1 β nor IFN- γ was detectable in WISH cells regardless of which stimulus was used (data not shown). A dose dependency for the induction of IL-32 was established for each of the stimuli by testing at least two concentrations (data not shown).

Exogenous IL-32 γ confers an antiviral state in WISH cells

To investigate the effect of exogenous IL-32, we used the γ -isoform of rIL-32, which has been shown to be the most active isoform in its activity both against viruses (13) as well as in other settings (18). We stimulated WISH cells with IL-32 γ and 20 h later challenged them with VSV. Protection was assessed by vital staining with crystal violet (as described in Ref. 6). We observed that nearly all WISH cells that were infected with VSV alone were killed by the virus (Fig. 2A, left panel). As expected, IFN- γ (Fig. 2A, left panel) and IFN- β (not shown) conferred a dose-dependent protection. Treatment with IL-32 γ also had a protective effect, but in contrast to IFN- γ , this effect exhibited a biphasic dose response: from 0.5–10 ng/ml IL-32 γ , the viability of the WISH cells increased; however, when we used concentrations >20 ng/ml, the protective effect abated and, at 50 ng/ml, was not detectable at all (Fig. 2A, right panel).

To assess whether exogenous IL-32 γ regulates cytokines in WISH cells, we assayed the supernatants and lysates from the experiments described above for IL-1 β , IL-6, IL-8, IFN- γ , and IL-1 α . Constitutive production of these cytokines ranged from 50–100 pg/ml for IL-6 and 5–10 pg/ml for IL-1 α , whereas IL-1 β , IL-8, and IFN- γ abundance was below limits of detection. The

abundance of each of these cytokines was unaffected by treatment with IL-32 γ (data not shown).

To quantify the VSV-induced cytopathic effect, we measured LDH levels in the supernatants of VSV-treated WISH cell cultures. Fig. 2B shows percent decreases in LDH release in the supernatants of the WISH cell bioassays. Cells with VSV alone exhibited the most cell death and release of LDH; this was set as baseline (0 in Fig. 2B). The decrease in LDH release associated with IL-32 γ stimulation was consistent with the increase in crystal violet staining (Fig. 2A). The inset in Fig. 2B shows the increase in LDH release induced by serial dilutions of VSV after 24 h of infection of WISH cell monolayers. The typical range between spontaneous LDH release (no virus, no stimulus) and maximum VSV-induced cell death (VSV alone, no stimulus) fell between 600 and 1800 arbitrary units. Using the information in the inset, we calculated that a 25% change in LDH translated into a 3- to 4-fold change in viral load.

Effects of specific blockade of type I IFN activity on antiviral functions of IL-32

We recently reported that IL-32 induces IFN- α in U1 cells (2). To demonstrate a similar regulation in WISH cells, we measured the production of type I and II IFNs (IFN- α , IFN- β , and IFN- γ) after treatment with IL-32 γ . IFN- α/β as well as IFN- γ levels were below detection limits of the ELISA (data not shown). However, failing to measure IFNs did not rule out a possible role for these cytokines in IL-32-induced antiviral activity, especially because IFNs can exert antiviral functions at low concentrations (Fig. 2). To resolve whether type I IFNs contributed to the antiviral activity of IL-32 γ , WISH cells were incubated with sol-IFN- α/β R. The effectiveness of sol-IFN- α/β R in our WISH-VSV model was tested by coincubating the inhibitor with IFN- β . As expected, we found that sol-IFN- α/β R dose-dependently decreased the antiviral activity of IFN- β (data not shown). When sol-IFN- α/β R was used alone, we observed a marginal increase in virus-induced cytopathic effect (data not shown). When coadministered with IL-32 γ (Fig. 3), sol-IFN α/β R increased LDH by ~50% compared with IL-32 γ alone. Therefore, this experiment indicates that IL-32 possesses both IFN-dependent as well as IFN-independent antiviral properties.

Regulation of PKR, eIF-2 α , and NO by IL-32 γ

Next, we investigated the antiviral PKR-eIF-2 α pathway. Fig. 4A shows that upon addition of IL-32 γ to WISH cell cultures, PKR levels were upregulated up to 13-fold as estimated by densitometry, with 5 and 10 ng/ml being the most effective concentrations, a finding consistent with the results from the bioassays (Fig. 2). As PKR activates eIF-2 α by phosphorylation, we performed a time

FIGURE 1. IL-32 is expressed and regulated in WISH cells. Except where indicated otherwise, WISH cells were incubated for 20 h with the indicated stimuli at the following concentrations: IL-1 β , 10 ng/ml; IFN- γ , 2 ng/ml; poly-IC, 100 μ g/ml; poly(U), 5 μ g/ml; and IFN- β , 2 ng/ml. **A**, One representative immunoblot of three independently performed is shown. **B**, IL-32 concentrations in WISH cell lysates; means \pm SEM are shown; $n = 3$. *** $p < 0.001$ for control (constitutive) versus stimulated conditions. **C**, IL-32 concentrations in WISH cell lysates stimulated with RNA-mimetic compounds; means \pm SEM are shown; $n = 3$. ** $p < 0.01$, *** $p < 0.001$ for control versus stimulated.

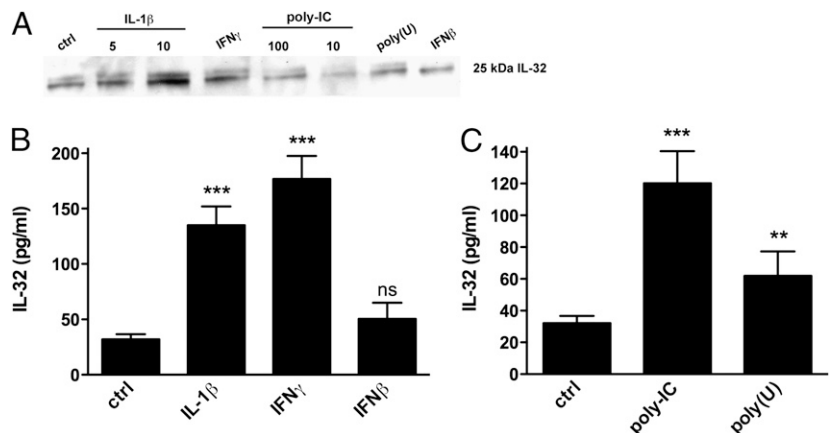
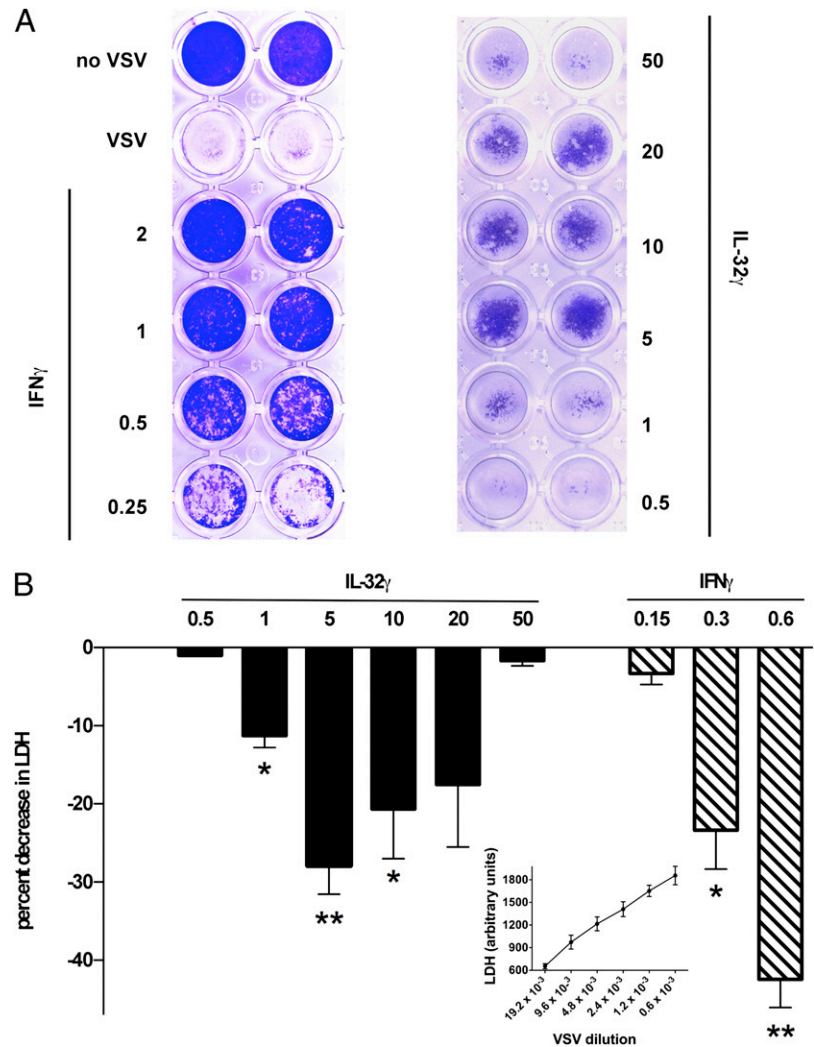


FIGURE 2. Exogenous IL-32 γ on WISH cells promotes an antiviral state. *A*, Antiviral bioassay. WISH cells were stimulated with IFN- γ , IL-32 γ (cytokine concentrations in ng/ml), or vehicle for 24 h, then VSV was added to each well except those designated “no VSV.” After 48 h of infection, viable cells were stained with crystal violet. Results from one representative plate of four are shown. *B*, Cytopathic effect of VSV on WISH cells stimulated with IFN- γ or IL-32 γ (both indicated in ng/ml) for 20 h, followed by a 24 h infection with VSV. Percent decrease in LDH with VSV alone is set at zero. Means \pm SEM are depicted; $n = 4$. *Inset*, VSV was serially diluted and incubated with WISH cell monolayers. LDH was measured from the supernatants 24 h postinfection; $n = 3$, means \pm SEM. * $p < 0.05$, ** $p < 0.01$ for control (baseline) versus IL-32 γ - or IFN- γ -stimulated conditions.



course of IL-32 γ stimulation in WISH cells (Fig. 4*B*). The blot shows WISH cell lysates after stimulation with 10 ng/ml IL-32 γ in which phosphorylated eIF-2 α is increased up to 5-fold. In contrast, unstimulated WISH cells exhibited no induction of phosphorylated eIF-2 α with the exception of a moderate increase at 24 h (data not shown).

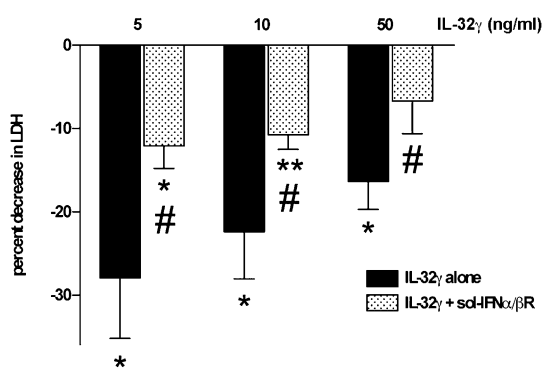


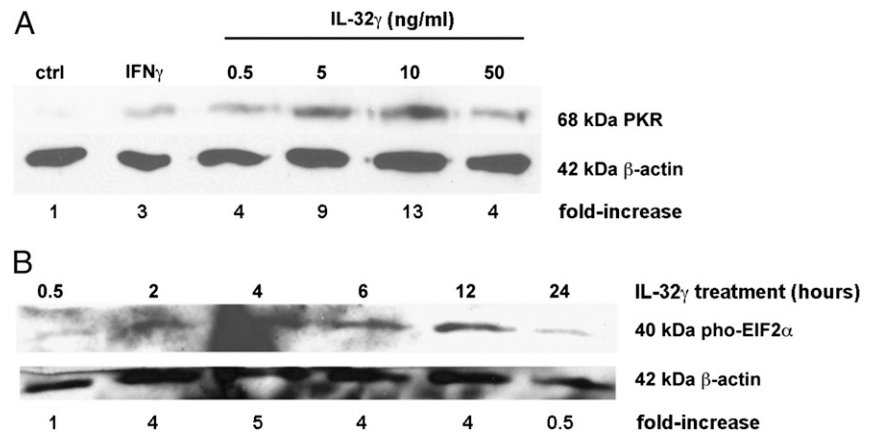
FIGURE 3. Effect of inhibition of type I IFNs on the antiviral activity of IL-32 γ . The graph shows percent decrease in LDH in WISH cells stimulated with IL-32 γ with or without sol-IFN- α/β R (added 30 min before IL-32 γ) for 20 h, then challenged with VSV for 24 h. LDH values in VSV alone controls are set at zero. Means \pm SEM are depicted; $n = 3$. * $p < 0.05$, ** $p < 0.01$ for VSV only versus IL-32 γ or IL-32 γ plus sol-IFN- α/β R, # $p < 0.05$ for IL-32 γ alone versus IL-32 γ plus sol-IFN- α/β R.

As others have reported an interplay between IL-32 and iNOS in the setting of viral infections (12) and neuroinflammation (19), we investigated whether this interaction occurred in WISH cells, in which iNOS is expressed and regulated (20). However, in contrast to the previous study, we did not observe any effect of IL-32 on NO or vice versa. Neither did the abundance of NO in WISH cells change with different concentrations of IL-32 γ , nor did L-NAME, an inhibitor of NO production, alter the antiviral state of WISH cells with or without IL-32 γ (data not shown).

Silencing of IL-32 reduces the efficacy of the antiviral response

To explore the antiviral functions of endogenous IL-32 in WISH cells, we knocked down the cytokine using specific siRNA in WISH cells. Using a pool of four different siRNAs at 25 nM each (siIL-32), we consistently achieved ~90% reductions of steady-state and inducible IL-32 protein, as shown in Fig. 5*A*. A pool of four nontargeting siRNAs at a similar concentration was used for comparison (scr). Consistent with the results obtained using exogenous IL-32 γ in WISH cells, silencing of IL-32 did not affect the levels of constitutive, IL-1 β -, or IFN- γ -induced IL-1 α , IL-6, IL-8, and IFN- γ (not shown). For instance, IL-1 β -induced IL-6 in transfected WISH cells increased by only 14% upon treatment with siIL-32 versus scrambled control ($p = 0.74$). In contrast, the efficacy of the antiviral responses triggered by poly-IC, poly(U), and IFN- β was reduced. As shown in Fig. 5*B*, LDH assays revealed that the cytopathic effects of VSV were more pronounced in siIL-32-transfected than in scr-transfected WISH cells. When

FIGURE 4. IL-32 γ activates the PKR–eIF-2 α pathway. One of three similar blots is shown in each panel. *A*, Immunoblot analysis of PKR in WISH cell lysates after stimulation for 20 h with the indicated concentrations of IL-32 γ , IFN- γ (2 ng/ml), or vehicle (ctrl). Fold increase in abundance of PKR over vehicle-stimulated conditions as assessed by densitometry is shown below the blot. *B*, Phospho(Ser⁵¹)–eIF-2 α –stained blots from WISH cells stimulated with 10 ng/ml IL-32 γ for the indicated period of time. Fold increase in phospho–eIF-2 α over 0.5 h of IL-32 γ as determined by densitometry is indicated below the blot.



endogenous IL-32 was reduced, the protection afforded by poly-IC or poly(U) was 48 and 88% less, respectively, which resulted in 2- to 3-fold more virus compared with the scrambled controls. IFN- β responses appeared less affected by the abundance of IL-32 (data not shown).

IL-32 interferes with HSV-2

Next, we tested whether IL-32 also participates in responses to the DNA virus HSV. Similar to VSV in WISH cells, HSV-2 has cyto-

pathic effects when propagated in Vero cells and HUVEC (17). To characterize this model, we established a kill curve for serially diluted HSV-2 in Vero cells (Fig. 6A, *inset*). In this model, a 26% change in LDH translated into a 4–10-fold change in viral load. As shown in Fig. 6A, a 20 h treatment of Vero cells with exogenous IL-32 γ before infection with HSV-2 resulted in a biphasic reduction in LDH release. The maximum protection, a 26% decrease in LDH compared with virus only, was observed at 5 ng/ml IL-32 γ .

Because the abundance of IL-32 was below detection limits of the assays in Vero cells, we employed HUVEC for the silencing experiments, as these cells produce considerable quantities of IL-32 (5) and are susceptible to HSV infection. After achieving the knockdown of IL-32 in HUVEC, the cells were treated with poly-IC, poly(U), or IFN- β . As shown in Fig. 6B, the viral cytopathic effect increased by 22% in poly-IC–stimulated conditions transfected with siIL-32 compared with scrambled-transfected controls. As per the *inset* of Fig. 6A, this change likely corresponded to a 6–8-fold increase in viral load. We also determined HSV DNA in these cultures by real-time quantitative PCR and observed that the loss of IL-32–mediated protection was assessed correctly by the LDH assays, as copies of HSV DNA increased 8-fold (Fig. 6C). Only slight changes in LDH values for IFN- β –stimulated conditions were noted (data not shown). The effect of poly(U) was not studied in this model, as TLR7 and -8 are not expressed in HUVEC (21).

Endogenous IL-32 is required for the production of IFN- α and MxA

To further characterize the role of endogenous IL-32 in the antiviral response, PBMC were transfected with siIL-32 and then stimulated with poly-IC, poly(U), or IFN- β . Fig. 7A shows an immunoblot from these lysates stained for the antiviral protein MxA. Upon silencing of IL-32, MxA protein production was reduced by ~50% in the poly(U)–stimulated conditions. Despite the moderate difference in poly-IC–stimulated conditions in Fig. 7A (which shows the blot that provides the best overview over all conditions), the abundance of MxA was in fact consistently reduced by siIL-32 (mean decrease 34% comparing poly-IC/scr to poly-IC/siIL-32). Constitutive and IFN- β –induced MxA appeared to be unaffected by changes in IL-32 levels.

To determine whether IL-32 participates in the production of IFN- α , we measured this cytokine in PBMC transfected with siIL-32 or scrambled siRNA. Fig. 7B demonstrates that in the absence of endogenous IL-32, the 3.7-fold increase in IFN- α production induced by a 20 h treatment with poly-IC was nearly abolished. Importantly, this effect was also observed after only 0.5 h of stimulation with poly-IC (1.4-fold increase in scrambled versus 0.6-fold decrease in siIL-32).

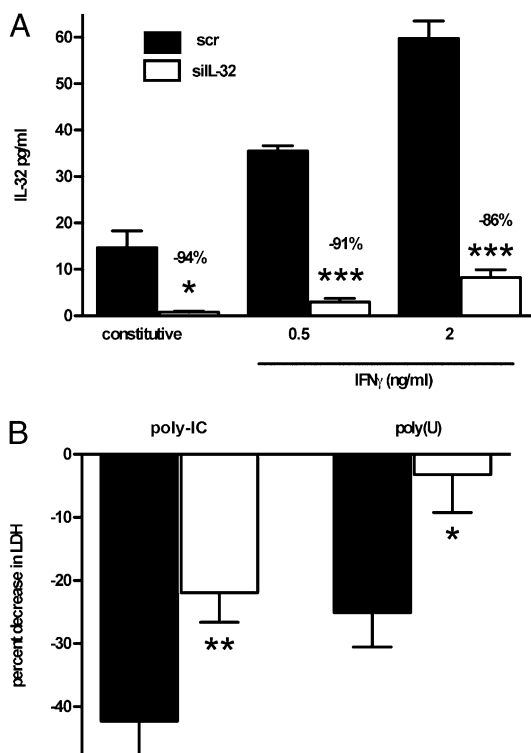


FIGURE 5. Silencing of IL-32 in WISH cells reduces responses to viral PAMPs. *A*, siRNA to IL-32 was transfected into WISH cells, and IL-32 protein was measured after 20 h of incubation with the indicated concentrations of IFN- γ (ng/ml) or vehicle (constitutive). Means \pm SEM are shown; $n = 4$. * $p < 0.05$, *** $p < 0.001$ for scrambled versus siIL-32–transfected conditions. Percent-decreases in IL-32 protein abundance conferred by siIL-32 are indicated above the empty bars. *B*, After transfection, WISH cells were treated with 100 μ g/ml poly-IC or with 5 μ g/ml poly(U) for 20 h, followed by infection with VSV. LDH was assessed 24 h postinfection. The graph shows means of percent differences in LDH comparing scr with siIL-32 \pm SEM. $n = 5$. * $p < 0.05$, ** $p < 0.01$ for scr vs siIL-32.

FIGURE 6. IL-32 augments the antiviral response to HSV-2. *A*, Mean percent decrease in LDH in Vero cells stimulated with the indicated concentrations of IL-32 γ (ng/ml), then infected with HSV-2 for 48 h. HSV-2 alone is set at zero. Means \pm SEM; $n = 4$. * $p < 0.05$, ** $p < 0.01$ for control (baseline) versus IL-32 γ -stimulated conditions. *Inset*, LDH release from Vero cells after 48 h of infection with different HSV-2 concentrations. Means \pm SEM are shown, $n = 2$. In *B*, HUVEC were transfected with siRNA to IL-32 or scr, incubated with poly-IC (100 μ g/ml) for 20 h, and then infected with HSV-2. Means of percent differences in LDH comparing scr with siIL-32 \pm SEM are shown; $n = 4$. * $p < 0.05$. *C*, Fold change in HSV-2 DNA normalized to total DNA \pm SEM from siRNA-transfected HUVEC infected with HSV for 24 h. Normalized C_T values in scrambled-transfected cells were set at 1. $n = 3$; *** $p < 0.001$ for scr versus siIL-32.

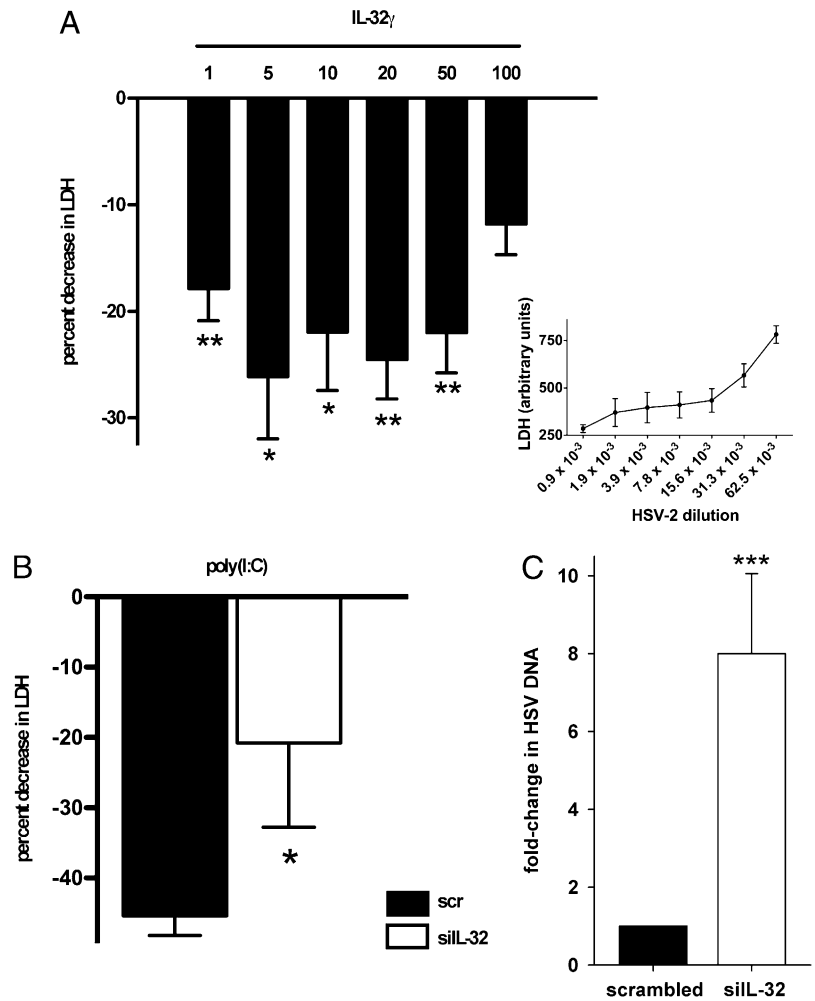


Fig. 8 provides an overview of the working hypothesis regarding the role of IL-32 in the immune response to DNA and RNA viruses based on the data presented above.

Discussion

When we first set out to investigate the role of IL-32 in viral infections, we expected this cytokine to increase the proliferation of viruses. This assumption was based on the proinflammatory properties of IL-32, which comprise signaling via the p38-MAPK, NF- κ B, and AP-1 pathways to upregulate IL-1 β , IL-6, IL-8, and TNF (1, 2). Under most circumstances, cytokines with similar activities stimulate viral reproduction, but not so for IL-32. Silencing of endogenous IL-32 in cells acutely or latently infected with HIV-1 resulted in up to 4-fold increases in the production of this virus, despite the fact that levels of IL-1 α , IL-6, IL-8, and TNF as well as the activity of NF- κ B and AP-1 in the same cultures were considerably reduced (2). These seemingly contradictory findings were explained by three key activities of IL-32: 1) this cytokine augmented the expression of type I and II IFNs, the prototypic antiviral cytokines (22); 2) silencing of IL-32 reduced the production of ligands of the HIV-1 coreceptors CCR5 and CXCR4 (2, 23); and 3) of Th1 cytokines (2), which are important during anti-HIV responses (2, 24, 25). Another group confirmed that IL-32 acts against HIV-1 in a different setting and that the abundance of IL-32 is regulated in human HIV infection (10).

We began our study by characterizing production and regulation of IL-32 in the well-established WISH-VSV bioassay (6). Baseline abundance of IL-32 in these cells was comparable to that in lung

epithelial A549 cells (reported in Ref. 1). Our results concerning regulation were also consistent with previous studies, as IFN- γ (1) and IL-1 β (26) increased IL-32 production in WISH cells. Next, we explored whether treatment with the synthetic analogs of viral ds- and ssRNA, poly-IC, and poly(U) affected the abundance of IL-32. These analogs are recognized by TLRs and signal via the NF- κ B and IRF-3/7 (27, 28) signaling pathways. Viral ds- and ssRNA strongly induce the production of antiviral cytokines, such as type I (IFN- α/β) (29) and III (IFN- λ) IFNs (30). Hence, the findings that both poly-IC and poly(U) increased the production of IL-32 as well as that the IL-32 promoter contains IRF consensus sites (although the functionality of these sites remains to be determined) underscore the importance of this cytokine in the immune response to viruses.

Having established that IL-32 is present and regulated by cytokines and viral components in WISH cells, we explored whether this cytokine imparted protection from the cytopathic effects of VSV infection. Indeed, treatment with exogenous IL-32 γ resulted in a marked, dose-dependent increase in viable WISH cells. Whereas this finding was consistent with the suppression of HIV-1 by IL-32 γ in U1 cells (2) and with antiviral activity of this cytokine against influenza A (13), we unexpectedly observed that when IL-32 γ concentrations were increased beyond 10–20 ng/ml, the protective activity progressively decreased. This biphasic fashion of protection was confirmed by an additional assay in which we quantified leakage of LDH from dying and dead cells. The cause for this biphasic pattern remains to be determined; however, one may speculate that, at higher concentrations, the

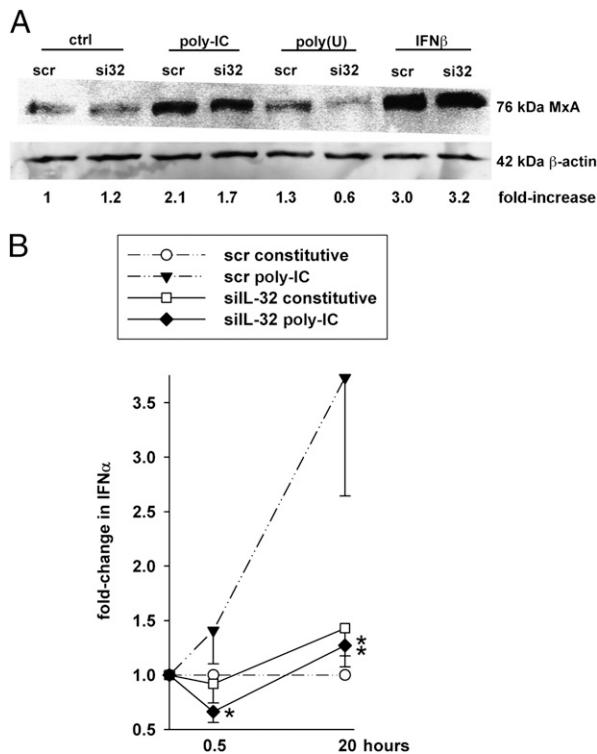


FIGURE 7. The presence of endogenous IL-32 is required for the production of antiviral regulators. In *A*, PBMC were transfected with siRNA to IL-32 or scrambled, then stimulated for 20 h with vehicle (ctrl), poly-IC (100 μ g/ml), poly(U) (5 μ g/ml), or IFN- β (2 ng/ml). One immunoblot representative for three blots with similar results from PBMC lysates stained against MxA is shown with multiple stimuli. The fold increase in MxA protein abundance compared with unstimulated, scr-transfected conditions as assessed by densitometry is indicated below the blot. Shown in *B* are the mean fold changes in IFN- α production \pm SEM in PBMC transfected with siRNA to IL-32 or scrambled and stimulated for the indicated periods of time with 100 μ g/ml poly-IC or vehicle. $n = 5$ donors. * $p < 0.05$, ** $p < 0.01$ for scrambled versus siIL-32.

induction of cytotoxic TNF (1, 31) or yet another proapoptotic mediator by IL-32 may supersede the protective effects in this model. In fact, PKR is known to induce apoptosis (32) and may play a role in this regard.

We hypothesized that treatment with IL-32 γ may upregulate other cytokines in the WISH cell cultures by which the protection from VSV may be mediated. But this was not the case, as the abundance of IL-1 α , IFN- α , and IFN- β , as well as of the effector cytokine IL-6, did not change with IL-32 γ -stimulation. [WISH cells are capable of producing these cytokines, but not IL-1 β and IFN- γ (33).] Because IFNs can protect WISH cells from VSV-induced cytopathic effects at low concentrations, we used sol-IFN- α / β R to block the activity of extracellular type I IFNs and observed a moderate decrease in the protection afforded by exogenous IL-32 γ . This result supports the conclusion that IL-32 indeed possesses IFN-independent antiviral properties. The finding that the antiviral program initiated by IL-32 γ appeared to be more transient than that triggered by IFNs (as shown by time-course analysis of eIF-2 α phosphorylation with a return to near baseline 24 h after IL-32 γ stimulation) also favors this hypothesis.

After characterizing the effects of exogenously added IL-32, we sought to establish causality for the role of IL-32 in antiviral responses. Unexpectedly and in contrast to PBMC (2) and endothelial cells (5), neither constitutive nor inducible IL-1 α , IL-6, IL-8, TNF, IFN- γ , or IFN- α / β were affected by changes in IL-32

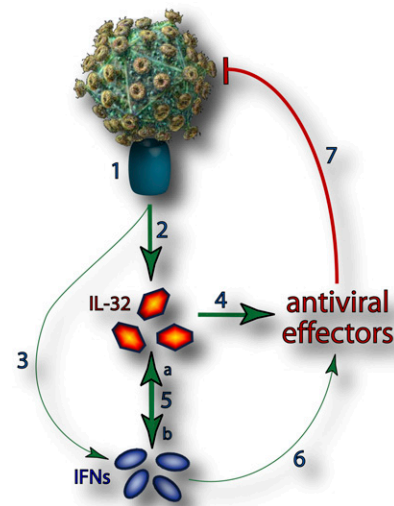


FIGURE 8. Working hypothesis for the mechanism of action of the antiviral properties of IL-32. Green arrows represent upregulation; the red connector symbolizes inhibition. Bold arrows show findings presented in this paper; thin arrows indicate that an effect was known previously (1). Recognition of a virus. A role for IL-32 in this process is possible, but currently unknown. IL-32 is induced (2) and, independent of IFNs (4), boosts cellular antiviral activity by triggering the PKR-eIF-2 α , the MxA, and possibly other pathways. Moreover, IL-32 is a crucial component of virally induced IFN- α (5*b*) and IFN- γ (Ref. 2) expression. However, some IFN is produced in the absence of IL-32 (3). 5*a*, IFNs can increase the production of IL-32, constituting a self-amplification cycle (5*a*, 5*b*) that initiates and sustains the antiviral machinery of the cell (4, 6, 7).

abundance. Although we cannot explain the absence of changes in cytokine levels at this time, this observation rules out that the antiviral effects of IL-32 are mediated by any of these cytokines. In contrast and consistent with the results obtained with exogenous IL-32 γ , silencing of endogenous IL-32 resulted in a marked reduction of the response to ss- and dsRNA viruses. Yet more importantly, one of the most impressive findings of this study was observed in PBMC, in which the induction of IFN- α was completely abolished when IL-32 was silenced. This observation suggests that IL-32 is crucial in the pathway that is initiated by dsRNA viruses and results in production of IFN- α .

Viruses trigger IFN production through several pathways. For RNA viruses, the detection event leads to phosphorylation and nuclear translocation of IRF-3 when detected by TLR3 (34) and IRF-3/7 via detection by RIG-I and/or MDA-5 (35, 36). These signals result in the production of type I and III IFNs (30, 37), which subsequently trigger the activation of IFN-stimulated genes such as PKR and MxA, as well as further upregulation of pattern recognition receptors (22). The signaling pathways of RNA and DNA viruses converge at the activation of NF- κ B and IRF-3, which subsequently elicit production of type I IFNs (38–40). Much less is known about the upstream events that lead to defense against DNA viruses, although recent studies provided evidence for the involvement of TLR 9 (27), DAI (39), AIM-2 (8), and RNA polymerase III (9). Therefore, the question of whether the protective properties of IL-32 pertain to DNA viruses is very intriguing. And indeed, consistent with the VSV-WISH model, exogenous IL-32 γ imparted a biphasic protection of Vero cells from HSV-2-induced cytopathic effects. More importantly, knockdown of IL-32 in HUVEC resulted in a 5- to 8-fold increase in HSV-2 viral load by LDH assay as well as by direct quantitative PCR analysis. To our knowledge, such regulation has not been reported for any cytokine except for IFNs.

To decipher the intricate interplay among IL-32 and IFNs, RNA and DNA viruses, and their synthetic mimetics poly-IC and poly (U), we explored the role of PKR-eIF-2 α and MxA, which are two prominent effectors in the antiviral repertoire of various cell types (41, 42). Upon reduction of IL-32 in PBMC, the production of poly-IC- as well as poly(U)-stimulated MxA was decreased considerably, suggesting that MxA may be one of the tools used by IL-32 to combat viruses. Regarding the regulation of PKR by exogenous IL-32 γ , we observed a biphasic pattern consistent with the IL-32-mediated protection of WISH and Vero cells from VSV and HSV-2, respectively. Using the fact that PKR phosphorylates and thus activates eIF-2 α (42), we provided evidence for a functional consequence of the induction of PKR by IL-32 by showing that IL-32 γ stimulation resulted in a rapid phosphorylation of eIF-2 α . This set of experiments provides mechanistic evidence for the antiviral properties of IL-32. In addition, the rapid nature of the effect suggests that IL-32 acts pointedly through the PKR-eIF-2 α pathway, as opposed to requiring the transcription and translation of an intermediate such as IFNs. In contrast to a previous study (12), NO and iNOS did not appear to play a role in the antiviral signaling of IL-32. This observation is likely due to different experimental approaches and opens this field to further investigation.

Collectively, these data identify IL-32 as a mediator that is crucial for the mounting of an efficient response to viral infection. As summarized in Fig. 8, we have explored the interplay between IL-32 and IFNs: not only does IL-32 upregulate the production of IFN- α/β (Fig. 8, 5), but it also controls their induction by viral PAMPs (Fig. 8, 1, 2, 5). Beyond the intermediate role in IFN regulation, we revealed that IL-32 controls key proteins that are directly involved in the antiviral response (Fig. 8, 4). By activating the PKR-eIF-2 α pathway, IL-32 likely suppresses the initiation of viral protein translation and furthermore may contribute to apoptosis of infected cells. Via upregulation of MxA, IL-32 may confer the neutralization of viral components (e.g., through the deactivation of viral nucleocapsid structures). We also showed that without IL-32, WISH and Vero cells as well as HUVEC are more susceptible to VSV- or HSV-2-induced cell death, respectively, and that HSV-2 thrives in an IL-32-deficient environment (absence of inhibition as in Fig. 8, 7). This study lays the foundation for a possible future therapeutic use of IL-32 or its signaling pathways in viral diseases in general and as a topical agent for HSV infections in particular.

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Disclosures

The authors have no financial conflicts of interest.

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