Molecular Signatures of CD101 Variants Provide Clues to HIV-1 Susceptibility

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BACKGROUND

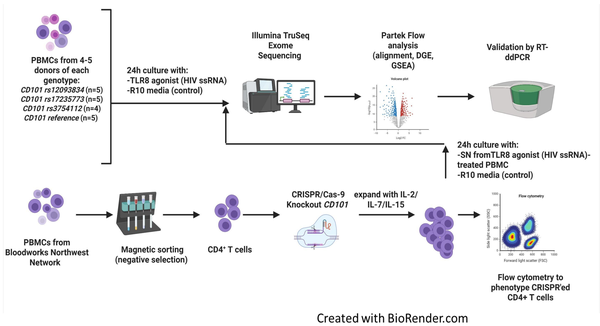
- Genetic variants in the gene ***CD101*** increase HIV risk (hazard ratio [HR]=4.3, p=1.9e^{-4})[1] while ***AXL*** variants reduce the risk (HR=0.4, p=4.7e^{-4})[2]. Nevertheless, the immunological consequences of *CD101* and *AXL* variants remain unclear at the cellular and molecular levels.
- **CD101** is a transmembrane regulator of T cell activation expressed on multiple immune cells. In T cells, it inhibits IL-2 secretion, activation, and proliferation[3].
- **AXL**, a receptor tyrosine kinase, inhibits toll-like receptor (TLR)-mediated innate responses by blocking interferon-stimulated genes (ISGs) in monocytes, resulting in inhibition of the inflammasome[4].
- We previously showed that peripheral blood mononuclear cells (PBMC) from individuals with *CD101* variant alleles exposed to a **TLR8 agonist** (HIV single-stranded RNA, HIV ssRNA) showed a larger reduction of *CD101* than in PBMC bearing reference *CD101* alleles[5].
- Our goal was to understand how *CD101* variants increase HIV-1 susceptibility compared to the reference.

METHODS (FIGURE 1)

- Using exome-targeted RNA-Seq, we evaluated transcriptomic profiles in PBMCs from individuals bearing each of the five missense variants (*rs3754112, rs17235773, rs116063197, rs12093834, rs34882009*) associated with increased HIV acquisition risk. We compared them to those from PBMCs bearing reference *CD101* alleles.
- We also used CRISPR to knock out *CD101* in primary CD4$^+$ T cells to test its impact on transcriptomics after challenge with HIV ssRNA.
- We compared: 1) Unstimulated PBMC from individuals with reference vs variant *CD101* genotypes (n=5 and 14, respectively), 2) *CD101* reference vs variant PBMC stimulated with HIV ssRNA – a TLR8 agonist, 3) primary CD4$^+$ T cells with *CD101* (*CD101*$^{+/+}$) vs with *CD101* knocked out (*CD101*$^{ko/ko}$) via CRISPR and 4) *CD101*$^{+/+}$ vs *CD101*$^{ko/ko}$ primary CD4$^+$ T cells each exposed to supernatants from HIV ssRNA-treated PBMC.
- Sequence alignment, differential gene expression (DGE), and gene set enrichment analyses were done using the Illumina Partek Flow pipeline.
- *AXL* and *CXCR4* gene expression validation was done using digital droplet PCR (ddPCR).

This study reveals that *CD101* and *AXL* are expressed in an HIV RNA-sensing inflammatory pathway with risk-associated *CD101* variants linked to both stronger *CD101* downregulation and increased *AXL* induction.

FIGURE 1. STUDY DESIGN



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RESULTS

FIGURE 2. EXPOSURE OF PBMC TO HIV ssRNA, A TLR8 AGONIST, UPREGULATES PROINFLAMMATORY GENES AND INTERFERONS

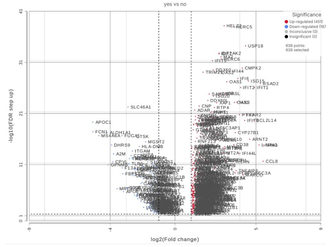


Figure 2. A. Volcano plot of differentially expressed genes (DEGs) identified following 24h of exposure of PBMCs to 5 µg/ml HIV ssRNA/LyoVec™ (FDR <0.05, |(Fold Change)| > 2). DEG analysis was done by combining the transcriptomic signatures of TLR8 agonist-treated PBMCs from 19 individuals and comparing the results to untreated PBMC controls. We identified a total of 638 DEGs. The x-axis represents the log2(Fold Change), while the y-axis represents the log10(statistical significance) for each gene. The red and blue dots denote up-regulated and down-regulated gene expression, respectively. **B. List of the 10 genes that were most significantly upregulated after TLR8 agonist activation. (by decreasing FDR).** LSMean(yes/no) is the calculated least squares mean with (yes) or without (no) HIV ssRNA.

B

		yes vs no					
Gene ID	Gene name	P-value	FDR step up	Ratio	Fold change	LSMean(yes)	LSMean(no)
ENSG00000130589	HELZ2	5.32e-43	6.79e-39	8.18	8.18	265.11	32.41
ENSG00000138646	HERC5	1.78e-42	1.14e-38	12.28	12.28	717.12	58.39
ENSG00000184979	USP18	1.38e-38	5.87e-35	20.42	20.42	166.22	8.14
ENSG00000055332	EIF2AK2	5.12e-37	1.63e-33	6.61	6.61	398.08	60.21
ENSG00000185507	IRF7	8.12e-37	2.07e-33	6.52	6.52	99.9	15.31
ENSG00000183486	MX2	3.74e-36	7.94e-33	7.16	7.16	491.79	68.68
ENSG00000138642	HERC6	1.26e-35	2.29e-32	7.25	7.25	243.37	33.57
ENSG00000152778	IFIT5	2.97e-35	4.73e-32	4.95	4.95	165.09	33.35
ENSG00000134326	CMPK2	8.75e-34	1.24e-30	17.79	17.79	100.36	5.64
ENSG00000137628	DDX60	2.34e-33	2.98e-30	5.15	5.15	549.4	106.73

FIGURE 3. *CD101* mRNA EXPRESSION WAS DOWNREGULATED FOLLOWING HIV ssRNA STIMULATION, WHEREAS THE *AXL* RESPONSE WAS VARIABLE

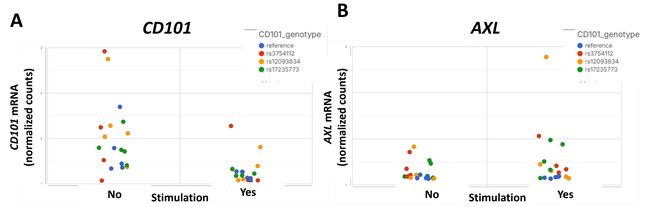


Figure 3. A. Dot plots depicting the level of expression of *CD101* (A) and *AXL* (B). TMM (trimmed mean of M-values)-normalized read counts for each gene are represented on the Y-axis. A point represents each sample; samples are separated by treatment (No=untreated control; Yes=24h HIV ssRNA stimulation) and by *CD101* genotype, identified by colors, as shown in the legend.

FIGURE 4. IN THE UNSTIMULATED CONDITION, GENES RELATED TO TASTE TRANSDUCTION WERE DOWNREGULATED IN THE *CD101* VARIANT GROUP COMPARED TO THE REFERENCE GROUP

A

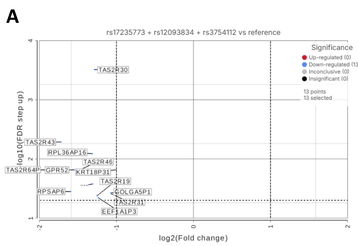
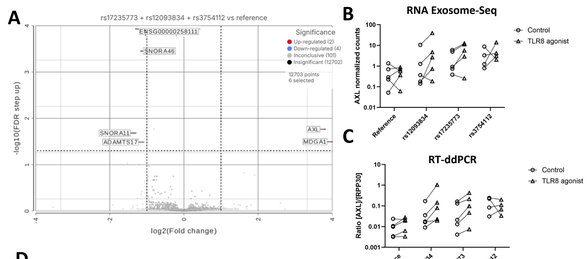


Figure 4. (A) Volcano plot of differentially expressed genes (DEGs) identified between untreated PBMC from individuals with versus without *CD101* variants (FDR <0.05, |(Fold Change)| > 2). DEG analysis was performed by combining the transcriptomic signatures of 14 individuals with *CD101* risk variants (rs17235773, n=5; rs12093834, n=5; and rs3754112, n=4) and comparing the results to those of 5 individuals bearing the *CD101* reference alleles. The x-axis represents the log2(Fold Change), while the y-axis represents the statistical significance for each gene. The blue dots denote down-regulated gene expression. **B. List of the top 13 genes that were the highest ones significantly downregulated in the *CD101* variant group compared to the reference group. (filtered by decreasing FDR).** LSMean is the least squares mean calculated by the Partek Flow package. The taste transduction pathway genes (indicated by red arrows) were overrepresented in this list. The enrichment score for this pathway (hsa04742, KEGG database) was 22.94 (P-value: 1.09e-10, FDR step up: 3.79e-8).

B

		rs17235773 + rs12093834 + rs3754112 vs reference					
Gene ID	Gene name	P-value	FDR step up	Ratio	Fold change	LSMean(rs17235773 + rs12093834...	LSMean(reference)
ENSG00000256188	TAS2R30	2.4e-8	0.00031	0.41	-2.43	7.95	19.35
ENSG00000255374	TAS2R43	8.06e-7	0.0052	0.3	-3.29	2.82	9.29
ENSG00000232228	RPL36AP16	0.0000025	0.0081	0.4	-2.49	1.9	4.72
ENSG00000249850	KRT18P31	0.0000072	0.015	0.5	-2.01	4.06	8.15
ENSG00000203737	GPR52	0.0000086	0.015	0.34	-2.92	2.08	6.09
ENSG00000256274	TAS2R64P	0.000011	0.015	0.26	-3.78	0.83	3.12
ENSG00000226761	TAS2R46	0.000012	0.015	0.36	-2.78	1.92	5.34
ENSG00000258111	---	0.0000024	0.027	0.4	-2.48	1.67	4.14
ENSG00000214629	RPSAP6	0.0000036	0.036	0.33	-3.02	1.45	4.38
ENSG00000251215	GOLGASP1	0.000041	0.037	0.48	-2.1	2.07	4.36
ENSG00000256436	TAS2R31	0.000045	0.039	0.48	-2.09	5.16	10.8
ENSG00000212124	TAS2R19	0.000055	0.042	0.42	-2.38	3.27	7.78

FIGURE 5. FOLLOWING HIV ssRNA STIMULATION, SIX GENES WERE DIFFERENTIALLY EXPRESSED BETWEEN THE *CD101* VARIANT GROUP AND THE REFERENCE



Gene set	Description	Enrichment score	P-value	FDR step up	Rich factor	Genes in set	Genes in list
path:hsa05168	Herpes simplex virus 1 infection	72.7103	2.64E-32	9.18E-30	0.853956	493	421
path:hsa05132	Salmonella infection	41.7052	7.72E-19	1.34E-16	0.868	250	217
path:hsa04141	Protein processing in endoplasmic reticulum	40.7766	1.95E-18	2.26E-16	0.911765	170	155
path:hsa05169	Epstein-Barr virus infection	37.4528	5.43E-17	4.71E-15	0.880597	201	177
path:hsa04144	Endocytosis	35.6366	3.34E-16	1.93E-14	0.849206	252	214
path:hsa05166	Human T-cell leukemia virus 1 infection	35.8006	2.83E-16	1.93E-14	0.864253	221	191
path:hsa05014	Amyotrophic lateral sclerosis	35.3556	4.42E-16	2.19E-14	0.810958	365	296
path:hsa05203	Viral carcinogenesis	34.8276	7.49E-16	3.25E-14	0.871287	202	176
path:hsa05131	Shigellosis	33.9961	1.72E-15	6.63E-14	0.846154	247	209
path:hsa04120	Ubiquitin mediated proteolysis	29.8907	1.04E-13	3.29E-12	0.894366	142	127
path:hsa03250	Viral life cycle - HIV-1	16.8186	4.96E-08	4.78E-07	0.920635	63	58

Figure 5. (A) Volcano plot of differentially expressed genes (DEGs) identified between TLR8-activated PBMC from individuals with versus without *CD101* variants (FDR <0.05, |(Fold Change)| > 2). DEG analysis was performed by combining the transcriptomic signatures of 14 individuals with *CD101* risk variants (rs17235773, n=5; rs12093834, n=5; and rs3754112, n=4) and comparing the results with those from 5 individuals bearing the *CD101* reference alleles. The x-axis represents the log2(Fold Change), while the y-axis represents the statistical significance for each gene. The red and blue dots denote up-regulated and down-regulated gene expression, respectively. **(B) AXL counts** determined by RNA-exosome Seq. Normalized values are shown on the y-axis; *CD101* genotypes are indicated on the x-axis. **(C) Validation of RNA-exosome results by RT-digital droplet PCR (RT-ddPCR).** The y-axis denotes the normalized copies of *AXL* (using *RPP30* counts as a housekeeper). **(D) Pathway enrichment analysis (KEGG datasets), filtered by decreasing FDR step up.** The pathways that were overrepresented in the variant groups are related to intracellular defense and protein folding.

ACKNOWLEDGEMENTS

This research was funded by NIH R01AI172479 (PIs: Jairam Lingappa and Florian Hladik) and the Gates Foundation (grant ID no. 47674; Partners PrEP Study). The authors want to thank Dr. Mary C. Reiger and the Genomics Core of the Institute for Stem Cell & Regenerative Medicine at the University of Washington for their support with the bioinformatics analysis.

FIGURE 6. COMPARISON OF TRANSCRIPTOMICS BETWEEN *CD101*$^{+/+}$ AND *CD101*$^{KO/KO}$ CD4$^+$ T CELLS

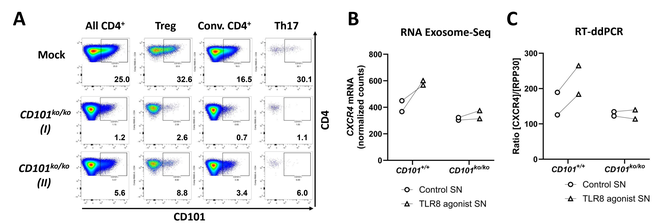


Figure 6. (A). Confirmation of *CD101* gene ablation by flow cytometry. *CD101* CRISPR-edited CD4$^+$ T cells were expanded in R10 media supplemented with IL-2, IL-7, and IL-15 and analyzed by flow cytometry. CD101 expression was measured at the cell surface along with lineage markers. Mock control was done by performing the nucleofection reaction without *CD101* sgRNAs. Two independent editing experiments ("I" and "II") were done on CD4$^+$ T cells from the same donor. "All -CD4$^{+/-}$"=CD45$^+$CD3$^+$CD4$^+$; "Conv. CD4$^{+/-}$"=CD45$^+$CD3$^+$CD4$^+$CD25$^-$CD127$^+$; "Treg"=CD45$^+$CD3$^+$CD4+CD25$^+$CD127$^-$FoxP3$^+$; "Th17"=CD45$^+$CD3$^+$CD4$^+$CD25$^-$CD127$^+$CCR6$^+$CD161$^+$ **(B) *CXCR4* counts** determined by RNA-exosome Seq. Normalized values are shown on the y-axis; *CD101* genotypes are indicated on the x-axis. **(C) Validation of RNA-exosome results by RT-digital droplet PCR (RT-ddPCR).** The Y-axis denotes normalized CXCR4 copies (using *RPP30* counts as a housekeeper).

CONCLUSIONS

- Exposure of PBMCs to HIV ssRNA resulted in a strong induction of proinflammatory and antiviral genes (Fig. 2), with concomitant downregulation of *CD101,* whereas *AXL* response was variable (Fig. 3).
- In unstimulated PBMCs, 13 genes were downregulated in the *CD101* variant compared to the reference group, with an over-representation of genes related to taste transduction (Fig. 4)
- Following TLR8 agonist stimulation, 6 genes (*AXL, SNORA46, SNORA11, MDGA1, ADAMTS17, and ATP5G1*; Fig. 5) were differentially expressed between variant and reference. Remarkably, *AXL* was upregulated 14-fold in PBMCs with *CD101* variants relative to the reference (FDR=0.02).
- Comparison of reference vs variant cells in both the stimulated and unstimulated conditions revealed overrepresentation of pathways related to protein processing and intracellular defense (Fig.5).
- Comparing *CD101*$^{+/+}$ to *CD101*$^{ko/ko}$ CD4$^+$ cells resulted in no DEGs. However, when the *CD101*$^{ko/ko}$ and *CD101*$^{+/+}$ cells were exposed to supernatants from the stimulated PBMC, *CXCR4* was down-regulated in the *CD101*$^{ko/ko}$ cells (1.7-fold change, p= 1.67E-6, Fig.6).

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PLAIN LANGUAGE SUMMARY

People who carry certain natural changes in the immune gene *CD101* show altered activity of other immune genes, including higher levels of the interferon response repressor *AXL*, which together may make it easier for HIV to establish infection. By removing the function of *CD101* in immune cells, we also observed changes in the HIV coreceptor *CXCR4*, suggesting that *CD101* reshapes inflammatory pathways in ways that can influence a person's vulnerability to HIV.