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S100A14 IS INCREASED IN ACTIVATED NK CELLS AND PLASMA OF HIV-EXPOSED SERONEGATIVE PEOPLE WHO INJECT DRUGS AND PROMOTES MONOCYTE-NK CROSSTALK.

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Abstract

Background: HIV-exposed sero-negative people who inject drugs (HESN-PWID) have been shown to have increased NK cell and myeloid activation when compared to control donors.

Methods: We investigated potential mechanisms maintaining NK activation by conducting quantitative proteome comparisons of NK cells from HESN-PWID subjects and control donors. Proteins upregulated in NK cells were measured in the plasma of HESN-PWID subjects by ELISA and further investigated for their ability to induce innate immune activation *in vitro*.

Results: The NK cell proteome comparison showed markedly higher levels of Interferon-stimulated proteins and S100 proteins, including S100A14. Consistent with these results, we observed significantly higher levels of S100A14 in the plasma of HESN-PWID subjects compared to controls ($p=0.033$, $n=25$). *In vitro*, the addition of recombinant S100A14 protein significantly activated NK cells in a PBMC mixture ($p=0.011$, $n=9$), but not purified NK cells alone. Treatment of purified monocytes with recombinant S100A14 protein induced secretion of TNF-alpha and led to significantly higher NK CD69 activation ($p=0.0156$, $n=7$) in a co-culture through a TLR4 dependent interaction.

Conclusions: Our study identified S100A14 as a novel protein increased within NK cells and plasma of HESN-PWID subjects with the capacity to sustain NK activation through TLR4-dependent activation of myeloid cells.

Keywords

HESN-PWID; NK cells; monocytes; S100A14; Interferon-alpha

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Introduction:

Since the discovery of HIV, there have been multiple reports of small groups maintaining natural resistance against HIV despite continued high-risk behavior. These groups are referred as HIV-1 exposed sero-negative (HESN) subjects and include: discordant couples¹⁻³, sex workers⁴⁻⁶, peri-natally exposed infants^{7,8}, and people who inject drugs⁹⁻¹². Host-mediated immune mechanisms of HIV-1 resistance have been proposed to explain the occurrence of HESN subjects, however, a unified protective mechanism in HESN subjects remains unlikely as both immune quiescence¹³⁻¹⁶, and immune activation^{1,2,8,9,17-19} have been identified depending on the route of exposure.

HIV-1 exposed sero-negative people who inject drugs (HESN-PWID) and share needles have been identified to possess activated myeloid dendritic cells¹², activated NK cells, and higher NK cell cytotoxic activity¹⁰⁻¹². Recently, we identified that low-risk non-sharing PWID subjects exhibit low levels of innate immune activation similar to control donors¹², suggesting that high-risk needle sharing activity, not the opioid drugs themselves, likely drives the activation state associated with HESN-PWID subjects. Here, we investigated the mechanism of immune activation in PWID subjects using proteomic analysis of isolated NK cells from HESN-PWID subjects and control donors. We identified that NK cells from high-risk needle-sharing HESN-PWID subjects possessed significantly increased expression of interferon-related proteins along with several proteins members of the S100 family, including S100A4, S100A6, S100P, and S100A14.

S100 proteins are involved in many cellular processes including: regulation of proliferation, differentiation, apoptosis, Ca²⁺ homeostasis, inflammation, and migration. Secreted S100s, such as S100A14, may function as danger-associated molecular patterns and mediate innate and adaptive immune responses by signaling mainly through Receptor for Advanced Glycosylation End Products (RAGE) and TLR4²⁰. The S100 family of proteins have been routinely described in cancer, but very little is known about their potential impact on NK cells or HIV. S100P was initially purified from placenta, and it has been described to be involved in cell proliferation, survival, and differentiation^{21,22}, while also serving as a prognostic biomarker in cancer²³. S100A4 and S100A6 were shown to be overexpressed specifically in CD57^{high}CD56^{dim} NK cells, a more mature subpopulation of NK cells, and recruited into the NK immune synapse^{24,25}. S100A14 has been described in cancer²⁶⁻³³, and according to its sequence and structure it differs from other members of the S100 family as it cannot bind Ca²⁺³⁴. The primary receptor for S100A14 in a cancer cell line has been identified as RAGE³³. Human NK cells express detectable RAGE on their surface, while TLR4 is generally expressed in NK cells at very low levels; but it can be enhanced by infection^{35,36}.

In this study, we describe S100A14 to be upregulated in NK cells by proteomic analysis and in the plasma of HESN-PWID compared to controls donors. We further investigated the role of S100A14 as a potential mechanism for the sustained activation of the NK cell in HESN-PWID. In control donors, *in vitro* treatment of PBMCs with extracellular S100A14 demonstrated that activation of NK cells is not direct, but it occurs in a monocyte-dependent manner and that monocyte activation is through TLR4 signaling.

Methods:

Subject Criteria

HIV-1 exposed, sero-negative people who inject drugs (HESN-PWID) were enrolled from the city of Philadelphia via community-based street outreach in specific neighborhoods previously identified as “risk pockets”³⁷. Risk pockets are defined as locations within neighborhoods with a high HIV-1 prevalence where injectable drugs are sold, used, and at times exchanged for sex¹². We utilized the “Prognostic Model for Sero-conversion Among Injection Drug Users”³⁸ to identify high-risk HESN-PWID subjects for our study based upon their frequency of injection and needle sharing behavior. Briefly, subjects from known risk pockets were identified as HESN-PWID if they remained HIV-1 IgG sero-negative despite a history of greater than 2 years of daily injection and frequent (weekly or greater) needle sharing with partners of unknown HIV status. Demographic characteristics and risk factors of the HESN-PWID subjects are summarized in Table 1. All HESN-PWID subjects screened for the study were referred to drug treatment programs, counseled to use the local needle-exchange program services to reduce their risk of exposure to blood borne pathogens and referred to additional health services as needed. Healthy control donors were recruited from The Wistar Institute Phlebotomy Program. Six ART-suppressed and 2 viremic HIV-1 infected subjects were recruited from the Presbyterian Hospital of Philadelphia; the age mean for these patients is 47.25 years and their CD4 counts range from 112–994 cells/mm³. All protocols were approved by institutional review boards of the National Institutes of Health and The Wistar Institute, and all participants provided informed consent prior to the blood draw. S100A14 plasma levels were measured by ELISA (Abexxa, Cambridge, UK) following the manufacturer instructions.

Sample preparation and proteome analysis

Purified NK cells from 6 HESN-IDU and 6 control donors were prepared for proteomic analysis by negative selection using magnetic beads (Miltenyi Biotech) to greater than 97% purity. NK cells (2×10^6 cells per donor) were lysed using 50 mM Tris-Cl, 1% SDS, pH 8.0. After centrifugation, the supernatant was loaded onto a 12 % SDS-gel and separated for 2.0 cm followed by fixing and staining with colloidal Coomassie. The region of the gel containing protein was excised into 10 equal pieces and digested with trypsin. Tryptic peptides were analyzed by LC-MS/MS on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific) coupled to a Nano-ACQUITY UPLC system (Waters). Samples were injected onto a UPLC Symmetry trap column (180 μ m i.d. x 2 cm packed with 5 μ m C18 resin; Waters), and tryptic peptides were separated by RP-HPLC on a BEH C18 nanocapillary analytical column (75 μ m i.d. x 25 cm, 1.7 μ m particle size; Waters) using a 2-hour gradient. Eluted peptides were analyzed in data-dependent mode where the mass spectrometer obtained full MS scans from 400 to 2000 m/z at a 70,000 resolution. Full scans were followed by MS/MS scans at a 17,500 resolution on the 20 most abundant ions. Peptide match was set as preferred, the exclude isotopes option and charge-state screening were enabled to reject singly and unassigned charged ions. MS/MS spectra were searched using MaxQuant 1.5³⁹ against the human Uniref database plus common contaminants. MS/MS spectra were searched using full tryptic specificity with up to two missed cleavages, static carboxamidomethylation of Cys, and variable oxidation of Met and protein N-terminal

acetylation. Consensus identification lists were generated with false discovery rates of 1% at peptide level and an initial 5% at the protein level. LFQ ratio was set at 1, main search at 4.5 ppm. Razor+unique and min ratio counts were both set at 1. Resulting data was processed in PERSEUS 1.5 and exported to Excel for further analysis. Data were further filtered by removing proteins identified by a single peptide and known contaminants. The final protein false discovery rate was approximately 1%. Proteins exhibiting at least a 2-fold change between groups and $p < 0.05$ (student T-test) were considered significantly changed.

Isolation and stimulation of cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation on Ficoll (GE Healthcare). Initial NK cell enrichment was performed with the Negative selection EasySep™ Human NK Cell Isolation Kit (StemCell; Vancouver, BC, CA). NK cells were then purified further to reduce the presence of contaminating myeloid cells by staining with CD56 PerCP Cy5.5 (BD) and CD3 APC (BD). CD56⁺CD3⁻ cells were sorted using a FACS Aria (BD Cytometry Systems, San Jose, CA) to greater than 99.5% purity. Monocytes were isolated using Negative selection EasySep™ Human Monocyte Isolation Kit (StemCell). To determine NK cells activation (from within PBMC or after NK cell isolation), cells were stimulated with either 10 µg/ml of recombinant S100A14 (ACROBiosystems; Newark, DE) or 5000U/ml of IFN- α (pbl Assay Science; Piscataway, NJ) for 18 hours. To determine monocyte activation (from within PBMC or after monocyte isolation), cells were stimulated for 5 hours with either 10 µg/ml of recombinant S100A14 (Acro Biosystems), or 100ng/ml of LPS in the presence of Monensin (BD Golgi Stop; 0.266 µl/10⁶ cells). To inhibit LPS, we added 15 µg/ml of Polymyxin B (Calbiochem; San Diego, CA) before addition of S100A14. For inhibiting TLR4, 10 µM of TAK-242 (Calbiochem) was added 30 minutes before the addition of S100A14. Cells were stained with the appropriate antibodies for 15 min at room temperature in the dark, washed twice with 0.09% Sodium Azide in PBS, and then fixed with Cytotfix Buffer (BD). All antibodies were used at manufacturer recommended concentrations including: CD3 BV510 (BD, UCHT1), CD56 PerCP Cy5.5 (BD, B159), CD69 BV421 (BD, FN50) for NK cells, and CD3 APC-H7 (BD, SK7), CD14 APC (BD, M5E2), and TNF- α PE (BD, Mab11) for monocytes. Samples were run in a 14-color LSR II (BD Biosciences), and a minimum of 100,000 events were recorded. Flow cytometry data was analyzed with the FlowJo Software v9.9.4 (TreeStar).

Trans-well Co-cultures

Isolated NK cells were cultured in 24-well plates; a trans-well insert with a 0.4-µm filter was placed in each well, and autologous isolated monocytes were added in a 1:1 ratio in media with or without S100A14 (10µg/ml). Expression of surface CD69 on NK cells was analyzed after 18 hours using multi-parameter flow cytometry using the antibodies describe above.

Statistical Analysis

All statistical analyses were performed using Prism 7 software (GraphPad Software, La Jolla, CA). No data was purposefully excluded from the analysis. Any missing data from any subject was solely due to technical/cell yield reasons. All graphs are presented using the mean and the $\pm$ standard error of mean (SEM). Mann-Whitney test was used to investigate

differences between two groups with an unpaired non-parametric analysis. To determine the differences between more than two groups with an unpaired non-parametric analysis, Kruskal-Wallis test was used. For statistical analysis of more than two groups with a paired, non-parametric analysis the Friedman test was used. Correlations between two variables were carried out using Spearman Correlation of untransformed data with a 95% confidence interval. All tests were done using a two-tailed analysis and results with a $p < 0.05$ were considered statistically significant.

Results:

Proteome of Purified NK cells from HESN-PWID subjects and control donors.

To investigate the protein profile of activated NK cells from HESN-PWID subjects, we performed label-free quantitative proteomics using Gel/LC-MS/MS on purified NK cells from six HESN-PWID subjects and six control donors. We identified a total of 5,818 proteins, from which 108 proteins had a fold change greater than ± 2.0 and a p-value lower than 0.05 (Supplementary Table 1). As shown in Figure 1, we observed the significantly increased expression of various Interferon-related proteins (depicted in blue) and proteins involved in NK cytotoxicity (depicted in green). We also observed an unexpected strong signature of S100 family proteins (depicted in red) including S100A4, S100A6, S100-P, and S100A14 (Figure 1). To investigate if the increased expression of proteins from the S100 family was linked to interferon stimulation, we performed a separate proteomic analysis of NK cells from control donors exposed to 5000 U/ml of Interferon-alpha (IFN-alpha) for 18 hours. Purified NK cells from control donors stimulated with IFN-alpha *in vitro* displayed a strong proteomic signature of Interferon-related proteins and proteins involved in NK cytotoxicity but did not show evidence of S100 proteins (Supplementary Table 2). Together, this data suggests that the Interferon-related response and S100 signature observed in the NK cells from HESN-PWID may represent distinct pathways linked to NK activation.

Of note, the median NK CD69 activation of the six HESN-PWID subjects and six control donors utilized for proteomic analysis was 7.7% and 1.8%, respectively, as determined by flow cytometry. We analyzed CD69 expression on CD56^{dim}CD3⁻ NK cell population using the gating strategy depicted in Figure 2A. This differential level of activation was representative of significant differences in NK activation evaluated in a larger cohort of samples between the HESN-PWID and control cohorts (Figure 2B). Due to the limited sample size of proteomic specimens, however, we could not determine if the intra-cellular expression of any of S100 proteins or IFN-induced proteins correlated directly with surface levels of NK activation.

Plasma S100A14 is higher in HESN-PWID subjects and correlates with intracellular NK cell S100A14 expression.

Amongst the unique S100 protein signature in the NK cells from HESN-PWID subjects, we set out to investigate the S100A14 protein due to its uniformly high fold change amongst HESN-PWID subjects and its reported potential to be secreted^{31,33}. We measured plasma levels of S100A14 in 27 HESN-PWID subjects (Table 1), 15 control donors, and 8 HIV-1 infected subjects (2 viremic and 6 ART-suppressed). We detected significantly higher levels

of S100A14 protein in the plasma of HESN-PWID subjects when compared to either control donors or HIV-1 infected subjects (Figure 2C). While we did not observe a correlation between surface CD69 protein expression in NK cells and S100A14 plasma levels (data not shown), we did observe that S100A14 levels in plasma by ELISA had a strong correlation ($r=0.951$, $p<0.0001$, $n=12$) with the expression of S100A14 in NK cells as detected by Proteomic analysis (Figure 2D). These data suggest that S100A14 plasma levels are associated with the up-regulation of S100A14 in NK cells, yet our data does not address if other cells or immune processes may also contribute to increased S100A14 plasma levels in HESN-PWID.

Recombinant S100A14 can activate NK cells in a monocyte-dependent fashion.

To investigate if extracellular S100A14 has a role in mediating innate immune activation observed in HESN-PWID subjects, we stimulated PBMCs from control donors with increasing doses of recombinant S100A14 for 18 hours and assessed NK activation by flow cytometry. Following stimulation of PBMC with S100A14, we observed a dose-dependent increase in CD69 activation among CD56^{dim}CD3⁻ gated NK cells (data not shown). Treatment of PBMC with 10 $\mu\text{g/ml}$ S100A14 induced significantly higher ($p=0.011$, $n=10$) CD69 activation on CD56^{dim}CD3⁻ gated NK cells compared to untreated PBMC (Figure 3A). As a positive control, we stimulated PBMCs with 5000 U/ml recombinant IFN- α . We observed that S100A14 and IFN- α both can induce comparable levels of CD69 upregulation (Figure 3A). Since we observed that S100A14 did not represent an Interferon-inducible protein by Proteomic analysis, we suspected that S100A14 induced activation might synergize with Interferon- α stimulation. Indeed, when we combined S100A14 and IFN- α together we observed an additive effect in the activation of NK cells that was greater than either stimulation alone (Figure 3A).

We next tested if recombinant S100A14 could directly activate NK cells or if it required the actions of other cells in the PBMC. We performed two rounds of NK isolation to achieve NK purity greater than 99.5% and stimulated NK cells directly with 10 $\mu\text{g/ml}$ S100A14. Unlike NK cells in a PBMC mixture, S100A14 did not directly activate isolated NK cells (Figure 3B). In contrast, Interferon- α stimulation induced isolated NK cells to significantly upregulate CD69 ($p=0.031$, $n=6$) as expected (Figure 3B). This data led us to test if other cell types in the PBMC respond to S100A14 and trigger NK cell activation indirectly. Due to the well described cross-talk between myeloid cells and NK cells, we tested if S100A14 could activate monocytes. We stimulated PBMCs from control donors with 10 $\mu\text{g/ml}$ S100A14 for 5 hours, and then measured production of TNF- α in CD14⁺HLA-DR⁺ gated monocytes by intracellular staining (Figure 3C). As shown in Figure 3D, the percentage of monocytes producing TNF- α was significantly increased after S100A14 exposure ($p=0.018$, $n=8$). The level of TNF- α produced by monocytes in response to S100A14 was comparable to levels induced by LPS stimulation, which is a potent trigger for monocyte activation. Unlike purified NK cells, S100A14 was able to induce production of TNF- α on isolated monocytes ($p=0.0099$, $n=7$) (Figure 3E). As activated monocytes can provide potent triggers for NK activation, we next measured if S100A14-activated monocytes could trigger NK activation. We co-cultured autologous purified monocytes and purified NK cells in a 1:1 ratio and observed that the presence of

monocytes and S100A14 could induce NK activation (Figure 3F). In contrast, the addition of monocytes to NK cells in the absence of S100A14 alone failed to trigger NK activation (Figure 3F). To investigate if monocyte-mediated activation was dependent on soluble factors (e.g. IL-12) or on cell-to-cell contact, we used a trans-well system for our NK-monocytes co-cultures. We observed that separation of NK cells and monocytes decreased NK cell activation in the presence of S100A14 (Figure 3F), indicating that S100A14-mediated activation of NK cells is augmented by direct contact with monocytes.

S100A14 drives TNF-alpha production in monocytes through the TLR4 receptor.

S100A14 has been reported to interact with both RAGE, and TLR4^{33,40}, therefore we next set out to investigate which potential receptor for S100A14 was required for monocyte-dependent NK activation. When PBMCs were pretreated with the TLR4 inhibitor, TAK-242, before the addition of S100A14, monocytes production of TNF-alpha was inhibited (Figure 4A). To rule out the possibility that the effects observed were due to LPS contamination independently of S100A14, we tested the effects of S100A14 in the presence of the LPS inhibitor Polymyxin B (PMB) and confirmed the activity of S100A14 on increasing TNF-alpha production in monocytes (Figure 4B). Together, this data indicate that S100A14 can activate monocytes through TLR4 which in turn can impact NK activation.

Discussion:

Here we identify for the first time a unique proteome profile in NK cells from HESN-PWID subjects compared to control donors. This included an IFN-induced protein signature as well as the presence of a number of proteins from the S100 family, including S100A14. The S100A14 protein was found to be increased in the plasma of HESN-PWID subjects compared to controls, and plasma levels of S100A14 were positively correlated with the expression of S100A14 in NK cells by Proteomic analysis. Furthermore, in our subset of HIV-infected subjects (n=8), S100A14 levels in plasma appear to be similar to those in normal donors, supporting the interpretation that increases in plasma S100A14 may be an enriched response in HESN-PWID. More studies are needed to address the importance of S100A14 during HIV infection and pathogenesis.

In vitro, recombinant S100A14 protein was shown to activate NK cells from control donors in a monocyte-dependent manner by signaling through TLR4. Together, these findings suggest that the S100A14 may be part of an NK/monocyte activation cycle promoting the activation phenotype seen in a subset of HESN-PWID subjects. However, the exact mechanism of the crosstalk between NK cells and monocytes in the context of S100A14 stimulation is still unknown. Since the crosstalk involves cell-to-cell contact, TLR4 (LPS)-mediated expression of MICA⁴¹, a ligand for engagement of the NKG2D receptor, in monocytes is a candidate mechanism for NK activation. NKG2D is an activator receptor mainly expressed in NK cells and CD8⁺ T cells, and it is used to recognize infected cells, tumor cells, or stressed cells, which leads to cell-mediated cytotoxicity^{42,43}. NK cells co-culture with LPS-stimulated monocytes results in a down regulation of NKG2D and increase IFN-gamma production in activated NK cells⁴¹.

Although our data shows that S100A14 can induce NK activation through TLR4-mediated activation of monocytes, added antiviral mechanisms mediated by TLR4 binding are likely. Previous studies have shown that pretreatment with LPS, a TLR4 ligand, can suppress HIV-1 replication in macrophages and monocytes by several distinct mechanisms; including blocking reverse transcription⁴⁴, downregulating the CD4 and CCR5 receptors^{45–47}, and inducing the production of pro-inflammatory cytokines and chemokines^{44,45,47,48}. Here, we describe the presence of a host protein that interacts with TLR4 that could potentially contribute to similar antiviral outcomes.

Altogether, we found evidence of increased expression of the S100A14 protein in both activated NK cells and the plasma from HESN-PWID subjects. Future research will need to investigate the effect of S100A14 NK/monocyte cross-talk on HIV-1 replication and potential NK-mediated clearance of infected target cells through S100A14 stimulation in HESN-PWID subjects. Elucidating the biological effect of S100A14 together with the triggers of S100A14 expression, could help to develop future therapies for prevention of HIV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Proteome of Purified NK cells from HESN-PWID subjects and control donors. Purified NK were obtained from 6 needle-sharing HESN-PWID subjects and 6 control donors by two rounds of selection to achieve greater than 99.5% purity. Samples were frozen and then prepared for label-free quantitative proteomics and sequenced using Gel/LC-MS/MS with EI. Volcano plot of shows the intensity of protein expression in HESN-PWID normalized relative to the median value for all the samples. Criteria for significantly differential expression of proteins in HESN-PWID was set up at a fold change higher than 2.0 or lower than -2.0 and a p value lower than 0.05 (dashed lines). S100 protein family members are shown in red. Interferon-related proteins are shown in blue. Proteins involved in NK cytotoxicity are shown in green.

Figure 2. Plasma S100A14 is higher in HESN-PWID subjects and correlates with intracellular NK cell S100A14 expression.

(A) Gating strategy for the flow cytometric analysis of CD69 expression on CD56^{dim}CD3⁻ NK cells. PBMCs were stained with fluorochrome-conjugated antibodies against CD3 (BV510), CD56 (PerCP Cy5.5), and CD69 (BV421). In the CD69 expression graph we present in red the histogram for the Fluorescence Minus One Control and in blue the constitutive expression of CD69 on a representative HESN-PWID subject. (B) Flow cytometry staining showing the constitutive expression of CD69 on NK cells (CD56^{dim}CD3⁻) in control donors (n=26) and HESN-PWID subjects (n=27). (C) ELISA measuring plasma S100A14 levels in control donors (n=15), HESN-PWID subjects (n=28), and 8 HIV-1 infected subjects including 6 that are ART-suppressed (shown in black) and 2 that are viremic (shown in red). Graphs are presented with mean and their standard error bars. Statistical analysis was carried out using a Mann-Whitney Test with a two-tailed p value. (D) Spearman correlation of the non-normalized LFQ intensity (raw data from Proteomic profile) and the plasma S100A14 levels in control donors (n=6, orange) and HESN-PWID subjects (n=6, red).

Figure 3. Recombinant S100A14 can activate NK cells in a monocyte-dependent fashion. (A-B) CD69 expression measured on CD56^{dim}CD3⁻ gated NK cells from within a PBMC mixture (n=10) (A) or on purified NK cells (n=6) (B) following an 18 hours exposure to 10 μ g/ml S100A14, 5000 U/ml IFN- α , or both stimuli together. (C) Gating strategy for the flow cytometric analysis of TNF- α production on CD14⁺HLA-DR⁺ monocytes. PBMCs were stained with fluorochrome-conjugated antibodies against CD3 (APC-H7), HLA-DR (FITC), CD14 (APC) and TNF- α (PE) using extracellular and intracellular staining protocols. In the TNF- α production graph we present in red the histogram for

the Fluorescence Minus One Control and in blue the production of TNF-alpha after 5 hours of 10ug/ml S100A14 stimulation. (D-E) TNF-alpha production measured on CD14⁺HLA-DR⁺ gated monocytes cells from within a PBMC mixture (n=8) (D) or on purified monocytes (n=7) (E) following a 5 hours exposure to 10µg/ml S100A14 or 100 ng/ml LPS. (F) Frequency of NK CD69 surface expression after co-culture of purified NK cells and monocytes at a 1:1 ratio in the presence or absence of 10 µg/ml S100A14 for 18 hours, and cultured either in direct contact (n=10) or in a trans-well insert (n=6) with a filter of 0.4-µm. All composite graphs are presented with their mean and their standard error of the mean. Statistical analysis was carried out using with a two-tailed p value. Significant values are shown in red. For analysis of two groups with a paired non-parametric analysis the Wilcoxon test was used. Friedman test was used to analyze differences between three or more paired groups and Kruskal-Wallis test for unpaired groups.

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Figure 4. S100A14 drives TNF-alpha production in monocytes through the TLR4 receptor. (A-B) TNF-alpha production measured on CD14⁺HLA-DR⁺ gated monocytes cells from within a PBMC mixture following a 5 hours exposure to 10 µg/ml S100A14 or 50 ng/ml LPS in the presence of TLR4 or LPS inhibitors. 10 µM TAK-242 (TAK) (n=8) (A), or 15 µg/ml Polymyxin B (PMB) (n=8) (B) was added 30 minutes prior to the 5 hours incubation to inhibit TLR4 and LPS, respectively. Graphs are presented with their mean and their

standard error of the mean. Statistical analysis was carried out using a Friedman test with a two-tailed p value.

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Table 1.

Demographic data and risk factors of HESN-PWID subjects.

HESN-PWID	
N	27
Age	44.1 ±10.8
Gender	
Male	22 (81.5%)
Female	4 (14.8%)
Transgender	1 (3.7%)
Frequency of Needle Sharing (Last 6 mo)	
1–24	22 (81.5%)
25–72	1 (3.7%)
73–144	4 (14.8%)
Co-infections	
HCV	23 (85.2%)
HBV	7 (25.9%)
Other Risks Factors	
Heroin	27 (100%)
Coc/Crack	17 (63%)
Unprotected Sex	21(77.8%)

* This table has been adapted from [12]