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Enhanced Immune Activation Linked to Endotoxemia in HIV-1 Seronegative Men who have Sex with Men

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Summary

This study assessed cellular and soluble markers of immune activation in HIV-1-seronegative men who have sex with men (MSM). MSM immune profiles were characterized by increased expression of CD57 on T cells and endotoxemia. Endotoxin presence was linked to recent high-risk exposure and associated with elevated cytokine levels and decreased CD4/CD8 T cell ratios. Taken together, these data show elevated levels of inflammation linked to periods of endotoxemia resulting in a significantly different immune phenotype in a subset of MSM at high risk of HIV-1 acquisition.

MSM represent approximately 2% of the US population, but account for approximately 63% of all new HIV-1 infections in the US [1]. Risk factors pertaining to MSM include anal intercourse [2], differences in transmission route and kinetics of viral dissemination [3], and inadequate access to preventative health care services [4-7]. Thus, MSM remain among the populations most highly affected by HIV-1 in both resource–rich and –poor settings [8].We recruited a cohort of MSM at high risk of HIV-1 acquisition to evaluate potential markers of immune activation and identify a potential high-risk immune profile in comparison to men at low risk of HIV-1 acquisition.

35 HIV-1 negative self-identified ‘high-risk’ MSM and 34 age- and ethnicity-matched self-identified ‘healthy, low-risk’ male control subjects were recruited at Fenway Health, Massachusetts General Hospital and Brigham and Women's Hospital under IRB-approved protocols and included in this study. There was no statistically significant difference in age

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(unpaired two-tailed t-test: p=0.1) or distribution of ethnicities (Chi-square test for trend: p=0.3) between the two groups. Peripheral blood samples were collected and PBMC isolated by density gradient centrifugation [9]. Plasma was analyzed for presence of LPS by LAL assay (Thermo Scientific, Pittsburgh, PA) and cytokine concentrations by 29-multiplex assay (HCYTMAG-60K-PX29, Millipore, Chicago, IL, USA). Plasma LPS was detected in 5/34 controls and 25/35 MSM (p<0.0001, Fisher's exact test) and levels of plasma endotoxin were significantly higher in MSM compared to controls (Figure 1A), but did not associate with history of STIs (p=0.9). Furthermore, levels of plasma LPS were inversely correlated with time since most recent sexual encounter without condom prior to date of blood draw (Figure 1B) and presence of LPS in plasma was associated with increased levels of plasma TNFα, Eotaxin, IP-10 and MCP-1 (Figure 1C). No other analytes differed significantly between LPS negative and positive MSM. Cells were stained with Fixable Live/Dead Stain, anti-CD4 (Invitrogen, Eugene, OR, USA), anti-CD3 (BioLegend, San Diego, CA, USA), anti-CD8, anti-CD38, anti-HLA-DR, anti-CD57, anti-CD25, anti-Ki-67 and anti-CD69 (BD Biosciences, San Jose, CA) and analyzed by flow cytometry. MSM presented with a significantly lower CD4/CD8 ratio compared to controls (Figure 1D). When stratified according to LPS levels, MSM with no detectable plasma endotoxin had higher CD4/CD8 T cell ratios compared to MSM with detectable plasma endotoxin (Figure 1D). Spearman analyses showed a negative correlation between LPS levels and CD4/CD8 ratio (r=-0.5; p=0.002). Overall, these data imply an altered immune profile in MSM compared to controls as signified by a decreased baseline CD4/CD8 T lymphocyte ratio, which can be indicative of systemic immune activation [10] or immune pathology [11].

To obtain a more comprehensive understanding of the differences in immune parameters between MSM compared to controls, we combined T cell markers and cytokine responses for analysis by partial least squares discriminant analysis (PLSDA) [12]. *Ex vivo* expression of %CD38, %HLA-DR, %CD25, %CD69, %CD57, %Ki67 and plasma cytokine concentrations were included in analysis. PLSDA showed separation according to latent variable (LV) scores (calibration error average of 0.18 and cross-validation error average of 0.22), suggesting differential association of various T lymphocytes marker and cytokine combinations with respect to MSM versus controls (Figure 1E). Specifically, higher plasma concentrations of Eotaxin, MCP-1, IP-10, and elevated bulk %$CD8^+$, %$CD57^+CD4^+$ and %$CD57^+CD8^+$ T cells were observed in MSM, while elevated bulk %$CD4^+$, %$CD25^+CD8^+$, %$CD69^+CD8^+$ and %$CD38^+CD4^+$ T cells were observed in controls. Heat map clustering using the GENE-E matrix visualization and analysis tool (Broad Institute, Cambridge, MA http://www.broadinstitute.org/cancer/software/GENE-E/) resulted in the same separation of immune markers for MSM versus controls (Figure 1F). Overall, these data demonstrate differential expression of several cytokines and markers of T cell activation and senescence in high-risk MSM compared to controls, as well as translocation of immune-activating bacterial products into the blood stream associated with decreased CD4/CD8 T cell ratio and elevated plasma cytokine concentrations.

In this cohort of MSM, the presence of elevated plasma endotoxin levels was associated with recent high-risk sexual encounter, decreased CD4/CD8 T cell ratio and increased plasma cytokine concentrations. We observed no difference in bulk %$CD3^+$ T cell

populations between MSM and controls, and others have shown that MSM and heterosexual men do not differ in their absolute CD4 counts [13]. The observed differences in CD4/CD8 T cell ratios, as well as elevated plasma cytokine levels and percentages of $CD57^+CD4^+$ and $CD57^+CD8^+$ T cell subsets in MSM might therefore be a reflection of immune activation following episodes of intermittent endotoxemia. The latter is supported by data from animal studies [14] and might contribute to alterations in immune homeostasis [15-17]. MSM furthermore presented with decreased percentages of $CD38^+CD4^+$ T cells, a phenotype linked to increased proliferative capacity but impaired cytokine production upon stimulation [18].

Several lines of evidence in HIV-1-exposed women suggest that enhanced levels of immune activation might lead to an increased risk of HIV-1 infection [19], and also be associated with faster disease progression should infection occur. Increased levels of TNFα can increase the risk of HIV-1 acquisition in women [20], and immune activation set point during early HIV-1 infection was shown to predict subsequent $CD4^+$ T cell levels independently of viral load [21]. In conclusion, our study identified a subset of MSM with plasma endotoxemia and an immune profile suggestive of chronic immune activation and immune exhaustion. These immunological changes should be taken into account in future vaccination or prevention studies as increased inflammation can represent a risk factor for HIV-1 acquisition, as recently shown for seronegative exposed women [20].

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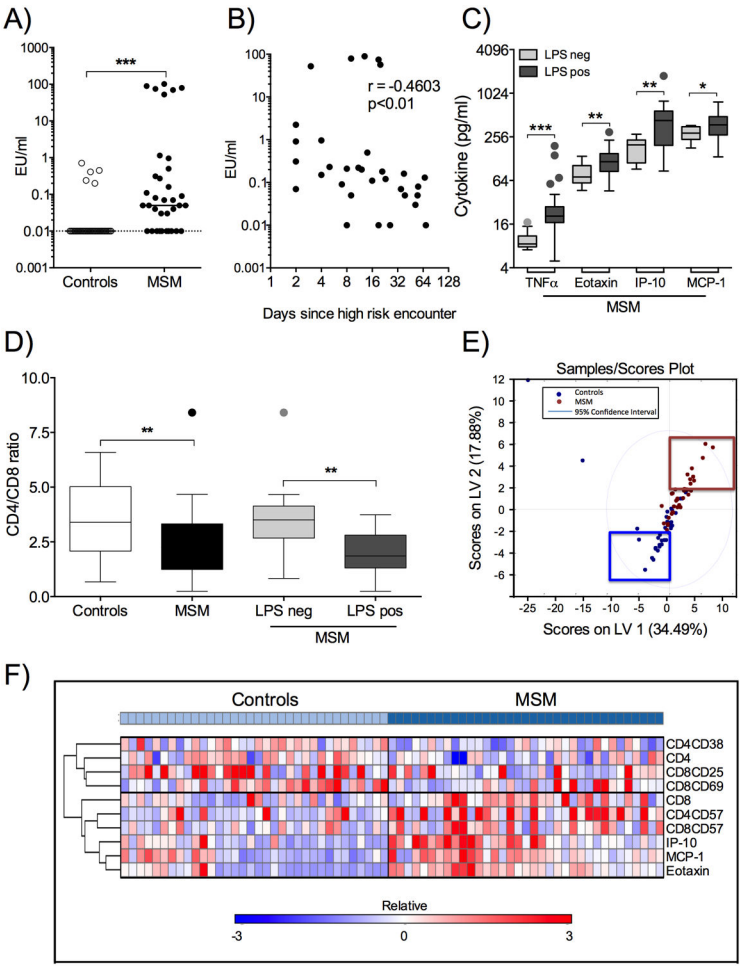

Figure 1. Analysis of Immune Profiles in MSM and controls

A) Plasma samples from MSM (n=35, black circles) and controls (n=34, clear circles) were analyzed for endotoxin by LAL assay. Values are presented as endotoxin units (EU)/ml for each individual on a $\log^{10}$ scale (EU/ml range: 0-0.71 (controls); 0–101.6 (MSM)). Lowest values set for logarithmic graphing is shown by black dotted line (=0.01 EU/ml). **B)** Spearman correlation of LPS levels (EU/ml, $\log^{10}$) versus days since most recent high-risk exposure in MSM (n=32). **C)** Cytokine concentrations in MSM samples were stratified into endotoxin negative (n=10) and positive (n=25). Tukey box and whiskers plots of TNFα, Eotaxin, IP-10 and MCP-1 concentrations (pg/ml, $\log^{2}$) in endotoxin negative (detection limit, light grey boxes) and endotoxin positive (0.03-101.61 EU/ml, dark grey boxes) samples showing median, 5-95 percentiles and individual outliers. **D)** Tukey box and whiskers plots of CD4/CD8 ratios and group median for controls (n=34, white box), total MSM (n=35, black box) and MSM divided into LPS negative (n=10, light grey box) and LPS positive (n=25, dark grey box) with outliers shown. **E)** T cell marker and cytokine data were combined (n= 69) and analyzed by PLSDA comparing MSM with controls. PLSDA scores plot showing MSM (n=35, red dots) and controls (n=34, blue dots). **F)** Heat map clustering analysis of immune markers identified by PLSDA. Expression levels are displayed on a relative scale (-3 to +3) for each individual marker adjusted to group mean

and standard deviation. Statistical significance is indicated as *p<0.05, **p<0.01 and ***p<0.001.