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Original Paper

Single-Cell Analysis Reveals That CD47 mRNA Expression Correlates with Immune Cell Activation, Antiviral ISGs and Cytotoxicity

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Key Words
Cd47 • Immune cells • Transcriptomic • Infection • Antiviral • Cytotoxic

Abstract
Background/Aims: Immune cells are reported to upregulate CD47 during infection, however, the role of CD47 in innate and adaptive immune cells remains unclear. ***Methods:*** To bridge this knowledge gap, we analysed our single cell (sc)-RNA dataset along with other publicly available sc-RNA datasets from healthy controls, people with HIV-1 (PWH) and COVID-19 patients. We characterized each immune cell based on low, intermediate, and high expression of *CD47*. ***Results:*** Our analyses revealed that *CD47*^{high} pDCs and monocytes exhibited relatively higher expression of IFN-α regulatory genes, antiviral interferon-stimulated genes (ISGs) and MHC-I associated genes compared to *CD47*$^{inter.}$ and *CD47*^{low} cells. Furthermore, *CD47*^{high} NK and CD8+ T cells showed higher expression of antiviral ISGs, as well as genes encoding for cytotoxic markers like granzyme B, perforin, granulysin, interferon gamma and NKG7. Additionally, *CD47*^{high} CD8+ T cells expressed higher levels of PD-1 and LAG-3 genes. Lastly, we found that *CD47*^{high} B cells had enriched expression of genes involved in cell activation and humoral responses. ***Conclusion:*** Overall, our analyses revealed that innate and adaptive immune cells expressing elevated activation and functional gene signatures also express higher *CD47* levels.

Introduction

Integrin associated protein (CD47) is a widely and lowly expressed glycoprotein on the surface of all healthy cells [1-4]. CD47 is the ligand for signal regulatory protein alpha (SIRPα),

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an inhibitory receptor predominantly expressed in macrophages and myeloid dendritic cells. Acting as a self-marker to maintain homeostasis and ensure normal cell survival, the engagement of CD47 with SIRPα induces an anti-phagocytic *"Don't Eat-Me"* signal [2, 5-7]. CD47/SIRPα binding results in the phosphorylation of immune-receptor tyrosine-based inhibitory motifs (ITIMs) within the cytoplasmic tail of SIRPα. Such phosphorylation leads to the recruitment and activation of inhibitory molecules such as Src homology 2 (SH2) domain- containing phosphatases (SHP-1 and SHP-2), which in turn regulate downstream signalling pathways, thus, resulting in inhibition of phagocytosis [8-11]. Another reported ligand of CD47 is thrombospondin-1 (TSP-1) [12]. The interaction between CD47 and TSP-1 reduces inflammation [13], limits primary CD8+ T cells antigen mediated activation and can cause differentiation of T cells into immunosuppressive phenotype [14].

Under normal physiological conditions, haematopoietic stem cells (HSCs) and progenitors are at constant risk of being phagocytosed by macrophages. To evade this attack, HSCs and progenitors transiently upregulate CD47 prior to and during migratory phases, and the level of CD47 determine the cells probability of survival [15, 16]. A similar immune evasion mechanism has also been observed in red blood cells (RBCs). Young RBCs overexpress CD47, while aged RBCs have reduced CD47 expression [17, 18] which may contribute to the clearance of aged RBCs.

CD47 has also become a hot research topic in oncology as it has been shown that tumor cells overexpress CD47 as a means to evade macrophage-mediated phagocytosis [19]. For instance, in ovarian cancer cells, haematological malignancies and other cancer types, elevated CD47 expression efficiently promotes tumor migration and tissue invasion. High CD47 expression is significantly correlated with poor prognosis in these cancer types [20-24]. In addition, observations in many different infections have shown that CD47 is upregulated in both immune cells and virus-infected cells, mainly due to Toll-like receptor (TLR) activation and induction by cytokines such as IFN-α, CXCL10, and TNF-α. Pro-inflammatory cytokines found in the plasma of hepatitis C patients were found to upregulate CD47 on uninfected dendritic cells. Importantly, *CD47* upregulation on immune cells was not limited to virus infected cells but also occurred on surveilling immune cells in response to pathogen recognition [1, 25]. Additionally, viruses have evolved to exploit inhibitory signaling pathways to evade host immune surveillance [26]. Studies have reported that poxviruses encode expression of CD47-like protein which enhances their virulence by decreasing both macrophage and T cells activation [27, 28].

While significant progress has been made in elucidating the role of CD47 in immune regulation and disease pathogenesis, several challenges remain in the field. A comprehensive understanding of the dynamic regulation of CD47 expression and its functional consequences in diverse cell types and disease contexts is essential for the development of targeted therapeutic strategies aimed at modulating CD47 signaling pathways in cancer and infectious diseases. Moreover, the precise mechanisms underlying CD47 dysregulation during viral infections and its impact on immune cell function represent critical areas requiring further investigation. Our study aims to provide insights into the intricate interplay between CD47 expression levels and immune cell activation during viral infections, thereby advancing our understanding of CD47-mediated immune regulation and its potential implication for therapeutic interventions.

Materials and Methods

sc-RNA-seq data pre-processing and quality control

Publicly available sc-RNA seq raw data were either downloaded or sent to us upon request. Using Seurat packages (version 4.0), we performed a clean-up and quality control based on cellular expression of mitochondrial genes (cells with a percentage of mitochondrial genes >5% were discarded). To be considered for further analysis, genes had to be expressed in more than ten cells, cellular barcodes had to be associated to at least 200 genes. We next identified doublets using the DoubletFinder algorithm and removed these cell doublets from the analysis. Each singlet file was saved for further analysis.

sc-RNA-seq data integration and identification of cell clusters

Next, we integrated individual singlet dataset of healthy individuals, HIV-1 infected individuals, COVID-19 patients or healthy with HIV-1 infected for the case of figure 1. In detail, data was log1p-normalized with the SCT normalizeData method using the "SCTransform" function, and subsequently scaled by the Pearson Residuals with a scale factor of 10, 000 as default using the "ScaleData" function. The top 2000 highly variable features were selected using the "SelectIntegrationFeatures" function, followed by finding the integration anchors using the "FindIntegrationAnchors" function, performing the integration of the data using the "IntegrateData" function. Following integration, principal component analysis was performed using the "RunPCA" function with default parameters, then both t-SNE (t-Distributed Stochastic Neighbor Embedding) and UMAP (Uniform Manifold Approximation and Projection) dimensionality reduction methods were conducted based on the top 20 principal components (PCs) using the "RunTSNE" and "RunUMAP" functions, respectively. Next, we normalized each integrated dataset, Variable genes were determined using Seurat's "FindVariableGenes" function with default parameters (selection.method = "vst", nfeatures = 2000). Clusters were identified via the "FindClusters" function (with a cluster resolution of 0, 3) implemented in Seurat using principal components with a P value < 0.01. To assign cellular identity, we applied graph-based clustering and a non-linear dimension reduction using uniform manifold approximation and projection (UMAP) or tSNE for cell cluster visualization. using the "RunTSNE" and "RunUMAP" functions (reduction = "pca"). Using the differentially expressed genes for known lineage markers, we annotated cell types based on these markers: CD8 T cells (*CD3G, CD3D, CD8A, IFIT2, GZMH), CD4 T cells* (*CD3G, CD3D, CD4, IL7R, CCR7),* monocytes (*LZY, S100A8, S100A9, S100A12*), B cells (*MS4A1, CD79A, CD74*), Dendritic cells (*IL3RA, ITGAX, LST1, FCER1G, FCER3A*), platelet (*PPBP*), NK cells, (*GNLY, NKG7*), pDC (*CLEC4C, LILRA4, IL3RA*).

Division of cell type in to CD47^{low}, CD47$^{inter.}$, and CD47^{high} cells

All immune cells, monocytes, pDC, NK cells, T cells and B cells were categorized based on low, intermediate and high *CD47* expression levels. This analysis was performed by first analyzing the *CD47* expression levels using volin plot and then set the criteria for the different categories. For instance. Immune cells were categorized into *CD47*^{low}, *CD47*$^{inter.}$, and *CD47*^{high} immune cells using the "Subset" function. Example:

```
DefaultAssay(lamin_all) <- "RNA"
lamin_all$CD47exp <- count [,"CD47"]
lamin_all$CD47_level <- "NA"
lamin_all$CD47_level [which(lamin_all$CD47exp < 0.5)] <- "CD47 Low"
lamin_all$CD47_level [which((lamin_all$CD47exp > 0.5) & (lamin_all$CD47exp < 1.5))] <- "CD47 Inter."
lamin_all$CD47_level [which(lamin_all$CD47exp > 1.5)] <- "CD47 High"
table(lamin_all$CD47_level)
Idents(lamin_all) <- "CD47_level"
levels(lamin_all)
levels(lamin_all)<- c("CD47 Low", "CD47 Inter.", "CD47 High")
```

Each dataset of *CD47*^{low}, *CD47*$^{inter.}$, and *CD47*^{high} immune cells were saved for further analysis. Similar criteria were applied to divide monocytes, pDCs, NK cells, T cells and B cells.

Differentially expressed genes (DEGs)

"FindAllMarkers" function implemented in Seurat v3 was used to identify DEGs across clusters with the options "min.pct = 0.25, logfc.threshold = 0.25". Multiple test correction for P value was performed using the Bonferroni method, and 0.05 was set as a threshold to define significance. Furthermore, for analysis of different *CD47* expressing levels, average gene expression was taken at the sample-level, followed by differential gene expression analysis using DESeq2 r package across the three groups (Wald test was applied for the analysis, followed by the visualization using pheatmap R package.

Gene ontology enrichment analysis

Gene Ontology (GO) analysis was performed using the clusterProfiler 4.0 package. The GO terms of selected genes were enriched in the database "org.Hs.eg.db" using "enrichGO" function because of the lack of study in pigs. Benjamini–Hochberg (BH) method was used for the multiple test adjustment.

Quantification And Statistical Analysis

Differential expression genes (DEGs) were analyzed using three tests, Wilcoxon-ranked sum test, t-test and t-test overestimated variance. DEGs were computed using the 'FindMarker' function of Seurat and the probability values were estimated with respect to all other clusters within each dataset.

Data availability

scRNA-seq data that support the findings of this study are been deposited in the Gene Expression Omnibus under (GEO accession number GSE157829), (accession code GSE228078), (GEO accession number GSE169346) and (Array Express with accession number: E-MTAB-9544).

Original codes used to generate data of this paper are publicly available at GitHub: https://github.com/laminbcham/CD47-upregulation-on-human-immune-cells/blob/main/codes

Results

$CD47^{high}$ immune cells exhibit higher expression of immune activation and functional genes

To investigate the role of *CD47* in host immune cells, we used a publicly available sc-RNA dataset (GEO accession number GSE157829) [29]. This study reported single-cell RNA sequencing data from peripheral blood mononuclear cells (PBMCs) from four healthy individuals (37, 847 cells) and six HIV-infected donors (28, 610 cells) [29]. We processed the sc-RNA dataset and identified distinct immune cell populations (Fig. 1A). To determine whether *CD47* is upregulated at transcriptomic level during HIV-1 infection, we analysed the *CD47* expression in healthy individuals compared to PWH. We found that *CD47* expression was upregulated during HIV-1 infection (Suppl. Fig. 1A). Next, we investigated *CD47* and *SIRPα* expression and found that *CD47* is expressed in all immune cells while its receptor (SIRPα) is predominantly expressed in monocytes (Fig. 1B). Furthermore, our analysis on *CD47* expression levels in each immune cell type revealed that Natural Killers (NK) cells, and CD8+ T cells have higher *CD47* expression compared to other immune cell types (Fig. 1C).

To determine the association between *CD47* expression levels and immune cell function, we categorized all cells based on their *CD47* expression level: low (*CD47*<0.5), intermediate (*CD47* ≥0.5 & *CD47*≤1.5) and high (*CD47*>1.5) (Fig. 1D). Detailed description is provided in the methods section. Next, we determined the average gene expression profile of various factors including IFN-α regulatory genes (e.g. *STAT1/2, IRF7, IRF9, TLR7*), antiviral ISGs (e.g. *IFI6, MX1, ISG15, LYE6*), cytotoxic-related genes (e.g. *IFNG, GZMA, GZMB, NKG7,KLRB1, GNLY, PRF1)* and exhaustion-associated genes (e.g. *PDCD1, LAG-3, TIGIT*) among $CD47^{high}$, $CD47^{inter.}$ and $CD47^{low}$ immune cells. Compared to $CD47^{inter.}$ and $CD47^{low}$, $CD47^{high}$ immune cells exhibited higher expression of IFN-α regulatory genes, antiviral ISGs, cellular activation, cytotoxic, and exhaustion related genes (Figures 1E, F). Collectively, these findings suggest that upregulation of *CD47* was associated with immune activation, antiviral response, cytotoxicity and exhaustion.

CD47 upregulation on monocytes and pDCs is associated with higher expression of IFN regulatory genes and antiviral ISGs

To investigate the impact of *CD47* expression levels on monocytes, we used a publicly available sc-RNA dataset of 81, 643 antigen-presenting cells (APCs), including monocytes and dendritic cell (DC) subsets from COVID-19 patients (GEO accession number GSE169346) [30]. After processing the sc-RNA dataset, our cluster analysis revealed three main immune subsets: monocytes (CD14 and CD16 monocytes), conventional dendritic cells (cDC) (CD1c, CLEC9A and AS dendritic cells) and pDCs (Suppl. Fig. 1B). Among these APC subsets, CD14+ monocytes were found to express lower CD47 levels compared to all other APC subsets (Suppl. Fig. 1C). To examine how *CD47* expression levels influence monocyte activation and function, we categorized monocytes based on low, intermediate, and high *CD47* expression levels and analysed the hallmark gene set. We found that $CD47^{high}$ monocytes had relatively higher

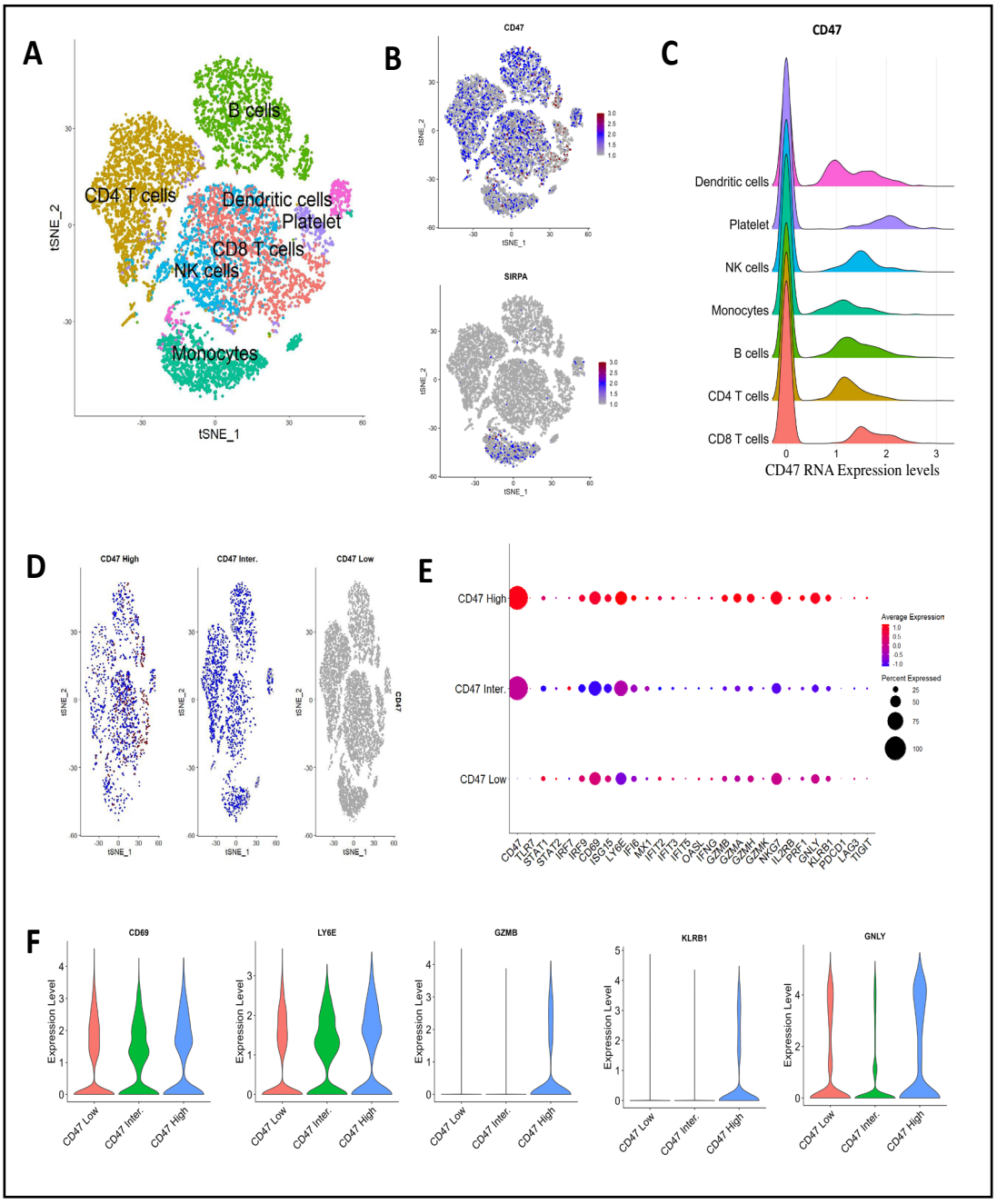

Fig. 1. CD47^{high} immune cells exhibit higher expression of immune activation and functional genes. (A) tSNE representation of immune cells (n= 43,522 cells) from healthy and PWH. (B) tSNE and feature plot of CD47 and SIRPa expression levels on immune cells. (C) Ridge plot of CD47 expression in each immune cell type. Immune cells were divided into low, intermediate, and high CD47 expression levels (D) tSNE plot representation of CD47^{low}, CD47$^{inter.}$, and CD47^{high} immune cells. (E) Dot plot representation of average and percentage expression of immune activation and functional genes and (F) Violin plot representation of CD69, LY6E, GZMB, KLRB1 and GNLY expression among CD47^{low}, CD47$^{inter.}$, and CD47^{high} immune cells.

expression of genes involved in the IFN-α response, complement response, inflammatory response, IFN-γ response, IL2_STAT5 response, and TNF-α response (Fig. 2A). Further, a differential gene expression and gene ontology analysis between *CD47*^{high} and *CD47*^{low} monocytes showed enrichment of genes involved in type I interferon production, cellular response to interferon and chemokine-mediated signalling pathways in *CD47*^{high} compared to *CD47*^{low} monocytes (Suppl. Fig. 1D, E). To further confirm the association between *CD47* upregulation and monocyte activation and function, we categorized monocytes based on low and high expression of *IRF7, IFNAR1, MX1, ISG15, CD86, IL1B, NKG7* and *TNF* genes and then analysed the *CD47* expression levels. We found that monocytes expressing high levels of these genes also upregulated their *CD47* expression (Fig. 2B), thus, indicating that highly activated and functional monocytes have elevated *CD47* expression levels.

Next, we investigated the role of *CD47* on pDCs using our sc-RNA dataset of enriched pDCs from PWH (GEO accession number GSE228078) [31]. After reprocessing the dataset, we performed cluster analysis (Suppl. Fig. 2A) and re-cluster the pDC subsets (Suppl. Fig. 2B). We found that pDC1 and cytotoxic-pDC cluster had higher expression of *CD47* (Suppl. Fig. 2C). Next, we categorized pDCs based on low, intermediate, and high *CD47* expression levels and evaluated the activation and functional gene signatures. We found that 69, 1% were classified as *CD47*^{low}, 18.3% as CD47$^{inter.}$ and 12.6% as *CD47*^{high} pDCs (Suppl. Fig. 2D). Our data showed that *CD47*^{high} and CD47$^{inter.}$ pDCs displayed higher expression of IFN-α regulatory genes compared to *CD47*^{low} pDCs. However, we observed higher expression of antiviral ISGs, and MHC-I associated genes in *CD47*^{high} pDCs compared to *CD47*^{inter} and *CD47*^{low} pDCs (Fig. 2C, D). Gene ontology analysis showed that *CD47*^{high} pDCs were enriched with gene transcripts involved in IFN production, cellular response to IFN and defense against virus (Fig. 2E). Overall, our results highlight that the upregulation of *CD47* in monocytes and pDCs is associated with enhanced innate immune activation and function.

CD47^{high} NK cells have higher expression of ISGs and cytotoxic encoding-genes

To determine the impact of CD*47* expression levels on NK cell activation and function, we interrogated NK cells from our sc-RNA dataset (Suppl. Fig. 2A) (GEO accession number GSE228078)[31]. The cluster analysis revealed four NK clusters: CD56^{dim}, CD56^{bright}, NKT and CD16^{negative} NK cells (Fig. 3A). We observed that CD56^{dim}, CD16^{neg} and NK/T cells have higher *CD47* expression levels compared to CD56^{bright} NK cells (Fig. 3B). To establish the association between *CD47* expression and NK cells activation and function, we categorized NK cells into low, intermediate and high *CD47* expression levels. Our analysis of antiviral and cytotoxic genes expression showed that *CD47*^{high} NK cells have relatively higher expression of *KLRD1, FCGR3A, MX1, ISG15, IFI6, NKG7, GNLY, PRF1, GZMB* (Fig. 3C). To further validate these findings, we categorized NK cells into low or high expression of *MX1, NKG7, PRF1, GZMB* and *GNLY,* and analysed their *CD47* expression levels. Our data revealed that NK cells expressing high levels of *NKG7 PRF1, GZMB* and *GNLY* also expressed higher levels of *CD47* (Fig. 3D). Together, our findings indicate that highly activated and cytotoxic NK cells also have elevated *CD47* expression.

CD47 upregulation on T cells is associated with increased activation, cytotoxicity and exhaustion

Similar to NK cells, we examined the impact of *CD47* expression on T cells. First, we selected the CD4+ and CD8+ T cells from our sc-RNA dataset from PWH (Suppl. Fig. 2A) (GEO accession number GSE228078)[31]. The cluster analysis identified CD4+ and CD8+ T cells subsets (Suppl. Fig. 3A and Fig. 4A). Our analysis showed that *CD47* expression was higher in cytotoxic CD4+ T cells compared to other CD4+ T cells subsets (Suppl. Fig. 3B). Similarly, effector CD8+ T cells showed elevated *CD47* and *GZMA* expression levels in comparison to other CD8+ T cells subsets (Fig. 4B). To determine whether the antiviral and cytotoxicity capabilities of CD8+ T cells is associated with *CD47* upregulation, we categorized CD8+ T cells into low, intermediate, and high *CD47* expression levels and analysed activation, cytotoxic and exhaustion encoding genes. Our data revealed that CD*47*^{high} CD8+ T cells have

relatively higher expression of *CD69, IFI6, ISG15, ISG20, TNFSF14, GZMB, NKG7, IL2RB, IFNG, PDCD1, LAG-3* etc., while *CD47*$^{inter.}$ CD8+ T cells exhibit higher expression of *CD38, OAS1* and *TNFRSF9* (Fig. 4C, D). To further confirm this association, we divided CD8+ T cells into low or high *ISG15* and *PRF1* expression and analysed *CD47* expression levels. We found that CD8+ T cells expressing high levels of *ISG15* or *PRF1* also have elevated *CD47* expression levels (Fig. 4E). Overall, our data showed that upregulation of *CD47* in T cells is associated with T cells activation, cytotoxicity, and exhaustion.

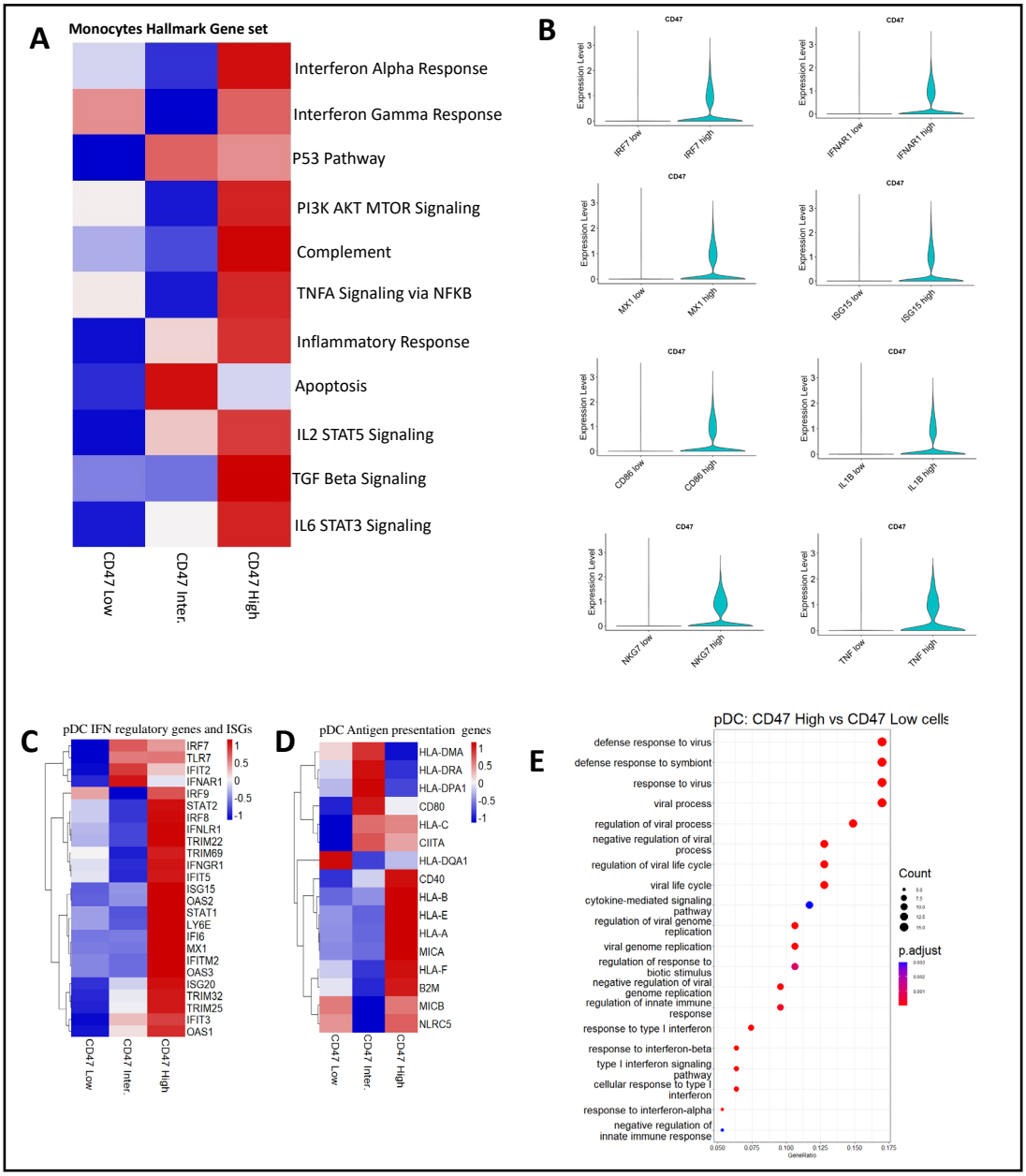

Fig. 2. CD47 upregulation on monocytes and pDCs is associated with higher expression of IFN regulatory genes and antiviral ISGs. Monocytes were divided into low, intermediate and high CD47 expression levels. (A) Heatmap illustration of relative gene expression levels of Hallmark gene set among CD47^{low}, CD47$^{inter.}$, and CD47^{high} monocytes. (B) Violin plot representation of CD47 expression levels among monocytes expressing low or high IRF7, IFNAR1, MX1, ISG15, CD86, IL1B, NKG7 and TNF. Heatmap illustration of relative gene expression levels of (C) pDCs IFN regulatory genes and ISGs and (D) pDCs antigen presentation genes among CD47^{low}, CD47$^{inter.}$, and CD47^{high} pDCs. (E) Dot plot representation of gene ontology between CD47^{low}and CD47^{high} pDCs.

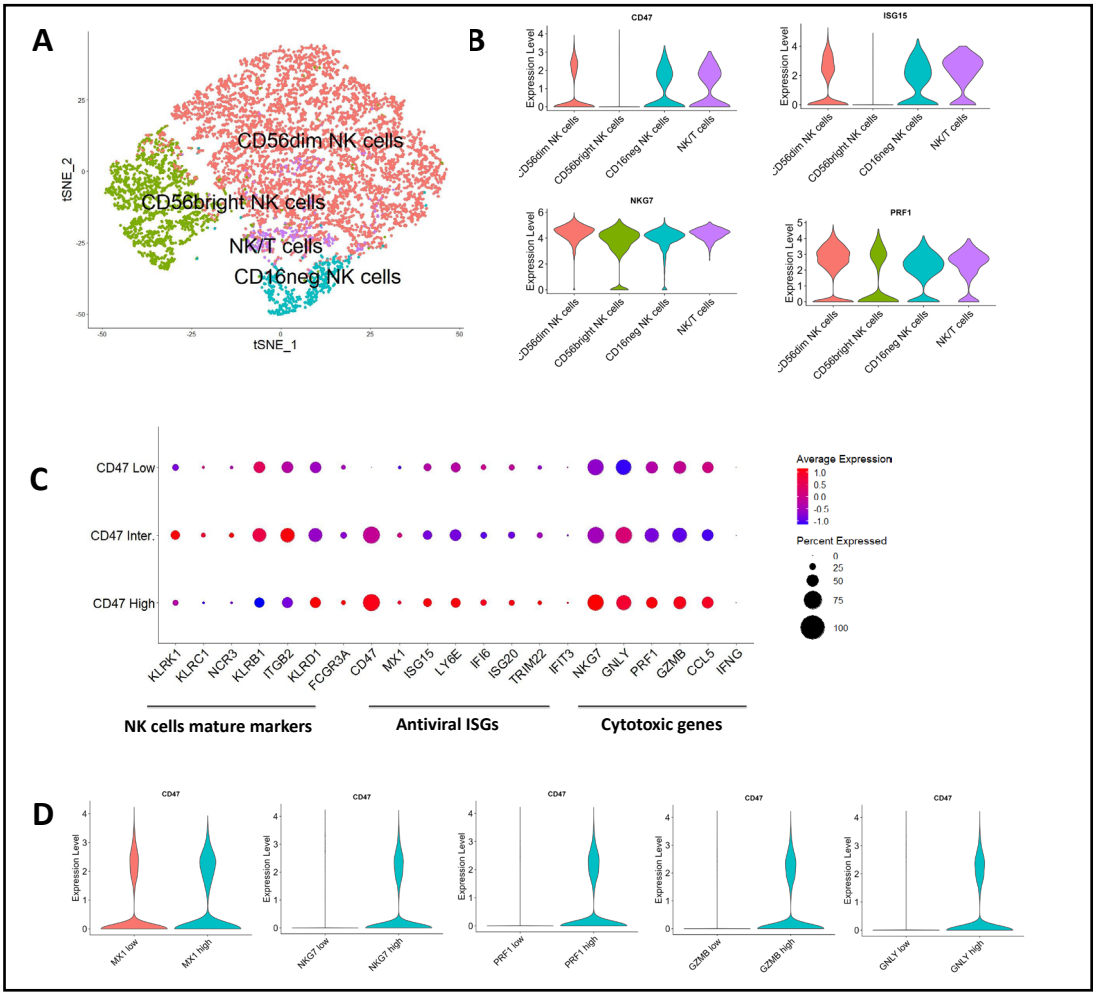

Fig. 3. CD47^{high} NK cells have higher expression of ISGs and cytotoxic encoding-genes. (A) tSNE illustration of NK cells subsets (n= 18,032 NK cells) from PWH. (B) Violin plot representation of CD47, ISG15, NKG7 and PRF1 expression among NK cells subsets. NK cells were divided into low, intermediate and high CD47 expression levels. (C) Dot plot representation of average and percentage expression of NK cell mature markers, antiviral ISGs and cytotoxic genes among NK CD47^{low}, CD47$^{inter.}$, and CD47^{high}. (F) Violin plot representation of CD47 expression levels among NK cells expressing low or high expression of MX1, NKG7, PRF1, GZMB, and GNLY genes.

B cells with elevated CD47 are enriched with genes involved in B cells activation and humoral response

To understand how *CD47* expression levels impact B cell activation and response, we used a publicly available sc-RNA dataset based on phenotypically sorted B cells (Array Express with accession number: E-MTAB-9544) [32]. This sc-RNA dataset enabled us to perform an in-depth characterization of a large number of B cells. After processing the sc-RNA dataset and performing cluster analysis, we identified five B cells subsets: naïve, transitional, double negative, classical memory and IgM memory B cells subsets (Fig. 5A). *CD47* expression was similar in all B cell subsets (Fig. 5B). Next, we categorized B cells based on low, intermediate, and high *CD47* expression levels and analysed B cells activation and functional encoding genes. These analyses showed that *CD47*^{high} B cells have relatively higher expression of *TLR9, IRF7, MZB1, ISG15, MX1, OAS1, CD40, CD80, HLA-C, IGHM*, while *CD47*^{inter} B cells displayed higher expression of *STAT1/2, IGHD, HLA-B, HLA-DMA* and *HLA-DR* compared to *CD47*^{low} B cells (Fig. 5C). Differential gene expression and gene ontology analysis showed that *CD47*^{high}

B cells were enriched for gene transcripts involved in the regulation of B cells activation, humoral response, immunoglobulin-mediated response, response to chemokine, cellular response to IFN-α and IFN-γ, antigen-mediated signalling, and programmed cell death (Fig. 5D). Overall, our data highlighted that the upregulation of *CD47* on B cells is associated with increased expression of activation and antiviral genes as well as genes involved in mediating humoral B cell response.

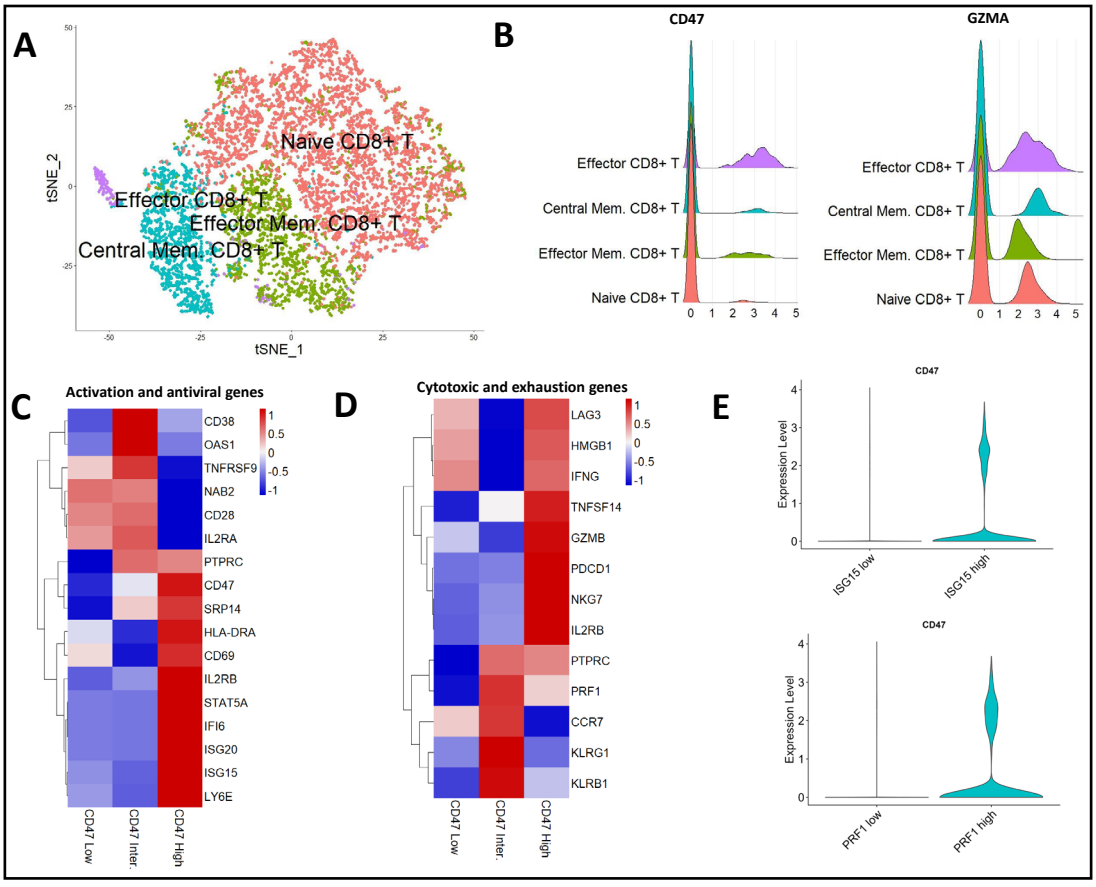

Fig. 4. CD47 upregulation on T cells is associated with increased activation, cytotoxicity and exhaustion. (A) tSNE plot illustration of CD8+ T cells subsets (n= 11,271 CD8+ T cells) from PWH. (B) Ridge plot of CD47 and GZMB expression among CD8+ T cells subsets. CD8+ T cells were divided into low, intermediate and high CD47 expression levels. Heatmap illustration of relative gene expression levels of CD8+ T cells (C) activation genes and ISGs and (D) cytotoxic and exhaustion genes among $CD47^{low}$, $CD47^{inter.}$, and $CD47^{high}$ CD8+ T cells. (F) Violin plot representation of CD47 expression levels among CD8+ T cells expressing low or high expression of ISG15, and PRF1 genes.

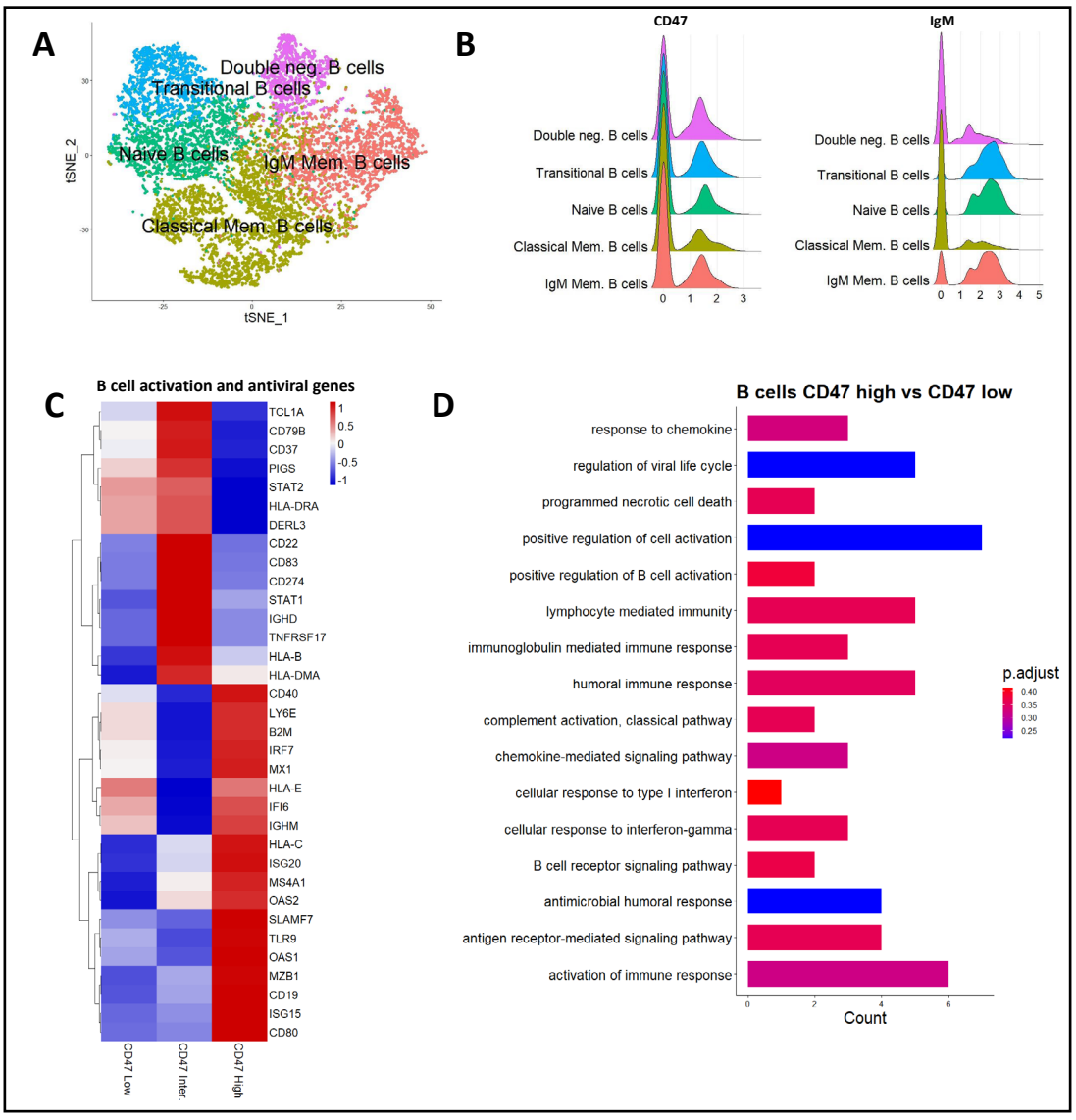

Fig. 5. B cells with elevated CD47 are enriched with genes involved in B cells activation and humoral response: (A) tSNE plot illustration of B cells subsets (n= 41,085 B cells) from healthy individuals. (B) Ridge plot of CD47 and IgM expression among B cells subsets. B cells were divided into low, intermediate and high CD47 expression levels. (C) Heatmap illustration of relative gene expression levels of B cells activation genes, ISGs and functional genes among $CD47^{low}$, $CD47^{inter.}$, and $CD47^{high}$ B cells. (D) Bar plot representation of gene ontology between $CD47^{low}$and $CD47^{high}$ B cells.

Discussion

Previous studies have demonstrated that immune cells and virus-infected cells upregulate CD47 during infection [25, 33]. In light of this, we investigated the association between *CD47* upregulation and immune cell activation and function using sc-RNA dataset from healthy controls, PWH and COVID-19 patients. Our comprehensive analysis revealed that *CD47* upregulation in monocytes and pDCs is associated with relatively higher expression of genes involved in IFN-α production, cellular response to IFN-α, as well as increased antigen presentation. Similarly, NK cell and T cells expressing high levels of *CD47* also exhibit high expression of genes encoding for cellular activation, antiviral ISGs, cytotoxicity, and exhaustion. Additionally, the upregulation of *CD47* on B cells is also linked to enhanced B cells activation and humoral response.

CD47 has previously been defined as an interferon-stimulated gene, and its upregulation is part of a coordinated program of host defence mechanisms triggered by IFN-α production [25, 34-36]. Our findings align with these studies, demonstrating that the upregulation of *CD47* in monocytes, pDCs, NK cells, T cells and B cells is associated with relatively higher expression of antiviral ISGs. Moreover, our study findings shed new light that upregulation of *CD47* on host immune cells is an indicating marker of activation, function and possibly exhaustion. Our data reveals that *CD47* upregulation is not just limited to IFN-α mediated ISGs induction but also highlights the upregulation of *CD47* in IFN-α producing cells. Monocytes and pDCs with intermediate or high expression of *CD47* have relatively higher expression of genes involve in type I interferon production, chemokine mediated signaling, complement activation and cellular response to other cytokines such as IFN-α and TNF-α. These findings suggest transient upregulation of CD47 upon TLR activation, consistent with previous report [25]. Similarly, NK cell and CD8+ T cells with intermediate or high *CD47* levels also express higher levels of *GZMB, IFNG, PRF1* and *NKG7*, clearly indicating that highly cytotoxic NK cells and T cells upregulate *CD47*. Further aligning with reports that *CD47* upregulation can also be induce by IFN-α and other unknown mechanisms [25]. Although PD-1 expression is linked to both T cell exhaustion as well activation [37, 38], our study reveals that $CD47^{high}$ CD8+ T cells express higher levels of *PDCD1* and *LAG-3* genes. In line with its effects on other immune cells, *CD47* upregulation on B cells is linked with enhanced B cells activation and humoral immune response. Therefore, suggesting a direct correlation between *CD47* expression levels and immune activation and function.

The question remains: why is *CD47* upregulated in highly activated and functional immune cells? Given the well-known anti-phagocytic protective function of CD47, it is reasonable to speculate that highly activated and functional immune cells increase CD47 expression as a defensive mechanism against phagocytosis. A recent study reported that *CD47* transcriptional interference by HIV-1 Viral protein U (Vpu) might promote the susceptibility of macrophages to viral infection via phagocytosis of infected $CD4^+$ T cells. The study highlighted that Vpu downregulates CD47 expression on infected $CD4^+$ T cells, leading to enhanced capture and phagocytosis by macrophages [39]. These findings support our hypothesis that activated and functional host immune cells upregulate *CD47* for protection. In addition, it has been reported that CD47-negative CAR-T cells fail to expand and persist *in vivo* due to continuous macrophage mediated phagocytosis, underscoring the importance of CD47 expression for CAR-T cell survival [40-42]. However, additional experiments are needed to elucidate how CD47 expression on each immune cell protects them from macrophage mediated phagocytosis.

Conclusion

In summary, our transcriptomic analysis of immune cells suggests a potential association between CD47 upregulation and cellular activation, antiviral ISGs, and cytotoxicity. Modulating *CD47* expression levels may offer a novel approach for host-directed therapies, such as downregulating CD47 in HIV-1 infected CD4+ T cells as a part of the ''shock and kill'' strategy or increasing CD47 expression on CAR-T cells therapy.

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Author Contributions

Conceptualization: L.B.C, and., O.S.S.; methodology, L.B.C., M.R.U., L.L., M.T. and O.S.S.; investigation, L.B.C., M.R.U., L.L., M.T. and O.S.S.; verification, L.B.C., L.L., M.T. and O.S.S.; formal analysis, L.B.C., L.L., and O.S.S.; writing—original draft, L.B.C., and O.S.S.; writing—review and editing, L.B.C., M.R.U., L.L., M.T. and O.S.S.; visualization, L.B.C. and O.S.S.; resources, O.S.S.; supervision, L.L. and O.S.S.; project administration, L.B.C. and O.S.S.; funding acquisition, O.S.S. All authors have read and agreed to the published version of the manuscript.

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