Humoral Dysregulation Associated with Increased Systemic Inflammation among Injection Heroin Users

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Abstract

Background

Injection drug use is a growing major public health concern. Injection drug users (IDUs) have a higher incidence of co-morbidities including HIV, Hepatitis, and other infections. An effective humoral response is critical for optimal homeostasis and protection from infection; however, the impact of injection heroin use on humoral immunity is poorly understood. We hypothesized that IDUs have altered B cell and antibody profiles.

Methods and Findings

A comprehensive systems biology-based cross-sectional assessment of 130 peripheral blood B cell flow cytometry- and plasma-based features was performed on HIV-/Hepatitis C-, active heroin IDUs who participated in a syringe exchange program (n = 19) and healthy control subjects (n = 19). The IDU group had substantial polydrug use, with 89% reporting cocaine injection within the preceding month. IDUs exhibited a significant, 2-fold increase in total B cells compared to healthy subjects, which was associated with increased activated B cell subsets. Although plasma total IgG titers were similar between groups, IDUs had significantly higher IgG3 and IgG4, suggestive of chronic B cell activation. Total IgM was also increased in IDUs, as well as HIV Envelope-specific IgM, suggestive of increased HIV exposure. IDUs exhibited numerous features suggestive of systemic inflammation, including significantly increased plasma sCD40L, TNF-α, TGF-α, IL-8, and ceramide metabolites. Machine learning multivariate analysis distilled a set of 10 features that classified samples based on group with absolute accuracy.
Conclusions
These results demonstrate broad alterations in the steady-state humoral profile of IDUs that are associated with increased systemic inflammation. Such dysregulation may impact the ability of IDUs to generate optimal responses to vaccination and infection, or lead to increased risk for inflammation-related co-morbidities, and should be considered when developing immune-based interventions for this growing population.

Introduction
Injection drug use continues to be a major public health issue, with rapid growth in recent years. The rate of heroin use and overdose doubled in the United States between 2010 and 2012 [1, 2]. Numerous co-morbidities are associated with injection drug use including increased risk of cardiovascular disease, chronic kidney disease, gastrointestinal diseases, and infections [3–5]. Along with HIV and Hepatitis C, which are primarily transmitted in IDUs through sharing of contaminated needles, there is increased incidence of bacteremia [4] and soft-tissue injection site infections that include Staphylococcus aureus, Group A streptococci, and Clostridial infections [6–8]. In addition to increased exposure to pathogens, prolonged injection drug use has been associated with immune dysregulation that may diminish effective innate and adaptive immune responses [9].

Opiates, including heroin, can directly act on immune cells through opioid receptors, and result in decreased in vitro T cell proliferation and cytokine production [10], and decreased phagocytosis and chemotaxis of macrophages [11]. In mice, morphine has been shown to inhibit antibody responses [11–13]. Although heroin dominates illicit injection drug use, polydrug use is substantial among IDUs, including use of cocaine and methamphetamine [14, 15], which prevents clear extrapolation of the impact of opiates on the immune system in animal models to the complexity of IDUs, necessitating a direct and comprehensive assessment of the injection drug user population.

Several studies have reported elevated inflammatory mediators associated with IDUs, including serum IL-1β, IL-6, and IL-8 in methadone-maintained IDUs [16] [17], and increased dendritic cell and natural killer cell activation in IDUs that is associated with needle sharing [18]. These findings suggest that multiple mediators may be contributing to altered immune homeostasis in IDUs. Direct assessment of immune responsiveness in IDUs has been limited, although diminished antibody responses to vaccinations, including Hepatitis A and B have been observed in IDUs [19, 20]. Studies thus far have primarily measured only serum IgG antibody, and have not evaluated potential qualitative differences among the serum antibody response or direct evaluation of the B cells, which may influence vaccine efficacy, particularly for HIV [21].

The primary objective of preventative vaccine strategies against HIV is the induction of a persistent humoral response that mediates sterilizing immunity. Although modest, encouraging results of a short-lived reduction in infections in low-risk individuals were obtained in the RV144 HIV vaccine efficacy trial [22] for high-risk individuals, including IDUs, no substantial reduction in infection has been observed in HIV vaccine efficacy trials [23–25]. Furthermore, correlates of protection associated with RV144 included the IgG3 antibody response to a particular epitope (V1V2) on the HIV Envelope protein, and not the overall IgG antibody response to the vaccine [26], highlighting the qualitative subtleties that may determine HIV vaccine efficacy. Most mechanistic vaccine studies, including those for HIV vaccines, are
primarily conducted with healthy subjects. This approach creates the vulnerability that advances in vaccine efficacy realized in healthy subjects may be difficult to translate to the IDU population, which is at high risk for HIV infection.

The impact of injection drug use on humoral responses is poorly defined. We sought to identify potential differences in the humoral profile of IDUs that could impact their immune responses and overall health. Through a cross-sectional assessment of peripheral blood B cells and plasma analytes, we have identified substantial alterations in IDUs that are associated with increased systemic inflammation, and which have the potential to influence the quality of the B cell and antibody response to vaccinations, infections, and contribute to inflammation-associated co-morbidities.

Material and Methods

Clinical samples and questionnaire

Peripheral blood samples were obtained from active IDUs recruited through the Trillium Health Syringe Exchange Program in Rochester, NY, during 2014. The IDUs were defined as having injected heroin within the previous 3 days and having a history of at least weekly heroin injection for a period of at least 3 months in duration within the previous 12 months. Healthy controls (HCs) with no history of substance abuse, no use of opiates (prescription or non-prescription), prescription stimulants, or illicit substances (excluding marijuana) within the previous 6 months were recruited at the University of Rochester Medical Center. All subjects were at least 110 lbs, 18–35 years old, HIV negative and Hepatitis C negative, and no history of immunization within the previous 2 weeks, as determined by self-report. All subjects provided signed, written informed consent. Competence and understanding of the informed consent was assessed throughout the process by study staff through a series of questions regarding study participation. Study subjects answered a series of questions regarding their demographics, health, substance use, sexual practices, and perspectives on vaccine research using a Research Electronic Data Capture (REDCap)–based questionnaire, hosted at the University of Rochester [27]. Peripheral blood was coded to blind the samples and was collected using CPT tubes (BD Biosciences, Franklin Lakes, NJ); peripheral blood mononuclear cells (PBMCs) and plasma were isolated and cryopreserved in a blinded manner as previously described [28] before subsequent analysis. All procedures and methods were approved by the University of Rochester Research Subjects Review Board.

Flow cytometry

For B cell phenotypic analysis PBMCs were stained similar to as previously described [28, 29] with anti-CD19-APC-Cy7 (SJ25C1, BD), anti-CD20-AlexaFluor 700 (2H7, Biolegend, San Diego, CA), anti-CD3-PacificOrange (UCHT1, Invitrogen, Carlsbad, CA), anti-IgD-FITC (IA6-2, BD), anti-IgG-APC (G18-145, BD), anti-IgM-PE-Cy5 (G20-127, BD), anti-CD27-Qdot655 (CLB-27/1, Invitrogen), anti-CD21-V450 (B-ly4, BD), anti-CD14-PerCP-Cy5.5 (M5E2, BD), anti-CD138-biotin (B-A38, Abcam, Cambridge, MA), streptavidin Qdot800 (Invitrogen), anti-CD4-Qdot705 (S3.5, Invitrogen), anti-CD38-Qdot605 (HIT2, Invitrogen), anti-CD95-PE-Cy7 (DX2, Biolegend), anti-CD24-PE-AlexaFluor610 (SN3, Invitrogen), anti-CD183-PE (1C6/CXCR3, BD) and Live/Dead fixable aqua dead cell stain (Invitrogen). One-to-two million total events per sample were collected on an LSRII instrument (BD Biosciences). Staining and analysis performed in a blinded manner, linking sample with experimental group after gating was completed using FlowJo software (Treestar, Inc, Ashland, OR). Total PBMC were gated on lymphocytes using FSC and SSC. Live/Dead stain, anti-CD3, anti-CD4, and anti-CD14 were used to exclude dead cells, T cells, and monocytes respectively.
Plasma antibody ELISAs and LAL

For detection of plasma total IgG and IgG subclasses ELISA plates were coated overnight with 1 μg/ml anti-human IgG (Jackson ImmunoResearch, 709-006-149, West Grove, PA) in PBS, blocked with 2% nonfat dry milk and 2% BSA in PBS for 1 h, then washed with 0.1% Tween 20 in PBS. Samples were serially diluted in triplicate in PBS containing 0.01% Tween 20 and 0.5% BSA, and incubated for 1 h. Plates were washed, and binding was detected using anti-human IgG-HRP (Jackson), anti-human IgG1-HRP (Southern Biotech, 9052–05, Birmingham, AL), anti-human IgG2-HRP (Southern Biotech, 9070–05), anti-human IgG3-HRP (9210–05), or anti-human IgG4-HRP diluted 1:2000 in PBS with 0.1% Tween 20. Total plasma IgM and IgA were detected using 1 μg/ml anti-human IgM (Jackson, 109-006-129) with 1:2000 anti-human IgM-HRP (Jackson, 109-035-129), and 1 μg/ml anti-human IgA (Jackson, 109-005-011) with 1:2000 anti-human IgA-HRP (Jackson, 309-035-011). ELISA plates were coated with 0.5 μg/ml Tetanus toxoid (Calbiochem, San Diego, CA), 10 μg/ml LPS (Sigma- St. Louis, MO Aldrich), or 1 μg/ml Sm antigen (Arotec Diagnostics, Wellington, New Zealand) and detected with 1:2000 anti-human IgG-HRP to measure Tetanus toxoid, LPS, and Sm specific IgG plasma antibody respectively. ELISA plates were coated with 1 μg/ml HIV SF162 gp140 protein or 1 μg/ml HIV p24 protein (NIH AIDS Reagent Repository) and detected with either anti-human IgG-HRP or anti-human IgM-HRP to measure HIV-specific antibody. Anti-dsDNA IgG ELISA was performed, testing samples in duplicate using a commercial kit according to manufacturer’s (Inova Diagnostics, San Diego, CA) recommendations. Plasma LPS was measured using the Kinetic-QCL chromogenic LAL assay (Lonza, Basel, Switzerland) according to manufacturer’s instructions.

Plasma analyte multiplex assay

Each plasma sample was tested in duplicate in a blinded manner using 25 μl plasma plus the addition of 5 μl of heteroblock (Omega Biologicals, Bozeman, MT) to minimize non-specific interactions [30] with a MilliPlex Human Cytokine/Chemokine assay (Millipore, Billerica, MA, MPXHCYTO60KPMX42) according to manufacturer’s instructions. Values that were below limit of detection were adjusted to non-zero value (10, 1, 0.1, or 0.01) depending on lower limit of detection for individual analyte to enable statistical analysis.

Ceramide profiling: Lipid extraction and LC/ESI/MS/MS analysis

Lipids were extracted from plasma using a modified Bligh and Dyer procedure as previously described [31]. The organic layer containing a crude lipid extract was dried using a nitrogen evaporator (Organomation Associates Inc., Berlin, MA, USA), and resuspended in pure methanol. Ceramides were detected by multiple reaction monitoring using a liquid chromatography coupled electrospray ionization tandem mass spectrometer (ESI/MS/MS) (API3000; AB Sciex Inc., Thornhill, ON, Canada) operated in positive mode. LC and MS/MS parameters have been previously described [32]. Slight differences in extraction efficiency and fluctuations in the efficiency of mass detection were normalized using a ceramide C12:0 internal standard (Avanti Polar Lipids, Alabaster, AL). Instrument efficiency was monitored daily, and at the end of the study individual plasma extracts were re-analyzed if the internal standard deviated more than 30% from the overall median internal standard value. Ceramide concentrations were determined by fitting the identified ceramide species to standard curves based on acyl-chain length. Ceramide standards C16:0, C18:0, C18:1 were purchased from Sigma. Ceramides C20:0, C24:0, C24:1 were purchased from Avanti Polar Lipids (Alabaster, AL). Palmitoyl-lactosyl ceramide C16:0-C16:0, stearoyl-lactosyl-ceramide C16:0-C18:0, lignoceryl-glucosyl-ceramide C16:0-C24:0, lignoceryl-galactosyl-ceramide C16:0-C24:0, and stearoyl-galactosyl-ceramide-
sulfate C18:1-C24:0 were purchased from Matreya Inc. (Pleasant Gap, PA). Instrument control and quantitation were performed using Analyst 1.4.2, and MultiQuant software (AB Sciex Inc. Thornhill, Ontario, Canada).

Statistical analysis

Univariate analysis of individual features was performed by the two-tailed Mann-Whitney test to compare groups using Prism 5.0 software (GraphPad Software, La Jolla, CA). No correction for multiple outcomes was performed due to the limited sample size and exploratory nature of the project. Heat maps, cluster analysis and principal component analysis were performed using Matlab (the Mathworks Inc., Natick MA).

Furthermore, a supervised machine learning classifier with wrapper method [33, 34] called support vector machine (SVM) recursive feature elimination [35, 36] was used to find small subsets of features that together have good predictive power to classify IDUs. In the first iteration, a SVM classifier was trained using the complete set of (216) features (S1 File). These features were then ranked based on their relative classification power in terms of their absolute weights. The feature with the smallest weight was removed iteratively, each time upon training a new SVM classifier. The performance of the classifier in each run was measured through 10-fold cross validation. IDU-23 was excluded from the training process because of its extreme outlier profile. IDU-27 was also excluded from the training process because of a missing "cell count" value. When these two subjects were independently tested on final SVM (built on top 10 features), the SVM was able to classify both of them accurately.

Results

Injection drug user population

Young adult HIV negative and Hepatitis C negative active IDUs (n = 19) were recruited through the local syringe exchange program. The majority of the IDU cohort was white (84%) and male at birth (68%) and similar in composition to the group of healthy controls (HCs) (Table 1). IDUs reported reduced self-perception of their overall health and satisfaction with their lives. Sexual risk behaviors did not significantly differ from HCs including past diagnosis of a sexually transmitted infection, number of sexual partners, or incidence of unprotected anal or vaginal intercourse. HIV testing rates were slightly higher among IDUs, as reported testing within the previous 6 months was 47% in IDUs vs 28% in HCs, and 68% in IDUs vs 50% in HCs within the previous year; likely driven by point-of-care HIV testing offered at the syringe exchange. Encouragingly, the majority (73%) of IDUs indicated they were agreeable to participating in a hypothetical future HIV vaccine trial, and participating in a hypothetical future research study that requires 10 visits (74%).

All IDUs had injected heroin within the preceding 72 hours of participation, and 56% reported to had used heroin every day for the preceding 3 months (Table 2). The combined use of heroin and cocaine was predominant, with 89% of IDUs reporting having injected cocaine within the preceding 30 days. Of the IDUs, 44% indicated they were intoxicated for most of the day for at least the majority of the days within the preceding 90 days. Occasional sharing of syringes in the last 30 days was reported by 20% of the IDUs, and the remainder indicated they had not shared needles in the past 30 days.

Increased total B cells and B cell activation among IDUs

As B cells are critical for protective immunity we investigated the phenotypic profile of the peripheral blood B cell compartment for altered homeostasis. There were no significant
Table 1. Participant Characteristics.

|                                | Healthy Control Group (n = 19) | Injection Drug User Group (n = 19) | p       |
|--------------------------------|--------------------------------|-----------------------------------|---------|
| Age (yrs)                      | 24.3 (18–35)                   | 28.4 (21–35)                      | 0.014   |
| Female (at birth)              | 42%                            | 32%                               | 0.2425  |
| Transgender                    | 5%                             | 5%                                | 1       |
| Black                          | 11%                            | 5%                                | 0.1913  |
| White                          | 79%                            | 84%                               | 0.4667  |
| Body mass index                | 29.4 (19–47)                   | 26.1 (21–38)                      | 0.1409  |
| “I seem to get sick a little easier than other people” (1 = definitely true, 5 = definitely false) | 3.8 (3–5)                      | 3.1 (2–5)                      | 0.005   |
| “In general how satisfied are you with your life?” (very dissatisfied to very satisfied) | 95%                            | 26%                               | <0.0001 |
| ≤ $15,000 annual income        | 47%                            | 74%                               | 0.0002  |
| College graduate               | 58%                            | 11%                               | <0.0001 |
| Men who have sex with men (of males) | 50%                            | 8%                                | <0.0001 |
| # of sexual partners in last 6 months | 3.2 (0–30)                      | 3.7 (0–42)                      | 0.8603  |
| Unprotected anal sex in last 6 months (not with main partner) | 5%                             | 5%                                | 1       |
| Sex for money, drugs, gifts, or services | 5%                             | 5%                                | 1       |
| New STI in last 6 months       | 5%                             | 0%                                | 0.0594  |
| Ever diagnosed with STI        | 21%                            | 26%                               | 0.5050  |
| HIV test w/in last 3 months    | 17%                            | 37%                               | 0.0023  |
| HIV test w/in last 6 months    | 28%                            | 47%                               | 0.0084  |
| HIV test w/in last year        | 50%                            | 68%                               | 0.0143  |
| Willing to participate in future HIV vaccine trial: agree or strongly agree | 68%                            | 73%                               | 0.5353  |
| Willing to participate in a future research study that requires 1 visit: agree or strongly agree | 100%                           | 84%                               | <0.0001 |
| Willing to participate in a future research study that requires 10 visits: agree or strongly agree | 63%                            | 74%                               | 0.128   |

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Table 2. Substance Use Characteristics.

|                                | Healthy Control Group (n = 19) | Injection Drug User Group (n = 19) | p       |
|--------------------------------|--------------------------------|-----------------------------------|---------|
| Tobacco use (any in last year) | 24%                            | 74%                               | <0.001  |
| Alcohol (at least 15 out of last 90 days) | 16%                            | 21%                               | 0.467   |
| Marijuana (at least 15 out of last 90 days) | 0%                             | 37%                               | <0.001  |
| Drunk or high most of the day (at least 1 day out of last 90 days) | 21%                            | 78%                               | <0.001  |
| Drunk or high most of the day (at least 16 out of last 90 days) | 0%                             | 61%                               | <0.001  |
| Drunk or high most of the day (at least 46 out of last 90 days) | 0%                             | 44%                               | <0.001  |
| Crack cocaine (at least 16 out of last 90 days) | NA                             | 26%                               |         |
| Non-crack cocaine (at least 16 out of last 90 days) | NA                             | 56%                               |         |
| Heroin (at least 16 out of last 90 days) | NA                             | 100%                              |         |
| Heroin (everyday of last 90 days) | NA                             | 56%                               |         |
| Injected cocaine (at least once in last 30 days) | NA                             | 89%                               |         |
| Most recent injection was both heroin and cocaine | NA                             | 42%                               |         |
| Never have shared needles (in last 30 days) | NA                             | 80%                               |         |
| Occasionally have shared needles (in last 30 days) | NA                             | 20%                               |         |
| Often or all the time shared needles (in last 30 days) | NA                             | 0%                                |         |

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differences in total lymphocyte count, frequency of CD14+ monocytes, or CD4+ T cells among the groups (not shown) however IDUs had approximately 2-fold higher frequency of total B cells, defined as CD19+ as compared to HCs (8.6% vs. 4.4%, p<0.005) (Fig 1A and 1B). This observation was most pronounced in the CD19+CD20+ B cell population (7.2% vs 3.8%, p<0.005), that is known to include nearly all peripheral blood B cell subsets. No significant difference was observed in the frequency of the CD19+CD20low/neg B cell population. The CD19+CD20low/neg population is primarily described as being enriched for plasmablasts, which are active antibody secreting cells, or pre-plasmablasts [37, 38]. A comprehensive phenotypic profiling of the B cell subsets (Fig 2) did not reveal significant phenotypic differences within the CD19+CD20+ B cell population, although there was a notable 40% increase in IgD +CD183(CXCR3)+ B cells among IDUs (p = 0.0935), suggesting there may be a low-level chronic inflammatory phenotype in IDUs [39–41].

Fig 1. Increased total peripheral blood B cells in IDUs. PBMC were analyzed by flow cytometry. (A) Representative plots gated on live, CD14-CD3-CD4- lymphocytes, the gate is colored red to highlight the expanded CD19+CD20+ total B cell population in IDUs. (B) Frequency of CD19+ (live, CD14-CD3-CD4-) populations among lymphocytes defined by FSC and SSC, each symbol is an individual subject, the red lines indicate mean. The singular CD19+ population is the combination of both the CD19+CD20low/neg population and the CD19+CD20+ population. Flow cytometry was conducted once per sample.
Although the frequency of the CD19+CD20low/neg B cell population was not significantly different between groups, numerous phenotypic differences were apparent within this population. Within the CD19+CD20low/neg B cell population, IDUs exhibited a 30% increase in CD21-CD27+ (p < 0.05), 30% increase in IgD-CD27+ (p < 0.05), with a reciprocal 50% decrease in CD21-CD27- (p < 0.005) and 40% decrease in IgD-CD27- (p < 0.005), in addition to a 40% increase in IgD-CD95+ (p < 0.005) ([Fig 2B and 2C]). The CD19+CD20low/neg plasmablast population has previously been defined by CD38 or CD138 expression [28, 38, 42], however, no significant differences were observed between groups among CD19+CD20low/neg CD27+CD38+ or CD138+ potential plasmablast populations ([Fig 2A]). The increased CD27 and CD95 expression among the CD19+CD20low/neg is consistent with an activated B cell phenotype in IDUs.

Skewed plasma antibody profile among IDUs

Observing such prominent differences in the phenotype of the B cells in IDUs, we evaluated if these alterations extended to the plasma antibody compartment. Comprehensive profiling of plasma antibodies revealed gross differences between IDUs and HCs ([Fig 3A]). IDUs exhibited significantly higher total IgM concentration (p < 0.05), and although total IgG concentrations were comparable between groups, IDUs had significantly higher IgG3 (p < 0.005) and IgG4 (p < 0.05) subclasses ([Fig 3B]). No significant difference in total IgA concentration was apparent. This skewing of plasma antibody profiles suggests a higher frequency of subclinical infections or ongoing inflammation in IDUs [43].

No significant differences in the incidence of auto-reactive IgG antibody (anti-Sm, anti-dsDNA) were observed ([Fig 3A]), suggesting that self-tolerance was being maintained in IDUs. Several IDUs had increased HepC-specific IgG that may suggest previous exposure to the virus. We next examined if there was any serum antibody reactivity to HIV; specifically we did not observe any significant differences in anti-HIV p24 IgG or anti-HIV gp140 IgG between the two groups; however, IDUs had significantly higher (p < 0.05) HIV gp140 IgM antibody titers ([Fig 3C]), which may suggest increased exposure to HIV within this population, and may reflect a history of exposure that did not result in HIV infection. Only one subject (IDU-23) exhibited anti-HIV p24 IgG and anti-HIV gp140 IgG antibody reactivities that were substantially higher than the group of HCs, this subject also had numerous elevated serum antibodies including anti-HepC IgG, -LPS IgG, -Sm IgG, and -dsDNA IgG, which could indicate undiagnosed HIV infection or high levels of poly-reactive serum antibodies.

Increased systemic inflammatory mediators in IDUs

To assess potential systemic alterations in innate immune mediators, an extensive panel of plasma cytokines, chemokines and soluble factors was measured ([Fig 4]). The group of IDUs had significantly increased plasma TGF-α (~10-fold, p < 0.005; an EGFR ligand), TNF-α (~5-fold, p < 0.005; a potent inflammatory cytokine); IL-8 (~10-fold, p < 0.001; a chemokine important for neutrophil migration, induction of phagocytosis and angiogenesis), and sCD40L (~5-fold, p < 0.0005; a potent inflammatory mediator produced primarily by platelets, which induces proliferation and class-switching of B cells [44]) ([Fig 4B]). We also found increased levels of plasma LPS (endotoxin) evident in several IDUs (p < 0.01) ([Fig 4A]), suggestive of bacterial infection or increased gut permeability [45, 46]. These results are consistent with a chronic systemic inflammatory phenotype in IDUs.
Fig 2. B cell phenotypic profile. (A) B cell profiles for live, CD14-CD3-CD4- CD19+CD20low/neg (left) and CD19+CD20+ (right). Heat maps show data corresponding to a particular B cell subset (column) and subject.
Fig 3. Plasma antibody profile. Relative concentrations of plasma antibody species was determined by ELISAs. (A) Antibody profiles are shown as a heat maps similar to Fig 1. Antibody concentrations were log-transformed prior to computing z-scores. (B) Plasma concentrations of total antibody isotypes and IgG subclasses and (C) select antigen-specific antibodies. Each symbol represents an individual subject, the red lines indicate mean.

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Altered ceramide profile in IDUs

Ceramides are bioactive lipids present in the circulation as components of lipoprotein complexes and exosomes [47–50]. This class of sphingolipid regulates intra- and intercellular signaling associated with inflammation, cellular growth, proliferation, differentiation, senescence, and apoptosis [51]. At low levels, ceramides are important for injury-induced cytokine production and for activating protein phosphatases and kinases involved in stress-related signaling cascades [51]. However, at high levels, ceramides inhibit cell division and induce cellular dysfunction and apoptosis. Ceramide metabolism is both impacted by, and contributes to inflammation [52, 53]. We measured plasma levels of simple and complex ceramides and found considerable up-regulation of numerous ceramide species in the IDUs (Fig 5A) that included significant increases of ceramide d18:1/16:0 (p < 0.005), monohexosylceramide 18.1/16.0 (p < 0.001), dihydroceramide d18:0/22:0 (p < 0.005), and lactosylceramide d18:1/22:0 (p < 0.005) (Fig 5B) consistent with inflammation in IDUs.

Machine learning identifies major distinguishing features of IDUs

To distill the features that best distinguish IDUs from HCs, a machine learning multivariate analysis approach was utilized that incorporated all measured features including cell phenotyping, plasma antibody, plasma cytokine/chemokine/factor, and plasma ceramide profiling data. A supervised recursive feature elimination approach was utilized to identify the top ten features that when combined, classified the samples as IDU or HC with 100% accuracy using 10-fold
cross-validation (Table 3). IDUs were classified as having increased CD19+CD20+ B cells, MIP1-β, sCD40L, TGF-α, TNF-α, anti-HIV gp140-specific IgM, total IgG4, MHC d18.1/16:0, and MHC d18.1/22:0 with decreased IgD-CD27-CD19+CD20low/neg cells as compared to HCs. Principal components analysis that considered only these ten selected features completely

| Ranked using SVM weights | Feature                                      | HC (mean) | IDU (mean) | P (univariate) |
|--------------------------|----------------------------------------------|-----------|------------|----------------|
| 1                        | sCD40L (pg/ml)                               | 120.2     | 249.5      | 0.0004         |
| 2                        | IgG4 (RU)                                    | 192.7     | 308.6      | 0.0310         |
| 3                        | CD19+CD20+ (% of lymphocytes)                | 3.8       | 7.2        | 0.0036         |
| 4                        | TGF-α (pg/ml)                                | 0.8       | 1.4        | 0.0056         |
| 5                        | TNF-α (pg/ml)                                | 2.6       | 4.1        | 0.0016         |
| 6                        | MIP-1β (pg/ml)                               | 4.4       | 13.2       | 0.0330         |
| 7                        | IgD-CD27- (% of 19+20low)                    | 48.0      | 27.6       | 0.0015         |
| 8                        | MonoHex Ceramide d18.1/16:0 (ng/ml)          | 0.04      | 0.07       | <0.0001        |
| 9                        | gp140-IgM (RU)                               | 634.9     | 1055.0     | 0.0529         |
| 10                       | MonoHex Ceramide d18:1/22:0 (ng/ml)          | 0.01      | 0.012      | 0.0269         |

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segregated samples as either IDU or HC (Fig 6). These results demonstrate the breadth of systemic alterations present in IDUs, and resolve a core set of features that may be of value to monitor in future investigations of IDUs.

**Discussion**

Our comprehensive humoral-centric immunological profiling study revealed dramatic dysregulation in active heroin IDUs. This was apparent at the gross level as increased total B cells and total plasma IgM in the IDUs, and finer analysis revealed altered B cell phenotypes; most prominent within the minor CD19+CD20low/neg IgD−CD27−, MIP-1β, gp140 IgM, IgG4, CD19+CD20+, sCD40L, TGF-α, MH: d18:1/16:0, TNF-α and MH: d18:1/22:0. PCA was computed on per-feature z-scores (based on all 38 samples) of log transformed data (except for B cell subset frequencies which were not log-transformed).

Increased inflammation in IDUs could be a consequence of numerous factors. Opioids have primarily been associated with decreased immune activation in studies of acute treatment and cessation of use, however increased plasma IL-1β, IL-6, IL-8, IL-10, and TNF-α were observed in heroin addicts undergoing methadone treatment as compared to healthy controls [16]. It has been postulated by others that in the setting of chronic opioid use, lymphocytes and other cell types may no longer be sensitive to opioids or perhaps could be hyper-responsive to...
opioids as a result of altered mu receptor expression and signaling [54, 55]. The majority (89%) of our cohort of IDUs reported frequent cocaine use, which has been associated with increased immune activation and inflammation in several studies [56–59]. The concomitant use of both heroin and cocaine among IDUs, drugs that have been reported to have grossly opposite immunological impacts, highlights the necessity of directly assessing this complex population. In studies by Tomescu et al. [18, 60], increased inflammation, including NK cell activation, plasma IP-10, and dendritic cell maturation were reported in IDUs, although mostly confined to needle-sharing IDUs and not significantly elevated in non-sharing IDUs, suggesting that inflammation may be driven by factors associated with the sharing of needles, such as increased exposure to blood-borne pathogens or allo-antigens. Our findings of increased inflammation in non-needle sharing IDUs do not necessarily contradict those of Tomescu et al. Perceived differences may be a result of our larger sample size and non-identical outcome measures, and together suggest that the immune dysregulation, such as increased total B cells and skewed plasma IgG subclasses we observed, may be further exacerbated in needle-sharing IDUs.

The detection of LPS in the plasma of several IDUs suggests increased bacterial exposure. This is consistent with increased bacterial infections, including sepsis, in IDUs [45], and may contribute to TLR-driven inflammation. Increased plasma LPS is commonly observed in HIV-infected patients and high-risk subjects, and has frequently been associated with, and suggested to contribute to increased systemic inflammation [61–65]. Plasma LPS in HIV-infected patients is suggested to be a consequence of microbial translocation, and is further elevated in HIV+ IDUs compared to HIV+ non-IDUs [46]. Thus, we speculate that increased LPS in our IDUs cohort may be the consequence of both exogenous bacterial exposure and microbial translocation.

Recent advances in lipidomics have begun to reveal disease, aging, and infection associated alterations in ceramides. Increased plasma ceramides have been associated with coronary artery disease, alcohol abuse, chronic kidney disease, diabetes, HIV-Associated Neurocognitive Disease, Alzheimer’s disease Parkinson’s disease, and depression [32, 66–73]. Elevated plasma ceramide in IDUs highlights potential intersections of injection drug use with declines in physiological and cognitive function [4, 74–80], although these associations were not directly tested in this study. Likewise, it is unclear if ceramides can directly impact the function of B cells, and these observations warrant further investigation. Interestingly, ceramides can facilitate viral entry, including HIV attachment, and the particular ceramide composition of virions generated by budding through the cell membrane can impact viral function [81], suggesting that up-regulation of ceramides may facilitate viral infections in IDUs.

The up-regulation of inflammatory mediators in IDUs likely act directly on their B cells; for example sCD40L and TNF-α may drive B cell expansion, perhaps in a polyclonal fashion [82], but may also exacerbate responses to insulting antigens (e.g. drug contaminants, allo-antigens, microbes) contributing to IgG3 and IgG4 skewing. This process may be amplified by indirect actions of TNF-α and IL-8 on accessory cells that shape the B cell response, such as T cells, antigen presenting cells, and neutrophils. Despite the remarkable 2-fold expansion of CD19+CD20+ B cells in IDUs, substantial phenotypic differences were not apparent, with the exception of increased IgD+CD183(CXCR3)+ in IDUs. CXCR3 expression on B cells has been suggested to indicate migration to sites of inflammation [39, 40], and increased CXCR3+ B cells have been observed in patients with chronic inflammation, including those with rheumatoid arthritis and systemic lupus erythematosus [41], indicating their increase in IDUs is consistent with an increased inflammatory state. The CD19+CD20low/neg compartment appears to be most sensitive to the increased inflammatory state of the IDUs, perhaps in-part driven by its relative small abundance (~0.5% of PBMC). This B cell compartment and its subsets including the CD19+CD20low/negCD27+ cells which are increased in IDUs, should be evaluated further.
for antibody secretion to determine if they are functionally pre-plasmablasts that have been described previously in other contexts [37, 75, 83, 84]. Additionally, immunoglobulin repertoire analysis may reveal the extent to which B cell alterations in IDUs are a function of antigen-driven or antigen-independent (e.g. TLR) expansion. The limited scope of our study did not directly address whether injection drug use alters the functions of B cells, such as their ability to proliferate, differentiate, or produce cytokines in response to stimulation. However, chronic inflammation may drive an exhausted-like immune response similar to that observed in chronic infections [85, 86], or conversely hyper-responsiveness, similar to what we observed in early-stage rheumatoid arthritis patients [28]. Both scenarios are likely to impact the characteristics and efficacy of the B cell response to pathogens and vaccines in IDUs.

HIV is a major infectious disease threat for IDUs, and the substantial B cell alterations we have observed in IDUs suggest they could have qualitatively distinct responses to HIV vaccine candidates, potentially compromising the efficacy of future HIV vaccine strategies in this population. The rigorous comparison by Yates et al. [26] of VAX003, the largest HIV vaccine efficacy trial conducted thus far in IDUs to RV144, which was conducted in predominantly low-risk subjects, may be the most compelling yet underappreciated example of how IDUs may generate qualitatively distinct HIV vaccine responses. Both trials utilized the same AIDSVAX gp120 protein immunogen, although RV144 included a canarypox prime (ALVAC) and VAX003 only repetitive AIDSVAX immunizations. Yates et al. [26] examined the V1V2-specific IgG3 response rate, which correlated with a decreased risk of infection in RV144. RV144 participants had a higher response rate than VAX003 recipients (~60% vs ~20%-40%), however, those who did respond in VAX003 had a significantly higher magnitude of response than the RV144 responders [26]. Also, >90% of VAX003 participants had a gp120-specific IgG4 response, in contrast to <20% of RV144 participants, which was suggested to be a consequence of the two additional AIDSVAX boosts that the VAX003 participants received [26]. In light of our findings of significantly higher total IgG3 and IgG4 in IDUs, another non-exclusive and complementary interpretation is that IDUs have a greater predisposition to generate vaccine-specific IgG3 and IgG4 responses, which we are actively investigating. A major detriment to exacerbated IgG3 and IgG4 responses is the short half-life of IgG3 and the inability of IgG4 to activate complement [43], which could dramatically impact the durability and functional activity of the humoral response, and may be a potential contributor to the increase in re-occurring infections observed among IDUs.

A common concern for detailed immune profiling studies on limited numbers of samples, such as ours is the extent to which false discovery or multiple outcomes can potentially accentuate biological features that are actually random and not authentic differences between groups. Our combined use of conventional univariate analysis and multivariate machine learning analysis revealed both features that have been previously reported to be impacted by injection drug use and novel features not previously identified. A follow-up study focused on these core features, that included both larger sample sizes and a longitudinal component would be ideal to thoroughly ascertain their true modulation in IDUs.

The elevated systemic inflammation in IDUs that we observed is consistent with previous studies [16, 17, 87–89], and may contribute to co-morbidities within this population. Chronic inflammation is associated with cardiovascular disease, kidney disease, accelerated aging, and neurodegenerative diseases, all of which are increased among IDUs [4, 74–79]. Although underlying HIV or Hepatitis C infections may also contribute to systemic inflammation and these co-morbidities, our results indicate that other factors may contribute. Not previously well-appreciated was the extent of humoral dysregulation among IDUs. In addition to potentially contributing to inflammation-mediated co-morbidities, the humoral alterations described here may have significant consequences for the protection of IDUs from pathogens,
including their ability to generate effective responses to vaccines. Investigations into the impact of anti-inflammatory interventions in IDUs are warranted, and may yield broad health benefits for this growing population.

Supporting Information

S1 File. SVM Features. List of features used in SVM analysis.

S2 File. Data. Contains study data.

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Author Contributions

Conceived and designed the experiments: MSP MS BC MK SBM DM NH WV MCK JJK. Performed the experiments: MSP MS BZ BC NH JJK. Analyzed the data: MSP MS BC AAK JT AFR NH JJK. Contributed reagents/materials/analysis tools: DM AAK JT CF AFR WV MCK. Wrote the paper: MSP AFR JT NH JJK.

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