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FIG. 1A

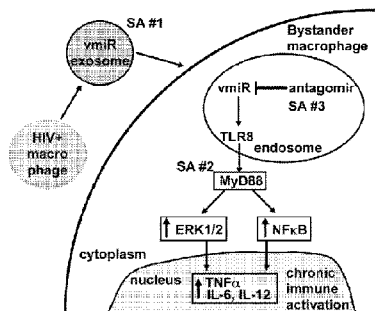
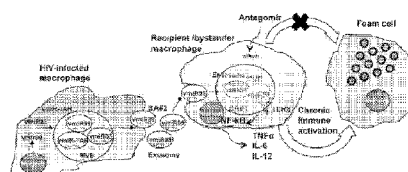


FIG. 1B



(57) Abstract: The invention provides compositions and methods for treating HIV-associated chronic immune activation in HIV-positive patients, or for preventing the same in patients at risk of being infected by HIV. The compositions of the invention include antagomirs that antagonize the function of certain HIV-derived viral microRNA (vmiR), which vmiR may lead to chronic immune activation in HIV-positive patients by non-canonical activation of certain pro-inflammatory cytokine pathways.

COMPOSITIONS AND METHODS FOR TREATING HIV-1-ASSOCIATED CHRONIC IMMUNE ACTIVATION

REFERENCE TO RELATED APPLICATIONS

This application claims priority to and the benefit of the filing date U.S. provisional application Nos. 61/824,764, filed on May 17, 2013, 61/876,832, filed on September 12, 2013, and 61/933,393, filed on January 30, 2014, the entire content of each of the three applications are incorporated herein by reference.

GOVERNMENT SUPPORT

This invention was made with U.S. government support under Grant No. R01-HL092811, awarded by the National Heart, Lung, and Blood Institute (NHLBI). The U.S. government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Despite effective highly active antiretroviral therapy (HAART), chronic infection in AIDS patients is becoming increasingly frequent, yet the stimuli inducing inflammation and the mechanisms accounting for its persistence remain incompletely understood.

HIV-associated chronic immune activation is a multifactorial phenomenon that involves a persistent and aberrant activation of immune cell types, increased plasma and tissue levels of pro-inflammatory mediators and high levels of activation-induced lymphocyte apoptosis. The causes of HIV-induced chronic immune activation are not fully defined, but likely include direct effects of viral proteins and nucleic acids, innate and adaptive viral responses to viral antigens, bystander activation of immune cells due to high levels of pro-inflammatory mediators, and translocation of microbial TLR ligands from the gut to the systemic circulation.

A key role for chronic immune activation in HIV pathogenesis is indicated since natural hosts of simian immunodeficiency virus (SIV) fail to develop immunodeficiency and AIDS despite high levels of virus replication, but surprisingly low levels of immune activation during the chronic stage of infection. In contrast, SIV infection of rhesus macaques and other non-natural hosts results in high levels of systemic immune activation, CD4 T-cell depletion and rapid progression to AIDS.

MicroRNAs (miRNAs) are increasingly recognized as important regulators of a wide range of cellular functions, such as proliferation, differentiation, metabolism, apoptosis and tumor progression. miRNAs are 18-22 nucleotides (nts) in length that regulate gene expression by binding to mRNAs leading to translational blockade or mRNA degradation.

Exosomes are lipid bilayer vesicles of 50-100 nm that form intracellularly upon inward invagination of endosome membranes and generated as intraluminal vesicles by reverse budding of membrane multivesicular bodies (MVBs). Fusion of MVBs limiting membrane with the plasma membrane triggers the release of exosomes into the extracellular milieu. Exosomes incorporate mRNAs, miRNAs and proteins that induce responses by target cells. Furthermore, exosomes internalized by dendritic cells (DC) localize to endosomes. Exosomes bearing miRNAs play a role in intercellular communication, although evidence is limited. Whether miRNAs are secreted in physiologically relevant amounts, and whether exosome-associated miRNAs influence canonical miRNA gene repression pathways or directly influence bystander immune cell signal transduction pathways has not been established.

Microsomes are vesicle-like re-formed pieces of the endoplasmic reticulum (ER). Microsomes can be concentrated and separated from other cellular debris by differential centrifugation when eukaryotic cells are broken-up. Microsomes usually have a reddish brown color, due to the presence of the iron-containing co-factor, heme (haem).

The canonical function of cellular and viral miRNAs is to control gene expression by repressing the translation of mRNAs into protein, a tightly regulated process in healthy cells but is dysregulated in cancerous and virus-infected cells. Epstein Barr virus (EBV) miRNAs induce a dose-dependent repression of an immunoregulatory gene *CXCL11/ITAC*, inducing EBV-associated lymphomas. HIV encodes low levels of vmiRs that are generated in HIV-infected cells, but a recent report did not detect HIV-1 encode miRNAs. To date, only four HIV-encoded miRNAs are identified and mapped to HIV TAR, Env, Nef, and U3 regions. In general, HIV-1 vmiRs seem to target viral transcripts.

In summary, HIV-associated chronic immune activation promotes AIDS pathogenesis, and contributes to the development of complications such as atherosclerosis, malignancy, and opportunistic infections. However, the mechanism(s) responsible for chronic immune activation remain incompletely understood. Thus there is a need for treating chronic immune activation associated with HIV disease.

SUMMARY OF THE INVENTION

In one aspect, the invention provides an oligonucleotide that inhibits binding of an endosomal (as opposed to a surface) TLR, such as TLR8 (Toll-like Receptor 8), to a microRNA encoded by an HIV-1 virus (vmiR), and/or inhibits activation of the TLR8 by the vmiR.

In certain embodiments, binding of TLR8 to the vmiR activates TLR8.

In certain embodiments, the vmiR is GU-rich (*e.g.*, $\geq 70\%$ GU).

In certain embodiments, the vmiR comprises a 7- or 8-nucleotide sequence consisting of at most one non-G non-U nucleotide.

In certain embodiments, the vmiR is any one of SEQ ID NOs: 1-3 (*e.g.*, 1-2) and 9-14.

In certain embodiments, the oligonucleotide inhibits activation of TLR8 by binding to / hybridizing with the vmiR.

In certain embodiments, the oligonucleotide binds to / hybridizes with the vmiR at a GU-rich region of the vmiR.

In certain embodiments, the oligonucleotide binds to / hybridizes with the vmiR with perfect base-pairing.

In certain embodiments, the oligonucleotide binds to / hybridizes with the vmiR with mismatch base-pairing.

In certain embodiments, the oligonucleotide is about 10-12 nucleotides in length, about 12-16 nucleotides in length, about 18-25 nucleotides in length, about 20, 21, or 22 nucleotides in length, or about 10-25 nucleotides in length.

In certain embodiments, the oligonucleotide comprises a modified sugar moiety (*e.g.*, 2-O-Me), a modified base moiety (*e.g.*, nebularine or xanthosine nucleotide), a modified inter-sugar linkage (*e.g.*, phosphorothioate), or combinations thereof.

In certain embodiments, the oligonucleotide comprises a locked nucleic acid (LNATM), a deoxyribonucleic acid (DNA), a ribonucleic acid (RNA), or a combination thereof.

In certain embodiments, the LNA base-pairs with a G or a U.

In certain embodiments, the HIV-1 virus is a Group M virus.

In certain embodiments, the HIV virus is of subtype B.

Another aspect of the invention provides a pharmaceutical composition comprising an oligonucleotide of the invention, or a polynucleotide encoding the oligonucleotide of the invention, and a pharmaceutically acceptable carrier and/or excipient.

In certain embodiments, the pharmaceutical composition comprises the oligonucleotide of the invention encompassed within an exosome or a microsome.

Another aspect of the invention provides a method of treating an inflammatory response in an individual infected by an HIV-1 virus, the method comprising administering to the individual a therapeutically effective amount of a pharmaceutical composition of the invention.

In certain embodiments, the inflammatory response is a symptom of chronic immune activation.

In certain embodiments, the inflammatory response is a symptom of an HIV-associated lung disease, atherosclerosis, a neurodegenerative disease, a malignancy, or an opportunistic infection.

In certain embodiments, the inflammatory response is induced by TLR8 activation.

In certain embodiments, TLR8 activation leads to increased activity of ERK1/2, increased activity of NF κ B, increased activity of a pro-inflammatory cytokine, and/or increased expression of an inflammatory marker.

In certain embodiments, the pro-inflammatory cytokine comprises TNF α , IL-6, or IL-12.

In certain embodiments, the inflammatory marker comprises VCAM-1 or ICAM-1.

In certain embodiments, the individual receives concurrent highly active antiretroviral therapy (HAART), has received HAART, or will receive HAART.

Another aspect of the invention provides a method of preventing or delaying the onset of an inflammatory response in an individual either suspected of or at high risk of being infected by an HIV-1 virus, the method comprising administering to the individual a prophylactically effective amount of a pharmaceutical composition of the invention.

Another aspect of the invention provides a method of inhibiting HIV elicited chronic immune activation in an individual infected by an HIV-1 virus, the method comprising administering to the individual a therapeutically effective amount of a pharmaceutical composition of the invention.

In certain embodiments, the HIV elicited chronic immune activation is inhibited by reducing differentiation into foam cells.

Another aspect of the invention provides a method of detecting the presence of HIV-1 infection in an individual, the method comprising isolating an exosome and/or a microsome from the individual and detecting the presence of a microRNA encoded by an HIV-1 virus (vmiR) in the exosome or microsome.

In certain embodiments, the exosome or microsome is isolated from serum of the individual.

In certain embodiments, the method further comprises quantitating the vmiR.

Another aspect of the invention provides an isolated microRNA or a cDNA thereof, wherein the microRNA is encoded by an HIV-1 virus (vmiR), and wherein the vmiR: (1) binds TLR8; (2) activates TLR8; (3) is GU-rich (*e.g.*, $\geq 70\%$ GU); (4) comprises a 7- or 8-nucleotide sequence consisting of at most one non-G non-U nucleotide; (5) is any one of SEQ ID NOs: 1-3 (*e.g.*, 1-2), 9-14, or any cloned vmiR sequences shown in Figure 11 or similar sequences (including any extension sequences of HIV origin, any intervening polyA sequences, and Universal Tag sequences, if present); and/or (6) is about 18-25 nucleotides in length, or about 20, 21, or 22 nucleotides in length, not counting any intervening polyA sequences and “universal tag” sequences (and optionally not counting any extension sequences of HIV origin). In other words, for sequences comprising extension sequences of HIV origin, polyA sequences, and/or Universal Tag sequences, polyA sequences and Universal Tag sequences (and optionally extension sequences) are not taken into account for (3) and (6). The universal tag sequence is from Exiqon’s miRCURY LNA PCR system (see below).

Another aspect of the invention provides an isolated pri-miRNA of the vmiR of the invention, or a cDNA thereof.

In certain embodiments, the pri-miRNA is in complex with Drosha / Pasha.

Another aspect of the invention provides an isolated pre-miRNA of the vmiR of the invention, or a cDNA thereof.

In certain embodiments, the pre-miRNA is in complex with Exportin-5 or Dicer.

Another aspect of the invention provides an isolated microRNA-protein complex, wherein the microRNA is the vmiR of the invention, and is in complex with an Argonaute protein (*e.g.*, AGO1, AGO2, AGO3, AGO4).

Another aspect of the invention provides an isolated exosome or microsome comprising a microRNA encoded by an HIV-1 virus (vmiR). In certain embodiments, the microRNA is not TAR miRNA.

It is contemplated that any embodiments described herein, including embodiments described in the examples and figures, and embodiments described under different aspects of the invention, can be combined with any one or more other embodiments where applicable.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic drawing showing one possible mechanism through which HIV-derived vmiR activates by-stander cells in an HIV-1 infected individual. Specifically, HIV-1 infected macrophage releases vmiR-containing exosomes, which delivers the encompassed vmiR to a previously uninfected by-stander cell (*e.g.*, a healthy macrophage). Inside the endosome of the by-stander macrophage, vmiR binds and activates a Toll-like receptor TLR8, which in turn produces an inflammatory response through a signaling cascade that may include MyD88, Erk1/2 and NFκB, which may activate the expression of pro-inflammatory cytokines such as TNFα, IL-6, and/or IL-12, leading to chronic immune activation in the HIV-1 positive individual.

Figure 1B shows a model of HIV miRNAs induction of foam cell in HIV+ individual. According to the model, HIV-derived vmiRs directly activate macrophage TLRs to induce pro-inflammatory cytokine release in non-canonical (non-RNAi) manner. Exosomes bearing HIV miRNA targeted to integrins deliver HIV miRNA to macrophages. vmiRs: HIV-derived vmiRs; MVB: multivesicular body.

Figure 2 shows that HIV-encoded miRNAs are produced in HIV-infected macrophages and induce TNFα release in alveolar macrophage (AM). Specifically, Figure 2A shows HIV replication in AM (obtained from healthy and asymptomatic HIV+ volunteers by bronchoalveolar lavage through a BIDMC institutional review board approved protocol and isolated as described in Tachado *et al.*, *Blood*, 115:3606-3615, 2010, incorporated herein by reference). AM were exposed to HIV particles, Ba-L strain (10 ng/0.1 mL Gag p24/10⁶ cells) for 3 hrs. and washed. p24 levels were assayed by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions (see Koziel *et al.*, *Am. J. Respir. Crit. Care Med.*, 160(6):2048-2055, 1999, incorporated herein by reference). HIV-1 p24 antigen ELISA was from Zeptometrix (Franklin MA). Data presented are AM infected with HIV-1 done in duplicate (n=4 subjects). Detection of viral miRs isolated from

HIV+AM with and without PMA, Mock infected and 15-day post-infection *in vitro*. Figure 2B shows miRNA copy numbers in cell lines U937 (American Type Culture Collection) and HIV+ U1 (HIV-infected U937 subclone; AIDS Research and Reference Reagent Program; Bethesda, Maryland) with and without PMA activation. Figure 2C shows absolute quantitation of miRs, which was standardized using synthetic miR oligos in the miRCURY LNA Universal RT microRNA PCR method (Exiqon) on an ABI 7500 Real Time PCR system. Data shown represent one experiment done in duplicate (n=4 subjects from each group). Figure 2D shows dose-dependent induction of TNF α by viral miRs in AM. AM were treated with different concentrations ($\mu\text{g/mL}$) of viral miRs and incubated for 24 hrs. TNF α released into the medium was assayed by ELISA. Data shown are representative of three independent experiments done in triplicate with similar results. Figure 2F shows an updated version of Figure 2D. *, p = <0.05 compared to unstimulated. N.S.: non-significant. Figure 2E shows miRNA standard curve generated from serially diluted synthetic oligonucleotides.

Figure 3 shows that exosomes from HIV-infected AM are enriched with HIV-derived miRNAs. Exosomes were precipitated from conditioned media using ExoQuick-TC polymer (System Biosciences, Mountain View, CA), and RNA extracted using SeraMir Exosome RNA Purification Columns (System Biosciences, Mountain View, CA). Figure 3A shows miRNA copy numbers in HIV+AM with and without PMA, mock infected and 15 days post HIV infection. Figure 3B shows miRNA copy numbers in U937 cells and HIV+U1 cells with and without PMA. Detection of viral miRs isolated from exosomes was done in duplicate (n=4 subjects) from each group. Figure 3C shows Western blot detection of the exosome marker CD63 in purified exosomes from conditioned media.

Figure 4A and 4B show that exosomal HIV-derived miRNAs are detected in serum from HIV-infected subjects. Exosomes were precipitated from serum of healthy and chronically HIV-infected subjects using SeraMir Exosome RNA Purification columns (Systems Biosciences, Mountain View, CA). Detection of viral miRs isolated from exosomes was done in duplicate (n=1 healthy subject; 3 HIV⁺ subjects). Figure 4A shows results from 3 HIV+ subjects and a healthy control. Figure 4B shows updated results from 14 samples obtained from 13 HIV+ subjects and a healthy control. Results for Figure 4B were obtained using archived human sera from consenting asymptomatic HIV+ persons with peripheral CD4⁺ T-lymphocytes counts < 200 cells/mm³ as detailed (Koziel *et al.*, *Am. J. Respir. Crit. Care Med.*, 160(6):2048-2055, 1999), and were available for exosome

preparation and HIV miRNA detection.

Figure 5 shows biogenesis of HIV-encoded miRNAs. Figure 5A shows that small RNAs processed from HIV-1 LTR region observed by SOLiD Deep Sequencing. Left peak (blue) shows small RNAs derived from TAR stem (miR TAR). Right peak (blue) shows a hotspot for small RNAs derived from R and U5 stem region. The GU-rich tract (right peak, green) encodes a family of viral miRs including vmiR88 and vmiR99. Modified from Shopman *et al.*, *Nucleic Acid Res.* 40:414, 2012. shRNA mirs are intermediates in biogenesis of mature vmiRs. Figure 5B: shRNA mirs are intermediates in biogenesis of mature vmiRs. shRNA reported for 43/9175 TAR (left). UNAFold software predicts folding of shRNAs vmiR88 (middle) and vmiR99 (right), which suggests the structures of intermediates in the biogenesis of the mature vmiR-TAR (black rectangle), vmiR88 (blue rectangle) and vmiR99 (red rectangle). UNAFold's thermodynamic calculations predict that all three shRNAs fold spontaneously ($\Delta G < 0$) into stable hairpins (high melting temperature, $T_M > 53.8^\circ\text{C}$ in 1M Na^+).

Figure 6 shows that HIV-encoded induction of $\text{TNF}\alpha$ in AM is dependent on TLR8. AM were pre-treated with siRNA targeted to human TLR8 (siTLR8) or non-silencing siRNA control (N.S). Cells were challenged with 1 $\mu\text{g/mL}$ vmiR99 and incubated for 24 hrs. Cell-free supernatant was assayed for $\text{TNF}\alpha$ by ELISA. Data shown are mean values + S.E. from four independent experiments done in triplicate (n=4 subjects).

Figure 7 shows that induction of $\text{TNF}\alpha$ by ssRNA40 is inhibited by ssRNA41 in a dose-dependent manner, and RNA40 forms a duplex with RNA41. In Figure 7A, monocyte-derived macrophages (MDM) were pre-treated with different concentrations of RNA40 for 1 hr., followed by treatment with ssRNA41 and incubation for 24 hrs. $\text{TNF}\alpha$ released into the medium was assayed by ELISA. Data shown are mean $\pm$ S. E. from 4 independent experiments in triplicate (n=4 subjects). *, $p < 0.05$ compared to RNA40 alone. HIV RNA40, GU-rich RNA; ssRNA41, control ssRNA with A replacing U. LA, lipid A (10 $\mu\text{g/mL}$) is positive control. Figure 7B shows that in the dsRNA40*RNA41 duplex, fluorescent dye (F) is quenched due to proximity of annealed quencher (Q). After melting, quencher strand dissociates freeing a highly-fluorescent ssRNA40-F strand. Figure 7C shows theoretical annealing/melting of fluorescent ssRNA40-F with excess ssRNA41 Q quencher strand. Figure 7D: Observed annealing of fluorescent ssRNA40-F with ssRNA41-Q in physiological buffer. Dashed line is the first derivative. Vertical line (red) at midpoint of thermal transition indicates melting temperature: $T_m = 35.6^\circ\text{C}$. Annealing was recorded using an ABI 7900HT

Fast Real Time PCR System.

Figure 8 shows that induction of TNF α by HIV-encoded miRNA is inhibited by antagomir99 in a dose-dependent manner. AM were pre-treated with different doses of antagomir99 for 1 hr. followed by 0.5 μ g/mL vmiR99 and incubated for 24 hrs. Cell-free supernatants were analyzed for TNF α by ELISA. Data shown are mean $\pm$ S.E. from four independent experiments in triplicate (n=4 subjects) *,p<0.05; **,p<0.01 when compared with unstimulated control (US).

Figure 9 shows that vmiRs of the GU-rich tract are highly conserved in HIV-1. Figure 9A shows that alignment of vmiR sequences of the GU-rich tract (*) is consistent with consensus genomic sequence from HIV-1, subtypes A-J, 533 isolates (**). Figure 9B shows that vmiR99 is 90-100% identical to 96% of HIV-1 sequences. GU tract, vmiR88, vmiR99, RNA40 (196, 201, 254, 272 genome sequences, respectively).

Figure 10 shows results of miRNA amplification using LNA-enhanced PCR primers for several HIV-1 miRs.

Figure 11 delineates the boundaries of mature viral miRNAs isolated from cell extracts and exosomal extracts. Sample cell extracts were *in vitro*-infected AM (healthy AM +HIV), HIV-positive U1 macrophages stimulated by PMA (U1 +PMA). Exosomal extracts were from exosomes of HIV+ human serum (HIV+ serum 10b). Total RNA was amplified by qRT-PCR, cloned into pCR4-TOPO vector, and DNA was sequenced. Sequences of vmiR88 and vmiR99 PCR products were aligned with sequences of plasmid (vector) and HIV-BaL strain.

Figure 12A shows that alignment of vmiR sequences of GU-rich tract are consistent with consensus genomic sequence from HIV-1 subtypes A-J, 533 isolates. In Figure 12B, vmiR99 is shown to be 90-100% identical to 96% of HIV-1 sequences. Sequences within the GU tract, vmiR88, vmiR99 and ssRNA40 were aligned with 196, 201, 254 and 272 genome sequences, respectively. In Figure 12C, genomic RNA of HIV-1 BaL strain was scanned for every 21-bp RNA segment and the distribution of base compositions (46.5 $\pm$ 11.8% G+U) is shown. VmiR-TAR is GU-poor (35%). VmiR88 and vmiR99 are GU-rich (71% and 76% G+U, respectively). Absolute quantitation of miRNAs was determined by Real Time RT-PCR. After first strand cDNA synthesis, amplification (Figs. 12D, 12F, 12H) and absolute quantitation (Figs. 12E, 12G, 12I) of vmiR-TAR (Figs. 12D-12E) vmiR88 (Figs. 12F-12G), vmiR99 (Figs. 12H-12I) and RNA40 (Fig. 12I) was standardized using synthetic miR oligonucleotides in the miRCURY LNA Universal RT microRNA PCR method (Exiqon) on

an ABI 7900HT FAST Real Time PCR system. ΔR_n is the change in normalized reporter fluorescence intensity. C_T is the threshold cycle where the amplification curve crosses the dashed horizontal line. Data depict a representative experiment done in duplicate.

Figure 13 shows that novel HIV-produced miRNAs are detected in HIV-infected human macrophages, and stimulate macrophage TNF α release *in vitro*. In Figure 13A, AM were exposed to HIV-1 particles, BaL strain (10 ng/0.1 mL Gag p24/10⁶ cells for 3h) and washed. HIV p24 levels were assayed by ELISA. Data presented are AM infected with HIV-1 done in duplicate ($n=4$ subjects). Quantitative PCR measurement of HIV miRNA from cell extracts of adherent (Figure 13B) human macrophage cell lines U937 and HIV+U1, and (Figure 13C) human primary alveolar macrophages (established *in vitro* HIV infection, or from asymptomatic HIV+ person), incubated in the absence or presence of PMA for 24 hr. Figure 13D: TNF α measurement (ELISA) in culture supernatants in AM from healthy volunteers following 24h incubation with HIV miRNA (vmiR-TAR, vmiR88, or vmiR99) over a concentration range (0.01 - 1.0 μ g/mL), lipid A (10 μ g/mL), or control GU-rich ssRNA40 or AU-rich ssRNA41. Figure 13E: TNF α measurement (ELISA) in culture supernatants from adherent human alveolar macrophages from healthy volunteers was treated with antagomir99 (1h) followed by vmiR99 (24h). At right, adherent AM were treated with pre-annealed antagomir99*vmiR99 duplex (**) for 24h. Figure 13F: TNF α measurement (ELISA) in culture supernatants from adherent human alveolar macrophages from healthy volunteers, in the presence of targeted TLR8 gene silencing (siTLR8) compared to control non-silencing RNAi (NS) following 24h incubation with novel HIV vmiR99. Data for each figure reflect a minimum of 4 experiments, performed in duplicate. *, $p < 0.05$.

Figure 14 shows that viral miRNAs stimulate THP-1 macrophages to release TNF α rapidly in a vmiR sequence-dependent manner, and release is inhibited by antagomirs. Figure 14A: THP-1 macrophages were treated with vmiR99 (1.0 μ g/mL) at the indicated time points (hr) or with Lipid A or ssRNA40 (24h). Conditioned medium was analyzed by ELISA. Total RNA was isolated from cell extracts, and expression of TNF (normalized by GAPD) was analyzed by qRT-PCR. Results are the average of three independent experiments done in duplicate. Figure 14B: THP-1 macrophages were pre-treated with antagomir (5.0 μ g/mL for 1h) followed by treatment with ssRNA40 (2.5 μ g/mL), vmiR88 (5.0 μ g/mL) or vmiR99 (5.0 μ g/mL for 24h), and conditioned medium analyzed by ELISA. Figure 14C: Sequence variants of vmiR88 and vmiR99 can elicit TNF α release by THP-1 macrophages. Variants of vmiR88 or vmiR99 were chemically synthesized by substituting

the uridine residues of U-rich motifs (boxed regions) for adenine residues. VmiRs and variants (5.0 $\mu\text{g/mL}$) were applied to cells for 24h. Supernatants of conditioned medium were assayed for TNF α by ELISA. *, $p < 0.05$.

Figure 15 shows that novel HIV-produced miRNAs are released by HIV-infected human macrophages and associated with exosomes *in vitro*, and detected in sera from asymptomatic HIV+ persons. Quantitative PCR measurement of HIV miRNA from exosomal preparation of cultured supernatants from adherent (Figure 15A) human macrophage cell lines U937 and HIV+U1, and (Figure 15B) human alveolar macrophages (established in vitro HIV infection, or from asymptomatic HIV+ person), incubated in the absence or presence of PMA for 24 hr. Western blot immediately beneath each bar graph demonstrates exosomal marker CD63 associated with corresponding sample. Data reflect a minimum of 4 experiments performed in duplicate. (Figure 15C) Quantitative PCR measurement of HIV miRNA in exosomal preparations from archived sera of asymptomatic HIV+ persons with peripheral blood CD4+ T-lymphocyte count $< 200 \text{ cells/mm}^3$. Data reflect measurements performed in duplicate. Results shown include exosome preparations isolated from HIV+ sera ($n=14$) sampled from HIV+ patients (numbered 1-13). Serum samples “10a” and “10b” were drawn on separate days from Patient 10. *, $p < 0.05$.

Figure 16 is a working model of HIV-produced miRNA activation of bystander cells in HIV+ persons. This figure provides a working model describing how HIV-produced miRNAs are encapsulated in exosomes and released from HIV-infected macrophages into the circulation. Host RNA polymerases transcribe HIV genomic RNA, which is then exported into the cytoplasm and packaged into infectious virions. However, some HIV transcripts can be processed in the host RNA interference pathway into mature viral miRNA. HIV miRNAs are packaged into multi-vesicular bodies and released by macrophages encapsulated in exosomes. Exosomes are disseminated either locally or systemically to be taken up by bystander macrophages, trafficking GU-rich vmiR88 and vmiR99 to the endosomal TLR8. Through a non-canonical function of miRNAs that is distinct from the well-established canonical role of miRNAs in RNA interference, vmiR88 and vmiR99 induce TLR8-mediated inflammatory signaling that releases TNF α leading to chronic immune activation.

Figure 17 shows that HIV-derived vmiRs (vmiR99 and vmiR88) stimulate human alveolar macrophage (AM) cells to differentiate into foam cells as measured by lipid accumulation (BODIPY 493/503 staining) with normalization for cell number by DAPI staining. Alveolar macrophages were plated and pretreated with different doses of

antagomir99 followed different doses of HIV vmiR99 and incubated for 24 hrs, and stained with BODIPY 493/503 and DAPI. Results are representative of four independent experiments with similar results. * $p < 0.05$ compared to unstimulated cells.

Figure 18A and 18B show HIV replication and foam cell formation in MDM. In Figure 18A, MDMs were exposed to HIV particles, Ba-L strain (10 ng/0.1 Gag p42/10⁶ cells for 3 hrs and washed. p24 levels were assayed by ELISA. In Figure 18B, MDMs were infected with HIV for 4 days and foam cell formation was analyzed by BODIPY 493/503 staining. Data shown is a representative experiment with similar results from four different healthy uninfected subjects. * $p < 0.01$ compared to mock infected control. “Fluorescence ratio” was calculated as the ratio of BODIPY fluorescence intensity to DAPI fluorescence intensity. The fluorescence ratio is a readout that measures lipid (BODIPY staining) and normalizes the signal to the number of cells (DAPI nuclear stain).

Figure 19 shows that HIV-derived ssRNA induces foam cell formation in a dose-dependent manner. MDMs were stimulated with different stimulants for 24 hrs and stained with either Oil Red O staining or BODIPY 493/503 staining. Conditions include unstimulated, ssRNA40, ssRNA41, ox-LDL, and Nef protein. Data shown is a representative experiment with similar results from four different healthy uninfected subjects. * $p < 0.01$, ** $p < 0.01$ compared to vehicle control (LyoVec; NS). ssRNA control (ssRNA 41) HIV-ssRNA (ssRNA 40).

Figure 20 shows that induction of foam cell formation by HIV-derived ssRNA is dependent on TNF α . MDMs were pre-treated with anti-TNF α for 1 hr, followed by ssRNA incubation for 24 hrs, and stained with either Oil Red O or BODIPY 493/503 staining. Data shown is a representative experiment with similar results from four different healthy uninfected subjects. * $p < 0.05$ compared to ssRNA control, TNF+IgG, ** $p < 0.01$ compared to ssRNA+IgG.

Figure 21 shows dependence of HIV-derived ssRNA-induced foam cell formation and TNF α in macrophages on endocytosis and endosomal acidification. MDMs were pretreated with 100 μ M chloroquine or 50 μ M dynasore for 1 hrs followed by incubation with 1 μ g/ml HIV-ssRNA for 24 hrs and stained with BODIPY and DAPI (Figure 21B). Cell free supernatant was analyzed for TNF α by ELISA and integrated fluorescence intensities for BODIPY in each cell of the confocal micrographs (Figure 21A). Data shown is a representative experiment with similar results from four different healthy uninfected subjects. * $p < 0.05$, ** $p < 0.01$ compared to vehicle control (LyoVec) or HIV-ssRNA alone. NS, non-

stimulated vehicle control (LyoVec); CQ, chloroquine; DY, dynasore.

Figure 22 shows an illustrative example of an exosome-based delivery system, which may be used to package miRs and the subject antagomirs into cell-homig exosomes for delivery to a target cell. Figure 22A shows an Adenovirus vector (AV) expressing an shRNA (vmiR88, vmiR99, miTAR, let etc.) and fusion protein tetraspanin-ligand-DsRed. The shRNA mir is processed into mature miRNA (miR) or antagomir. In Figure 22B, following MVB biogenesis, miRs are packaged into exosomes as cargo, and fusion protein is targeted to exosome membrane to display the homing ligand on the exosome surface. The exosome cartoon is adapted from BBA 1820:940-948 (2012).

Figure 23 shows dependence of foam cell formation in MDMs on TLR8 activation by HIV ssRNA. Figure 23A shows intracellular expression of TLR8 in MDMs. MDMs were incubated with PE-conjugated anti-TLR8 or isotype matched antibody control. Intracellular expression was determined by flow cytometry. Representative profiles were similar in four independent experiments (n=4 subjects). Figure 23B shows that functional silencing of human TLR8 leads to diminution of foam cell formation in MDMs. BODIPY staining of MDMs after pretreatment with TLR8 siRNA or nonsilencing control is shown. Cells were challenged with HIV ssRNA or ssRNA control for 24 hrs. Bar graph shows integrated fluorescence intensities of BODIPY per cell of the confocal micrographs. Results are representative of four independent experiments with similar results. **p<0.01 compared to NS siRNA + ssRNA41 control.

Figure 24 shows that induction of foam cell formation in MDMs by HIV ssRNA is dependent on MyD88. MDMs were pretreated with MyD88 siRNA and nonsilencing control. Cells were challenged with HIV ssRNA or ssRNA control, and incubated for 24 hrs. Cell free supernatants were assayed for TNF α by ELISA (Figure 24A) and BODIPY staining (data not shown). Western blot analysis of MyD88 after gene silencing with the use of MyD88 siRNA and nonsilencing siRNA control. β -actin was used to monitor loading after stripping the membrane (Figure 24B). Bar graph shows integrated fluorescence intensities of BODIPY per cell of the confocal micrographs (FIG. 24C). A representative blot shows results from one experiment with similar results obtained in four independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

1. Overview

The invention described herein provides novel isolated microRNA of viral origin

(vmiR), particularly of human immunodeficiency virus type 1 (HIV-1) origin, or cDNA thereof. The vmiRs of the invention originates from a GU-rich region of the HIV-1 long terminal repeat (LTR) region, and surprisingly possess the non-canonical function of binding to a Toll-like receptor, such as TLR8, and eliciting downstream pro-inflammatory responses as a result of receptor activation.

The invention described herein further provides antagomir oligonucleotides that inhibit the binding of such vmiRs to the Toll-like receptors, inhibit the activation of the Toll-like receptors, or otherwise antagonize the function of the Toll-like receptors. Such antagomirs, and pharmaceutical compositions comprising the antagomirs, may be used to treat or prevent a pathological condition associated with HIV-1 infection, such as chronic immune activation in HIV-positive subjects, or other HIV-associated conditions, such as lung diseases, atherosclerosis, neurodegenerative diseases, malignancy, or opportunistic infections.

The invention described herein further provides a novel approach to diagnose the presence of HIV infection, or to monitor the status of HIV infection (*e.g.*, before and/or after a treatment). This aspect of the invention is partly based on the surprising finding that certain HIV-1 encoded vmiRs accumulate in exosomes shed from HIV-infected host cells. Thus the presence and level of vmiRs in exosomes, such as exosomes in patient serum, may be indicative of disease status and may provide valuable indication for further treatment.

The invention described herein additionally provides methods of making the subject antagomirs and pharmaceutical compositions comprising the same, including an exosome-based delivery system for antagomirs, as well as methods of using the same.

With the inventions generally described herein, the sections below provide details for the various aspects of the invention.

2. *Antagomirs*

As used herein, “antagomirs” (also known as “anti-miRs”) include oligonucleotides that antagonize a function of a target microRNA (miR), such as by preventing the target microRNA from binding to a target molecule (*e.g.*, an mRNA or a Toll-like receptor). Thus an antagomir of the invention may bind to a target miR and prevent the silencing of a target mRNA of the miR, or bind to a target miR and prevent its non-canonical binding and activation of a Toll-like receptor, such as TLR8.

Alternatively or in addition, an antagomir of the invention may inhibit the function or activation of a Toll-like receptor that can be activated by a vmiR, such as vmiR88 or vmiR99

or variants / analogs thereof (or collectively “vmiR” hereinbelow). In this embodiment, the antagomir may directly antagonize the signaling of the Toll-like receptor activated by a vmiR, regardless of whether the antagomir is complementary, partially complementary, or non-complementary to the vmiR. In this embodiment, one antagomir may simultaneously inhibit Toll-like receptor signaling activated by two or more related or unrelated vmiR(s) (*e.g.*, vmiR88 and vmiR99), regardless of whether the antagomir is complementary, partially complementary, or non-complementary to one or more of said vmiR(s).

An antagomir of the invention may be a small oligonucleotide, *e.g.*, a synthetic DNA or RNA, with or without various modifications, as described in further details in a separate section. The antagomir oligonucleotide of the invention may be perfectly or imperfectly complementary (or, in some embodiments, non-complementary) to a target miRNA target (*e.g.*, vmiR88 or vmiR99). In certain embodiments, the antagomir of the invention may have one or more features that inhibits cleavage by AGO2 in the RISC complex (RNA-interference silencing complex). Such features may include mispairing at the cleavage site of AGO2, and/or modification in the oligonucleotide sequence (see modifications on the base, sugar, or phosphate backbone, and other modifications below), such as 2'-methoxy modifications and phosphorothioates, *etc.*

Antagomirs have been used to constitutively inhibit the activity of specific miRNAs. For example, antagomirs against miR-21 have been successfully used to inhibit fibrosis of heart (Adam *et al.*, *Basic Res. Cardiol.* 107(5):278, 2012) and lung (Pandit *et al.*, *Am. J. Respir. Crit. Care Med.* 182(2):220-229, 2010).

Thus one aspect of the invention provides an oligonucleotide that inhibits binding of a Toll-like receptor, such as TLR7 (Toll-like Receptor 7) or TLR8 (Toll-like Receptor 8), to a microRNA encoded by an HIV-1 virus (vmiR).

In a related aspect, the invention provides an oligonucleotide that inhibits activation of a Toll-like receptor, such as TLR7 (Toll-like Receptor 7) or TLR8 (Toll-like Receptor 8), by a microRNA, such as one encoded by an HIV-1 virus (vmiR) or a variant / analog thereof.

In certain embodiments, the oligonucleotide is substantially complementary (*e.g.*, at least about 90, 95, or 100%), partially complementary (*e.g.*, at least about 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85%), or non-complementary (*i.e.*, less than 30, 25, 20, 15, 10%) to the vmiR.

In certain embodiments, the vmiR is encoded by the Long Terminal Repeat (LTR) region of an HIV-1 virus.

In certain embodiments, the HIV-1 virus is a Group M virus. In other embodiments, the HIV-1 virus is a Group N, O, or P virus.

In certain embodiments, the HIV-1 virus is a Group M, subtype B virus. In other embodiments, the HIV-1 virus is a Group M, subtype A, C, D, E, F, G, H, I, J, or K virus.

In certain embodiments, the vmiR bound by the antagomir oligonucleotide of the invention binds to and activates TLR8. The activated TLR8 may elicit downstream signaling (see, for example, Figure 1) that leads to the production of pro-inflammatory cytokines, such as TNF α , IL-6, and/or IL-12.

In certain embodiments, the vmiR bound by the antagomir oligonucleotide of the invention is GU-rich, *e.g.*, $\geq 70\%$ GU, $\geq 75\%$ GU, $\geq 80\%$ GU, $\geq 85\%$ GU, $\geq 90\%$ GU, or $\geq 95\%$ GU. In certain embodiments, the antagomir oligonucleotide of the invention does not include antagomir that has GU percentage less than 70%.

The GU percentage of a given antagomir oligonucleotide of the invention can be calculated by determining the percentage of all G and U nucleotides over the total number of nucleotides in the oligonucleotide. For the purpose of this calculation, nucleotides modified based on G (*e.g.*, an rG with a 2'-O-methyl modification, or a LNA linkage, or a phosphorothiate backbone) or U (including T) are considered the same as G or U, respectively. Thus a 20-mer having a total of 14 G and U nucleotides has a GU percentage of 70%.

In certain embodiments, the vmiR bound by the antagomir oligonucleotide of the invention comprises a (stretch of) 5-, 6-, 7- or 8-nucleotide sequence consisting of at most one non-G non-U nucleotide. Thus the stretch of 5, 6, 7- or 8-nucleotide sequence may contain 0 or 1 non-G non-U nucleotide. Such stretch of vmiR nucleotide sequence may also be referred to as vmiR sequence elements, including Box 4-8 (UGCUU on vmiR88), Box 13-20 (UAGUGUGU on vmiR88 and vmiR99), and Box 26-31 (UCUGUU on vmiR99, also on RNA40). Such sequence elements are highly conserved among different strains of HIV viruses, particularly Box 13-20 and Box 26-31. See Fig. 9A.

As used herein, "non-G non-U nucleotide" includes those nucleotides that are not G and not U. Modified G or U (or T) are considered G or U, respectively. Thus the "non-G non-U nucleotide" may be a nucleotide with A or C base, or bases related to A or C, or based not related to either G or U/T.

In certain embodiments, the vmiR (RNA) bound by the antagomir oligonucleotide of the invention is represented by:

5'-GAGUGCUUCAAGUAGUGUGUG-3' (SEQ ID NO: 1, vmiR88);

5'-GUAGUGUGUGCCCGUCUGUUG-3' (SEQ ID NO: 2, vmiR99);

5'-GCCCGUCUGUUGUGUGACUC-3' (SEQ ID NO: 3, RNA40);

Certain variant sequences of vmiR88 include:

vmiR88 extended at the 3'-end by a 15-nt HIV RNA sequence, *i.e.*, 5'-GAGUGCUUCAAGUAGUGUGUGCCCGUCUGUUGUGUG-3' (SEQ ID NO: 9); or by a 12-nt HIV RNA sequence, *i.e.*, 5'-GAGUGCUUCAAGUAGUGUGUGCCCGUCUGUGUG-3' (SEQ ID NO: 10).

Certain variant sequences of vmiR99 include:

vmiR99 missing the 3' end G, *i.e.*, 5'-GUAGUGUGUGCCCGUCUGUU-3' (SEQ ID NO: 11); vmiR99 missing the most 3' end GUUG sequence, *i.e.*, 5'-GUAGUGUGUGCCCGUCU-3' (SEQ ID NO: 12); vmiR99 extended at the 3'-end by a 15-nt HIV RNA sequence 5'-GUAGUGUGUGCCCGUCUGUUGUGUGACUCUGUUG-3' (SEQ ID NO: 13); or by a 8-9 nt HIV RNA sequence 5'-GUAGUGUGUGCCCGUCUGUUGCCUCUGUUG-3' (SEQ ID NO: 14).

Additional variants of vmiR88 and vmiR99 may include any of the non-vector cloned sequences in Figure 11, including the above-described variant sequences, with or without the intervening polyA sequences, and with or without the "Universal Tag sequences."

In certain embodiments, the antagomir oligonucleotide of the invention is represented by:

5'-CACACACUACUUGAAGCACUC-3' (SEQ ID NO: 4, antagomiR88);

5'-CAACAGACGGGCACACACUAC-3' (SEQ ID NO: 5, antagomiR99);

5'-GAGUCACACAACAGACGGGC-3' (SEQ ID NO: 6, antagomir40);

5'-CACACAACAGACGGGCACACACUACUUGAAGCACUC-3' (SEQ ID NO: 15, antagomiR88 variant 1);

5'-CACACAGACGGGCACACACUACUUGAAGCACUC-3' (SEQ ID NO: 16, antagomiR88 variant 2);

5'-AACAGACGGGCACACACUAC-3' (SEQ ID NO: 17, antagomiR99 variant 1);

5'-AGACGGGCACACACUAC-3' (SEQ ID NO: 18, antagomiR99 variant 2);

5'-CAACAGAGGAGACGGGCACACACUAC-3' (SEQ ID NO: 19, antagomiR99 variant 3); and,

5'-CAACAGAGUCACACAACAGACGGGCACACACUAC-3' (SEQ ID NO: 20, antagomiR99 variant 4).

In certain embodiments, antagomirs of the invention also includes those of a pre-determined length, and comprising partly or entirely the extension sequences of HIV origin. In certain embodiments, the pre-determined length may be 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 nucleotides or more. In certain embodiments, the pre-determined length is shorter than the length required for Dicer cleavage, or the length required for a functional guide sequence in a RISC complex, or less than 20, 19, or 18 nucleotides.

In certain embodiments, antagomirs of the invention comprise a sequence complementary to one or more of vmiR sequence elements Box 4-8, Box 13-20, and Box 26-31, or homologous vmiR sequence elements thereof. In related embodiments, sequences of antagomirs of the invention may comprise non-complementary sequences (with respect to a vmiR) outside Box 4-8, Box 13-20, or Box 26-31.

In certain embodiments, the antagomir oligonucleotide of the invention is represented by:

5'-CACACACACACACACACACAC'3' (SEQ ID NO: 7, antagomirCA);

5'-ACACACACACACACACACACA'3' (SEQ ID NO: 8, antagomirAC).

An additional sequence related to RNA40, named RNA41, has every U in SEQ ID NO: 3 replaced by A. Example 13 shows that RNA40 and RNA41 forms a duplex based on fluorescent quenching data, and thus RNA41 functions as an antagomir (at least when in molar excess) to block RNA40 function.

In certain embodiments, the antagomir oligonucleotide of the invention is represented by an RNA having a sequence of any one of SEQ ID NOs: 4-6 (*e.g.*, 4-5), and 15-20. In certain embodiments, the antagomir oligonucleotide of the invention is represented by an oligonucleotide having DNA, RNA, modification thereof (*e.g.*, LNA or PNA *etc.*), and/or combinations thereof, and having a sequence of any one of SEQ ID NOs: 4-6 (*e.g.*, 4-5), and 15-20 (for example, in this embodiment, "G" in any of SEQ ID NOs: 4-6 (*e.g.*, 4-5) may represent RNA (rG), DNA (dG), or LNA with a G base).

For simplicity, as used herein below, unless specified explicitly, antagomir sequences, such as SEQ ID NO: 4, 5, 6, 15, 16, 17, 18, 19, or 20, may include all RNA sequences, DNA sequences, modifications thereof (*e.g.*, LNA or PNA, phosphorothioate backbone

modifications, or 2'-modifications at one or more positions), or combinations thereof (*e.g.*, hybrid nucleotides having DNA, RNA, LNA, *etc.*) having the specified bases shown in the SEQ ID NOs, and may not be limited to RNA without modification.

In certain embodiments, SEQ ID NO: 4, 5, 6, 15, 16, 17, 18, 19, or 20, comprises one (or more) modification(s) on a base, a sugar, a phosphate backbone, or a combination thereof. In certain embodiments, where two or more modifications are present, the modifications may be present on the same nucleotide or on different nucleotides.

In certain embodiments, all nucleotides in SEQ ID NO: 4, 5, 6, 15, 16, 17, 18, 19, or 20, are RNA having 2'-modification, such as 2'-O-methyl modification.

In certain embodiments, all nucleotides in SEQ ID NO: 4, 5, 6, 15, 16, 17, 18, 19, or 20, are RNA having backbone modification, such as phosphorothioate linkages.

In certain embodiments, all nucleotides in SEQ ID NO: 4, 5, 6, 15, 16, 17, 18, 19, or 20, are RNA having 2'-modification, such as 2'-O-methyl modification, and having backbone modification, such as phosphorothioate linkages.

In certain embodiments, SEQ ID NO: 4, 5, 6, 15, 16, 17, 18, 19, or 20, comprises one (or more) LNA modification(s). In certain embodiments, the LNA modification(s) appears on nucleotides base-pairing with the respective miR. In certain embodiments, oligonucleotides of the invention comprising LNA modifications have T_m that is at least about 10, 20, 30, 40, 50, 60, 70, 80, 90°C higher than counterpart oligonucleotides without any LNA modification.

In certain embodiments, the antagomir oligonucleotide of the invention is represented by an oligonucleotide comprising 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 continuous nucleotides of any one of SEQ ID NOs: 4-6 (*e.g.*, 4-5) and 15-20. In certain embodiments, the antagomir oligonucleotide of the invention has 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotides. In certain embodiments, the antagomir oligonucleotide of the invention is about 10-12 nucleotides in length, about 12-16 nucleotides in length, about 18-25 nucleotides in length, about 20, 21, or 22 nucleotides in length, or about 10-25 nucleotides in length.

In certain embodiments, the antagomir oligonucleotide of the invention is represented by an oligonucleotide comprising 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 continuous nucleotides of any one of SEQ ID NOs: 4-6 and 15-20, and may have 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotides, as appropriate.

For example, an antagomir oligonucleotide of the invention may have 25 nucleotides, 21 of which are identical to SEQ ID NO: 4 or 5. The remaining 4 nucleotides may be all 5' or 3' to the 21 identical nucleotides, or flank the 21 identical nucleotides (three 5' and one 3', 2 on both sides, *etc.*). In another example, an antagomir oligonucleotide of the invention may have 15 nucleotides (15-mer), 10 of which are identical to a stretch of 10 consecutive nucleotides of SEQ ID NO: 4, 5, or 6, while the remaining 5 may be all 5' or 3' to or flanks the 10 matching nucleotides, do not match the corresponding nucleotides in SEQ ID NO: 4, 5, or 6.

Similarly, for antagomir oligonucleotide of the invention corresponding to vmiR variants of SEQ ID NOs: 9-14, the antagomir sequences may include a portion or the entire extension sequences of HIV origin (and excludes polyA sequences and Universal Tag sequences). Such antagomirs may additionally include 5' or 3' sequences flanking the matching nucleotides. The flanking sequences may not match the corresponding nucleotides in SEQ ID NOs: 9-14.

In certain embodiments, the antagomir oligonucleotide of the invention comprises a sequence that can bind to or hybridize with the vmiR at a GU-rich region of the vmiR. In certain embodiments, the antagomir oligonucleotide of the invention binds to or hybridizes with the vmiR at a GU-rich region of the vmiR at an acidic pH environment, such as the acidic pH environment of endosome (*e.g.*, 4.5-6.0, or microphage endosome pH of about 5-5.5). The GU-rich regions of vmiR may be the sequence elements Box 4-8, Box 13-20, and Box 26-31.

In certain embodiments, the antagomir oligonucleotide of the invention binds to or hybridizes with the vmiR at a GU-rich region of the vmiR. The GU-rich region may consist essentially of all G and U bases, or modifications or analogs thereof.

In certain embodiments, the antagomir oligonucleotide of the invention can bind to or hybridize with the vmiR with perfect base-pairing.

In certain embodiments, the antagomir oligonucleotide of the invention can bind to or hybridize with the vmiR with imperfect or mismatch base-pairing. For example, the mismatch may be outside a GU-rich region consisting essentially of all G and U bases, or modifications or analogs thereof.

In certain embodiments, the antagomir oligonucleotide of the invention hybridizes with the vmiR with sufficient sequence complementary such that binding to and activation of the Toll-like receptor by vmiR are blocked by the antagomir oligonucleotide of the invention.

Designing sequences in terms of size and complementarity to optimize binding to target sequences is well known in the art. The antagomir may have 100% sequence identity to the vmiR. However, 100% identity is not required for antagomir function. In certain embodiments, there is greater than 80% sequence identity, *e.g.*, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100% sequence identity, between the antagomir and the vmiR.

Sequence identity may be determined by sequence comparison and alignment algorithms known in the art. To determine the percent identity of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the first sequence or second sequence for optimal alignment). The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical positions/total # of positions x 100), optionally penalizing the score for the number of gaps introduced and/or length of gaps introduced.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using any of many art-recognized mathematical algorithm. In one embodiment, the alignment generated over a certain portion of the sequence aligned having sufficient identity but not over portions having low degree of identity (*i.e.*, a local alignment). A non-limiting example of a local alignment algorithm utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-68, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-77. Such an algorithm is incorporated into the BLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10.

In another embodiment, the alignment is optimized by introducing appropriate gaps and percent identity is determined over the length of the aligned sequences (*i.e.*, a gapped alignment). To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25(17):3389-3402.

In another embodiment, the alignment is optimized by introducing appropriate gaps and percent identity is determined over the entire length of the sequences aligned (*i.e.*, a global alignment). A non-limiting example of a math algorithm for global comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is

incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package.

Alternatively, the antagomir may be defined functionally as an oligonucleotide sequence that is capable of hybridizing with at least a portion of the target vmiR under preferred hybridization conditions, *e.g.*, 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours; followed by washing. Additional suitable hybridization conditions include hybridization at 70°C in 1×SSC or 50°C in 1×SSC, 50% formamide followed by washing at 70°C in 0.3×SSC or hybridization at 70°C in 4×SSC or 50°C in 4×SSC, 50% formamide followed by washing at 67°C in 1×SSC.

The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature (T_m) of the hybrid, where T_m is determined according to the following equations. For hybrids less than 18 base pairs in length, $T_m(^{\circ}\text{C}) = 2(\# \text{ of A+T bases}) + 4(\# \text{ of G+C bases})$. For hybrids between 18 and 49 base pairs in length, $T_m(^{\circ}\text{C}) = 81.5 + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G+C}) - (600/N)$, where N is the number of bases in the hybrid, and $[\text{Na}^+]$ is the concentration of sodium ions in the hybridization buffer ($[\text{Na}^+]$ for 1×SSC = 0.165 M).

Additional examples of stringency conditions for polynucleotide hybridization are provided in Sambrook, Fritsch, and Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, chapters 9 and 11, and *Current Protocols in Molecular Biology*, 1995, F.M. Ausubel *et al.*, eds., John Wiley & Sons, Inc., sections 2.10 and 6.3-6.4, and subsequent revisions thereof, all incorporated herein by reference.

In certain embodiments, the antagomir oligonucleotide of the invention comprises a modified sugar moiety (*e.g.*, 2-O-Me), a modified base moiety (*e.g.*, nebularine or xanthosine nucleotide), a modified inter-sugar linkage (*e.g.*, phosphorothioate), or combinations thereof. In certain embodiments, the oligonucleotide comprises a locked nucleic acid (LNATM), a deoxyribonucleic acid (DNA), a ribonucleic acid (RNA), or a combination thereof. In certain embodiments, the LNA base-pairs with a G or a U.

Additional modifications suitable for the antagomir oligonucleotide of the invention are described further in a section entitled “modified nucleotides.”

3. Modified Nucleotides

The oligonucleotide of the invention may comprise one or more modified nucleotides

or chemical modifications to, for example, enhance a desired property, such as to enhance its stability (*e.g.*, to prevent degradation), to promote its cellular uptake, to enhance targeting efficiency and/or affinity to its binding partner (*e.g.*, vmiR), to improve patient tolerance, and/or to reduce toxicity, *etc.*

As used herein, “nucleoside” includes the unit made up of a heterocyclic base and its sugar. “Nucleotide” includes a nucleoside having a phosphate group on its 3’ or 5’ sugar hydroxyl group. “Oligonucleotide” includes a plurality of joined nucleotide units formed in a specific sequence from naturally occurring bases and pentofuranosyl groups joined through a sugar group by native phosphodiester bonds. These nucleotide units may be nucleic acid bases such as guanine (G), adenine (A), cytosine (C), thymine (T), or uracil (U). The sugar group may be a deoxyribose or ribose. This term also includes both naturally occurring and synthetic species formed from naturally occurring subunits.

In certain embodiments, oligonucleotide may also include “oligonucleotide analogue” that includes moieties which function similarly to oligonucleotides but which have non-naturally occurring portions. Oligonucleotide analogues may have altered sugar moieties, altered base moieties (*e.g.*, inosine, xanthine, hypoxanthine, isocytosine, isoguanine, diaminopurine (DAP), diaminopyrimidine, 2’-deoxyinosine (hypoxanthine deoxynucleotide) derivatives, nitroazole analogues, hydrophobic aromatic non-hydrogen-bonding bases, the thymine analogue 2,4-difluorotoluene, the adenine analogue 4-methylbenzimidazole, isoquinoline, pyrrolo[2,3-b]pyridine, size extended adenine, 2-amino-6-(2-thienyl)purine, pyrrole-2-carbaldehyde), or altered inter-sugar linkages (*e.g.*, phosphoramidate, phosphorothioate, phosphorodithioate, O-methylphosphoroamidite linkages, peptide nucleic acid backbones and linkages, positive backbones; non-ionic backbones, and non-ribose backbones, including those described in U.S. Pat. Nos. 5,235,033 and 5,034,506, which are incorporated by reference). For the purposes of this invention, an oligonucleotide analogue having non-phosphodiester bonds, *i.e.*, an altered inter-sugar linkage, can alternately be considered as an “oligonucleoside.” Such an oligonucleoside thus refers to a plurality of joined nucleoside units joined by linking groups other than native phosphodiester linking groups. Thus oligonucleotide of the invention may include a series of nucleosides or nucleoside analogues that are joined together via either natural phosphodiester bonds or via other linkages, including phosphorothioate linkages or the four atom linkers described in, for example, US 5,610,289. Generally, while the linkage is from the 3’ carbon of one nucleoside to the 5’ carbon of a second nucleoside, in some embodiments, the linkage may also include

other linkages such as a 2'-5' linkage.

Oligonucleotide or analogues thereof may also comprise other modifications consistent with the spirit of this invention, and in particular such modifications as may enhance cellular uptake, nuclease resistance, and hybridization properties or other useful properties. For example, when the sugar portion of a nucleoside or nucleotide is replaced by a carbocyclic or other moiety, it is no longer a sugar. Moreover, when other substitutions, such a substitution for the inter-sugar phosphodiester linkage are made, the resulting material is no longer a true nucleic acid species. All such modifications, however, are denominated as oligonucleotide analogues or simply oligonucleotides. Throughout this specification, reference to the sugar portion of a nucleic acid species shall be understood to refer to either a true sugar or to a species taking the traditional space of the sugar of natural nucleic acids. Moreover, reference to inter-sugar linkages shall be taken to include moieties serving to join the sugar or sugar analogue portions together in the fashion of natural nucleic acids.

The oligonucleotide of the invention may be modified at the 5' end, 3' end, both 5' and 3' ends, and/or at one or more internal nucleotides, or any combinations thereof.

In one embodiment, the oligonucleotide of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) end modifications, which end modifications may be at the 5' end, the 3' end, or both ends.

In certain embodiments, the internal nucleotides of the oligonucleotide of the invention are modified. As used herein, an "internal" nucleotide is one occurring at any position other than the 5' end or 3' end of a nucleic acid molecule, polynucleotide or oligonucleotide. An internal nucleotide can be within a single-stranded molecule or within either strand of a duplex or double-stranded molecule.

In one embodiment, the oligonucleotide of the invention is modified in at least one internal nucleotide, *e.g.*, in at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more, or all internal nucleotides.

In one embodiment, the oligonucleotide of the invention is modified in all nucleotides.

In another embodiment, the oligonucleotide of the invention is modified in at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% of the internal nucleotides, or 100% of the nucleotides.

Modifications to the internal or end nucleotides can include, for example, sugar modifications, base modifications, backbone modifications, or combinations thereof.

In one embodiment, the oligonucleotide of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more) backbone-modified nucleotides (*i.e.*, modifications to the sugar phosphate backbone). For example, the phosphodiester linkages of natural DNA or RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. In certain backbone-modified oligonucleotides, the phosphodiester group connecting adjacent nucleotides may be replaced by a modified group, *e.g.*, a phosphorothioate group.

In one embodiment, the oligonucleotide of the invention comprises one or more sugar-modified nucleotides. Sugar-modified nucleotides can include modifications to any substituents of the sugar portion of the nucleotide, *e.g.*, the 2' moiety of the ribose sugar in a ribonucleotide. The 2' moiety can be, but is not limited to, H, OR, R, halo, SH, SR, NH₂, NHR, NR₂ or CN, wherein R is C₁-C₆ alkyl, alkenyl, or alkynyl, and halo is F, Cl, Br, or I. In certain embodiments, the modifications are 2'-fluoro, 2'-ammo, and/or 2'-thio modifications. The nucleic acid may comprise a 2'-ribose replacement such as a 2'-O-methyl and 2'-fluoro group, as described in U.S. Patent No. 7,138,517, the contents of which are incorporated herein by reference. Modified nucleotides also include nucleotides conjugated with cholesterol through a hydroxyprolinol linkage as described in Krutzfeldt et al., *Nature*, 438:685-689 (2005), Soutschek et al., *Nature*, 432:173-178 (2004), and U.S. Patent Publication No. 2005/0107325, which are incorporated herein by reference.

In certain embodiments, modifications include uridines or cytidines modified at the 5-position, *e.g.*, 5-(2-amino)propyl uridine, 5-bromo uridine; adenosines and guanosines modified at the 8-position, *e.g.*, 8-bromo guanosine; deaza nucleotides, *e.g.*, 7-deaza-adenosine; O- and N-alkylated nucleotides, *e.g.*, N6-methyl adenosine are suitable.

In certain embodiments, sugar modifications include 2'-fluoro-cytidine, 2'-fluoro-uridine, 2'-fluoro-adenosine, 2'-fluoro-guanosine, 2'-amino-cytidine, 2'-amino-uridine, 2'-amino-adenosine, 2'-amino-guanosine, 2,6-diaminopurine, 4-thio-uridine, and/or 5-amino-allyl-uridine. In certain embodiments, the 2'-fluoro ribonucleotides are every uridine and cytidine. Additional exemplary modifications include 5-bromo-uridine, 5-iodo-uridine, 5-methyl-cytidine, ribo-thymidine, 2-aminopurine, 2'-amino-butyryl-pyrene-uridine, 5-fluoro-cytidine, and 5-fluoro-uridine. In addition, 2'-deoxy-nucleotides and 2'-O-Me nucleotides can also be used in the oligonucleotide of the invention. Additional modified residues

include, deoxy-abasic, inosine, N3-methyl-uridine, N6, N6-dimethyl-adenosine, pseudouridine, purine ribonucleoside and ribavirin. In one embodiment, one or more 2' moiety is a methyl group (2'-O-methyl oligonucleotide). In one embodiment, the 2'-O-methyl modified nucleotide occurs on alternating positions (*e.g.*, on the odd or even number nucleotides, over a part or the entire oligonucleotide). In one embodiment, the 2'-O-methyl modified nucleotide does not occur on alternating positions (*e.g.*, on the odd or even number nucleotides) over any stretch of 4 or more consecutive nucleotides, or over the entire length of the oligonucleotide.

In certain embodiments, the oligonucleotide of the invention comprises one or more Locked Nucleic Acids (LNAs). LNAs comprise sugar-modified nucleotides that resist nuclease activities (thus highly stable), and possess single nucleotide discrimination for mRNA (Elmen *et al.*, *Nucleic Acids Res.*, 33(1): 439-447, 2005; Braasch *et al.*, *Biochemistry*, 42:7967-7975, 2003; Petersen *et al.*, *Trends Biotechnol.*, 21:74-81, 2003). These molecules have 2'-O,4'-C-ethylene-bridged nucleic acids, with possible modifications such as 2'-deoxy-2''-fluorouridine. Moreover, LNAs increase the specificity of oligonucleotides by constraining the sugar moiety into the 3'-endo conformation, thereby pre-organizing the nucleotide for base pairing and increasing the melting temperature of the oligonucleotide by as much as 10°C per base.

In certain embodiments, the oligonucleotide of the invention comprises Peptide Nucleic Acids (PNAs). PNAs comprise modified nucleotides in which the sugar-phosphate portion of the nucleotide is replaced with a neutral 2-amino ethylglycine moiety capable of forming a polyamide backbone, which is highly resistant to nuclease digestion, and imparts improved binding specificity to the molecule (Nielsen *et al.*, *Science*, 254:1497-1500, 2001).

In certain embodiments, the oligonucleotide of the invention comprises Morpholino nucleic acid analog, or "PMO" (phosphorodiamidate morpholino oligo). Morpholinos are synthetic nucleic acid analogs that bind to complementary sequences of RNA by standard nucleic acid base-pairing. Structurally, Morpholinos are similar to DNA in that Morpholinos have standard nucleic acid bases. However, those bases are bound to morpholine rings instead of deoxyribose rings and linked through phosphorodiamidate groups instead of phosphates. Replacement of anionic phosphates with the uncharged phosphorodiamidate groups eliminates ionization in the usual physiological pH range, so Morpholinos in organisms or cells are uncharged molecules. The entire backbone of a Morpholino is made from these modified subunits.

In certain embodiments, the oligonucleotide of the invention comprises Glycol Nucleic Acid (GNA), which is a synthesized polymer similar to DNA or RNA but differing in the composition of its backbone. Specifically, DNA and RNA have a deoxyribose and ribose sugar backbone, respectively, whereas GNA's backbone is composed of repeating glycol units linked by phosphodiester bonds. The glycol unit has just three carbon atoms, yet still shows Watson-Crick base pairing, and the Watson-Crick base pairing is much more stable in GNA than its natural counterparts DNA and RNA as it requires a high temperature to melt a duplex of GNA. The 2,3-dihydroxypropyl nucleoside analogues were first prepared by Ueda et al. (1971).

In certain embodiments, the oligonucleotide of the invention comprises Threose Nucleic Acid (TNA), which is a synthetic nucleic acid analog similar to DNA or RNA but differing in the composition of its backbone. Specifically, TNA's backbone is composed of repeating threose units linked by phosphodiester bonds. TNA can hybridize with RNA and DNA in a sequence-specific manner. TNA is also capable of Watson-Crick pair bonding, and forming a double helix structure.

In certain embodiments, the oligonucleotide of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more) base-modified nucleotides (*i.e.*, the nucleotides contain at least one non-naturally occurring base instead of a naturally occurring base). Bases may be modified to block the activity of adenosine deaminase. Exemplary modified bases include, but are not limited to, uridine and/or cytidine modified at the 5-position (*e.g.*, 5-(2-amino)propyl uridine, 5-fluoro-cytidine, 5-fluoro-uridine, 5-bromo-uridine, 5-iodo-uridine, and 5-methyl-cytidine), adenosine and/or guanosines modified at the 8 position (*e.g.*, 8-bromo guanosine), deaza nucleotides (*e.g.*, 7-deaza-adenosine), and O- and N-alkylated nucleotides (*e.g.*, N6-methyl adenosine). Base-modified nucleotides for use in the present invention also include, but are not limited to, ribo-thymidine, 2-aminopurine, 2,6-diaminopurine, 4-thio-uridine, and 5-amino-allyl-uridine and the like. It should be noted that the above modifications may be combined.

In certain embodiments, the oligonucleotide of the invention, with or without modification, comprises a sequence wherein at least a portion (*e.g.*, the miRNA binding / hybridizing moiety) contains one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) mismatches with the target polynucleotide (*e.g.*, miRNA). In one embodiment, the oligonucleotide of the invention, with or without modification, may bind to its target

sequence (*e.g.*, vmiR), and may optionally contain one or more mismatches or bulges.

In certain embodiments, the oligonucleotide of the invention comprises any combination of two or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) modifications as described herein. For example, the oligonucleotide of the invention can comprise DNA, RNA, LNA, PNA, or a combination thereof. The oligonucleotide of the invention may comprise phosphorothioate linkages throughout, and may additionally comprise one or more sugar-modified nucleotides, such as 2'-fluoro modified ribonucleotides (*e.g.*, 2'-fluoro uridine or 2'-fluoro cytidine), 2'-deoxy ribonucleotides (*e.g.*, 2'-deoxy adenosine or 2'-deoxy guanosine), and/or 2'-O-methyl modified ribonucleotides.

Other modified nucleotides are described in US 5,610,289 (incorporated by reference), which describes therapeutic oligonucleotide analogues having improved nuclease resistance and improved cellular uptake. According to US 5,610,289, replacement of the normal phosphodiester inter-sugar linkages found in natural oligomers with four atom linking groups forms unique di- and poly- nucleosides and nucleotides useful in therapeutics. More specifically, oligonucleotides or analogues thereof may have at least portions of their backbone linkages modified. In these modifications, the phosphodiester linkage of the sugar phosphate backbone found in natural nucleic acids has been replaced with various four atom linking groups. Such four atom linking groups maintain a desired four atom spacing between the 3'-carbon of one sugar or sugar analogue and the 4'-carbon of the adjacent sugar or sugar analogue. Oligonucleotide analogues so made are comprised of a selected sequence which is specifically hybridizable with a preselected nucleotide sequence of single stranded or double stranded DNA or RNA. Such oligonucleotides are synthesized conveniently, through known solid state synthetic methodology, to be complementary to or at least to be specifically hybridizable with the preselected target nucleotide sequence of the RNA or DNA. Nucleic acid synthesizers are commercially available and their use is generally understood by persons of ordinary skill in the art as being effective in generating nearly any oligonucleotide or oligonucleotide analogue of reasonable length which may be desired.

According to the invention, the oligonucleotide of the invention should be modified as necessary, in part, to improve stability, to prevent degradation *in vivo* (*e.g.*, by cellular nucleases), to improve cellular uptake, to enhance target efficiency, to improve efficacy in binding (*e.g.*, to the targets), to improve patient tolerance, and/or to reduce toxicity.

In one embodiment, the oligonucleotide of the invention has a target binding moiety or portion of about 5-10 nucleotides in length, 10-15 nucleotides in length, 15-20 nucleotides

in length, about 20, 21, or 22 nucleotides in length, or about 25-50 nucleotides in length.

In certain embodiments, the targeting moiety or portion is on the 5' end or the 3' end of the oligonucleotide of the invention, or roughly the middle of the oligonucleotide of the invention.

In certain embodiments, the oligonucleotide of the invention may be linked to other moieties, such as fluorescent dyes, and may have additional modifications in such other moieties.

The oligonucleotide of the invention or portions thereof may be produced enzymatically and/or by partial/total organic synthesis, and any modified nucleotides may be introduced by *in vitro* enzymatic or organic synthesis.

In one embodiment, the oligonucleotide of the invention is prepared chemically. Methods of synthesizing oligonucleotide molecules are known in the art, in particular, the chemical synthesis methods as described in, for example, Verma and Eckstein (1998) *Annul. Rev. Biochem.*, 67:99-134.

Alternatively, the oligonucleotide of the invention can be prepared by enzymatic transcription from synthetic DNA templates or from DNA plasmids isolated from recombinant host, such as a bacteria. For example, phage RNA polymerases may be used for RNA oligonucleotides, including T7, T3 or SP6 RNA polymerase (Milligan and Uhlenbeck (1989) *Methods Enzymol.*, 180:51-62). The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to inhibit annealing, and/or promote stabilization of the single strands.

In another embodiment, oligonucleotide of the invention is synthesized directly either *in vivo*, *in situ*, or *in vitro*. An endogenous RNA polymerase in the cell may mediate transcription of the oligonucleotide of the invention *in vivo* or *in situ*, or a cloned RNA polymerase can be used for transcription of the oligonucleotide of the invention *in vivo* or *in vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region (*e.g.*, promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the oligonucleotide of the invention. Inhibition of the target vmiR may be targeted by specific transcription in an organ, tissue, or cell type (*e.g.*, T-cells, macrophages, *etc.*); stimulation of an environmental condition (*e.g.*, infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. A transgenic organism that expresses an oligonucleotide of the invention from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or

another multipotent cell derived from the appropriate organism.

The invention also provides recombinant expression vectors comprising recombinant nucleic acids operatively linked to an expression control sequence, wherein expression, *i.e.*, the transcription and optionally further processing, results in one or more oligonucleotide of the invention or a precursor molecules thereof. The vector may be a DNA vector, *e.g.*, a viral vector or plasmid, particularly an expression vector suitable for nucleic acid expression in eukaryotic, more particularly mammalian cells. The recombinant nucleic acid contained in aid vector may be a sequence which results in the transcription of the oligonucleotide of the invention as such, a precursor or primary transcript thereof, which may be further processed to give the oligonucleotide of the invention.

In certain embodiments, the vector may be administered *in vivo* to thereby initiate therapeutic or prophylactic treatment, by expression of one or more copies of the oligonucleotide of the invention. In certain embodiments, use of vectors may be advantageous because the vectors can be more stable than oligonucleotides and thus effect long-term expression of the oligonucleotide of the invention.

Vectors may be designed for delivery of multiple oligonucleotides of the invention capable of antagonizing multiple target vmiRs. Accordingly, in one embodiment, a vector is contemplated that expresses a plurality of the oligonucleotide of the invention.

In one embodiment, the vector encodes about 2, 3, 4 or more oligonucleotide of the invention, each of which may target the same or different vmiR.

In one embodiment, expression of the oligonucleotide of the invention is driven by a RNA polymerase III (pol III) promoter (T.R. Brummelkamp *et al.*, *Science*, (2002) 296:550-553; P.J. Paddison *et al.*, *Genes Dev.*, (2002) 16:948-958). Pol III promoters are advantageous because their transcripts are not necessarily post-transcriptionally modified, and because they are highly active when introduced in mammalian cells. In another embodiment, expression of the oligonucleotide of the invention is driven by a RNA polymerase II (pol II) promoter. Polymerase II (pol II) promoters may offer advantages to pol III promoters, including being more easily incorporated into viral expression vectors, such as retroviral and adeno-associated viral vectors, and the existence of inducible and tissue specific pol II dependent promoters.

4. *Pharmaceutical Composition*

One aspect of the invention provides a pharmaceutical composition comprising an

antagomir oligonucleotide of the invention (such as those described herein above), or a polynucleotide encoding the antagomir oligonucleotide of the invention, and a pharmaceutically acceptable carrier and/or excipient.

In certain embodiments, the antagomir oligonucleotide of the invention is encompassed within an exosome for delivery to a target cell, such as a cell of an HIV-1 infected individual.

In certain embodiments, the pharmaceutical composition may comprise one and only one antagomir of the invention, or a polynucleotide encoding the same.

The antagomir oligonucleotide of the invention (with or without modification) can be transfected directly into a host / packaging cell for exosome production, or can be encoded by an expression vector which can be transfected into the host / packaging cell and be used to express the antagomir inside the packaging cell. The advantage of directly transfecting the antagomir is that the antagomir may comprise synthetic oligonucleotides, such as those comprising one or more modification. The advantage of transfecting antagomir-encoding expression vector is that the expression of the antagomir can be controlled (*e.g.*, inducible expression using any art recognized inducible promoters), and may be produced stably, and/or at a very high level.

In certain embodiments, antagomir or expression vector thereof is first transfected into a host / packaging cell, such as THP-1 cells. The medium is then replaced with fetal bovine serum-free medium, and the cells are incubated with phorbol ester to allow differentiation. Exosome may be isolated using ultracentrifugation, and/or precipitation using ExoQuick-TC. Optionally, total RNA may be extracted from the exosome and analyzed for antagomir sequence using qRT-PCR as quality control. In certain embodiments, exosomal markers such as CD63, CD9, CD81, and/or Hsp70 may be checked as quality control for the exosome preparation. In certain embodiments, presence of acetylcholinesterase (AChE) activity may be checked as quality control for the exosome preparation. In certain embodiments, to exclude the possible presence of minute amounts of infectious HIV, exosome fractions / preparations are tested by infectivity assay or TaqMan Real Time PCR assay as quality control for the exosome preparation.

Isolated or purified exosome preparations may be prepared in a medium suitable for administering to a host. For example, a suspension of antagomir exosome fraction may be prepared in phenol red-free RPMI medium for *i.v.* injection. Optimum concentrations may be determined based on *in vitro* assays. To monitor treatment efficacy, inflammatory markers

such as VCAM-1, ICAM-1, TNF α , IL-12, IL-6 and viral load may be monitored periodically (*e.g.*, every week).

Thus the antagomir oligonucleotide of the invention can be incorporated into pharmaceutical compositions suitable for administration (such as *i.v.* injection). Such compositions typically comprise the antagomir oligonucleotide of the invention formulated in a pharmaceutically acceptable carrier.

As used herein, “pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Thus in certain embodiments, the pharmaceutical composition of the present invention includes an antagomir oligonucleotide of the invention and an agent suitable for delivery to a subject. Alternatively, the invention includes an antagomir oligonucleotide of the invention encompassed within a delivery vehicle (such as exosome) suitable for delivery to a subject. Suitable delivery agents include, but are not limited to, proteinaceous agents (*e.g.*, peptides), hydrophobic agents or lipid-based agents.

A pharmaceutical composition of the invention may be formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, intraperitoneal, intramuscular, oral (*e.g.*, inhalation), transdermal (topical), and transmucosal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like.

In many cases, it may include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and

swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer. Such methods include those described in U.S. Patent No. 6,468,798.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The pharmaceutical composition can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery. The pharmaceutical composition can also be administered by transfection or infection using methods known in the art, including but not limited to the methods described in McCaffrey *et al*, *Nature* 418:38-39, 2002 (hydrodynamic transfection); Xia *et al*, *Nature Biotechnol.*, 20:1006-1010, 2002 (viral-mediated delivery); or Putnam, *Am. J. Health Syst. Pharm.*, 53:151-160, 1996, erratum at *Am. J. Health Syst. Pharm.*, 53:325, 1996).

The pharmaceutical composition can also be administered by any method suitable for administration of nucleic acid agents, such as a DNA vaccine. These methods include gene guns, bio injectors, and skin patches as well as needle-free methods such as the micro-particle DNA vaccine technology disclosed in U.S. Patent No. 6,194,389, and the mammalian transdermal needle-free vaccination with powder-form vaccine as disclosed in U.S. Patent No. 6,168,587. Additionally, intranasal delivery is possible, as described in, *inter alia*,

Hamajima *et al.* (1998), *Clin. Immunol. Immunopathol.*, 88(2):205-210. Liposomes (*e.g.*, as described in U.S. Patent No. 6,472,375) and microencapsulation can also be used.

Biodegradable targetable microparticle delivery systems can also be used (*e.g.*, as described in U.S. Patent No. 6,471,996).

In one embodiment, the active pharmaceutical composition is prepared with carriers that will protect the composition against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can also be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid.

Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811. It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit large therapeutic indices are preferred. Although compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the EC₅₀ (*i.e.*, the concentration of the test compound which achieves a half-maximal response) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

A therapeutically effective amount of a composition containing a compound of the invention (*e.g.*, an antagomir oligonucleotide of the invention) (*i.e.*, an effective dosage) is an amount that inhibits Toll-like receptor mediated pro-inflammatory response, which may be measured by reduction in production of a pro-inflammatory cytokine, such as TNF1, IL-6, or IL-12, by at least about 20 percent. Higher percentages of inhibition, *e.g.*, 30, 40, 50, 60, 70, 80, 90 percent or higher may be desired in certain embodiments.

Exemplary doses include milligram or microgram amounts of the molecule per kilogram of subject or sample weight (*e.g.*, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram). The compositions can be administered one time per week for between about 1 to 10 weeks, *e.g.*, between 2 to 8 weeks, or between about 3 to 7 weeks, or for about 4, 5, or 6 weeks. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments.

It is furthermore understood that appropriate doses of a composition depend upon the potency of composition with respect to the expression or activity to be modulated. When one or more of these molecules is to be administered to an animal (*e.g.*, a human) to modulate expression or activity of a vmiR, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate

response is obtained. In addition, it is understood that the specific dose level for any particular subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

As described above, the antagomir nucleic acid molecules of the invention can also be inserted into expression constructs, *e.g.*, viral vectors, retro viral vectors, expression cassettes, or plasmid viral vectors, *e.g.*, using methods known in the art, including but not limited to those described in Xia et al, (2002). Expression constructs can be delivered to a subject by, for example, inhalation, orally, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see, *e.g.*, Chen *et al* (1994), Proc. Natl. Acad. Sci. USA, 91, 3054-3057). The pharmaceutical preparation of the delivery vector can include the vector in an acceptable diluent, or can comprise a slow release matrix in which the delivery vehicle is imbedded. Alternatively, where the complete delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system such as exosome.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. Kits comprising the pharmaceutical composition are also contemplated as part of the invention.

5. *Method of Treatment or Prevention*

One aspect of the invention provides a method of treating an inflammatory response in an individual infected by an HIV-1 virus, the method comprising administering to the individual a therapeutically effective amount of a pharmaceutical composition of the invention.

In certain embodiments, the inflammatory response is a symptom of chronic immune activation associated with HIV infection.

One major hallmark of HIV infection is chronic immune activation that promotes viral replication and drives CD4⁺ T-cell depletion, although the mechanisms underlying such immune activation are not well understood. In treated HIV infection, chronic immune activation persists, and is implicated in non-infectious clinical complications of HIV that account for most of the morbidity and mortality in patients treated with anti-retroviral therapy. Chronic immune activation has been associated with poor CD4 T-cell recovery,

non-AIDS defining illnesses, and mortality during combination antiretroviral therapy (cART). Measures of chronic immune activation, namely T-cell activation and more recently monocyte activation and plasma inflammatory and thrombotic biomarkers, have all been shown to remain elevated despite years of suppressive cART.

While not wishing to be bound by any particular theory, Applicants have provided evidence that HIV associated chronic immune activation, including chronic immune activation in individuals showing efficacious HAART treatment response, is at least partly related to vmiR stimulated Toll-like receptor activation and the resulting pro-inflammatory cytokine production.

Continuous immune stimulation in turn likely creates a permissive environment for further viral replication, while temporarily allowing successful replenishment of the T-cell pool. Thus in certain embodiments, treatment of such chronic immune activation may further facilitate the treatment of HIV infection, and may be included as a combination therapy with other HIV therapy aimed at inhibiting viral replication. That is, in certain embodiments, the individual receiving the subject method of treatment also receives concurrent highly active antiretroviral therapy (HAART), has received HAART, or will receive HAART.

In other embodiments, the treatment method of the invention may be used in combination with other methods or agents known to be efficacious to treat chronic immune activation, including: statins (*e.g.*, Crestor); chloroquine and hydroxychloroquine; selective cyclooxygenase-2 (COX-2) inhibitors (*e.g.*, those which modulate T-cell activation via inhibition of prostaglandin E2 and the cyclic adenosine 30,50-monophosphate (cAMP) protein kinase A pathway, such as celecoxib); Leflunomide; certain biological agents including bovine colostrum, micronutrients and pre-/probiotics; antiretroviral treatment (*e.g.*, raltegravir; maraviroc); anti-CMV treatment (*e.g.*, valgancyclovir or Valcyte); anti-HCV treatment (*e.g.*, interferon gamma and ribavirin).

Additional combination therapy may include immune suppressants (*e.g.*, prednisone, hydroxyurea, cyclosporine, and mycolic acid), Renagel or colostrum supplements, Motrin, Aleve, Serostim, anti-rejection drug (*e.g.*, Sirolimus), IL-7, Esbriet, resveratrol, telomerase activators, vitamin D and omega-3 fatty acids.

In certain embodiments, the inflammatory response is a symptom of HIV-associated lung disease, liver disease, kidney disease, atherosclerosis, neurodegenerative disease, malignancy, cardiovascular disease, bone disease (*e.g.*, bone loss), or an opportunistic infection.

In certain embodiments, the individual may have high CD4 count, and may have heart, liver, and/or kidney diseases in association with HIV infection, or at high risk of developing such heart, liver, and/or kidney diseases.

In certain embodiments, the individual may have undetectable or low level of viral load, and/or CD4 count of above 200.

In certain embodiments, the inflammatory response is induced by TLR8 activation. For example, the TLR8 activation may lead to increased activity of ERK1/2, increased activity of NF κ B, increased activity of a pro-inflammatory cytokine, and/or increased expression of an inflammatory marker. In certain embodiments, the pro-inflammatory cytokine comprises TNF α , IL-6, or IL-12. In certain embodiments, the inflammatory marker may comprise VCAM-1 or ICAM-1.

In certain embodiments, the efficacy of the treatment is measured by decreased levels (*e.g.*, in blood or urine) of one or more markers of inflammation, such as C-reactive protein (CRP), interleukin-6, D-dimer, TNF α , MCP-1, CD27, CD40, and/or RANTES. In certain embodiments, the subject method of treatment leads to a decrease, in the treated individual, of at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or higher of one or more such markers of inflammation.

In certain embodiments, the method is for treating atherosclerosis, coronary artery disease (CAD), or coronary heart disease (CHD) associated with HIV infection in the individual. The individual may or may not have received combined anti-retroviral therapy (cART).

In certain embodiments, the CHD/CAD comprises pathogenesis of atheromatous plaque, or is characterized by the presence of atheromatous plaque. In certain embodiments, the CHD/CAD comprises increased formation of foam cells, such as those formed from circulating mononuclear phagocytes and tissue macrophages, including monocyte-derived macrophages (MDM). The formation of foam cells represents a critical step in the pathogenesis of atheromatous plaque, the hallmark of atherosclerotic disease that characterizes CHD/CAD.

Clinical presentations of CHD in HIV infection are distinct from CHD due to traditional risk factors. For example, HIV patients are generally a decade younger with a mean of 50 years, and, unlike non-HIV patients, tend to have a single vessel affected rather than multiple vessels. Moreover, in HIV patients whose infection is controlled without receiving cART (“elite controllers”), they also have more extensive carotid narrowing than

age-matched controls. HIV infection by itself is also implicated with an increased risk of myocardial infarction.

Using HEK-293 cells overexpressing TLR8 and clinically relevant human healthy monocyte-derived macrophages (MDMs), as well as primary alveolar macrophages (AMs), several examples described herein demonstrate that HIV infection *per se* is sufficient to induce foam cell formation. Specifically, *in vitro* infection of human MDM with monocyte-tropic (R5) B-al HIV isolate promotes foam cell formation, in part dependent on active HIV replication. Stimulation of TNF α release causes MDM to differentiate into foam cells, and foam cell formation is inhibited by blocking anti-TNF α antibody. Furthermore, HIV-derived ssRNA promotes concentration-dependent MDM foam cell formation, dependent on TNF α release, mediated in part through the TLR8 and MyD88 signaling pathway, and involves direct binding of ssRNA to TLR8. In addition, novel HIV-derived miRNAs can also promote foam cell formation in tissue macrophages, and foam cell formation can be blocked using specific antagomirs targeting the HIV-derived miRNA.

The data presented herein demonstrates that HIV infection is sufficient to induce macrophage foam cell formation, independent of cART medications, and in part mediated by HIV-derived ssRNA through TLR8-dependent TNF α release. Thus, targeting HIV-derived ssRNA according to the instant invention provides a therapeutic means to limit the development of accelerated atherosclerotic disease in HIV+ persons.

The data presented herein also suggests that blocking the function of certain signaling molecules or steps between HIV infection and foam cell formation can effectively treat atherosclerosis, coronary artery disease (CAD), or coronary heart disease (CHD) associated with HIV infection. Thus the invention also provides a method of treating atherosclerosis, coronary artery disease (CAD), or coronary heart disease (CHD) associated with HIV infection in an individual, the method comprising administering to the individual a therapeutically effective amount of a pharmaceutical composition of the invention, and/or an inhibitor of the signaling pathway from HIV infection to foam cell formation, either alone or in combination.

For example, the signaling pathway from HIV infection to foam cell formation may include TLR8, TNF α , dynamin, and MyD88, and an inhibitor of any one of these signal pathway intermediates may include an antisense polynucleotide, a small RNA that inhibits gene function by RNA interference mechanism (e.g., siRNA, miRNA, shRNA), a ribozyme, an antibody (such as a human antibody, humanized antibody, chimeric antibody, mouse

antibody) or antigen-binding fragment thereof (*e.g.*, Fab, Fab', and F(ab')₂, Fv, scFv, minibodies, diabodies, tribodies, tetrabodies), or an antibody mimic thereof, such as protein scaffolds based on a consensus sequence of fibronectin type III (FN3) repeats (or "Centyrins," see US 2010/0255056, incorporated herein by reference), Ankyrin Repeat Proteins (or DARPins, see US 2004/0132028, US 2009/0082274, US 2011/0118146, US 2011/0224100, all incorporated herein by reference), or fibronectin domain scaffold proteins (or Adnectins, see US 2007/0082365, US 2008/0139791, incorporated herein by reference).

In a related aspect, the invention also provides a method of preventing or delaying the onset of an inflammatory response in an individual either suspected of or at high risk of being infected by an HIV-1 virus (including individuals that may be in latency period post HIV infection), the method comprising administering to the individual a prophylactically effective amount of a pharmaceutical composition of the invention.

In certain embodiments, the method is for preventing or delaying the onset of coronary artery disease (CAD) associated with HIV infection. The individual may or may not have received combined anti-retroviral therapy (cART).

In any of the subject methods, in certain embodiments, only one antagomir of the invention is administered.

6. *Diagnostic Methods*

Applicants provide evidence herein that HIV-1 encoded vmiR selectively accumulate in exosomes shed from HIV-infected cells. Such exosomes contain significant amounts or copies of vmiR, and may provide a novel source to detect the presence of virus in an infected subject. It has been reported that HIV-1 microRNAs were not identified in *in vitro* infections by HIV-1 (Whisnant *et al.*, 2013). However, the authors do not appear to have analyzed the presence of vmiRs in exosomes, particularly the 15-days post infection time point Applicants have shown the presence of vmiR in exosome isolated from patient serum.

Thus, the invention also provides a method of detecting the presence of HIV-1 infection in an individual, the method comprising isolating an exosome from the individual and detecting the presence of a microRNA encoded by an HIV-1 virus (vmiR) in the exosome.

Exosomes are 60-120 nm membrane vesicles secreted by most cell types *in vivo* and *in vitro*. Exosomes may be found in various body fluids, such as blood, urine, amniotic fluid,

or malignant ascite fluid, as well as tissue culture media of cultured cells.

Thus in certain embodiments, exosomes can be isolated (*e.g.*, precipitated) from patient sample or tissue (*e.g.*, serum) by using, for example, ExoQuick (optimized for serum use) or ExoQuick-TC polymer (for tissue culture medium or urine samples), or equivalents thereof. The ExoQuick and ExoQuick-TC exosome precipitation reagents are commercially available formulations from System Biosciences, Inc. (Mountain View, CA). According to the manufacture, ExoQuick-TC has been optimized for exosome isolation from media and urine samples, while ExoQuick is optimized for exosome isolation from serum sample. As little as 100 μ l of serum may be sufficient using the ExoQuick reagent, while 5 or 10 mL tissue culture media or urine sample may be sufficient for ExoQuick-TC.

RNA can be isolated from the exosomes using, for example, SeraMir Exosome RNA Purification Columns (System Biosciences, Mountain View, CA), or equivalents thereof. According to the manufacture, the SeraMir kit includes all reagents necessary to accurately and sensitively measure RNAs isolated from serum samples. Specifically, exoRNAs from isolated exosomes can be purified using a phenol-free lysis buffer and rapid spin columns. The SeraMir kit enables the 3' tailing and simultaneous tagging of both 5' and 3' ends during cDNA synthesis, which can be used in downstream analysis such as qPCR, cDNA amplification, T7 IVT, microarrays, and NextGen Sequencing. Primers for PCR amplification are also included for highly sensitive applications.

Optionally, in certain embodiments, ultracentrifugation on 6-18% iodixanol (GE Healthcare) density gradient may be used in conjunction with the method above to further distinguish exosomes from any contaminating viral particles.

In certain embodiments, the diagnostic method of the invention further comprises quantitating vmiR. For example, vmiR may be quantitated by using the miRCURY LNATM Universal RT microRNA PCR method of Exiqon Inc. (Woburn, MA).

According to the manufacturer, the miRCURY LNATM Universal RT microRNA PCR system is a microRNA-specific, LNATM-based system designed for sensitive and accurate detection of microRNA by quantitative real-time PCR using SYBR[®] Green. The method is based on universal reverse transcription (RT) followed by real-time PCR amplification with LNATM enhanced primers. The miRCURY LNATM Universal RT microRNA PCR offers solutions both for high-throughput microRNA expression profiling and for quantification of individual microRNAs.

In certain embodiments, the individual is HIV+ (positive), suspected to be HIV positive, or has a high risk of being HIV positive (such as one in contact with an HIV+ individual, *e.g.*, a healthcare worker). In certain embodiments, the individual is asymptomatic HIV+ persons. In certain embodiments, the individual has peripheral blood CD4⁺ T-lymphocyte count < 200 cells/mm³, or < 250 cells/mm³, or < 300 cells/mm³, or < 350 cells/mm³, or < 400 cells/mm³.

7. *Isolated Compositions*

Another aspect of the invention provides an isolated microRNA encoded by an HIV-1 virus (vmiR), or cDNA thereof (*e.g.*, cDNA of SEQ ID NOs:1-3 (*e.g.*, 1-2) or 9-14 or any of the cloned sequences shown in Figure 11 or similar sequences), wherein the vmiR is characterized by one or more of the following, including combination thereof: (1) binds TLR8 and/or TLR7; (2) activates TLR8 and/or TLR7; (3) is GU-rich (*e.g.*, ≥70%, 75%, 80%, 85%, 90%, 95% GU); (4) comprises a 7- or 8-nucleotide sequence consisting of at most one non-G non-U nucleotide; (5) is any one of SEQ ID NOs: 1-3 (*e.g.*, 1-2), or 9-14, or any of the cloned sequences shown in Figure 11, or similar sequences, such as a sequence variant thereof from a related HIV-1 virus with at least about 90% sequence identity or 95% sequence identity; and/or (6) is about 18-25 nucleotides in length, or about 20, 21, or 22 nucleotides in length. For sequences comprising extension sequences of HIV origin, polyA sequences, and/or Universal Tag sequences, polyA sequences and Universal Tag sequences (and optionally extension sequences) are not taken into account for (3) and (6).

Not counting polyA sequences and Universal Tag sequences (and optionally not counting any extension sequences), the vmiR sequence may comprise from 13-33, 18-24 or 21-23 nucleotides. The vmiR may also comprise a total of at least 5-40 nucleotides. The sequence of the vmiR may be the first 13-33 nucleotides of its corresponding pre-miRNA. The sequence of the vmiR may also be the last 13-33 nucleotides of the corresponding pre-miRNA.

In a related aspect, the invention also provides an isolated pri-miRNA of the vmiR of the invention (*e.g.*, vmiR88 or vmiR99), or a cDNA encoding the same. The pri-miRNA is a precursor of the respective vmiR, and is transcribed from the HIV-1 template inside the nucleus of an infected cell (*e.g.*, macrophage). The pri-miRNA may be processed by RNase III family nuclease Drosha and/or Pasha to produce a pre-miRNA.

The pri-miRNA may form a hairpin structure. The hairpin may comprise first and

second nucleic acid sequence that are substantially complementary. The first and second nucleic acid sequence may be from 37-50 nucleotides. The first and second nucleic acid sequence may be separated by a third sequence of from 8-12 nucleotides. The hairpin structure may have a free energy less than -25 Kcal/mole as calculated by the Vienna algorithm with default parameters, as described in Hofacker et al., *Monatshefte f. Chemie*, 125:167-188 (1994), the contents of which are incorporated herein. The hairpin may comprise a terminal loop of 4-20, 8-12 or 10 nucleotides.

In certain embodiments, the isolated pri-miRNA is in complex with Drosha / Pasha. That is, the invention also provides a complex between the pri-miRNA and Drosha / Pasha.

In yet another related aspect, the invention provides an isolated pre-miRNA of the vmiR of the invention (*e.g.*, vmiR88 or vmiR99), or a cDNA encoding the same. The pre-miRNA is the process product of pri-miRNA by Drosha / Pasha, and can be exported from nucleus of the infected cell to the cytoplasm by the RAN-GTPase Exportin 5. Once inside the cytosol, the pre-miRNA may be further bound and processed by another RNase III Dicer to produce a double-stranded complex of vmiR and its complementary sequence, which vmiR is subsequently loaded into the mi-RISC complex, and forms a complex with an Argonaute protein (*e.g.*, AGO1, AGO2, AGO3, AGO4).

Thus in certain embodiments, the pre-miRNA is in complex with Exportin-5 or Dicer.

In still another aspect, the invention provides an isolated microRNA-protein complex, wherein the microRNA is the vmiR of the invention (*e.g.*, vmiR88 or vmiR99), and is in complex with an Argonaute protein (*e.g.*, AGO1, AGO2, AGO3, AGO4).

In a further related aspect, the invention provides an isolated exosome comprising a subject microRNA encoded by an HIV-1 virus (vmiR).

8. Other Reagents

In a further related aspect, the invention also provides labeled oligonucleotides, such as any of the vmiR, cDNA thereof, and antagomir oligonucleotides of the invention, with or without base, sugar, backbone modifications.

In certain embodiments, the labels may be a fluorescent label on the 5' end, 3' end, or both ends. An exemplary fluorescent label is the green fluorescent dye Alexa Fluor[®] 488 (Life Technologies Corp., Grand Island, NY), which may be linked to the 3' end via an NHS ester linkage. Another exemplary fluorescent label is the red fluorescent dye Alexa Fluor[®] 647 (Life Technologies Corp., Grand Island, NY), which may be linked to the 5' end via an

NHS ester linkage. Many other Alexa Fluor[®] dyes with different excitation / emission wavelengths are also available from Life Technologies Corp. Paired oligonucleotides (such as vmiR and its antagomir) labeled with different fluorescent dyes may be used in FRET (Fluorescence Resonance Energy Transfer) based binding assays.

For example, one of such fluorescent dyes (*e.g.*, Alexa Fluor[®] 488) may be conjugated to the 3' end of a vmiR (such as RNA40, vmiR88, vmiR99), while another fluorescent dye (*e.g.*, Alexa Fluor[®] 647) may be conjugated to the 5' end of a corresponding antagomir for the vmiR (such as antagomir40, antagomir88, antagomir99). When the labeled antagomir and vmiR bind or hybridize to each other, the two fluorescent dyes will be brought to close proximity such that FRET can occur. On the other hand, no FRET is indicative of no (stable) binding or hybridization under the condition tested. Any pairs of fluorescent dyes suitable for FRET (such as Cy3 and Cy5; Alexa555 and Alexa647; Atto555 and Atto647N) may be used in conjugating to the vmiR / antagomir pairs to detect binding or hybridization.

In certain embodiments, the labels may be a fluorescent quencher that may be conjugated to either the 5'- or 3'-end of an oligonucleotide. Exemplary quenchers include the Iowa Black[®] quenchers (IDT) Iowa Black[®] FQ (ideal for use with fluorescein and other fluorescent dyes that emit in the green to pink spectral range) and Iowa Black[®] RQ (ideal for use with Texas Red[®], Cy5[™], and other fluorescent dyes that emit in the red spectral range).

The fluorescent quencher may be used with a corresponding fluorescent dye (*e.g.*, Alexa Fluor[®] 488), in a similar manner as the two fluorescent dyes in FRET, except that, when the quencher is brought to close proximity to the fluorescent dye due to oligonucleotide hybridization, fluorescent is quenched by the quencher.

Thus for the use above, the fluorescent dyes (*e.g.*, Alexa Fluor[®] 488) may be conjugated to the 3' end of a vmiR (such as RNA40, vmiR88, vmiR99), while the quencher (*e.g.*, Iowa Black[®] quencher) may be conjugated to the 5' end of a corresponding antagomir for the vmiR (such as antagomir40, antagomir88, antagomir99).

Other label configurations with vmiR and antagomir labeled by different dyes or quenchers at different ends are also within the scope of the invention.

In a further related aspect, the invention also provides vmiR oligonucleotides comprising one or more modification on the sugar, base, or backbone. Any of the modifications described above in the *Modified Nucleotide* section may be present in the modified vmiR oligonucleotide.

In certain embodiments, the modified vmiR is identical to any one of SEQ ID NOs: 1-3 (*e.g.*, 1-2), and 9-14, except that all backbone linkages are phosphorothioate, and all bases are RNA except for the two nucleotides at the 3' end being 2'-O-methyl modified RNA.

In certain embodiments, the 5'-end of any of the oligonucleotides described herein above has a 5' phosphate group. In other embodiments, the 5' end is -OH.

9. *Exosome-based delivery system*

Another aspect of the invention provides an exosome-based delivery system, *e.g.*, one for delivering a molecule, including a nucleic acid, a protein, a lipid, or a therapeutic molecule based on nucleic acid or protein, the delivery system comprising a first expression vector that expresses a targeting ligand, wherein the targeting ligand is expressed on the surface of an exosome, and comprises a moiety on / outside the exterior surface of the exosome for binding to a target on a recipient cell.

In a related aspect, the invention provides an exosome-based delivery system for delivering a therapeutic molecule, the delivery system comprising an exosome expressing a targeting ligand on the surface of the exosome, and comprising the therapeutic molecule in the lumen of the exosome, wherein the targeting ligand comprises a moiety on / outside the exterior surface of the exosome for binding to a target on a recipient cell.

In certain embodiments, the delivery system further comprises a second expression vector encoding a sequence for expressing a nucleic acid, a protein, or an enzyme that produced a lipid. In an alternative embodiment, the sequence is present on the first expression vector (not on the second expression vector).

For example, the nucleic acid encoded / expressed by the sequence may be a vmiR (*e.g.*, vmiR88, vmiR99, or any other vmiR described herein), an antagomir of the vmiR (*e.g.*, antagomir of vmiR88 or vmiR99), or a precursor thereof (such as an shRNA) that can be processed to the vmiR.

In certain embodiments, the first and/or the second expression vector is a plasmid or a viral vector, such as adenoviral vector, AAV vector, retroviral vector, lentiviral vector, or any other viral vector known in the art.

In certain embodiments, the targeting ligand is a fusion protein. For example, the fusion protein may be a fusion between a tetraspanin family protein fused to the moiety, such that the moiety is expressed in one of the extracellular loops (*e.g.*, EC2 domain, see below) of the tetraspanin family protein.

Numerous tetraspanin family proteins can be used for the instant invention, including any of TSPAN1-TSPAN34. In certain embodiments, the tetraspanin family protein is TSPAN28 (or CD81), TSPAN29 (or CD9), or TSPAN30 (or CD63). Tetraspanins, also called tetraspans or the transmembrane 4 superfamily (TM4SF), are a family of membrane proteins found in all multicellular eukaryotes. They have four transmembrane domains, intracellular N- and C-termini and two extracellular domains, one short (called the small extracellular domain or loop, SED/SEL or EC1) and one longer, typically 100 amino acid residues (the large extracellular domain/loop, LED/LEL or EC2). Although several protein families have four transmembrane domains, tetraspanins are defined by conserved domains listed in the Protein Families database under pfam00335.12. The key features are four or more cysteine residues in the EC2 domain, with two in a highly conserved “CCG” motif.

In certain embodiments, the moiety is selected based on target specificity or recipient cell specificity. For example, the moiety may be a small disintegrin, such as the 5.4 kDa echistatin that binds target integrin (*e.g.*, $\alpha_v\beta_3$ or $\alpha_5\beta_1$).

In certain embodiments, the tetraspanin family protein is further fused to a marker protein or a sequence tag for ease of detection or isolation. Such marker proteins may include a fluorescent marker (such as GFP, DsRed, YFP, BFP, EGFP, CFP, see Kremers *et al.*, JCS 124:157-160, 2011, incorporate by reference), or an enzyme catalytic domain (such as a luciferase, an aequorin, a uroporphyrinogen (urogen) III methyltransferase (UMT), or an enhanced chemiluminescence (ECL) enzyme such as a horseradish peroxidase enzyme (HRP), etc.). The sequence tag may include a polyhistidine-tag, a chitin binding protein (CBP) tag, a maltose binding protein (MBP) tag, and a glutathione-S-transferase (GST) tag.

An illustrative but non-limiting embodiment of the delivery system of the invention is depicted in Fig. 22. One of skill in the art can readily envision other systems according to the teachings of the invention with minor modifications.

In a related aspect, the invention provides a method to deliver a molecule, including a nucleic acid, a protein, a lipid, or a therapeutic molecule based on nucleic acid or protein, using the subject exosome-based delivery system, the method comprising: (1) harvesting exosomes from a cultured packaging cell line, wherein the packaging cell line is introduced with a subject first expression vector (optionally further comprising the second expression vector); and (2) administering the exosomes harvested in step (1) to an individual.

The harvested exosomes are expected to express the targeting ligand on the exterior surface, which targeting ligand is expected to home to the target on the recipient cell, and

promote the fusion of the exosome with the recipient cell, thereby delivering the content of the exosome (including any expressed or packaged molecules) to the recipient cell.

In yet another related aspect, the invention provides a method to treat a symptom of HIV infection in an individual in need to treatment, the method comprising delivering a subject antagomir (*e.g.*, antagomir88 or antagomir99) using the subject exosome based delivery system that expresses the subject antagomir.

In certain embodiments, the method inhibit the formation of atherosclerotic plaques in said individual; inhibit the formation of or differentiation into foam cells in said individual; or inhibit the onset or progression of atherosclerosis, coronary artery disease (CAD), or coronary heart disease (CHD) associated with HIV infection.

As an alternative to the exosome based delivery system, the polynucleotides (*e.g.*, antagomirs) of the invention may also be delivered using LyoVec or other similar products that are commercially available. The polynucleotide / LyoVec complex can be formed according to the manufacture's recommendation, or with minor modification without departing from the spirit of the invention. See, for example, Example 18 below and other examples in which such polynucleotide / LyoVec complex is formed (incorporated herein by reference).

EXAMPLES

This invention is further illustrated by the following examples, which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

Example 1 HIV-Derived vmiRs Are Released by Activated Macrophages via an Exosomal Pathway

Preliminary data shows that HIV vmiRs were present in both cellular and extracellular compartments (Figures 2B-C; and 3A-B), and individual vmiRs were mostly concentrated in exosomes than in cells. It is believed that this could be an explanation why recent report failed to detect HIV-encoded miRNAs in infected cells (Whisnant *et al. MBio* 4(2):e00193-13, 2013). Furthermore, 100 to 1,000,000 copies/mL of exosomal HIV vmiRs were detected in the serum from chronically HIV-infected subjects (Figures 4A and 4B). The data

demonstrates that HIV vmiRs are secreted via the exosomal pathway.

Specifically, Figure 4B shows quantitative PCR measurement of HIV miRNA in exosomal preparations from archived sera of asymptomatic HIV+ persons with peripheral blood CD4⁺ T-lymphocyte count < 200 cells/mm³. Data reflect measurements performed in duplicate. Results shown include exosome preparations isolated from HIV+ sera (n=14) sampled from HIV+ patients (numbered 1-13). Serum samples “10a” and “10b” were drawn on separate days from Patient 10. *p< 0.05.

In addition, it was found that the infection rate of macrophages in HIV-infected patients was <10%, and did not correlate with chronic immune activation. Thus miRNAs transferred through exosomes may play an important role in intercellular communication by inducing TLR-mediated inflammatory responses in the >90% uninfected bystander cells.

Example 2 HIV vmiRs Are Associated with Intracellular Vesicles Destined to Become Exosomes

Components of the RNA-interference silencing complex (RISC) such as GW182, ARGONAUTE2 and mature miRNA associate with multivesicular bodies (MVBs) and exosomes. This example demonstrates that HIV vmiRs colocalize in HIV-infected monocyte-derived macrophages (MDM) with proteins that are known to be enriched in exosomes, such as CD63 and CD81. Human MDM are used here as a clinically relevant cell model for chronic immune activation.

Exosome-producing HEK 293 cells expressing GFP-CD63 (SBI, Mountain View, CA), GFP-CD81, or vector control (OriGene, Rockville, MD) are treated with increasing concentrations of HIV vmiRs (labeled with fluorescent Alexa Fluor 647-labeled HIV vmiRs (vmiR99-AF647, vmiR88-AF647, RNA40-AF647) formulated with LyoVec. LyoVec has been used previously to successfully deliver HIV ssRNA to macrophages. Upon nuclear counterstain (DAPI, blue), colocalization is determined by confocal microscopy. A positive result indicates that HIV vmiRs are associated with intracellular vesicles destined to become exosomes.

Colocalization of vmiRs to exosomes are also confirmed by Amaxa transfection of fluorescently labeled vmiRs with HEK cells expressing GFP-CD63 (OriGene, Rockville, MD) or GFP-CD81 or vector control (SBI, Mountain View, CA), with exosome detection using Nanosight LM10 (Duxbury, MA).

These experiments are expected to demonstrate colocalization of HIV vmiR with HEK cells that overexpress CD63 and CD81, while no colocalization is expected in the vector control. Such a result suggests that HIV vmiRs could be incorporated into intracellular vesicles destined to become exosomes.

Example 3 Inclusion of HIV-Derived vmiRs into Exosome is CD81-Dependent

CD81 is first genetically silenced by siRNA-mediated knockdown (Dharmacon, Boulder, CO). Specifically, monocyte-derived macrophages (MDM) are isolated from buffy coat of healthy subjects using Percol Hypaque, and cultured for 7-10 days in the presence of macrophage colony stimulating factor (M-CSF). Healthy MDM are transfected with On Target Plus siRNA CD81 or non-silencing siRNA as a negative control.

Cells are then infected *in vitro* with various strains of HIV-1, including HIV-1_{Ba-L} or HIV-1_{CM235} (R5-tropic), and HIV-1_{C7/86} (R5/X4 dual-tropic; NIH Reagent Suppository Program), and are incubated for 3-15 days. Past experience indicates that the percentage of cells infected *in vitro* with HIV is between 80-85%.

HIV replication is confirmed by culture supernatant p24 levels using ELISA. Exosomes are isolated and probed with exosomal marker CD63 using NanoSight LM10 or ExoELISA (SBI, Mountain View, CA) followed by detection of HIV-derived vmiRs in both silenced and non-silenced cells. It is expected that, in CD81-silenced macrophages, HIV-derived vmiR expression is reduced (if not abolished) compared to non-silenced cells.

ICAM-1 serves as a positive control for this experiment, as its expression is reduced in CD81-silenced cells.

Alternatively or in addition, CD81 knockdown is carried out via stable transfection using vectors coding shRNA mir precursor sequences under Zeocin selection. shRNA expression is induced with tetracycline (Life Technologies, Foster City, CA).

Example 4 Trafficking of HIV vmiRs into Exosomes

Lentiviral shRNA transfection is used in this experiment to allow DROSHA processing of immature shRNA (pre-mir88, 99 and TAR) in the nucleus, and subsequent exporting of the products to the cytosol.

Specifically, HEK 293 cells are treated with lentiviral vectors expressing immature

shRNA (pre-vmiR88, 99 and TAR), or empty vector as negative control. Expression of vmiR sequences is confirmed by qRT-PCR. Exosomes are then harvested from supernatants and purified using ExoQuick-TC and evaluated for the presence of exosomal marker CD63.

The presence of mature vmiR88, vmiR99, and vmiR-TAR sequences are determined by qRT-PCR, and confirmed by TOPO TA cloning and sequencing the PCR products.

It is expected that qRT-PCR products of the expected size are only present in exosomes of cells expressing vmiR shRNA sequences processed into mature vmiRs, but not with empty vector.

Example 5 HIV vmiRs Are Delivered to and Internalized by Healthy Primary MDM via Purified Exosomes

Preliminary data showed that HIV vmiRs in serum exosomes occur in high copy numbers (Figures 4A and 4B). Thus HIV vmiRs may function outside HIV-infected macrophages by being delivered into physiologically relevant recipient cells.

In this experiment, exosome transfer into primary macrophages is monitored by adding purified exosomes (green label, lipid dye PKH67; Sigma) from HIV-infected macrophages detectable by confocal microscopy through capture by HLA-DR-coated beads or with pHrodo, a dye that becomes fluorescent at acidic pH (Life Technologies, Carlsbad, CA). Acceptor MDMs are plated and then combined with different doses of purified labeled exosomes, followed by time dependent (*i.e.*, 1-2 hrs.) incubation at either 4°C or 37°C. Exosomal uptake is expected to be inhibited by disruption of actin filaments (cytochalasin D), or by blocking the vacuolar proton ATPase (bafilomycin A1).

Exosomes can be separated from viral particles by ultracentrifugation on 6-18% iodixanol (GE Healthcare) density gradients. According to previously reported data, exosomes are present in low-density fractions (presence of acetylcholinesterase (AChE) activity as marker of exosome vesicles) while HIV virions are located in high-density fractions (absence of AChE activity).

In addition, to exclude the possible presence of minute amounts of infectious HIV, exosome fractions are tested by infectivity assay or TaqMan Real Time PCR assay.

Example 6 HIV vmiRs Are Delivered to and Internalized by Healthy Primary MDM via Exosomes - Transwell Experiment

Transwell experiments are performed with PKH67-labeled HIV-infected or non-infected MDM placed in the top well and primary MDM (recipient cells) placed in the bottom well. The 1 μ m porous membrane allows transfer of fluorescent exosomes but not MDM. Transference of exosomes through the membrane is confirmed by adding purified PKH67-labeled exosomes in the top chamber. Internalized labeled exosomes are measured after 6 hrs. and 24 hrs. of co-culture by FACS and spectrofluorometer. The presence of vmiRs in the recipient cells is analyzed by qRT-PCR in both recipient cells as well as the culture supernatants.

The data is expected to show that HIV-vmiRs are disseminated to and accumulated in primary MDM through internalization of exosomes secreted by HIV-infected MDM. The source of the exosomes (*e.g.*, whether they are shed from HIV-infected macrophages) is determined by analyzing the culture supernatant for the presence of exosomes in the lower chamber without cells. Furthermore, culture supernatant is also analyzed for MDM by probing for CD14, a macrophage marker. The exosomes are probed for the presence of exosomal marker CD63 by Western blotting. In addition to determine the presence of HIV-vmiRs in exosomes, exosome-containing vmiRs are analyzed by qRT-PCR.

Example 7 HIV vmiRs from Internalized Exosomes Regulate Inflammatory Response

This experiment demonstrates that HIV vmiR transferred from exosomes induce inflammatory responses in bystander cells, as measured by the presence of TNF α , IL-6, IL-12 in cell-free supernatants comparing HIV-infected and non-infected co-cultured cells.

Pre-vmiR88, 99 in lentiviral vectors (Life Technologies, Foster City, CA) are transfected to HEK 293 cells, and vmiR TAR serves as a negative control. These cells are placed in the upper chamber of a transwell, and analyzed for cytokines released by MDM in the bottom chamber. To distinguish the source of cytokines (either infected cells vs target cells), cytokines are measured in both the upper and bottom chambers.

The data demonstrates that HIV vmiRs shuttled through exosomes that are released from HIV-infected MDM regulate inflammatory responses.

About 10^2 - 10^6 exosomal copies of vmiRs/mL have been detected in serum. To show

that the amounts are large enough to stimulate TLR8-induced signaling, or to determine the number of copies sufficient to stimulate TLR8 signaling pathway, exosomes are isolated and purified from HIV-infected macrophages, and the number of vmiR copies/exosome is determined by qRT-PCR and NanoSight LM10. MDM is then incubated with a range of concentrations of exosomes bearing vmiR88, 99 to determine the optimum concentration for inducing cytokine release from macrophages. vmiR TAR is used as negative control, and ssRNA40 formulated in LyoVec is used as positive control.

Data from these studies determines the number of copies required to stimulate an inflammatory response.

Example 8 HIV-Encoded miRNAs Are Present in the Serum of HIV-Infected Persons

Applicants believe that novel HIV-derived small non-coding RNAs (such as miRNAs and ssRNA) in HIV-infected macrophages can activate innate immune cells. Upon reviewing published Deep Sequencing data for HIV-infected cells, Applicants identified a hotspot for small RNAs derived from R and U5 stem regions within the LTR region of HIV genome (Figure 5A). In addition, recognizing that folding of shRNA mir intermediates are critical in miRNA biogenesis, Applicants used UNAFold software to predict RNA hairpins within the hotspot for miR synthesis. These RNA hairpins arise from structured regions in the HIV genome that facilitate DROSHA- DICER- and ARGONAUTE-mediated processing (Figure 5B). Applicants focus on a hotspot with corresponding GU-rich sequences (Figure 5A, in green peaks) and with RNA secondary structures (Figures 5C and 5D). Applicants showed that GU-rich ssRNA induced a robust TNF α release in macrophages. Other hotspots were also detected, but they lack these structures, and thus those regions of the HIV genome were excluded.

Applicants identified novel HIV-derived mature candidate miRs named “vmiR88” and “vmiR99” from R/U5 and U5 region, respectively (Figures 5C and 5D, highlighted in blue).

Applicants confirmed the UNAFold software folding prediction of shRNAs vmir88 and vmir99, which suggests the structures of intermediates in the biogenesis of the mature vmiR-TAR, vmiR88 and vmiR99. UNAFold's thermodynamic calculations predict that all three shRNAs fold spontaneously ($\Delta G < 0$) into alternative hairpins that are stable (high melting temperature, $T_M > 53.8^\circ\text{C}$ in 1M Na⁺).

Furthermore, Applicants also detected the abundant TAR-derived vmiR43/9175 that has been already reported (Figure 5B). Critical preliminary data detected these novel HIV-derived miRNAs in cultured supernatants of activated macrophages and in serum from HIV-infected persons (Figures 2B and 4) using sensitive quantitative RT-PCR enhanced by using LNA primers (Figure 2B and 2C). Applicants detected 10 and 50 copies of vmiR-TAR and vmiR99 respectively in HIV+ AM, provided the cells were activated with phorbol myristate (PMA).

Specifically, to address the issue of detection limitations, a highly sensitive Real Time PCR method enhanced by Locked Nucleic Acid (LNA) primers was developed for single-copy detection of HIV miRNAs. The assays were standardized using chemically synthesized miRNAs for absolute quantitation. Synthetic miRNA standards were reverse transcribed into cDNA, and real Time PCR was performed using 10-fold serial dilutions of cDNA. This provided a concentration-dependent lag that precedes discernible exponential amplification as shown by monitoring normalized fluorescence intensity (Fig. 12D, F, H). The standard curves for vmiR-TAR, vmiR88, vmiR99 and ssRNA40 (Fig. 12E, G, I) demonstrate a seven log analytical range including single-copy detection ($\log(\text{copies/well})=0$). Thermal denaturation provided experimental melting temperatures (T_M) of SYBR Green-bound PCR products in good agreement with the manufacturer's expected values (Table I). Thus, sensitive LNA-enhanced Real Time PCR methodology offers a powerful approach to detect and analyze HIV-produced miRNAs of low-abundance.

Based on this technology, in AM infected *in vitro* for 15 days, Applicants detected the vmiR-TAR, vmiR88, vmiR99, but not ssRNA40, whereas no vmiRs were detected in mock-infected AM (Figure 2B). These data demonstrate that HIV-encoded miRNAs are present in the serum of HIV-infected persons and are produced in HIV-infected macrophages.

Example 9 Endocytosis Is Required for HIV vmiRs to Reach Endosomal TLR8

When mature miRNAs were added to macrophages, release of TNF α was robust and was induced only by vmiR88, and vmiR99 but not by vmiR-TAR suggesting a mechanism driven by sequence differences (Figure 2D). Importantly, on a molar basis, these novel vmiR88 and 99 molecules are more potent in inducing TNF α compared to endotoxin lipid A (Gram-negative bacteria-derived TLR4 ligand implicated in the gut translocation hypothesis of HIV chronic immune activation) (Figure 2D). This is the first demonstration that HIV-

encoded vmiRs are generated in HIV-infected macrophages that induce TNF α release by macrophages.

Preliminary data show that HIV vmiR-induced TNF α release is robust and dependent on endosomal TLR8 (Figure 2D and 6). Since TLR8 is located in the endosome, this study demonstrates that this signaling event is dependent on dynamin-mediated endocytosis.

Healthy MDM are pre-treated in the presence or absence of guanosine triphosphatase (GTPase) dynamin inhibitor (Dynasore, Sigma) followed by challenge with vmiRNAs (Alexa 488, green color; vmiR88, 99, TAR, synthesized by Harvard Core Facility, LyoVec formulation). Cells are incubated with HIV vmiR (0.01-1.0 μ g/ml) at 37°C to allow internalization for 1-4h, and during the final 30 mins., the endosomal probe LysoTracker Red DND99 (Invitrogen) is added. Cells are fixed and nuclear counterstained with DAPI (blue, Invitrogen). Images are captured using Zeiss laser scanning confocal microscope. ssRNA40 is used as positive control.

Due to possible off-target effects of pharmacological inhibitors, experiments are repeated with MDM following dynamin-targeted gene silencing (On Target Plus siRNA, or non-silencing siRNA as control (Dharmacon, Boulder, CO) using Amaxa system (Amaxa, Cologne, Germany). Gene knockdown efficiency is monitored using antibody against dynamin 2 and analyzed by Western blotting (BD Transduction Labs). Targeted and non-targeted cells are then similarly treated as above. These studies demonstrate that HIV vmiRs are taken up via endocytosis and colocalize with endosomal TLR8.

To show that endocytosis is required for vmiR-mediated cytokine release, adherent macrophages will be pre-treated in the presence or absence of dynasore followed by vmiRs and incubated for 24h. Release of TNF α , IL-12, and IL-6 will be measured by ELISA from cell-free supernatant. TLR4 ligand lipid A is used as a positive control and GA-rich ssRNA41 (InvivoGen) is used as a negative control. These studies demonstrate that HIV-vmiR-mediated induction of TNF α in macrophages is dependent on dynamin-mediated endocytosis.

To determine further whether HIV infection is dependent on dynamin-mediated endocytosis, MDM from healthy subjects are infected *in vitro* with macrophage-tropic HIV-1 (R5 HIV-BaL isolate) in the presence or absence of dynasore and compared to MDM without *in vitro* infection for 3-15 days. HIV replication is monitored for p24 levels using ELISA. These studies determine the effect of dynasore has in inhibiting HIV replication in HIV-

infected macrophages.

Example 10 Mechanism of HIV miRNA Activation of Endosomal TLR8

Preliminary data show that HIV-encoded miRNAs such as vmiR88 and vmiR99 induce the release of TNF α in a dose-dependent manner (Figure 2D), and vmiR99-mediated TNF α release is dependent on TLR8 (Figure 6). This is the first evidence that HIV-derived miRNAs induce TNF α production in AM.

Immunoprecipitation (IP) assays are used to show that exogenous HIV-encoded vmiRs can activate endosomal TLR8. Specifically, healthy MDM are treated with LyoVec (InvivoGen) alone and LyoVec-vmiR88, vmiR99 and vmiR-TAR (Table 1), and incubated for different times. Anti-TLR8 antibody (Imgenex, San Diego, CA) are used to generate the immunoprecipitates from cell lysates followed by vmiR determination using quantitative Real-Time RT-PCR. TNF α , IL-6, and IL-12 are analyzed from the supernatant using ELISA. The above experiment is validated by treating HEK-293 cells expressing GFP-TLR8 with LyoVec alone or with LyoVec formulations of 5'-biotinylated vmiR88, 99, and vmiR-TAR and generate the immunoprecipitate. As a positive control, ssRNA40 activates TLR8 to release TNF α , whereas ssRNA41 was used as a negative control. These data demonstrates that TLR8 recognizes and bind HIV-encoded vmiRs to induce a pro-inflammatory response.

Immunoprecipitation is a valid methodology in assessing binding, but it may be limited by sequestration of unrelated proteins in the pellet due to inadequate washing that might give false positive results. Part of the problem may be solved by using Fluorescence Resonance Energy Transfer (FRET) as an independent method to validate binding of HIV vmiR with TLR. Specifically, HEK cells expressing TLR8, TLR7, TLR3 or vector control (Imgenex, San Diego, CA) are treated with increasing concentrations of FRET donor: fluorescent Alexa Fluor 488-labeled oligos (vmiR99-AF488, vmiR88-AF488, RNA40-AF488) formulated with LyoVec, and vmiR-TAR-AF488 as a negative control. Cells are permeabilized and labeled with FRET acceptor: Alexa 647 conjugates of monoclonal antibody (anti-TLR8 clone 44C143 or anti-TLR3 clone 716G10.15) Imgenex). Ligand binding is quantified by exciting the FRET acceptor (oligo-Alexa488, green color) and detecting emission by the adjacent FRET acceptor (anti-TLR MAb Alexa 647 conjugate, red color) using flow cytometry, confocal microscopy and spectrofluorometer. The specificity of receptor-ligand interaction is confirmed by competitive binding. Alternative unlabeled

competitors including TLR8 agonist R848 and TLR3 agonist poly (I:C) are also tested and assess specificity. With two-color imaging, sub-cellular RNA ligand and TLR receptor localization is assessed by immunofluorescence microscopy (Nikon Eclipse E800 with SPOT RT3 camera), and a third color can assess co-localization to acidic compartments using LysoTracker Blue stain.

Fluorescence Polarization is also used as an independent method to measure HIV miRs binding to TLR. This is a sensitive homogenous binding assay. Briefly, proteoliposomes displaying full-length recombinant TLR8, TLR3, TLR9 or null from Abnova (Walnut, CA) are treated with increasing concentrations of fluorescent Alexa 488-labeled phosphorothioate oligos (vmiR99, vmiR88, RNA40, RNA41). Binding is obtained by using a range of acidic cacodylate buffers, pH ~ 5.2-7.2, to mimic the acidic compartment in which ssRNA ligand engages a TLR receptor. To quantify binding of fluorescent vmiR to TLR receptor, the concentration dependence of fluorescence polarization readings (Molecular Devices M5 plate reader, Core facility) are converted to fluorescence anisotropy and the EC_{50} calculated using GraphPad Prism software (San Diego, CA). The specificity of receptor-ligand interaction is then confirmed by competitive binding to measure IC_{50} . Alternative unlabeled competitors including other vmiRs, TLR8 agonist R848 and negative controls (dsRNA and TLR3 agonist poly (I:C)) are also tested to determine IC_{50} and assess specificity and selectivity.

Example 11 Signaling Molecules that Mediate HIV miRNA Activation of TLR8 Signaling

While not wishing to be bound by any particular theory, Applicants believe that in macrophages TLR8 activation elicits increases in pro-inflammatory cytokine release, and TLR8 activation requires involvement of TLR8 signaling mediator.

The intracellular adaptor protein MyD88 is downstream from TLR8. MyD88 is expressed in macrophages and is required in mediating TLR4 induction of TNF α . To show that induction of pro-inflammatory cytokines occurs via MyD88, siRNA-mediated knockdown of MyD88 is performed using synthetic siRNA (Dharmacon, Boulder, CO). Following MyD88-targeted gene silencing or non-silencing control (On Target Plus siRNA MyD88 or non-silencing siRNA), healthy MDM are challenged with LyoVec alone or with LyoVec-HIV-containing vmiRs 88, 99, TAR in LyoVec formulations for 24h and culture

supernatants are analyzed for the presence of TNF α , IL-6, and IL-12 levels by ELISA. EGF is used as a positive control, and HEK cells are used as negative control.

To verify the specificity of the MyD88 knock down, cells are challenged with TLR-independent stimuli (PMA) and culture supernatants analyzed for cytokines by ELISA.

The above studies are validated by performing an NF- κ B reporter assay in HEK-293 cells. HEK-293 cells expressing TLR8 (Imgenex, San Diego, CA) are treated with LyoVec-miR88, 99, TAR (or LyoVec alone) followed by NF- κ B reporter assay. Gardiquimod and ssRNA40 are used as positive controls, which are specific ligands of TLR7 and TLR8, respectively. The signaling triggered by HIV-derived miRs is further characterized by analyzing the whole-cell lysates extracts for MAP kinase activity using a TLR8-linked downstream signaling cassette. Activation (phosphorylation) of ERK MAP kinase is also evaluated. ssRNA40 is used as a positive control, while ssRNA41 is used as a negative control.

These experiments demonstrate that neither LyoVec alone nor LyoVec-miR-TAR could induce cytokine release. Similarly, irrespective of whether or not TLR8 and or MyD88 are silenced, they do not stimulate ERK MAP kinase or NF- κ B activity. However, it is expected that cytokine release induced by both vmiR88, and -99 are markedly reduced as compared to that in the non-silencing control.

Taken together, induction of pro-inflammatory cytokines induced by vmiR88 and -99 in human macrophages is anticipated to be dependent on the TLR8 signaling pathway.

Example 12 Activation of TLR8 Dependent on Evolutionarily Conserved GU-Rich Motif

Preliminary data showed that HIV-derived vmiR88 and vmiR99 (but not vmiR-TAR) induced a robust TNF α release in AM, establishing signaling specificity among structurally distinct vmiRs (Figure 2D).

Furthermore, upon careful examination of vmiR sequences, Applicants noted that vmiR88 and 99 have GU-rich core that is lacking in vmiR-TAR (Table1). This GU-rich core is highly conserved amongst HIV-1 strains (Figure 5A, green peak; Figure 9), and GU motifs are predominant in the TLR8-activating ssRNA40 (Table 1).

Specifically, the consensus sequence of the 46-nt GU-rich tract is strongly conserved with two or fewer mismatches in 84% of HIV genomic isolates (196 independent isolate

sequences examined; Fig. 12A) of which 37% are completely identical to the consensus. Therefore, the GU content and sequence conservation of the candidate miRNAs were evaluated. By scanning every miRNA-sized (21-bp) segment in the genomic RNA of HIV-1_{BaL}, it was determined that these segments have base compositions of $46.5 \pm 11.8\%$ G+U. TAR miRNA with only one UG is relatively GU-poor (35% G+U; Fig. 12C). However, within the R and U5 regions, the two candidate mature miRs have very high G+U base compositions (vmiR88, 71% G+U and vmiR99, 76% G+U), which were over two standard deviations above average for HIV-1_{BaL} strain (Fig. 12B). Moreover, the individual candidate miRNAs are highly conserved. VmiR99 is identical to 82% of genomic HIV sequences and has two or fewer mismatches in 96% of genomic sequences from 254 independent isolates (Fig. 12B). Similarly, vmiR88 is identical to 45% of genomic HIV sequences and has 0-2 mismatches in 82% of genomic sequences from 201 independent isolates (Fig. 12B).

The sequence conservation of the GU-rich tract, which overlaps the alternative RNA secondary structures, the poly(A) hairpin, would support the important function of producing active vmiR88 and vmiR99. Sequence variants of vmiR88 and vmiR99 differ in potency for eliciting TNF α release in macrophages. By contrast, the normal function of the poly(A) hairpin is governed by the stability of the RNA secondary structure rather than its sequence, because mutations can destabilize the hairpin and cause loss of function, but compensatory mutations on the opposite strand of the stem restore the stability of the RNA secondary structure and function.

To determine the structural features in the sequence of vmiR88 and vmiR99 that confer the ability to activate TLR8, the GU motifs are disrupted by substituting bases 15-21 in vmiR88, and bases 4-10 or 14-22 in vmiR99. In addition, an antagomir scan using a series of short 10-to 12-mer antagomirs (including a number of LNA residues to improve duplex stability) is performed to delineate which nucleotide sequences are specific for blocking vmiR-induced TNF α release. Their ability to induce TNF α , IL-6, IL-12 in MDM is then tested, with vmiR-TAR as negative control and ssRNA40 as positive control. The results above are validated using TLR8-HEK-293 cells by measuring TLR8-mediated activation of NF- κ B.

These experiments are expected to demonstrate that vmiR induction of pro-inflammatory cytokines as well as NF- κ B activation in MDM are dependent on the GU-rich core, as evidenced by strong inhibition of these effects when the GU core was substituted (Figure 7A). In addition, replacement of GU-rich motif inhibits the inflammatory response

mediated by GU-rich HIV vmiRs. Thus the presence of GU-motif in HIV-derived miRNAs is required for pro-inflammatory cytokine release and NF- κ B activation. HIV vmiRs maybe an important pathogenicity determinant of chronic immune activation in HIV infection.

Overall, the data shows the specific nature and position of nucleotides in mature sequence of HIV-derived miRNAs involved in TLR8 activation.

Example 13 Mechanism of Antagomir Inhibition of HIV miRNA-Mediated Activation of TLR8

Preliminary data showed that RNA41 (RNA40 with all U residues substituted by A) inhibits the effect of RNA40 on MDM cells (Figure 7A). Applicants have developed a duplex formation assay using RNA coupled to Alexa Fluor 488 at the 3' end of one RNA strand and a fluorescence quencher dye (Iowa Black) coupled to the 5' end of another RNA strand (Figure 7B). Preliminary data demonstrate annealing of fluorescent ssRNA40-F (100 nM) to a 10-fold molar excess of ssRNA41-Q quencher strand, which indicates formation of a RNA40·RNA41 duplex with a $T_m=36^\circ\text{C}$ (Figure 7D).

To narrow down the site of the critical duplex vs. ssRNA flanking arms; a shortened 10-mer fragment of ssRNA40 was annealed with ssRNA41, with exhibited decreased stability ($T_m=13^\circ\text{C}$; data not shown). This *in vitro* duplexing assay enables the mapping of the critical region(s) of the vmiR that is hybridized by the biologically active short antagomirs.

The critical regions of vmiR99 is determined by mapping the minimal pro-inflammatory core of vmiR99. The core is mapped using short overlapping antagomirs as probes that had been demonstrated to inhibit TNF α release (Figure 8). Specifically, a set of overlapping 10- to 12-mer antagomir99Q strands are annealed to vmiR99F and analyzed for T_m in comparison to the full-length duplex vmiR99·antagomir99. The critical region of vmiR99 that hybridizes to signal-blocking short antagomirs is confirmed by biochemical analysis.

The vmiR99*10-mer antagomi99 is expected to be thermally stabile (as assessed by T_m) at physiological temperature, pH, and ionic strength that is permissive of stable duplex formation, after judicious incorporation of a few LNA residues into the antagomir sequence.

Activation of TLR8 was shown to inhibit HIV replication in *ex vivo* infected lymphoid tissue while inducing virion release from transformed cell lines. To determine whether vmiR99 or vmiR99 antagomir influences HIV infection or replication in primary

macrophages, macrophages are incubated with vmiR99 or vmiR99 + antagomir99 for 24 hrs., followed by HIV infection for 3 hrs. Infected cells are then washed and incubated with or without vmiR99 or vmiR99 + antagomir99 for different times (3-12 days). Extracellular release of HIV p24 into the supernatant is detected by ELISA.

These studies show that activation of TLR8 by vmiR99 or inhibition of TLR8 by vmiR99 antagomir inhibits HIV replication.

Since TLR3 is activated by dsRNA, duplexing between vmiR and antagomir might act as TLR3 agonist. Thus, HEK cells overexpressing TLR3 are incubated with the duplex complex and analyzed for NF- κ B activation. Poly(I:C) is used as positive control, and ssRNA40 is used as negative control. To complement the experiment above, TLR3 in MDM is knocked down, and the cells are challenged with duplex molecules before measuring pro-inflammatory cytokines.

These experiments demonstrate that HIV-derived vmiRs hybridize with the antagomir oligonucleotides, suggesting that duplexing might mask the binding site of the HIV vmiRs with TLR8, thereby inhibiting TLR8 signaling. Therefore, antagomirs are good candidates for inhibiting inflammatory response elicited by vmiRs in macrophages, and could be useful in containing chronic immune activation in HIV+ subjects. Furthermore, vmiR99 will decrease HIV replication through TLR8 activation.

To confirm specificity of the vmiR-induced proinflammatory response, an antagomir was designed complementary to vmiR99. vmiR99-mediated macrophage TNF α release was significantly inhibited in the presence of specific antagomir either pre-annealed to vmiR99 before addition to cells or by pre-treatment of macrophages with antagomir followed by vmiR99 challenge (Fig. 13E).

Example 14 Exosome-Mediated Antagomir Delivery *In vivo*

Transfection of RNA molecules in human monocytic leukemia THP-1 cells are shed via exosomes. For clinical application, the instant invention provides a delivery system using exosomes to deliver antagomir *in vivo*.

Specifically, antagomir99 or control miRNA is first transfected into THP-1 cells. The medium is then replaced with fetal bovine serum-free medium, and the cells are incubated with phorbol ester to allow differentiation.

Exosome is isolated using ultracentrifugation, and total RNA is extracted from the exosome and analyzed for antagomir sequence using qRT-PCR.

To test the effects of antagomir99 on macrophage soluble inflammatory markers induced by HIV in humanized mice, mice are infected, and treatment commences at the peak of infection (7-15 days) and continues for 4-8 weeks.

Mice receive a suspension of antagomir99 exosome fraction in phenol red-free RPMI injected intravenously (optimum concentrations are determined based on the *in vitro* assay) every three days. BLT mice are sorted into five mice per group. The mice are bled three days after start of injection and every week thereafter. Inflammatory markers such as VCAM-1, ICAM-1, TNF α , IL-12, IL-6 and viral load are monitored every week. These studies show that novel unique antagomirs can dampen the inflammatory response in HIV infection model *in vivo*.

It is expected that serum inflammatory markers are increased in HIV-infected mice compared to mock infection, and are dampened by antagomir99. The novel antagomir delivery system avoids problems with RNAi delivery *in vivo*.

These data suggests a therapeutic role for antagomirs in preventing chronic immune activation in HIV+ subjects and targeted delivery via exosomes.

Example 15 Sequencing of Cloned Mature vmiR88 and vmiR99

To delineate the boundaries of mature viral miRNAs, cell extracts and exosomal extracts were analyzed. Sample cell extracts were *in vitro*-infected AM (healthy AM +HIV), HIV-positive U1 macrophages stimulated by PMA (U1 +PMA). Exosomal extracts were from exosomes of HIV+ human serum (HIV+ serum 10b).

Analysis of RNA from HIV-infected U1 cells and *in vitro* infected AM cells revealed full-length vmiR88 and vmiR99 (Fig. 11). One clone from U1 cells showed a missing 3'-terminal G, like synthetic vmiR99. Furthermore, analysis of exosomal RNA exhibits full-length vmiR88 in clinical HIV+ serum of an asymptomatic person and in *in vitro* infected AM. However, exosomal miRNAs also demonstrated some longer variants. Exosomes from PMA-stimulated U1 cells produced vmiR88 with a 3'-terminal 15-nt HIV RNA extension (Fig. 11) and vmiR99 with a 3'-terminal 13-nt HIV RNA extension (Fig. 11). Analysis of vmiRs in exosomes from HIV+ serum of an asymptomatic person exhibited sequences from vmiR99 with four 3' nucleotides substituted for 9 nt of HIV RNA (Fig. 11). Observed DNA

sequencing of vmiR88 or vmiR99 have differing 3' termini followed by polyadenylation that may have occurred *in vivo* and/or prior to first strand cDNA synthesis by *in vitro* polyadenylation. The 3' termini of vmiR88 and vmiR99 lie downstream from the classical poly A site (Fig. 11). Moreover, the observed vmiR88 sequence spans the classical poly(A) site (Bohnelein *et al.*, *J. Virol.*, 63: 421-4, 1989), suggesting a mechanism of alternative RNA folding and cleavage produces mature vmiR88. Interestingly, vmiR88 (HIV-1 poly(A) hairpin) was shown to regulate polyadenylation (Das *et al.*, *J. Virol.*, 73: 81-91, 1999). Observation of vmiR99 sequences from HIV-infected samples suggests a similar mechanism for vmiR99 biogenesis.

In summary, sequences of qRT-PCR products of vmiR88 and vmiR99 from cell extracts were confirmed and exhibited 3' termini that are distinct from the reported 3'-terminal polyadenylation site of HIV genomic RNA. Longer variants of vmiR88 and vmiR99 bearing 3' extensions of viral sequence were detected in exosomes, which may be related to selective miRNA packaging into exosomes or have other functions.

Example 16 Stimulation of THP-1 Macrophages by vmiRs is Rapid, and is Inhibited by Complementary, Partially Complementary, and Non-complementary Antagomirs

To determine the time course for HIV vmiRNA-mediated macrophage TNF α release, human THP-1 macrophages were incubated with vmiR99 and TNF α release measured in cell culture supernatants over 2-24h. THP-1 macrophages treated with vmiR99 resulted in half-maximal TNF α by 2 hr and maximal release of TNF α by 6 hr (Fig. 14A). Treatment with controls (Lipid A and ssRNA40) induced robust release of TNF α (Fig. 14A).

Specifically, in response to vmiR99, half-maximal TNF α release was observed by 2h with maximal release by 6h, which remained stable through 24h (Fig. 14A). By contrast, vmiR99-induced gene expression was slow and transient; induction of TNF mRNA did not exhibit a statistically significant increase until 12 hr followed by a decline to basal expression by 24h (Fig. 14A). Thus, vmiR99-mediated human macrophage TNF α release was rapid and consistent with release of pre-formed TNF α protein, suggesting direct stimulation rather than requiring vmiR99-targeted gene transcription, translation, post-translational modification, trafficking, externalization of TNF α protein or vmiR99-targeted silencing of genes regulating the pathway leading to activation of TNF α release.

The specificity of antagomirs was tested. ssRNA40, vmiR88 and vmiR99 stimulated TNF α release by THP-1 macrophages (Fig. 14B). Pre-treating macrophages by either antagomir88 or antagomir99 provided equal inhibition of TNF α release for all three signaling ligands (Fig. 14B). Treatment with perfectly complementary vmiR / antagomir pairs (vmiR88 / antagomir88 and vmiR99 / antagomir99) inhibited TNF α release (Fig. 14C).

Specifically, pre-treatment with antagomir (1h) followed by vmiR treatment (perfectly complementary vmiR/antagomir pairs are strongly favored to form full-length 21-nt dsRNA duplexes (vmiR88/antagomir88 and vmiR99/antagomir99; $\Delta G = -31.0$ and -38.4 kcal/mole, respectively) inhibited TNF α release (Fig. 14B).

Surprisingly, half complementary vmiR/antagomir pairs that can form half-length 10-nt dsRNA duplexes (vmiR88/antagomir99 and vmiR99/antagomir88; both with $\Delta G = -13.8$ kcal/mole; calculated $T_M = 39^\circ\text{C}$) were inhibited to the same extent as perfectly complementary pairs (Fig. 14B). Further, ssRNA40/antagomir88 have minimal complementarity (6-nt dsRNA duplex) but TNF α release was inhibited as well as perfectly complementary vmiR/antagomir pairs. Because an ssRNA40•antagomir88 duplex has poor thermodynamic stability ($\Delta G = -8.6$ kcal/mole), inhibition by duplexing the signaling ligand (ssRNA40) can be ruled out in this case, suggesting an alternative mechanism of inhibition.

Overall, the fact that TNF α release was inhibited by antagomir88 and antagomir99 even with partial or no complementarity to the ssRNA ligand, suggests that these antagomirs may function as receptor antagonists instead of their intended function as ligand antagonists. The data may also suggest that targeted duplexing of vmiRNA with specific antagomirs is not essential to impairing vmiR88 or vmiR99-mediated TNF α release, but may require alternate mechanisms such as competitive binding to the ligand-binding site of cellular receptors such as TLR8.

Example 17 Stimulation of THP-1 Macrophages by vmiRs Dependent on vmiR Sequence Motifs

The example shows that sequence motifs of vmiRs affects stimulation of THP-1 macrophages to release TNF α . Variants of vmiR88 and vmiR99 were chemically synthesized. Small molecule ligands of TLR8 (R848, CL075 and CL097; Tanji *et al.*, 2013) are purine analogs that mimic the structure of adenine, suggesting that adenine residues may be important components of functional ligands of TLR8. By contrast, ssRNA41 (ssRNA40

with all U residues substituted for A) fails to stimulate TNF α release (Han et al., 2012).

To test the hypothesis that nucleotide substitutions for A may upregulate or downregulate TNF α release, GU-rich motifs were prepared to substitute U residues with A in three major blocks (nt 4-8, 13-20 or 26-31) or throughout the entire vmiR88 or 99 (Fig. 14C). Unstimulated and control ssRNA41-treated macrophages released little TNF α . VmiR88, 99 and positive control ssRNA40 stimulated TNF α release, and variants of either vmiR88 or vmiR99 bearing alterations of the middle block (U13-20A modification) stimulated higher TNF α release than either wild-type vmiR (Fig. 14C). Substitution of all U residues to A (vmiR88 U4-20A) stimulated lessened TNF α release compared to vmiR88 (Fig. 14C). Substitution of all U residues to A (vmiR99 U13-31A) stimulated greater TNF α release than vmiR99 (Fig. 14C).

Example 18 HIV-derived vmiRs stimulate lipid accumulation in human alveolar macrophage (AM) cells

Primary AM cells were isolated from healthy volunteers by clinical bronchoscopy followed by cell adhesion onto 12-well microplates. Viral miRNAs and antagomir99 were formulated in LyoVec and applied at the indicated concentrations (μ g/mL) for 24 hr. Cells were washed, fixed and stained for lipid content with the lipophilic dye, BODIPY 493/503 (10 μ g/mL in PBS) and for cell number using the nuclear stain, DAPI.

Figure 17 shows that lipid accumulation was stimulated by vmiR88 and in a dose-dependent manner by vmiR99, but was not stimulated by vmiR-TAR. Pre-treatment with antagomir99 (1 hr before applying vmiR99) blocked lipid accumulation to that of the unstimulated control. Fluorescence intensities for BODIPY and DAPI were measured using a spectrofluorometer (SpectraMax Gemini EM, Molecular Devices, Sunnyvale, CA). Results depict mean+SEM. *, $p < 0.05$ using Dunn's multiple comparisons test.

The result shows that primary human alveolar macrophages treated with vmiRs can be stimulated to differentiate into foam cells. Applicants believe that macrophage differentiation into foam cells is the mechanism by which HIV elicits chronic immune activation in long-term HIV patients. By blocking vmiR stimulated foam cell differentiation, the subject antagomirs may be used to delay, reduce the severity, or even abolish HIV elicited chronic immune activation in long-term HIV patients.

Unless otherwise provided specifically, the examples above were performed using the following methods and reagents.

Reagents. HIV RNA oligoribonucleotide vmiR-TAR (Table I) and novel HIV RNA oligonucleotides vmiR88 and vmiR99 (Table I) were chemically synthesized (Integrated DNA Technologies, Coralville, Iowa). ssRNA 40/Lyovec and ssRNA41/Lyovec were purchased from Imgenex (San Diego, CA). Lipid A, protease inhibitor mixture, and phorbol myristic acid (PMA) were purchased from Sigma (St. Louis, MO). Cytokine ELISA kits were from R&D Systems (Minneapolis, MN). Oligonucleotides were complexed (50 µg/ml) in LyoVec according to the manufacturer's instructions (Imgenex, San Diego, CA) prior to treating cells.

Table I. Oligoribonucleotides in quantitative Real Time RT-PCR and melting analysis of PCR products.

ORN	Synthetic RNA sequence	qPCR product T_M (°C)	
		Observed	Expected
vmiR88	5'-PO ₄ -G*A*G*U*G*C*U*U*C*A*A*G*U*A*G*U*G*U*G*mU*mG-3'	70.5 ±0.2	71
vmiR99	5'-PO ₄ -G*U*A*G*U*G*U*G*U*G*C*C*C*G*U*C*U*G*U*mU*mG-3'	70.2 ±0.3	70
vmiR-TAR	5'-PO ₄ -C*U*A*A*C*U*A*G*G*G*A*A*C*C*C*A*C*U*mG*mC-3'	69.1 ±0.2	70
ssRNA40	5'-PO ₄ -G*C*C*C*G*U*C*U*G*U*U*G*U*G*U*G*A*C*mU*mC-3'	70.5 ±0.4	71
antagomir88	5'-mC*mA*mC* mA*mC*mA* mC*mU*mA* mC*mU*mU* mG*mA*mA* mG*mC*mA* mC*mU*mC -3'	--	--
antagomir99	5'-mC*mA*mA*mC*mA*mG*mA*mC*mG*mG*mG*mC*mA*mC*mA*mC*mA*mC*mU*mA*mC-3'	--	--

“*”: phosphorothioate linkage

“m”: 2'-O-methyl modification

Human macrophage cell lines

Human promonocytic THP-1 cells and macrophage cell line U937 (American Type Culture Collection), and HIV-infected U1 (HIV-infected U937 subclone; AIDS Research and Reference Reagent Program; Bethesda, Maryland), were differentiated as described (Tachado *et al.*, *Am. J. Respir. Cell Mol. Biol.*, 33(6):610-21, 2005, incorporated herein by reference), using exosome-depleted FBS (System Biosciences, Mountain View, California).

Monocyte cell lines were harvested during exponential growth phase, washed, and then incubated in complete medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units of penicillin, and 100 µg/ml streptomycin), differentiated with PMA (100 nM) for 24h, adherent cells washed three times *with complete medium*, and then cultured in exosome-depleted FBS medium (System Biosciences, Mountain View, California). Macrophage differentiation was confirmed by CD11b expression and enhanced granularity by flow cytometry. Cells in complete medium were stimulated with oligoribonucleotide/LyoVec complexes or Lipid A (10 µg/mL) for 24 hr (37°C, 5% CO₂), and conditioned medium was collected for TNFα analysis by ELISA.

Human alveolar macrophages (AM). Primary human AM cells were obtained from healthy 18-55 year old volunteers (or from asymptomatic HIV+ volunteers) by bronchoalveolar lavage (BAL) using standard techniques (Tachado *et al.*, *Blood*, 115:3606-3615, 2010, incorporated herein by reference). All procedures were performed on consenting adults following protocols approved by the Beth Israel Deaconess Medical Center Institutional Review Board and Committee for Clinical Investigations. Healthy subjects were without HIV risk factors and confirmed HIV seronegative by ELISA. BAL cells were separated from the pooled alveolar lavage fluid, and AM isolated by adherence (Tachado *et al.*, *Am J Respir Cell Mol Biol* 33: 610-21, 2005, incorporated herein by reference). AM viability was determined using trypan blue dye exclusion, and demonstrated >95% positive nonspecific esterase staining. AM in complete medium were stimulated with oligoribonucleotide/LyoVec complexes or Lipid A (10 µg/mL) for 24 hr (37°C, 5% CO₂), and conditioned medium was collected for TNFα analysis by ELISA.

Isolated AM were infected *in vitro* with R5 HIV Ba-L isolate (or mock infection), and maintained in culture for 2 weeks, and HIV infection confirmed by HIV p24 release into culture supernatant as described (Koziel *et al.*, *Am. J. Respir. Crit. Care Med.*, 160(6):2048-2055, 1999, incorporated herein by reference).

Human sera. Archived sera from consenting asymptomatic HIV+ persons with peripheral CD4+ T-lymphocytes counts <200 cells/mm³ as detailed (Koziel *et al.*, *Am. J. Respir. Crit. Care Med.*, 160(6):2048-2055, 1999, incorporated herein by reference) were available for exosome preparation and HIV miRNA detection.

ELISA. TNFα measurements of cell-free macrophage cultured supernatants were determined by ELISA (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions and absorbance was measured at 450 nm using an Emax ELISA

plate reader with multi-point data analysis using SoftMax Pro software (Molecular Devices, Sunnyvale, California). The detection limit for TNF α is 15.6 pg/mL. HIV-1 p24 antigen ELISA was from Zeptometrix (Franklin, Massachusetts). All measurements were performed in duplicate, and mean values of four measurements were used for statistical analysis.

Time course analysis. THP-1 macrophages in complete medium were treated with vmiR99 (1.0 μ g/mL) formulated in LyoVec at the indicated time points (37°C, 5% CO₂). Conditioned medium was collected for TNF α analysis by ELISA. For analysis of cytokine mRNA, adherent cells were treated with Trizol (Applied Biosystems, Foster City, California) and total RNA was prepared according to the manufacturer's instructions. First strand cDNA was synthesized with the High Capacity RNA-to-cDNA kit (Applied Biosystems) using a GeneAmp PCR System 9600 (Perkin Elmer) set for 37°C for 60 min, 95°C for 5 min followed by 4°C. Real Time PCR was performed in 20- μ L reactions with SYBR Select Master Mix (Applied Biosystems) using an ABI 7000 system programmed for 50°C (2 min), 95°C (2 min) followed by 40 cycles of 95°C (15 s) denaturation, 55°C (15 s) annealing and 72°C (1 min) extension. For cytokine expression analysis, qPCR of TNF cDNA was performed using forward primer 5'-TGCCTTGGCTCAGACATGTTT-3' and reverse primer 5'-GCTACATGGGAACAGCCTATTGT-3'. For normalization, GAPD was detected using forward primer 5'-GGAGTCCACTGGCGTCTT-3' and reverse primer 5'-AGGCTGTTGTCATACTTCTCAT-3'. Relative quantitation of TNF gene expression was calculated using the $\Delta\Delta C_T$ method.

TLR8 gene silencing in macrophages. To determine the non-canonical function in HIV miRNA-mediated TNF α release in macrophages targeted TLR8 gene silencing of human AM was performed as previously described (Han *et al.*, *J. Biol. Chem.*, 287:13778-13786, 2012, incorporated by reference).

Exosome isolation. Exosomes were isolated from sera and cell culture conditioned medium using ExoQuick/ExoQuick-TC reagents (System Biosciences, Mountain View, California), according to the manufacturer's instructions. First flow-through fraction was analyzed for exosomal marker using anti-CD63 primary antibody, anti-rabbit HRP conjugate (System Biosciences) and ECL Select Western blotting detection (Amersham).

RNA isolation. Total RNA was isolated from cells using the mirVana miRNA Isolation Kit (Life Technologies, Foster City, California) according to the manufacturer's instructions. Total RNA was extracted from exosomes using a SeraMir Exosome RNA Purification Column kit (System Biosciences) according to the manufacturer's instructions.

Total RNA was estimated by ratio of absorbance at 260 nm and 280 nm, and analyzed by agarose gel electrophoresis.

Real Time RT-PCR. First strand cDNA was synthesized from total RNA using GeneAmp PCR System 9600 (Perkin Elmer), and PCR amplification was performed using the miRCURY LNA Universal RT microRNA PCR system (Exiqon Inc., Woburn, Massachusetts) on an ABI 7500 or ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, California). Chemically synthesized microRNAs were used to standardize the assays in Absolute Quantitation mode. Briefly, RNA samples were pre-diluted to 5 µg/mL in nuclease-free water. RNA is further diluted into a reverse transcriptase master mix using the provided reagents (Reaction Buffer, synthetic spike-in RNA UniSp6 and enzyme mix). The RT mixtures (20 µL/well) are incubated for 60 min at 42°C, 5 min at 95°C, and the cDNA products were cooled to 4°C. The miRCURY LNA Universal RT microRNA PCR system (Exiqon Inc., Woburn, Massachusetts) was used for Real Time PCR analysis. Ten-fold serial dilutions of cDNA from synthetic microRNA were prepared in nuclease-free water as standards. cDNA from standards and samples were pre-diluted 80-fold into nuclease-free water containing ROX dye (50 nM for ABI 7500 instrument or 500 nM for ABI 7900HT; Applied Biosystems). The qPCR master mix was prepared from the provided reagents (SYBR Green master mix and PCR primers). Diluted standards and samples were combined with qPCR master mix and the plate was centrifuged 1500xg for 1 min at RT. The Real Time PCR instrument was configured for absolute quantitation of each amplicon. The instrument was set for 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 60°C for 1 min (ramp rate=1.6°C/s; 100% ramp rate in Standard mode) with detection of SYBR Green fluorescence. After cycling, melt curves were monitored to measure T_M of each PCR product, and absolute quantitation of microRNA from Real Time PCR was measured using SDS software (Applied Biosystems). Selected qRT-PCR products were cloned into a pCR4-TOPO vector and chemically transformed into One Shot TOP10 chemically competent *Escherichia coli* (Applied Biosystems). Transformants bearing inserts were selected by spreading onto LB ampicillin plates, and individual clones were grown in LB ampicillin medium. Plasmids were purified using the PureLink Quick Plasmid Miniprep Kit (Applied Biosystems), annealed with M13(-21) forward primer (5'-TGTAACGACGGCCAGT-3') or M13 reverse primer (5'-CAGGAAACAGCTATGAC-3') followed by extension and chain termination with fluorescent dye labeled dideoxy nucleotides. DNA sequencing in both directions was analyzed by capillary electrophoresis

using an ABI 3730 DNA Analyzer (Dana Farber Molecular Biology Core facility, Boston, MA) and Sequence Scanner Software (Applied Biosystems).

Amplification and sequencing of HIV miRNAs. PCR and cloning was used to confirm the sequences and ends of the candidate HIV miRNAs. Total RNA was isolated from HIV-infected cells and from exosomes and analyzed using miRCURY LNA Universal RT microRNA PCR (Exiqon, Woburn, MA). First strand cDNA was synthesized from total RNA by poly(A) tailing, annealing a universal primer (5' Universal Tag, central poly(T) and 3' degenerate anchor (Exiqon, Woburn, MA)) and reverse transcription with dNTPs. The Universal Primer accommodates RT of any RNA template (including miRNA) to identify free 3' termini of RNA with or without polyadenylation. PCR was performed using a pair of miR-specific primers containing Locked Nucleic Acids (LNA) from Exiqon (Woburn, MA). PCR products were cloned using pCR4-TOPO vector (Applied Biosystems, Foster City, CA). DNA sequencing of synthetic vmiR88 shows full length sequence followed by a 15-nt poly(A) and Universal Tag sequence (Fig. 1C). Similarly, synthetic vmiR88 showed full length sequence (missing the final G) followed by a 15-nt poly(A) and Universal Tag sequence.

Statistical Analysis. Group comparisons were performed using Student's t-test (two sample test) or one-way ANOVA using InStat statistical software (GraphPad Software, San Diego, CA). Non-parametric data is analyzed by Mann-Whitney U test, or ANOVA. Results expressed as mean±SEM. Statistical significance was accepted for $p < 0.05$.

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All references cited herein are incorporated by reference.

Example 19 HIV Infection Is Sufficient to Induce Foam Cell Formation in Monocyte-Derived Macrophages

Atherosclerosis incidence is consistently higher among HIV⁺ patients with or without cART treatment than that in the HIV-negative population. This example demonstrates that HIV infection (*e.g.*, in monocyte-derived macrophages) by itself promotes foam cell formation.

Specifically, MDM were incubated with HIV-1 particles Ba-L strain (10 ng/0.1 ml Gag p24/10⁶ cells), and cells were washed three hours after incubation. Viral infection was then assessed based on p24 measurements in the supernatant.

Viral replication starts at 4 days and peaks at 8 days post infection and plateaus thereafter (Fig.18A). Importantly, foam cell formation was induced in HIVinfected cells as well as cells treated with a protease inhibitor (PI) ritonavir measured by BODIPY staining (Fig.18B). These results show that HIV infection alone is sufficient to induce foam cell formation in MDM.

Example 20 HIV-1 ssRNA Induces Foam Cell Formation in Monocyte-Derived Macrophages Human Macrophages in a Dose-Dependent Manner

Soluble HIV proteins such as Nef, Tat, and Vpr have been detected in the serum of HIV-infected patients. They are probably released into the circulation by infected or apoptotic cells, which may interact with macrophages to drive an inflammatory response.

Applicants have shown above that HIV-derived ssRNA40 induces TNF α release in macrophages, and that two novel GU-rich HIV-produced miRNAs in HIV-infected macrophages induce TNF α release by macrophages in a TLR8-dependent manner. These data suggest release of novel HIV-derived miRNAs by HIV-infected macrophages potentially induces a TNF α release through TLR8 stimulation, and may contribute to chronic immune activation.

This example demonstrates that such uridine-rich HIV ssRNA is able to induce foam cell formation, using two independent methodologies (i.e., Oil red O staining and BODIPY). Specifically, HIV-derived ssRNA is sufficient to promote foam cell formation in macrophages as indicated by dose-dependent increases in Oil Red O staining and BODIPY staining (data not shown). As a negative control, an inactive variant of GU-rich ssRNA40 was synthesized as AU-rich ssRNA41. With this change, its addition to macrophages failed to induce foam cell formation. Their appearance was the same as that of unstimulated macrophages (data not shown). These effects were validated by using instead oxidized LDL (ox-LDL), as a positive control, which induces foam formation (data not shown). Interestingly, Nef protein (another molecular component of HIV) also induced foam cell formation in macrophages (data not shown).

Taken together, GU-rich ssRNA from HIV is sufficient to induce foam cell formation in macrophages, and the effect is specific because when uridine residues were replaced with adenine, this response is lost. These results suggest that increases in foam cell formation by HIV ssRNA40 could be the result of HIV-induced inflammation.

Example 21 Blocking HIV ssRNA-Mediated TNF α Release Inhibits Foam Cell Formation in MDMs

A critical early step in atherosclerosis is the migration of monocytes into the developing atherosclerotic plaques and their development into inflammatory lipid-laden foam cells. Applicants have demonstrated that signaling by HIV ssRNA40 induces TNF α release in macrophages.

To demonstrate whether inflammatory factors including TNF α mediate foam cell formation, induction of foam cell formation in MDMs was investigated in the absence or presence of anti-TNF α antibody. Pre-treatment of human macrophages with anti-TNF α antibody significantly reduced HIV ssRNA40 mediated foam cell formation by <75% (Fig. 20). Activation of TNF α receptor induces robust foam cell formation compared to unstimulated cells (Fig. 20), which was inhibited in the presence of anti-TNF α antibody (Fig. 20). As a positive control, Ox-LDL induced robust foam cell formation (Fig. 20).

These data suggest that inflammatory factors such as TNF α may also act on macrophages to cause them to become foam cells and potentiate early atherogenic processes.

Example 22 HIV-1 ssRNA40 Induction of Macrophage Foam Cell Formation Is Dependent on Endosomal Acidification and Endocytosis and Endosomal Acidification

Applicants have demonstrated that HIV-1 ssRNA40 signaling induces TNF α release by macrophages that are dependent on TLR8 expression.

Applicants also demonstrated that endosomal acidification is required for HIV ssRNA-mediated foam cell formation in MDMs. It has been shown that intracellular TLR signaling is inhibited by chloroquine through inhibition of lysosomal acidification. Pretreatment of MDMs with chloroquine markedly inhibited HIV-ssRNA-mediated foam cell formation (data not shown) and in a dose-dependent manner (Fig. 21B). Interestingly, chloroquine inhibited TNF α release by MDMs down to unstimulated levels (Fig. 21A), suggesting that induction of TNF α by ssRNA40 plays a role in foam cell formation.

Applicants further investigated the role of endocytosis in HIV-1 ssRNA40 induction of foam cell formation in macrophages, in the presence or absence of an inhibitor of the guanosine triphosphatase (GTPase) dynamin (dynasore). TLR8 is expressed in the luminal

aspect of the endosomal membranes. This suggests that HIV-1 ssRNA40 undergoes endocytosis in order to engage TLR8. Pre-treatment of human macrophages with dynasore significantly reduced foam cell formation induced by HIV ssRNA40 in MDMs in a dose-dependent manner. Reduction of foam cell formation at 10 μ M was ~20% and at 50 μ M ~50% (Fig. 21B), suggesting that dynamin-mediated uptake of HIV ssRNA in MDMs contributes to foam cell formation.

Taken together, these data demonstrate that, in MDMs, HIV-1 ssRNA40 induces foam cell formation through endocytosis, followed by endosomal acidification and TLR8 signaling.

Example 23 HIV-1 ssRNA40 Binds TLR8 Protein

This experiment provides direct evidence that ssRNA40 binds to TLR8, based on a fluorescence resonance energy transfer (FRET) assay in a recombinant cell system using anti-TLR8 antibody clone 44C143 (Imgenex, San Diego, CA) raised against a KLH-conjugated synthetic peptide from human TLR8 (residues 750-850), which includes an accessible α -helix (residues 781-789) as possible epitope.

Specifically, HEK-293 cells were stably transfected to express human TLR8, and were treated with ssRNA40 Alexa 546 conjugate. Cells were fixed, permeablized and intracellular antigen TLR8 detected using Alexa 488 conjugates of anti-TLR8 antibody or isotype control.

To evaluate the proportions of labeled cells, Applicants used dual excitation (488 nm and 561-nm lasers). Direct excitation of both fluorophores in conventional flow cytometry demonstrates that HEK-TLR8 had specific labeling with 2.11% of cell population positive for TLR8 (anti-TLR8 vs. IgG1 (green)) and 54.2% of cells had taken up the ssRNA40 Alexa 546 conjugate (ssRNA40(gold)). Double-positive cells (1.38% of population were labeled for TLR8 (Alexa 488) and uptake of ssRNA40 Alexa 546 (ssRNA40 + anti-TLR8 vs. IgG1 + ssRNA40 (red)).

Cells were further analyzed for FRET by flow cytometry (data not shown) with excitation at 488 nm and emission at 530 nm (FRET Donor; Alexa 488) or 575 nm (FRET Acceptor; Alexa 546).

Untreated cells show minimal background fluorescence intensity for both TLR8 (Alexa 488 signal) and for ligand (Alexa 546). Isotype antibody contributes little background

fluorescence intensity (data not shown, IgG1). HEK-TLR8 cells stained with anti-TLR8 antibody Alexa 488 (FRET acceptor) indicate cells positive for the TLR8 receptor (data not shown), whereas isotype control antibody contributes minimal background fluorescence intensity (data not shown, IgG1). HEK-TLR8 cells that had taken up ssRNA40 Alexa 546 (FRET acceptor) were excited at 488 nm showing a fraction of cells (7.46%) were fluorescent (data not shown, ssRNA40), which was comparable to 7.39% of cells that had taken up ssRNA40 Alexa 546 followed by staining with isotype control antibody (data not shown, ssRNA40 + IgG1). Excitation (488 nm) of HEK-TLR8 cells labeled with both FRET donor and FRET acceptor exhibited total emission within the FRET gate in 1.92% of the cell population (data not shown, ssRNA40 + anti-TLR8 FRET), and subtracting the isotype control background 0.62% (data not shown, ssRNA40 + IgG1) reveals specific FRET emission in 1.3% of the total cell population.

To evaluate the proportions of labeled cells, dual excitation (488 nm laser and re-enabling excitation by the 561-nm laser) were used in conventional flow cytometry. Direct excitation of both fluorophores demonstrates that HEK-TLR8 had specific labeling with 2.11% of cell population positive for TLR8 (data not shown, anti-TLR8 vs. IgG1) and 54.41% of cells had taken up the ssRNA40 Alexa 546 conjugate (data not shown, ssRNA40). Double-positive cells (1.38% of population were labeled for TLR8 (Alexa 488) and uptake of ssRNA40 (Alexa 546)) are shown in data not shown TLR+RNA vs. IgG1 + ssRNA40. Thus, ~94% of TLR8-positive cells that had also taken up ssRNA40 demonstrated detectable FRET signals indicative of ssRNA40 binding to TLR8 receptor in the flow cytometry FRET assay.

Example 24 Foam Cell Formation in Human Macrophages Is Dependent on TLR8 Activation by HIV-1 ssRNA40

As HIV-1 ssRNA40, derived from Long Terminal Repeats and enriched in uridine, activates TLR7 in murine dendritic cells and TLR8 in human macrophages, Applicants first demonstrated that TLR8 is expressed intracellularly in MDMs using flow cytometry analysis (FIG. 23A), then showed that TLR8 engages HIV-1 ssRNA40 to induce foam cell formation in MDMs. Foam cell formation induced by HIV-1 ssRNA was markedly reduced in TLR8-silenced MDM compared to that in the non-silencing control (FIG. 23B). Furthermore, an inactive ssRNA41 control failed to induce foam cell formation in non-silenced cells (FIG. 23B).

Taken together, stimulation of foam cell formation by HIV-1 ssRNA40 in MDMs is

dependent on TLR8 activation.

Example 25 Foam Cell Formation Mediated by HIV-1 Derived ssRNA40 in Macrophages Is Dependent on Induced MyD88 Signaling

MyD88 is an adaptor protein that is recruited to mediate TLR8 signaling events leading to the release of cytokines such as TNF α . This example demonstrates that MyD88 is required in TLR8-linked signaling in MDM cells, foam cell formation, and TNF α release, based on MyD88-targeted gene silencing.

Specifically, following exposure to non-silencing siRNA, HIV ssRNA stimulated TNF α release in a dose-dependent manner (FIG. 24A). Similarly, cells transfected with MyD88 siRNA also elicited such a response, but TNF α release was reduced compared to that in non-silenced cells. Similarly, TNF α release induced by TLR2 agonist, BLP (10 μ g/ml) was diminished in the MyD88-silenced cells (FIG. 24A). Silencing of MyD88 gene resulted in ~65% reduction of MyD88 protein (FIG. 24B). Furthermore, foam cell formation was markedly reduced in MyD88-silenced cells (FIG. 24C). To rule out off-target effects, foam cell formation is similar in non-silencing control compared to MyD88 silenced cells exposed to Ox-LDL (FIG. 24C).

Taken together, in MDMs, TLR8 activation by HIV-1 ssRNA stimulates TNF α release and foam cell formation through a MyD88-dependent signaling pathway.

Example 26 HIV-Derived miRNA Induces Foam Cell Formation in a Dose-Dependent Manner and Is Inhibited by a Specific Antagomir

Having established the effects of HIV ssRNA stimulation on foam cell formation, Applicants showed here the ability of HIV-derived miRNA to stimulate foam cell formation in human macrophages.

The data demonstrates that HIV vmiR99 induced foam cell formation in a dose-dependent manner in AM cells (data not shown). Foam cell formation began at 0.01 μ g/ml, and reached a maximum level at 1 μ g/ml, whereas, GU-poor HIV-TAR miRNA did not elicit a response (data not shown). Interestingly, this response was inhibited by antagomir99, which has a completely complementary sequence of its target.

Taken together, in macrophages, HIV-derived miRNA can induce foam cell formation as well as TNF α release in a dose-dependent manner, and can be inhibited by a specific antagomir.

The reagents and detailed procedures for Examples 19-26 are described below, if not already covered earlier.

Reagents. ssRNA40/LyoVec and ssRNA41/Lyovec were purchased from InvivoGen (San Diego, CA). Lipid A (the biologically active component of LPS, and specific TLR4 ligand) from *Escherichia coli* F583 Rd mutant, protease inhibitor cocktail and phorbol myristic acid (PMA) were purchased from Sigma Chemical Company (St Louis, MO). Bacterial lipopeptide (BLP) was from EMD Millipore (Billerica, MA). Oxidized LDL was from Biomedical Technologies, Inc. (Ward Hill, MA). Dynasore and chloroquine were purchased from Calbiochem (San Diego, CA). Paraformaldehyde solution (4% in Phosphate Buffered Saline) was from Fisher Scientific. BODIPY 493/503 dye was from Life Technologies (Carlsbad, CA). Saponin (molecular biology grade) was from Sigma. Phosphate Buffered Saline without calcium or magnesium was from Cellgro (Herndon, VA). Nef, was purchased from Trinity Biotech (Wicklow, Ireland). The phosphorothioate oligoribonucleotides, GU-rich ssRNA40 from LTR of HIV (5'-GCCCCGUCUGUUGUGUGACUC-3', and GA-rich ssRNA41 (5'-GCCCCGACA GAAGAGAGACAC-3'), were purchased from Invivogen, San Diego, CA. Phosphorothioate oligoribonucleotide conjugate Alexa 546-ssRNA40 (5'-Alexa Fluor 546 GCCCCGUCUGUU GUGUGACUC-3') was from Integrated DNA Technologies (Coralville, IA). The phosphorothioate oligoribonucleotides vmiR88, vmiR99 and vmiRTAR derived from HIV-1 sequence and antagomir99 as described above were from Integrated DNA Technologies (Coralville, IA).

Antibodies. Anti-TLR7-PE and anti-TLR8-PE antibodies were purchased from Imgenex, (San Diego, CA). Anti-MyD88, and β -actin antibodies were purchased from Cell Signaling (Beverly, MA). Fluorescent conjugates of monoclonal antibody against TLR8/CD288 (clone 44C143; mouse IgG1/ κ), Alexa Fluor 488 conjugate of mouse IgG1/ κ isotype control (clone MOPC-31C) were from Imgenex (San Diego, CA). Blocking anti-TNF α antibody was from Abcam (Cambridge, MA). Cytokine ELISA kits were from R&D Systems (Minneapolis, MN). HIV-1 p24 antigen ELISA kit was from Zeptometrix (Franklin, MA).

Alveolar macrophages (AM). To determine the clinical relevance of the study, select experiments were carried out using human AM. Recruited healthy subjects had no active pulmonary disease and normal spirometry. They were confirmed to be HIV seronegative by ELISA and had no known risk factors for HIV infection. Using standard techniques, bronchoalveolar lavage (BAL) was performed to obtain lung immune cells. All procedures were performed on consenting adults following protocols approved by Beth Israel Deaconess Medical Center Institutional Review board and Committee for Clinical Investigations. Cells were separated from the pooled BAL fluid and AM were isolated. AM were isolated by adherence to culture plates in medium (RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B), and yielded cells that were >98% viable as determined by trypan blue dye exclusion, and demonstrated >95% positive nonspecific esterase staining.

Monocyte-derived macrophages (MDMs). Healthy individuals were confirmed to be HIV seronegative by ELISA and had no known risk factors for HIV infection. Using standard techniques, venipuncture was performed to obtain peripheral blood. All procedures were performed on consenting adults following protocols approved by Beth Israel Deaconess Medical Center institutional review board and Committee for Clinical Investigations. Healthy MDM was isolated from buffy coat of healthy subjects using Percol Hypaque and cultured for 7-10 days in medium (RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B) containing macrophage colony stimulating factor (MCSF) and non-adherent cells were washed away followed by addition of fresh medium prior to experimentation.

Cells and Macrophage cell lines. HEK-293 stable cell line expressing full-length human TLR8 was from Imgenex. Macrophages were differentiated from human promonocytic THP-1 (American Tissue and Cell Company, ATCC). THP-1 cells were harvested during exponential growth phase, pelleted, resuspended and then incubated in complete medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin). To induce macrophage differentiation, THP-1 cells were incubated with 100nM PMA at 37°C in a humidified atmosphere containing 5% CO₂ for 24h. Adherent cells were then washed three times with medium (to remove PMA) and incubated (37°C, 5% CO₂) in complete medium (without PMA) for use in experiments.

Western blot analysis. Western blotting was performed as described. Briefly, adherent human macrophages were treated with indicated reagents, washed 2 × with ice-cold PBS (pH 7.4). Cells were lysed in lysis buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Sigma Chemicals; St. Louis, MO), placed on ice for 20 min. Cells were harvested by scraping, followed by centrifugation at 40°C for 15 min at 14,000 rpm. Equal amounts of cell lysates were subjected to SDS/PAGE and Western blot analysis with designated antibodies and detected by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences; Piscataway, NJ). Resolved bands were quantified by densitometry (Amersham Biosciences; Piscataway, NJ).

Small Interfering RNA (siRNA)- mediated knockdown in macrophages. To determine the functional relevance of TLR8 signaling pathway in foam cell formation, RNAi-mediated knockdown of TLR8 was performed using synthetic duplex RNA oligonucleotides including On-Target Plus Smart Pool short interfering RNA (siRNA) TLR8 (Thermo Scientific). Target sequences for TLR8 were GAACGGAAAUCCCGGUAUA, CAGAAUAGCAGGCGUAACA, GUGCAGCAAUCGUCGACUA, and CUUCCAAACUUAUCGACUA. The non-targeting irrelevant siRNA (ON-TARGETplus Non-targeting siRNA#1 from Thermo Scientific) was used as control. Macrophages were electroporated with 100nM siRNA using Amaxa Nucleofactor system following the manufacturer's protocol (Amaxa, Cologne, Germany). TLR8-mediated knockdown was determined by Western blot probed with anti-TLR8, or by flow cytometry using anti-TLR8-PE 24-48h after transfection.

Binding assay by flow cytometry – fluorescence resonance energy transfer (FC-FRET). HEK-293 cells expressing TLR8 were plated in 6-well plates and grown in DMEM containing glucose (4.5 g/L), 10% FBS, L-glutamine (4 mM), sodium pyruvate (1 mM), penicillin (100 units/mL), streptomycin (100 µg/mL) and blasticidin (10 µg/mL) in humidified atmosphere (37°C, 5% CO₂). After aspiration, fresh medium was applied (1.0 mL/well) without blasticidin. Oligonucleotide ssRNA40-Alexa 546 was prepared at 50 µg/mL in LyoVec (Invivogen, San Diego, CA) according to manufacturer's instructions and applied to wells (1.0 µg/mL) for 20 min at 37°C, 5% CO₂. Medium was aspirated, and cells were treated with trypsin/EDTA. Medium (2 mL) was applied to the cell suspension and pelleted in centrifuge tubes. Cell pellets were re-suspended in paraformaldehyde (4% in PBS) for 20 min at RT and then washed once in PBS. Cell pellets were re-suspended and

permeablized in PBS containing 0.1% saponin for 15 min at RT. Cells were pelleted and supernatant aspirated. Cells were re-suspended in PBS/0.1% saponin containing anti-TLR8 Alexa 488 or mouse IgG1/ κ Alexa 488 (40 μ g/mL) in the dark for 30 min at RT. Cells were washed in PBS/0.1% saponin, centrifuged, re-suspended in PBS containing 0.5% paraformaldehyde and placed in the dark at 4°C. Cells were analyzed using a SORP LSR II flow cytometer system (BD Biosciences, San Jose, CA) configured with a solid state 50-mW 488-nm laser and a Coherent Compass 25-mW 561-nm laser, and gating was performed using forward and side scatter parameters of unstained cells. For direct detection of labeled cells, Alexa 488 was excited (488-nm laser) through the 505-nm longpass dichroic mirror and emission detected through the 530/30 bandpass filter. Direct detection of Alexa 546 was achieved by exciting with the 561-nm laser with emission detected through the 585/42 bandpass filter. For FRET assay, the 561-nm laser was disabled and its light path physically blocked to ensure that FRET excitation was provided exclusively by the 488-nm laser. FRET Donor (Alexa 488) was detected using the 488-nm laser with emission through the 505-nm longpass dichroic mirror and emission detected through the 530/30 bandpass filter. The FRET Acceptor (Alexa 546) was detected via 550-nm longpass dichroic mirror and emission through the 575/26 bandpass filter. Thus the presence of antibody-Alexa 488 and ssRNA-Alexa 546 was confirmed by direct excitation using 488-nm and 561-nm lasers, respectively. After disabling the 561-nm laser, FRET was assessed by exciting with the 488-nm laser and detecting emission by FRET Donor (Alexa 488) and FRET Acceptor (Alexa 546). A minimum of 10,000 events was collected for each sample, and data were analyzed using FCS Express Flow Cytometry software (De Novo Software, Los Angeles, CA).

TLR analysis by flow cytometry. Cell surface expression of TLRs was determined by Epics XL flow cytometer (Beckman/Coulter, Miami, FL) with laser power of 5.76 mW. The instrument was calibrated before each measurement with standardized fluorescent particles (Immunocheck; AMAC, Inc. Westbrook, ME). Fluorescence signals of the cells were measured simultaneously by 3 photomultiplier tubes and optical filters and shown as the mean of the log fluorescence intensity of the cell population within each gate. Macrophages were incubated with an anti-TLR4 antibody on ice for 60 min, washed three times, incubated with a FITC-conjugated secondary antibody for 30 min on ice protected from light, fixed in Optilys (Beckman/Coulter, Miami, FL) at RT for 5-10 min, and analyzed by flow cytometry. Human macrophages were first identified by the characteristic forward and side scatter parameters on unstained cells, and confirmed by staining with PE-conjugated primary

anti-human HLA-DR (Beckman/Coulter, Miami, FL). Data were expressed as a mean relative fluorescence units (RFU) and the percentage of cells staining positive. Isotype primary conjugated antibodies served as a negative control. Samples were prepared and analyzed in duplicate, and a minimum of 5,000 cells was counted for each sample.

ELISA. After cell stimulation, supernatants were collected, centrifuged to remove cellular debris, and assayed immediately or stored at -80°C until assayed. Cytokine measurements were performed using commercially available ELISA (R&D Systems, Minneapolis, MN) following manufacturer's instructions, and absorbance (450 nm) was measured using an Emax ELISA plate reader with multi-point data analysis by SoftMax Pro software (Molecular Devices, Sunnyvale, CA). The detection limit for TNF- α was 15.6 pg/ml. All measurements were performed in duplicate, and mean values of the two measurements were used for statistical analysis.

Detection of lipid accumulation in macrophages. Accumulation of lipid droplets in macrophages and foam cell formation: For detecting lipid accumulation in macrophages, lipophilic staining by Oil Red O and by BODIPY 493/503 were performed. Macrophages were incubated in growth medium for incubation overnight on chamber slides (Nalge Nunc International). Cells were then treated with ssRNA (2 $\mu\text{g}/\text{ml}$) for 24h. For Oil Red O staining, neutral lipids were stained using 0.5% Oil-Red-O (Sigma) in isopropanol for 60 min. The Oil- Red-O-stained lipids were morphologically evaluated by microscopy. For BODIPY 493/503 fluorescence staining of cholesterol ester, after incubation for indicated time, the cells were washed twice in ice-cold PBS, followed by paraformaldehyde fixation (2% in PBS) for 1h at room temperature. The cells were stained with BODIPY 493/503 working solution (10 $\mu\text{g}/\text{ml}$ in PBS) for 2h at room temperature. The cells were rinsed twice with PBS, counterstained with DAPI and mounted on the slides. Micrographs were recorded using an SM 510 META inverted confocal system (Carl Zeiss Microimaging, Inc.) using a 30-mW argon ion laser (excitation wavelength, 488 nm) and emission wavelengths from 505 to 635 nm. To measure lipid accumulation in individual cells, total BODIPY staining intensity per cell was measured from confocal micrographs using SigmaScan Pro 4.0 image analysis software (SPSS Inc.). A second method to measure lipid accumulation involved the fluorescence intensity ratio (BODIPY/DAPI) in microplate assays using a Gemini EM spectrofluorometer (Molecular Devices, Sunnyvale, CA). The ratio of BODIPY fluorescence intensity (excitation 490 nm, emission 520 nm, cutoff 515 nm) to DAPI fluorescence intensity (excitation 355 nm, emission 460 nm, cutoff 455 nm) is a readout that measures

lipid (BODIPY staining) and normalizes the signal to the number of cells (DAPI nuclear stain).

Statistical Analysis. Group comparisons were performed using Student's t-test (two sample test) or one-way ANOVA. Calculations were performed with StatView (SAS Institute, Inc; Cary, NC) and INSTAT2 (GraphPad Software, San Diego, CA) software package. Results are given as mean $\pm$ SEM. Statistical significance was accepted for $p < 0.05$.

Example 27 Exosome-Based Delivery System for Antagomir

The therapeutic potential and clinical application of many nucleic acid-based drugs has long been limited, in part by the lack of appropriate or optimal delivery systems. Exosomes or microvesicles are small endosomally derived vesicles that are secreted by a variety of cell types and tissues. They are released into the extracellular milieu upon fusion of multivesicular bodies (MVB), with the plasma membrane.

This example provides an advanced delivery system for expression and packaging a molecule, such as a nucleic acid or protein based therapeutic molecule, into engineered exosomes that can target specific cells, for delivering the therapeutic molecule (*e.g.*, the miRNA and oligonucleotide therapeutics / antagomirs of the invention).

While not wishing to be bound by any particular theory, Applicants believe that exosomes display certain homing ligands on their surface, and thus miRNA encapsulated in exosomes can be delivered to specific target cells, where the exosomes engage cell surface receptors before they are taken up by target cells, thereby delivering the enclosed biologically active miRNAs. These exosomes function as natural carriers of miRNA, and can be exploited as an RNA delivery system for delivering drugs (such as protein or nucleic acid based drug, including the antagomirs of the invention).

For example, the subject HIV miRNAs (vmiR88 or vmiR99 etc.) or antagomirs thereof can be overexpressed from a heterologous vector, in donor cells for packaging into cell-homing exosomes. Targeting can be achieved by engineering the donor cells to express CD81 (tetraspanin) fused to a disintegrin (Fig. 22). Delivery of exosomes is expected to depend on exosomal surface antigens that promote initial docking to specific receptors on the surface of recipient cells, followed by its uptake.

Exosomes display surface markers may comprise engineered tetraspanin family proteins including CD63, CD81 and CD9. For exosome homing, ligand-fused tetraspanin

can be engineered into the DsRed site of Adeno-X Adenoviral vector (Clontech) so that exosomes can display high affinity ligands (including small disintegrins, e.g., 5.4 kDa echistatin) that bind receptors (such as integrin $\alpha v \beta 3$ or $\alpha 5 \beta 1$) on recipient cells, and promote exosome fusion with the cell membrane. Cells comprising the construct will express shRNA (which can then be processed to mature vmiR88, vmiR99 or vmiR-TAR, and the host miRNA let-7, etc., before being packaged into exosomes). The construct may also express tetraspanin-disintegrin fusion (which is displayed on the surface of exosomes). Exosomes can be purified from conditioned medium and then applied to recipient / target cells. Delivery efficiency is assessed by the percentage of DsRed-positive cells.

To demonstrate specificity, receptors of recipient cells are blocked by pre-treating them with competitors in the form of soluble ligands or antibodies, e.g., disintegrin echistatin (Sigma), cyclic RGD peptide (AnaSpec, Inc.), or anti- $\alpha v \beta 3$ integrin antibody clone LM609 (Millipore) or anti- $\alpha 5 \beta 1$ integrin antibody, followed by incubation with exosomes and analysis of conditioned medium for cytokine release.

The Adeno-X Adenoviral vector will drive doxycycline-inducible expression of HIV vmiR shRNA and antigens for display on the surface of exosomes. Following DROSHA processing, shRNA in the nucleus is exported to the cytosol for DICER and AGO processing to yield mature vmiRs in the cytoplasm.

To this end, THP-1 monocytes are treated with vectors expressing shRNA (pre-vmir88, vmiR99) or negative control (TAR hairpin or empty vector). Expression of HIV miRNA sequences is checked by qRT-PCR. Exosomes are harvested from supernatants and purified using ExoQuick-TC as well as evaluated for the presence of exosomal marker CD63. Total RNA is prepared, and mature vmiR88, vmiR99, and vmiR-TAR sequences are assayed by qRT-PCR. The specificity of the detected signal is confirmed by TOPO TA cloning and sequencing the PCR products. Different doses of exosome-bearing miRNAs are added to freshly isolated MDM from healthy subjects and analyzed for TNF α and IL-6 release as well as foam cell formation by BODIPY 493/503 staining. These procedures determine whether individual exosomal HIV miRNA induces the release of inflammatory cytokines and transition of MDM into foam cells. Similarly, to demonstrate specificity, constructs expressing antagomirs produce exosome-encapsulated antagomirs, with which we will pre-treat MDM before challenge with LyoVec (Invivogen, San Diego, CA) formulations of vmiR88, 99 or ssRNA40. Cytokines are analyzed by ELISA, and the extent of foam cell formation is determined.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

WE CLAIM:

1. An oligonucleotide that inhibits binding of TLR8 (Toll-like Receptor 8) to a microRNA encoded by an HIV-1 virus (vmiR), and/or inhibits activation of the TLR8 by the vmiR.
2. The oligonucleotide of claim 1, wherein binding of TLR8 to the vmiR activates TLR8.
3. The oligonucleotide of claim 1, wherein the vmiR is GU-rich (*e.g.*, $\geq 70\%$ GU).
4. The oligonucleotide of claim 1, wherein the vmiR comprises a 7- or 8-nucleotide sequence consisting of at most one non-G non-U nucleotide.
5. The oligonucleotide of claim 1, wherein the vmiR is any one of SEQ ID NOs: 1-2 and 9-14.
6. The oligonucleotide of claim 1, wherein the oligonucleotide inhibits activation of TLR8 by binding to / hybridizing with the vmiR.
7. The oligonucleotide of claim 6, wherein the oligonucleotide binds to / hybridizes with the vmiR at a GU-rich region of the vmiR.
8. The oligonucleotide of claim 6, wherein the oligonucleotide binds to / hybridizes with the vmiR with perfect base-pairing.
9. The oligonucleotide of claim 6, wherein the oligonucleotide binds to / hybridizes with the vmiR with mismatch base-pairing.
10. The oligonucleotide of claim 1, wherein the oligonucleotide is partially complementary or non-complementary to the vmiR.
11. The oligonucleotide of claim 1, wherein the oligonucleotide is about 10-12 nucleotides in length, about 12-16 nucleotides in length, about 18-25 nucleotides in length, about 20, 21, or 22 nucleotides in length, or about 10-25 nucleotides in length.
12. The oligonucleotide of claim 1, wherein the oligonucleotide comprises a sequence complementary to one or more of vmiR sequence elements Box 4-8, Box 13-20, and Box 26-31.
13. The oligonucleotide of claim 1, wherein the oligonucleotide comprises a modified sugar moiety (*e.g.*, 2-O-Me), a modified base moiety (*e.g.*, nebularine or xanthosine

- nucleotide), a modified inter-sugar linkage (*e.g.*, phosphorothioate), or combinations thereof.
14. The oligonucleotide of claim 1, wherein the oligonucleotide comprises a locked nucleic acid (LNATM), a deoxyribonucleic acid (DNA), a ribonucleic acid (RNA), or a combination thereof.
 15. The oligonucleotide of claim 14, wherein the LNA base-pairs with a G or a U.
 16. The oligonucleotide of claim 1, wherein the HIV-1 virus is a Group M virus.
 17. The oligonucleotide of claim 16, wherein the HIV virus is of subtype B.
 18. A pharmaceutical composition comprising an oligonucleotide of claim 1 or a polynucleotide encoding the oligonucleotide of claim 1, and a pharmaceutically acceptable carrier and/or excipient.
 19. The pharmaceutical composition of claim 18, comprising the oligonucleotide of claim 1 encompassed within an exosome.
 20. The pharmaceutical composition of claim 18, comprising one oligonucleotide of claim 1 or a polynucleotide encoding the oligonucleotide of claim 1.
 21. A method of treating an inflammatory response in an individual infected by an HIV-1 virus, the method comprising administering to the individual a therapeutically effective amount of a pharmaceutical composition of claim 18.
 22. The method of claim 21, wherein the inflammatory response is a symptom of chronic immune activation.
 23. The method of claim 21, wherein the inflammatory response is a symptom of an HIV-associated lung disease, atherosclerosis, coronary artery disease (CAD) or coronary heart disease (CHD), neurodegenerative disease, malignancy, or opportunistic infection.
 24. The method of claim 21, wherein the inflammatory response is induced by TLR8 activation.
 25. The method of claim 24, wherein said TLR8 activation leads to increased activity of ERK1/2, increased activity of NFκB, increased activity of a pro-inflammatory cytokine, and/or increased expression of an inflammatory marker.

26. The method of claim 25, wherein said pro-inflammatory cytokine comprises TNF α , IL-6, or IL-12.
27. The method of claim 25, wherein said inflammatory marker comprises VCAM-1 or ICAM-1.
28. The method of claim 21, wherein the individual receives concurrent highly active antiretroviral therapy (HAART), has received HAART, or will receive HAART.
29. A method of preventing or delaying the onset of an inflammatory response in an individual either suspected of or at high risk of being infected by an HIV-1 virus, the method comprising administering to the individual a prophylactically effective amount of a pharmaceutical composition of claim 18.
30. A method of detecting the presence of HIV-1 infection in an individual, the method comprising isolating an exosome and/or microsome from said individual and detecting the presence of a microRNA encoded by an HIV-1 virus (vmiR) in the exosome or microsome.
31. The method of claim 30, wherein the exosome is isolated from serum of said individual.
32. The method of claim 30, further comprising quantitating the vmiR.
33. An isolated cDNA encoding a microRNA encoded by an HIV-1 virus (vmiR), wherein the vmiR:
 - (1) binds TLR8;
 - (2) activates TLR8;
 - (3) is GU-rich (*e.g.*, $\geq 70\%$ GU) without taking into consideration any extension sequences of HIV origin, and polyA sequences, and any Universal Tag sequences;
 - (4) comprises a 7- or 8-nucleotide sequence consisting of at most one non-G non-U nucleotide;
 - (5) is any one of SEQ ID NOs: 1-2 and 9-14; and/or
 - (6) is about 18-25 nucleotides in length, or about 20, 21, or 22 nucleotides in length, without taking into consideration any extension sequences of HIV origin, and polyA sequences, and any Universal Tag sequences.
34. An isolated cDNA encoding a pri-miRNA of the vmiR of claim 33.

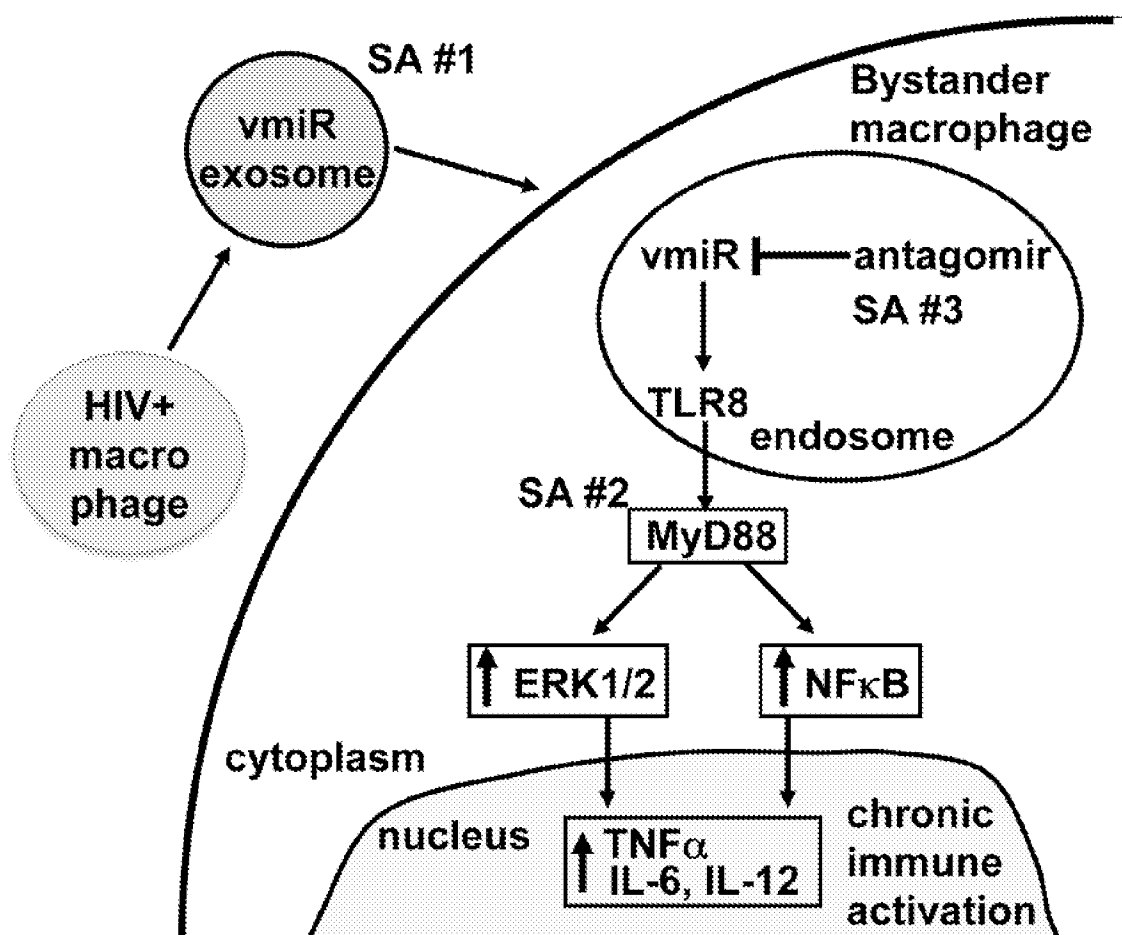
35. The isolated cDNA of claim 34, wherein the pri-miRNA is in complex with Drosha / Pasha.
36. An isolated cDNA encoding a pre-miRNA of the vmiR of claim 33.
37. The isolated cDNA of claim 36, wherein the pre-miRNA is in complex with Exportin-5 or Dicer.
38. An isolated microRNA-protein complex, wherein the microRNA is the vmiR of claim 33, and is in complex with an Argonaute protein (*e.g.*, AGO1, AGO2, AGO3, AGO4).
39. An isolated exosome comprising a microRNA encoded by an HIV-1 virus (vmiR).
40. The isolated exosome of claim 39, wherein the microRNA is not TAR miRNA.
41. An exosome-based delivery system for delivering a therapeutic molecule, the delivery system comprising a first expression vector that expresses a targeting ligand, wherein the targeting ligand is expressed on the surface of an exosome, and comprises a moiety on / outside the exterior surface of the exosome for binding to a target on a recipient cell.
42. The exosome-based delivery system of claim 41, further comprising a second expression vector encoding a sequence for expressing a nucleic acid, a protein, or an enzyme that produced a lipid.
43. The exosome-based delivery system of claim 41, wherein the first expression vector further encodes a sequence for expressing a nucleic acid, a protein, or an enzyme that produced a lipid.
44. The exosome-based delivery system of claim 42 or 43, wherein the nucleic acid encoded / expressed by the sequence is a vmiR (*e.g.*, vmiR88, vmiR99), an antagomir of the vmiR (*e.g.*, antagomir of vmiR88 or vmiR99), or a precursor thereof (such as an shRNA) that can be processed to the vmiR.
45. The exosome-based delivery system of claim 41 or 42, wherein the first and/or the second expression vector is a plasmid or a viral vector (*e.g.*, adenoviral vector, AAV vector, retroviral vector, lentiviral vector).
46. The exosome-based delivery system of claim 41 or 42, wherein the targeting ligand is a fusion protein, wherein the fusion protein is a fusion between a tetraspanin family

protein and a moiety expressed in one of the extracellular loops (*e.g.*, EC2 domain) of the tetraspanin family protein.

47. The exosome-based delivery system of claim 46, wherein the tetraspanin family protein is any one of TSPAN1-TSPAN34, such as TSPAN28 (or CD81), TSPAN29 (or CD9), or TSPAN30 (or CD63).
48. The exosome-based delivery system of claim 46, wherein the tetraspanin family protein is further fused to a marker protein or a sequence tag
49. The exosome-based delivery system of claim 41 or 42, wherein the moiety is selected based on target specificity or recipient cell specificity.
50. An exosome-based delivery system for delivering a therapeutic molecule, the delivery system comprising an exosome expressing a targeting ligand on the surface of the exosome, and comprising the therapeutic molecule in the lumen of the exosome, wherein the targeting ligand comprises a moiety on / outside the exterior surface of the exosome for binding to a target on a recipient cell.
51. A method to deliver a molecule (*e.g.*, a nucleic acid, a protein, a lipid, or a therapeutic molecule based on nucleic acid or protein) using the exosome-based delivery system of claim 41 or 42, the method comprising:
 - (1) harvesting exosomes from a cultured packaging cell line, wherein the packaging cell line is introduced with the first expression vector of claim 41; and
 - (2) administering the exosomes harvested in step (1) to an individual.
52. A method to treat a symptom of HIV infection in an individual in need to treatment, the method comprising delivering an oligonucleotide of claim 1 using the exosome based delivery system of claim 41 or 42 that expresses the oligonucleotide.

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FIG. 1A



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FIG. 1B

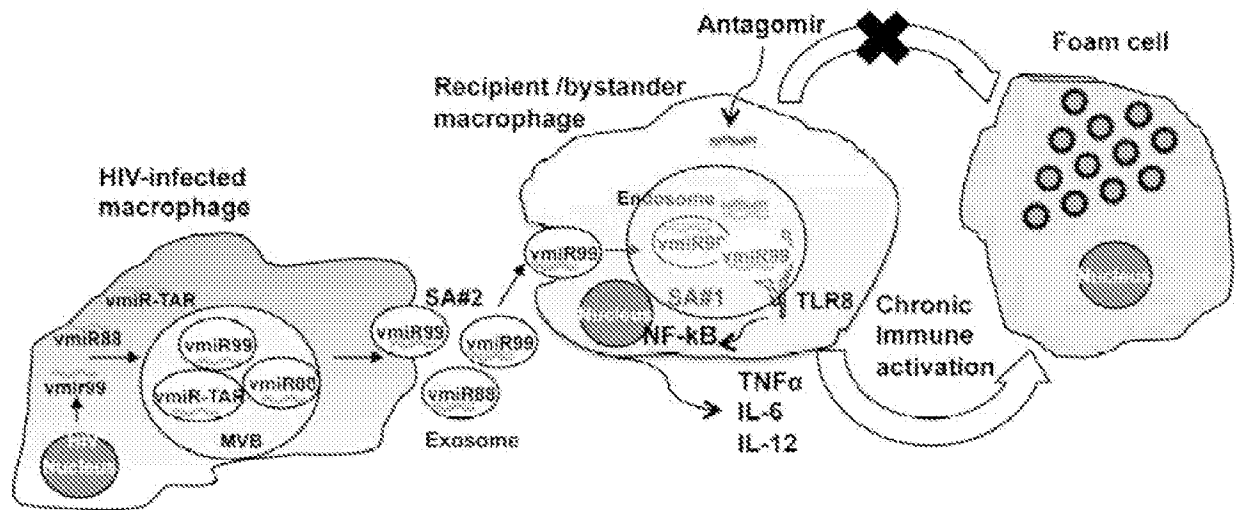
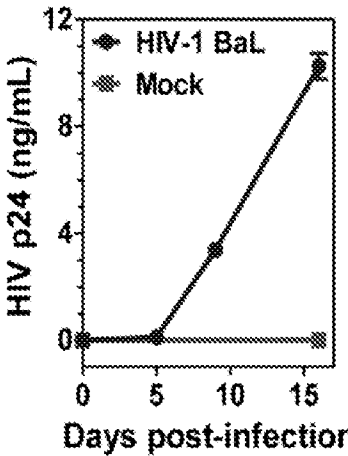
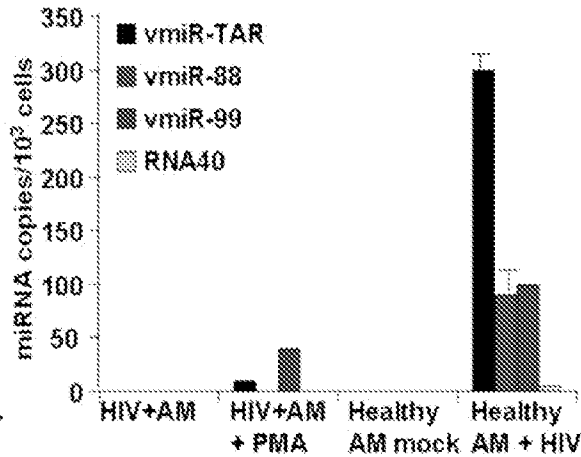


FIG. 2

2A



2B



2C

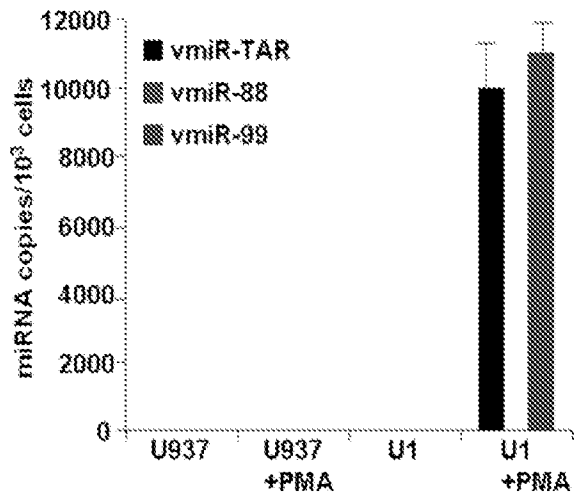
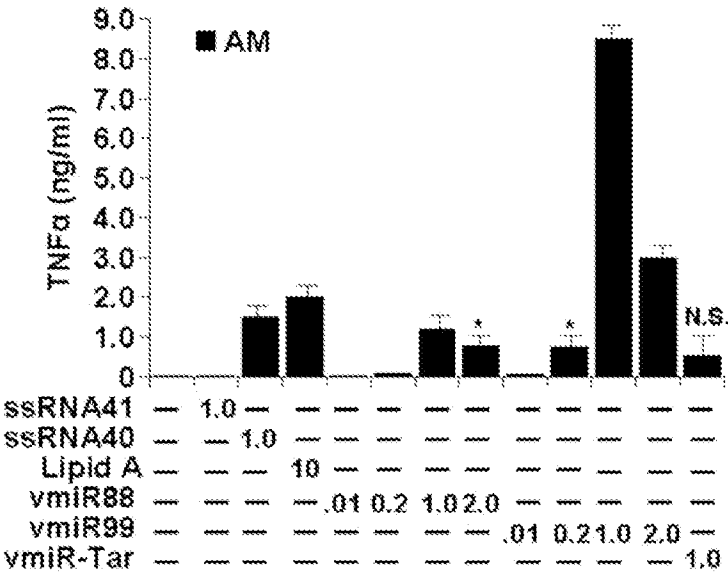
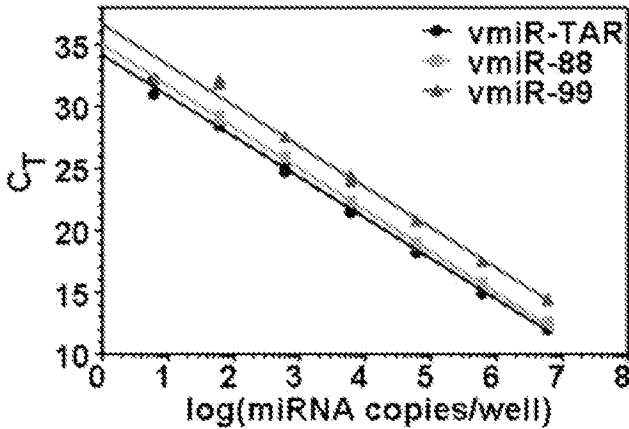


FIG. 2 – Cont'd.

2D

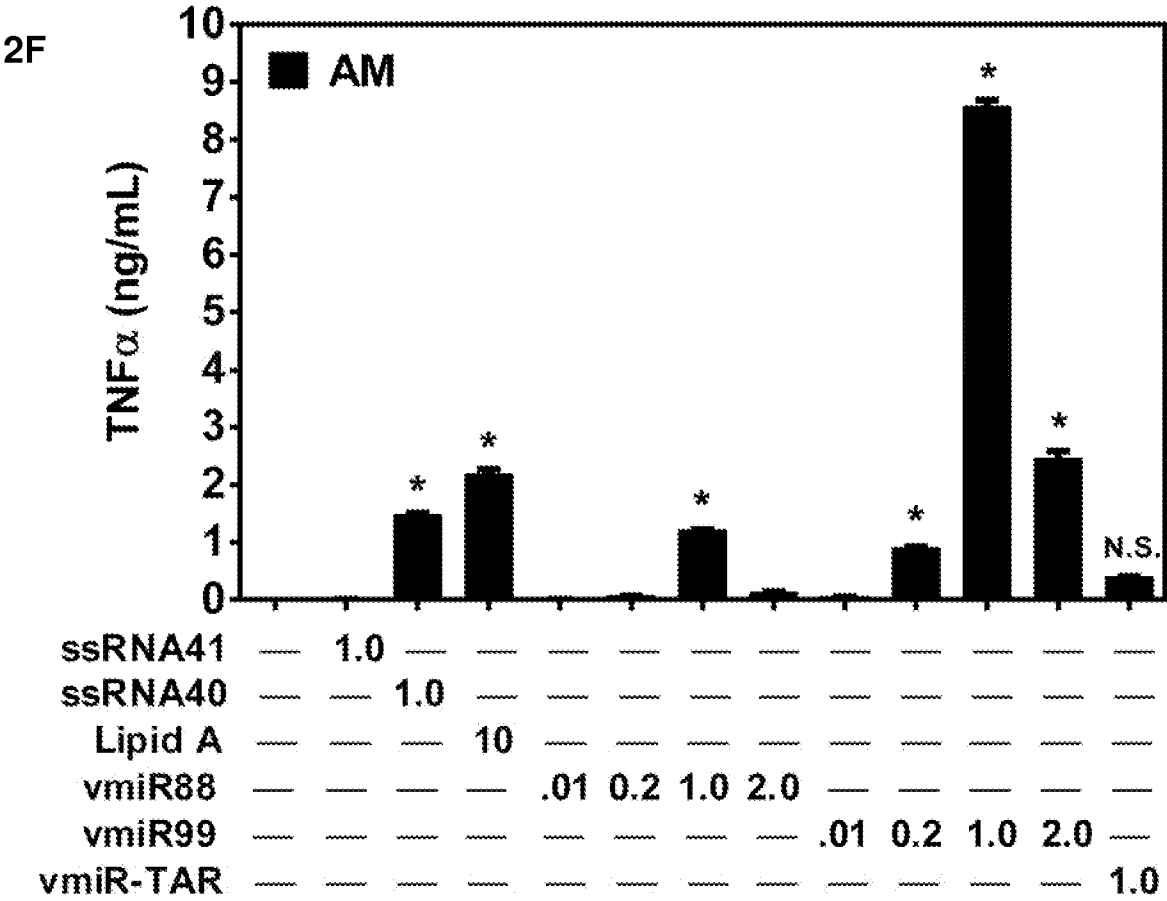


2E



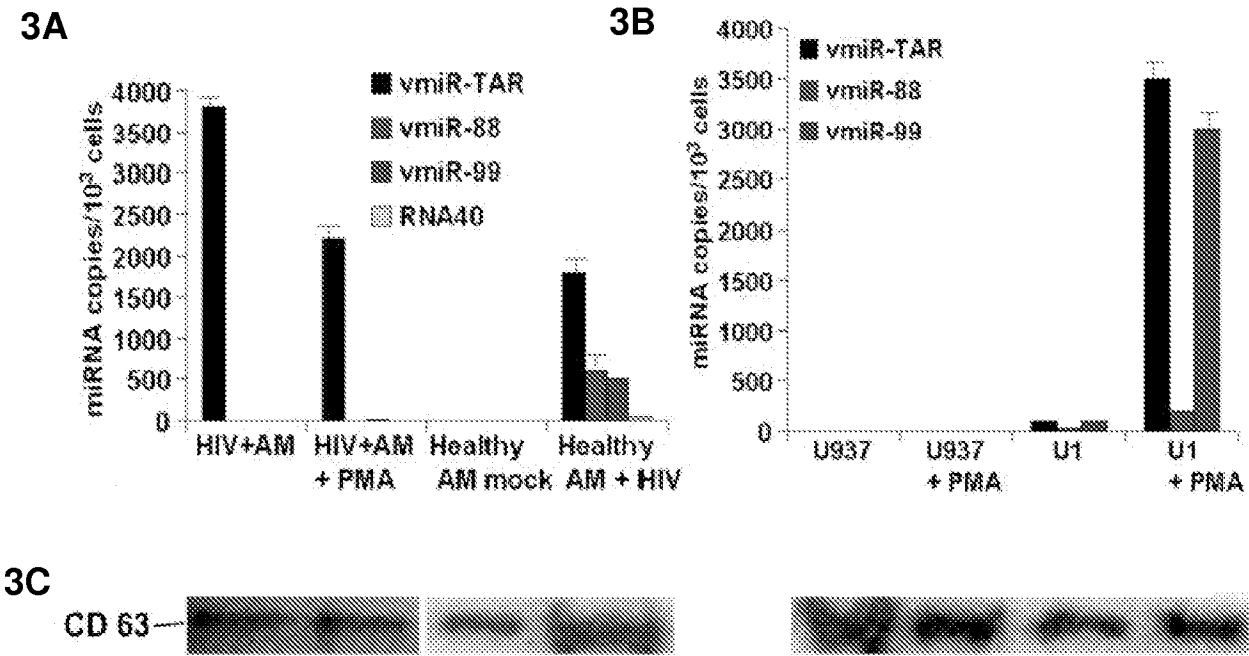
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FIG. 2 – Cont'd.



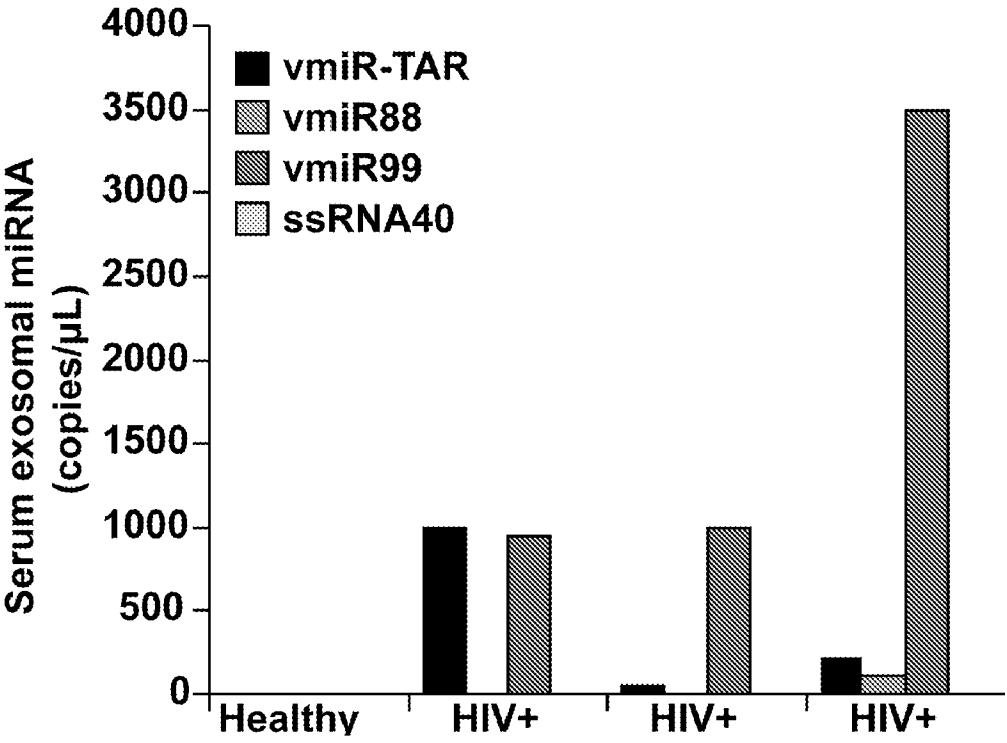
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FIG. 3



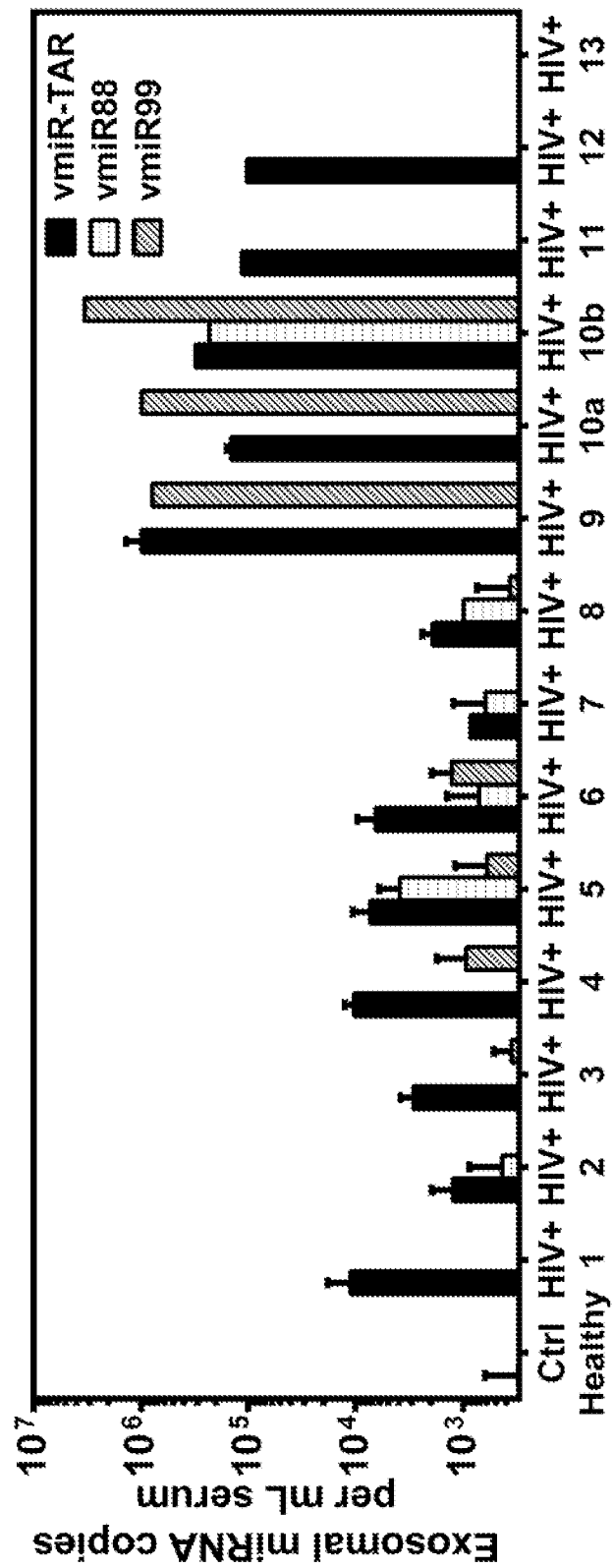
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FIG. 4A



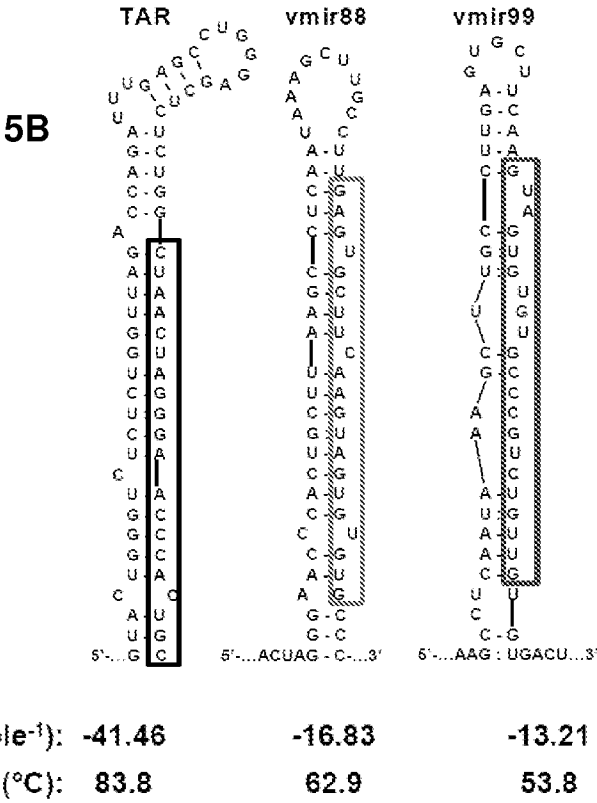
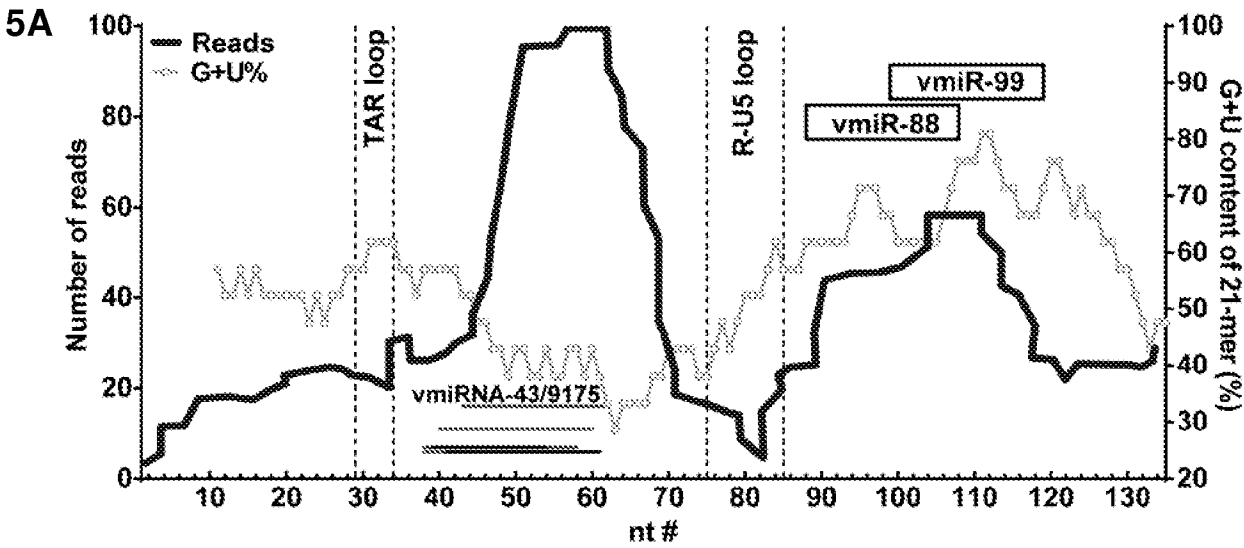
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FIG. 4B



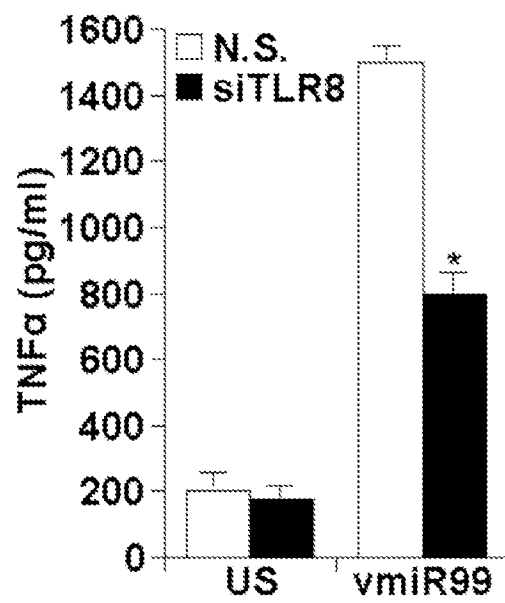
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FIG. 5



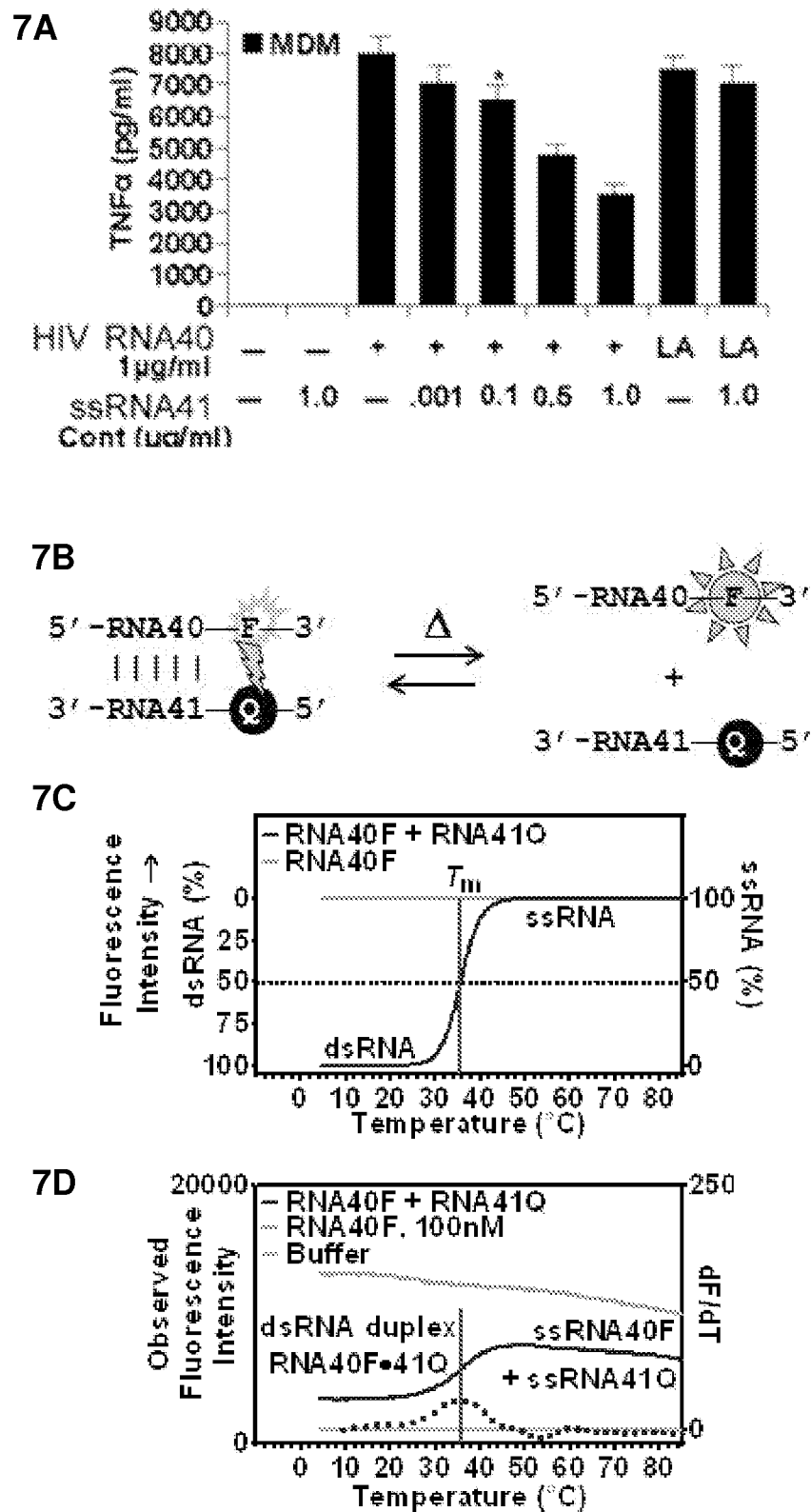
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FIG. 6



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FIG. 7



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FIG. 8

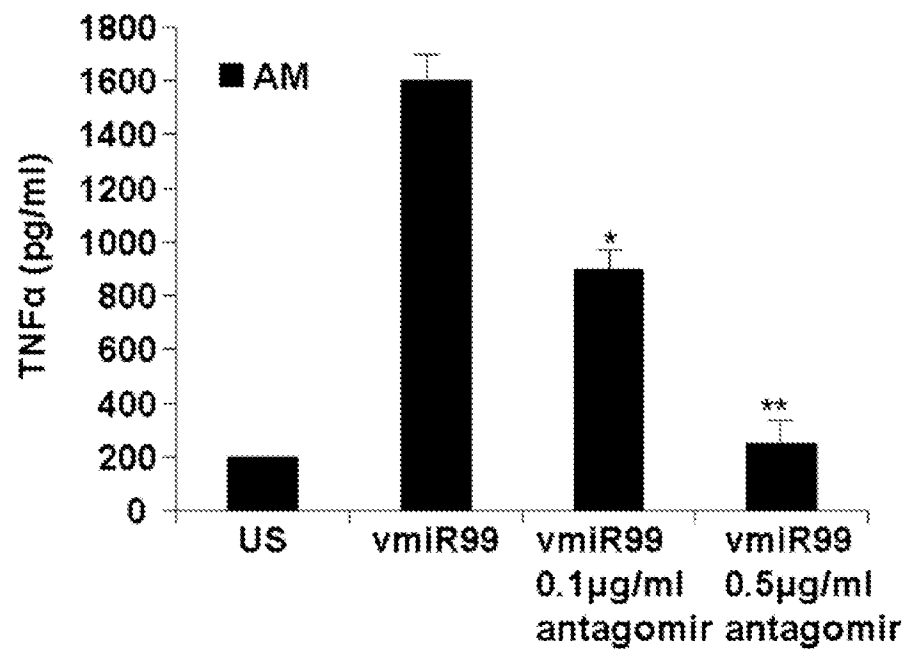
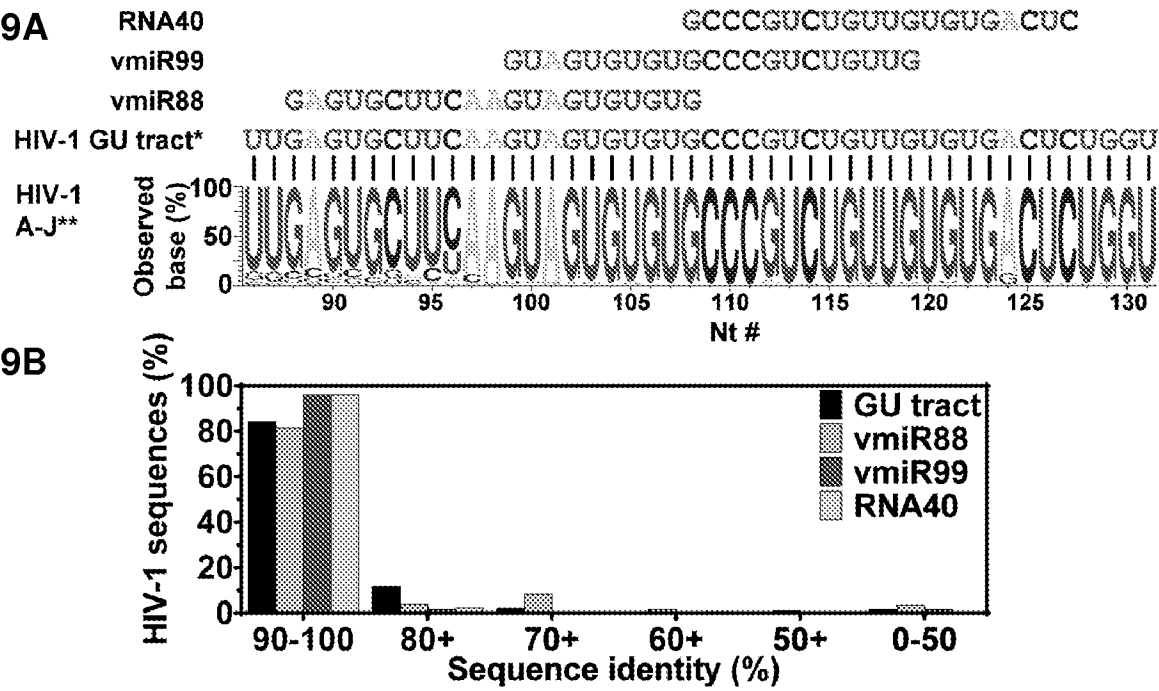


FIG. 9



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FIG. 10

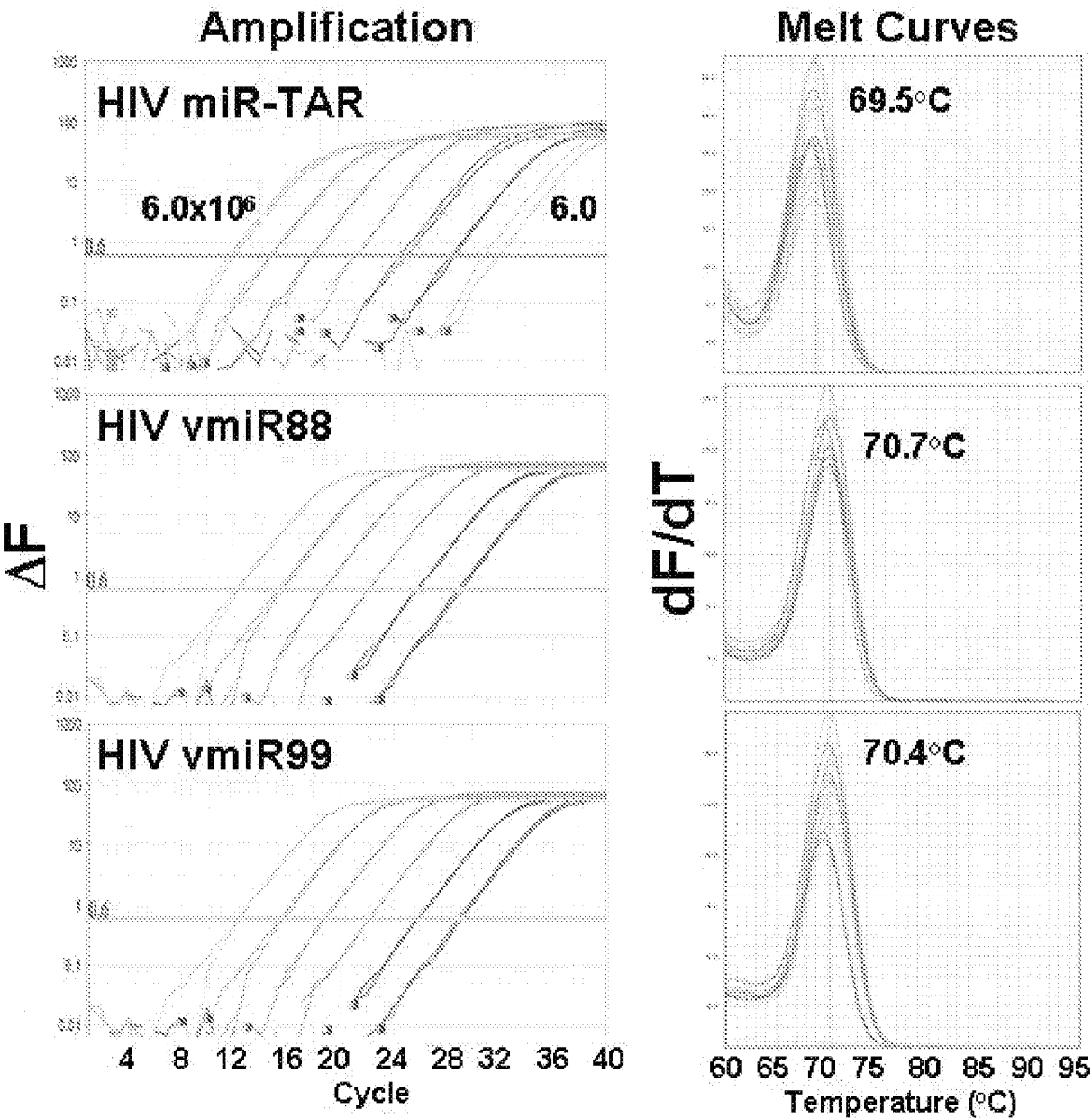
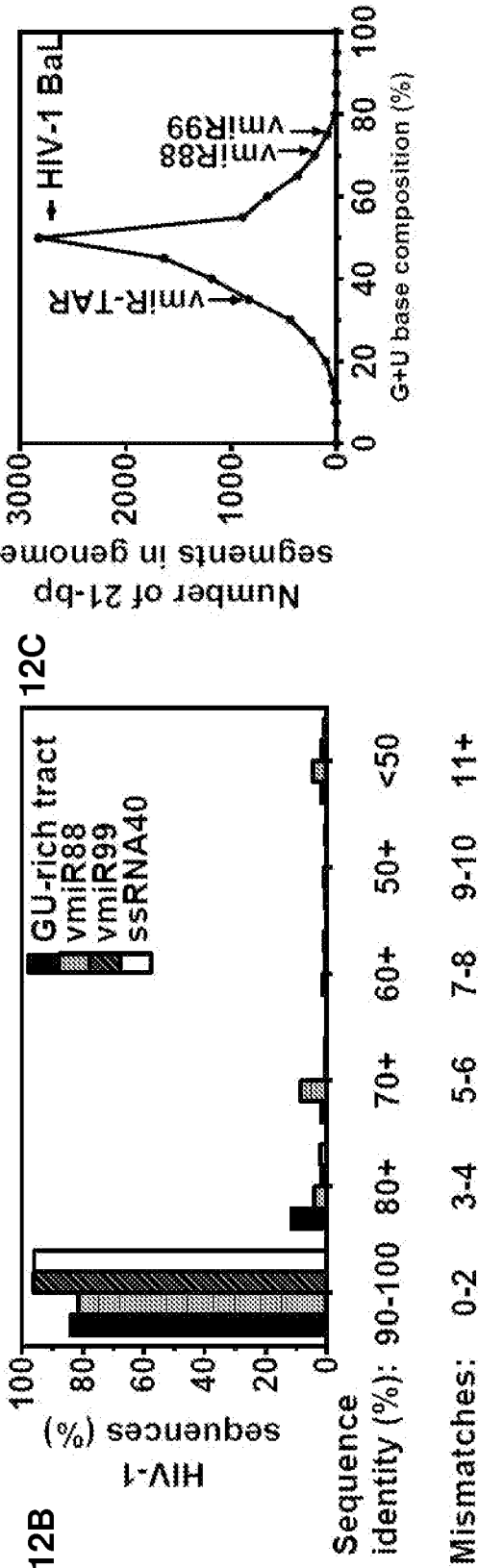
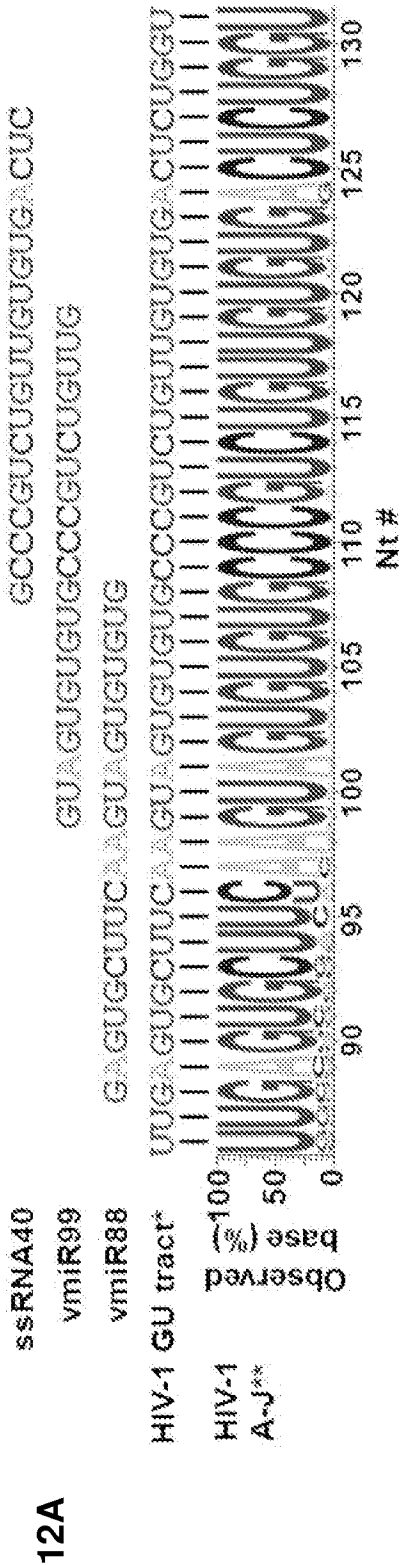


FIG. 12



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FIG. 12 - Continued

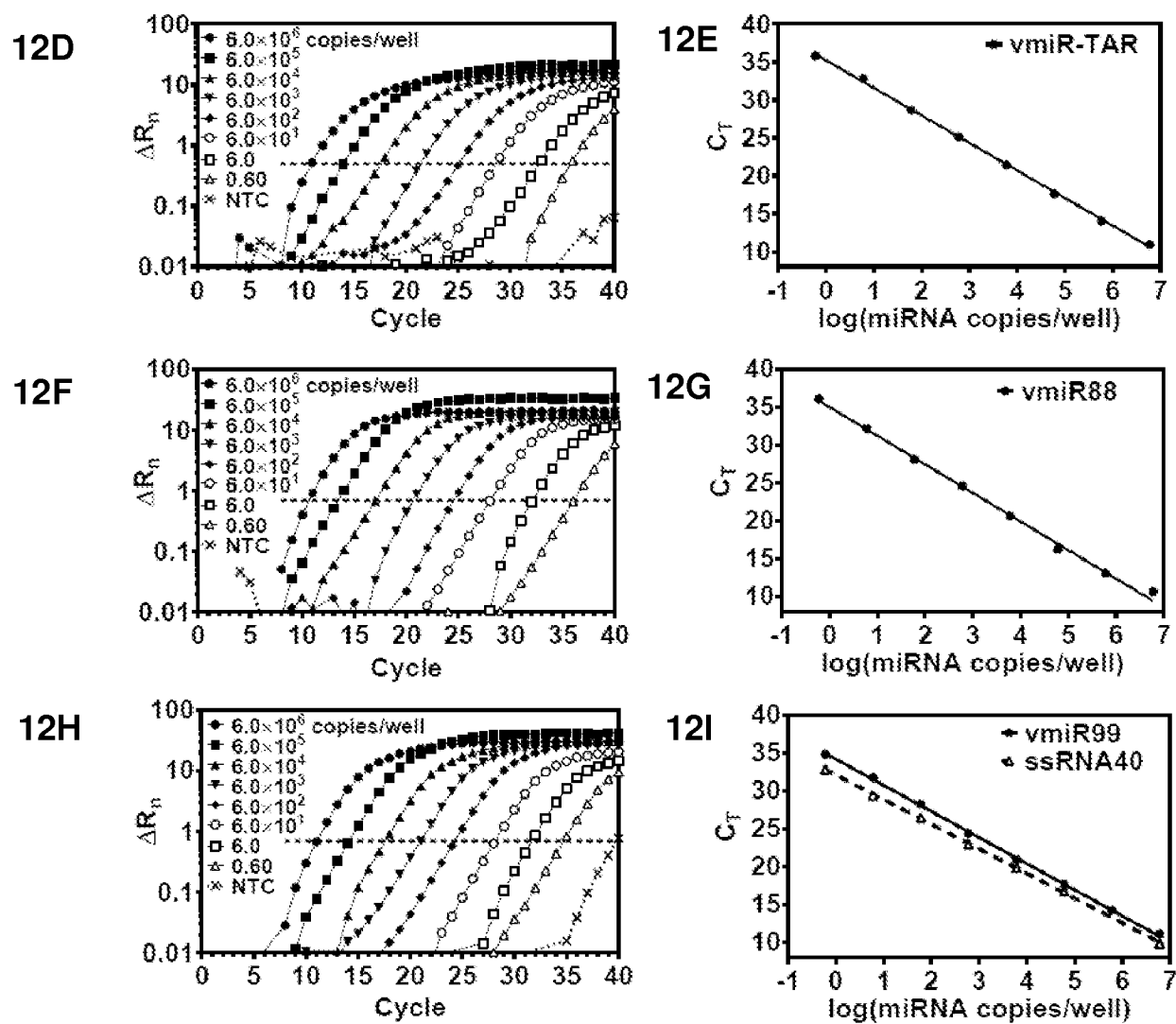


FIG. 14

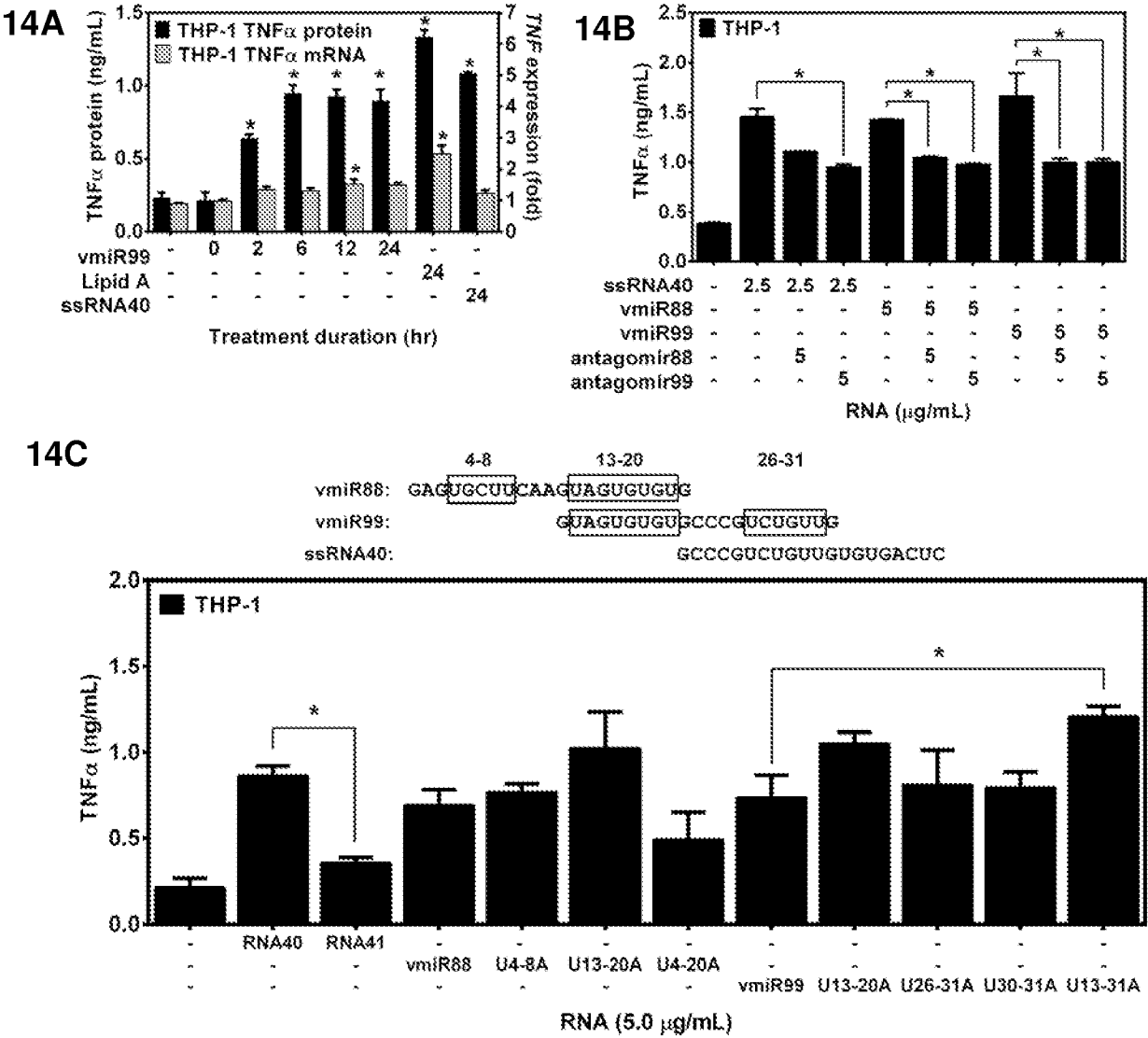


FIG. 15

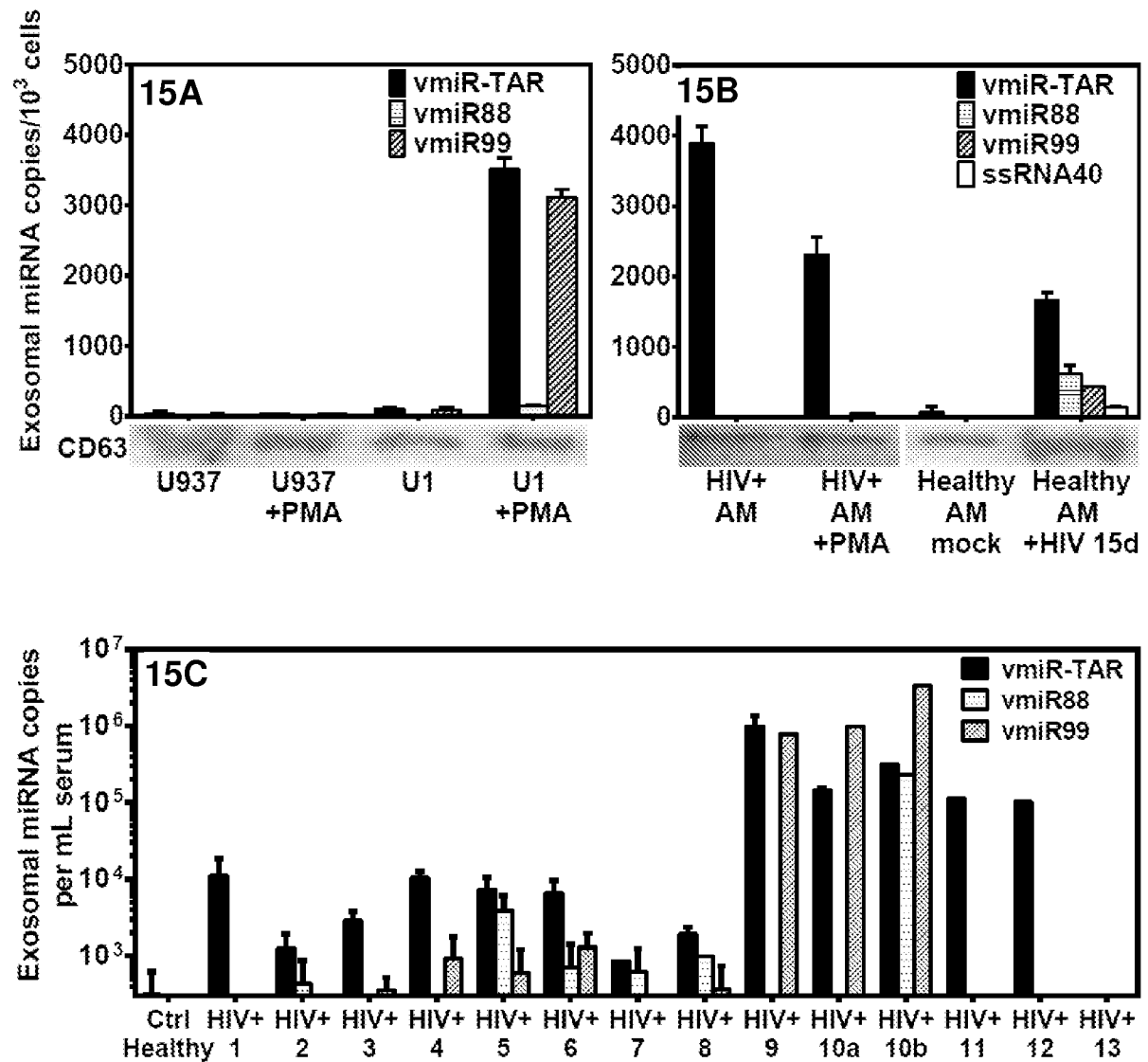
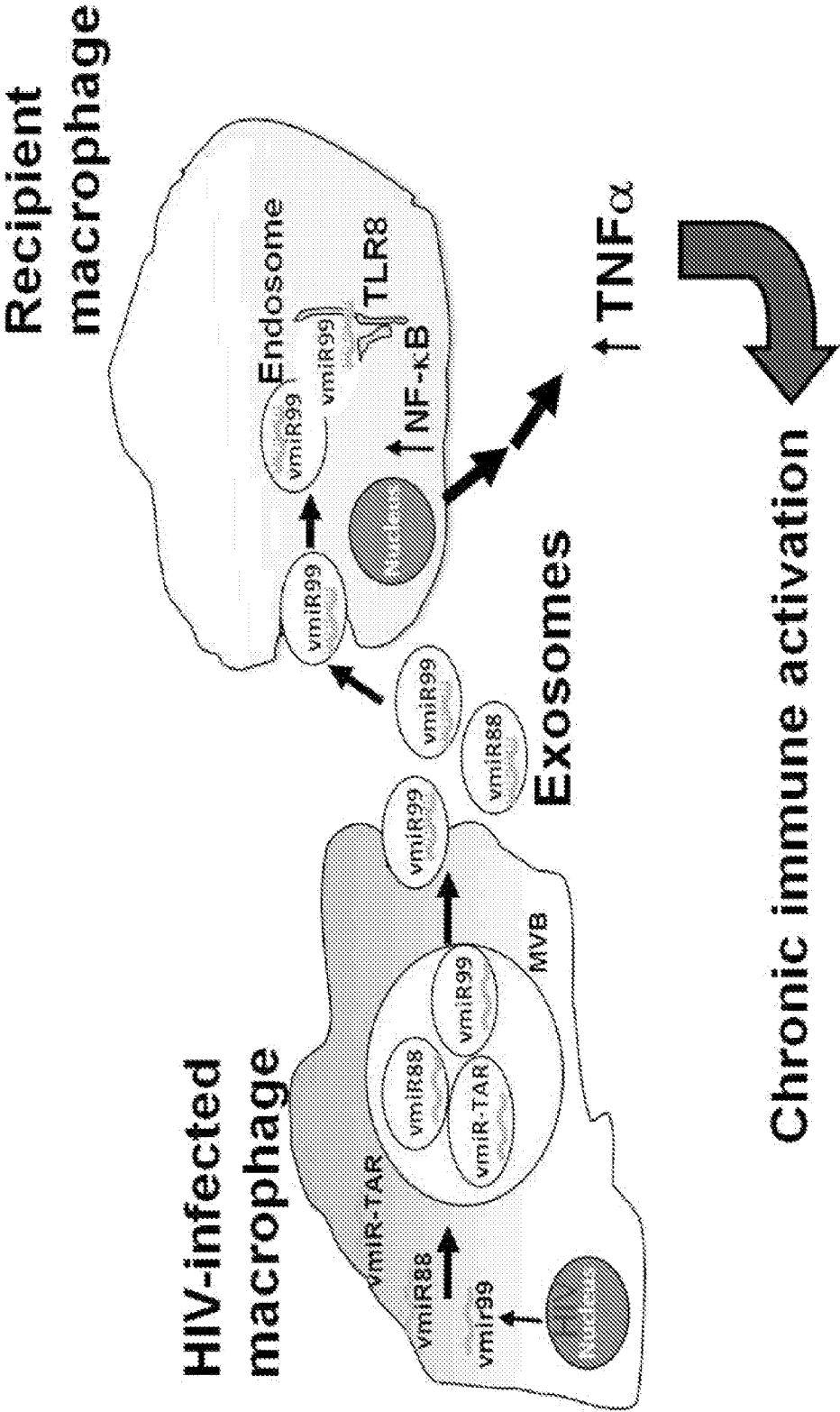


FIG. 16



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FIG. 17

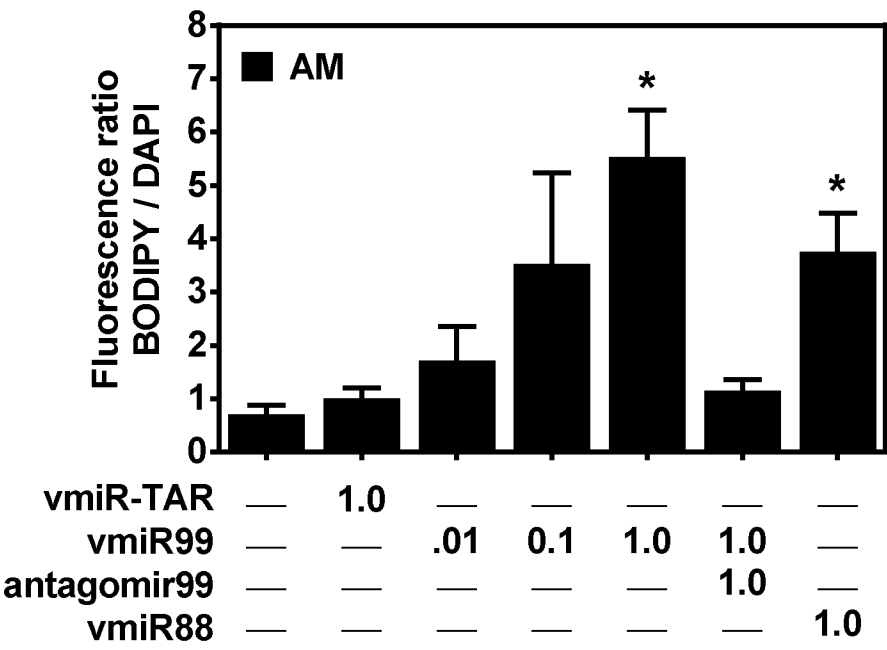


FIG. 18

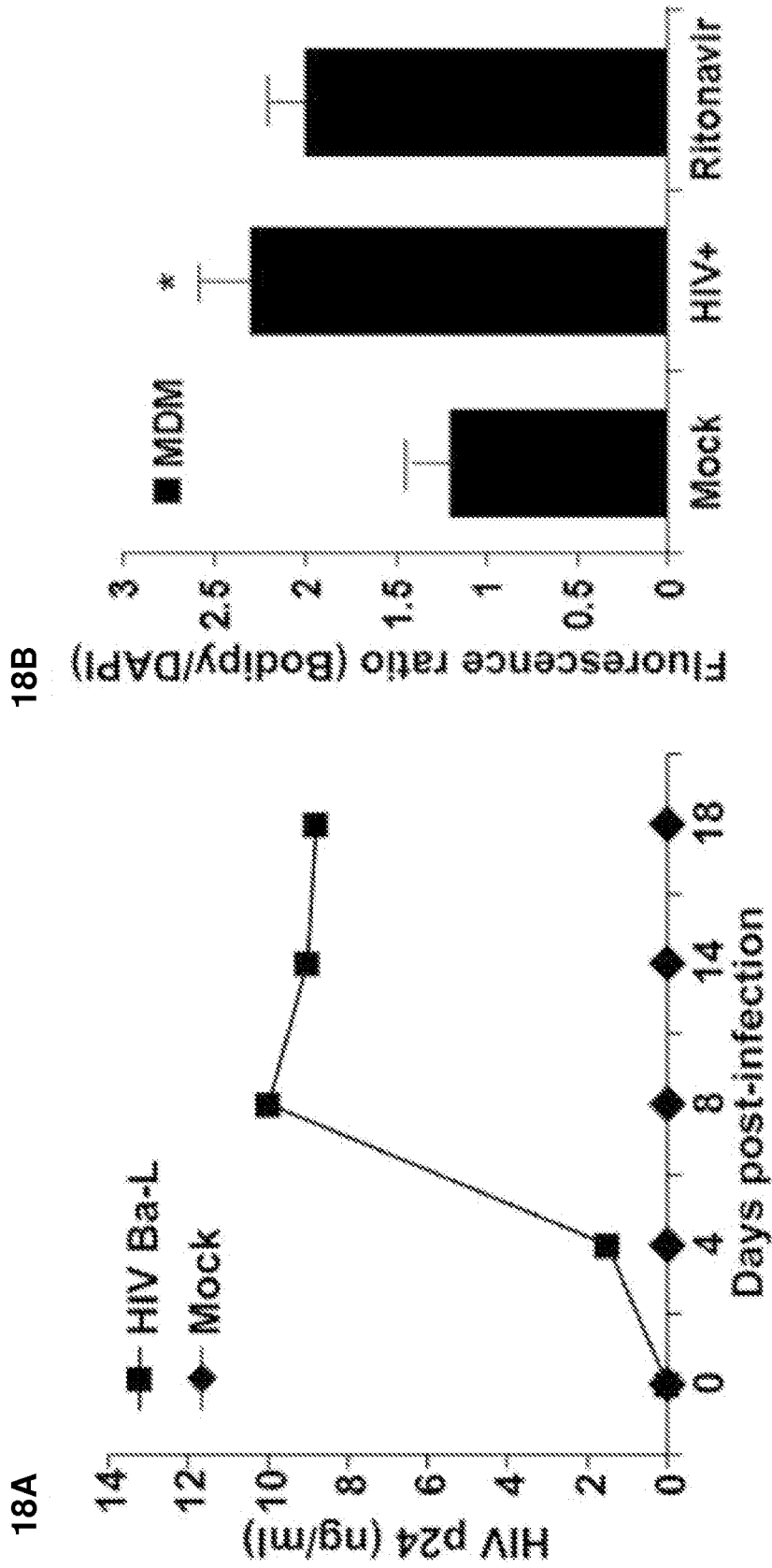
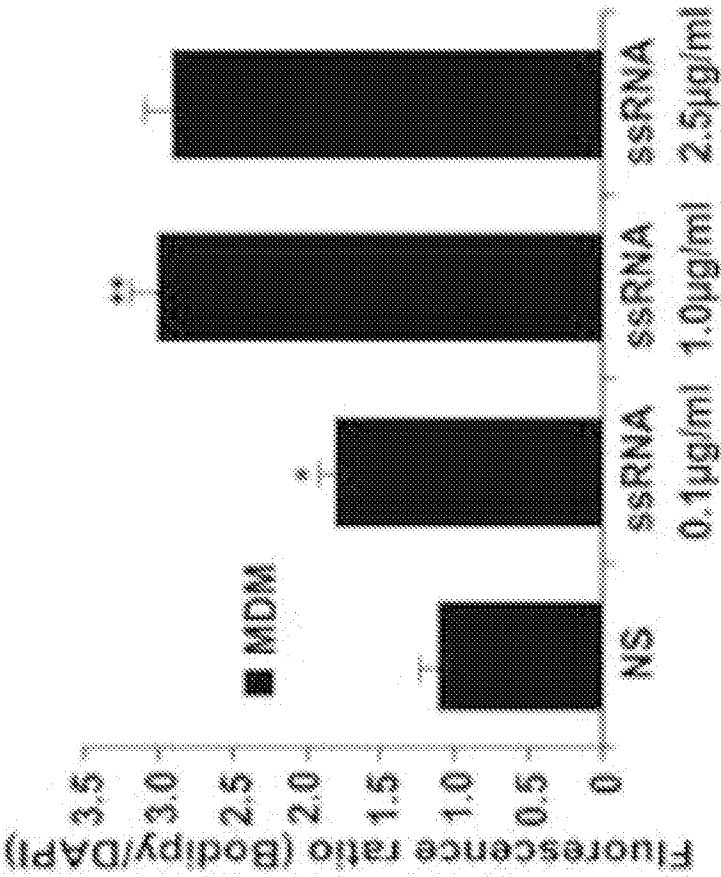


FIG. 19



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FIG. 20

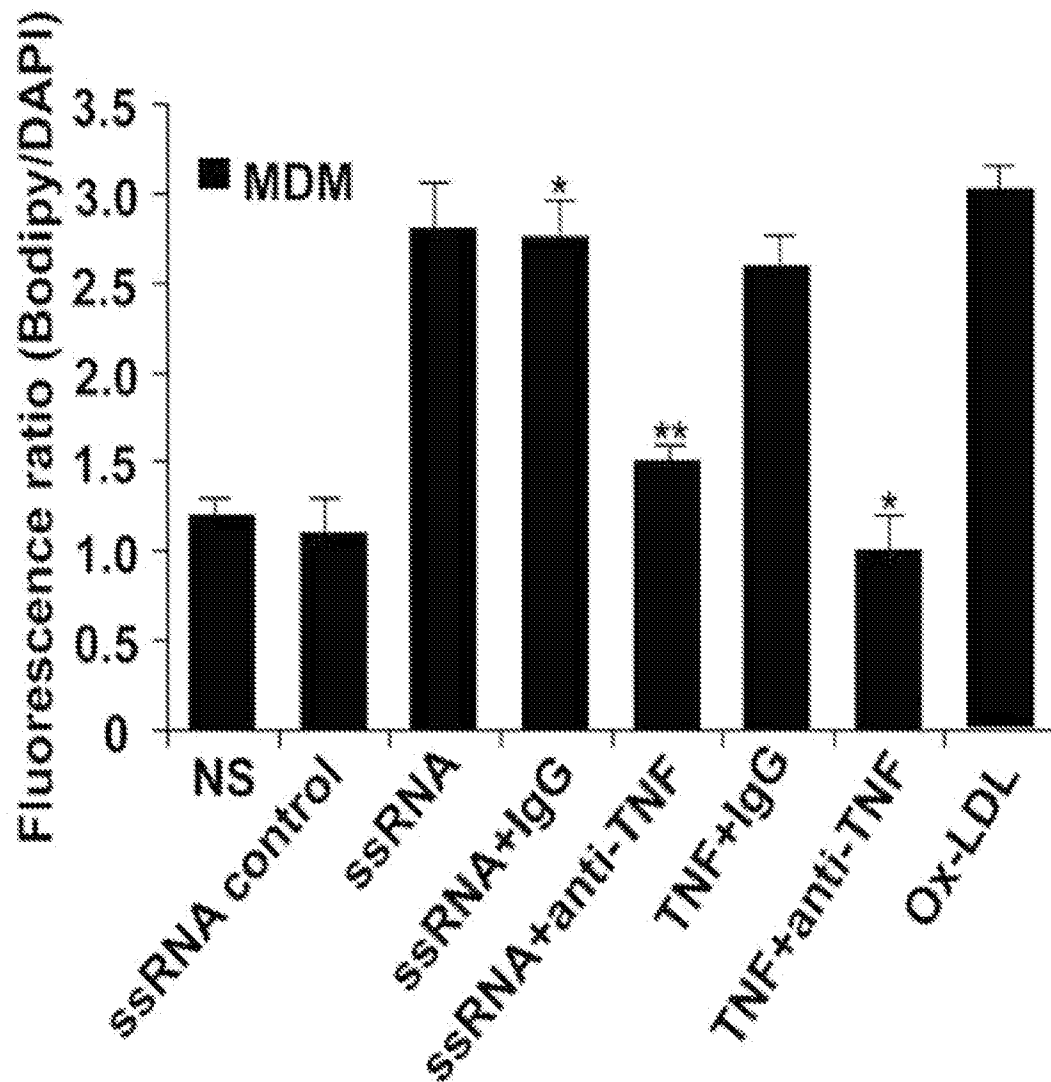
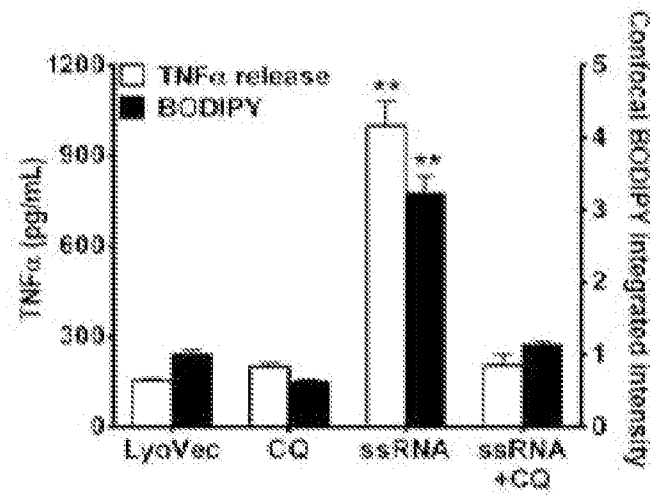


FIG. 21

21A



21B

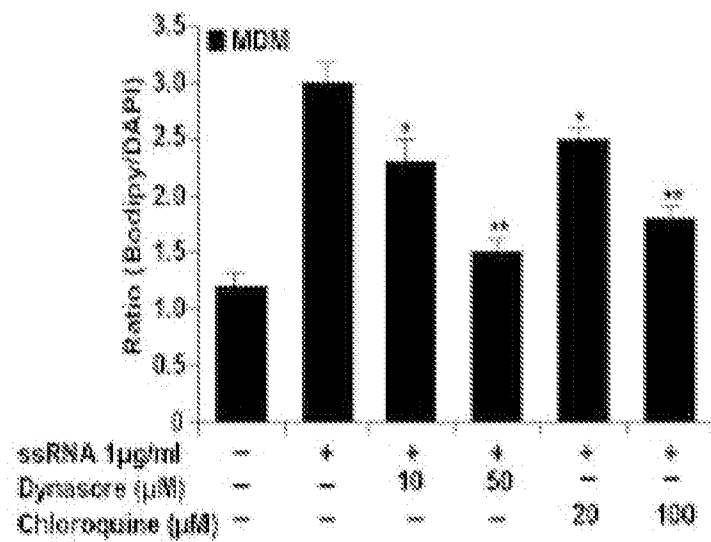


FIG. 22

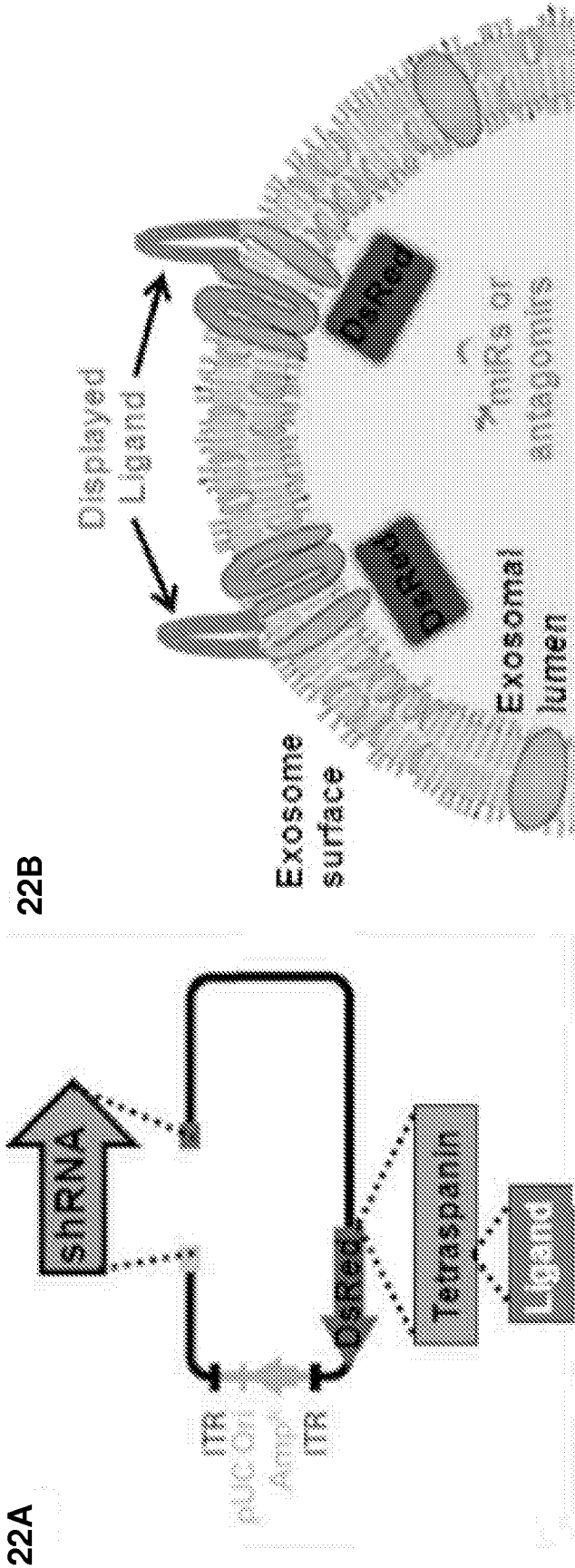
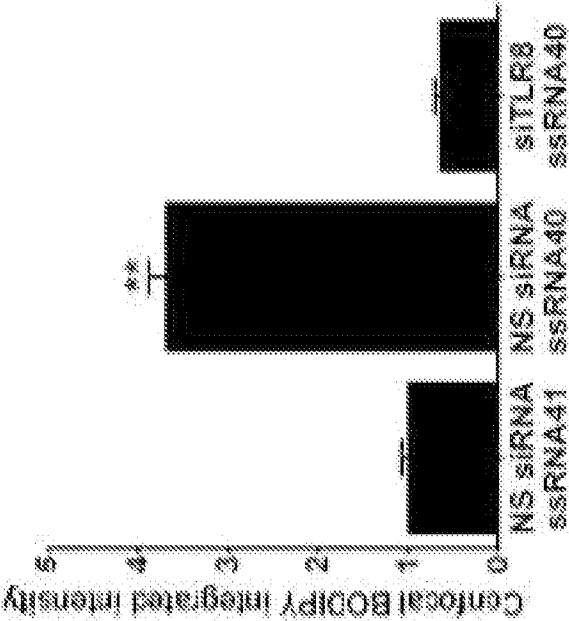


FIG. 23

23B



23A

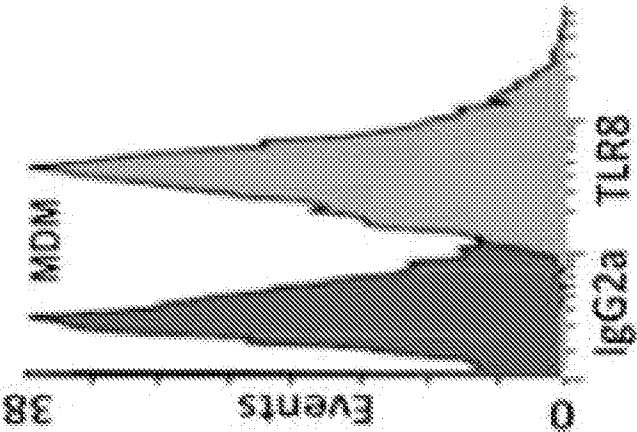
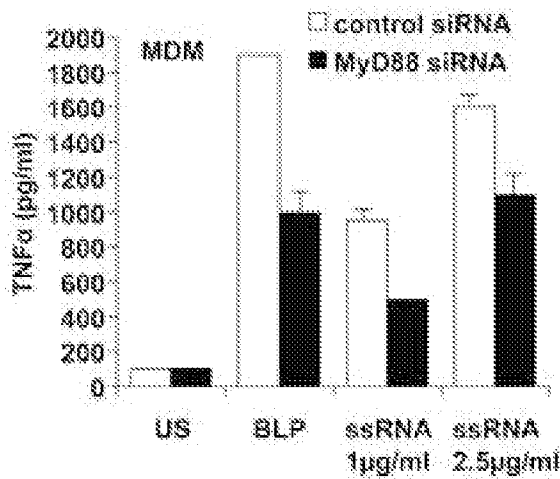
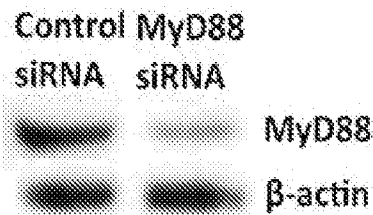


FIG. 24

24A



24B



24C

