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Case Report

Elevated Notch ligands in serum are associated with HIV/TB coinfection

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ABSTRACT

Objective: There is a clear need for improved biomarkers to diagnose HIV/TB coinfection. Although numerous tests can identify the existence of both of these microbes within the host, a parallel assessment of the host response to HIV/TB coinfection may prove as useful confirmation in cases where microbiological tests are inconclusive. To this end we assessed the levels of Notch ligands found in serum samples of patients with TB, HIV or HIV/TB coinfection. The Notch system is involved in almost every stage of development, including the maturation of the immune response. Upon exposure to a pathogen, the innate immune system will increase expression of Notch ligands Delta-like 1 and Delta-like 4. Previous research has demonstrated that Notch ligand expression is increased on monocytes from patients diagnosed with tuberculosis. We hypothesized that if Notch ligands were present in the peripheral blood of individuals diagnosed with TB, they may serve as a novel marker for infection.

Design: Serum samples from patients with HIV, TB or HIV/TB coinfection were compared to serum from uninfected individuals to determine levels of DLL1 and DLL4 in a case controlled study.

Methods: DLL1 and DLL4 were measured by ELISA. Linear regression with post tests were used to determine if levels of DLL1 and DLL4 were increased in individuals with HIV/TB coinfection as compared to individuals infected with either HIV or TB or healthy controls.

Results: Delta-like 1 and Delta-like 4 were significantly increased in the serum of patients with HIV and HIV/ M. tuberculosis coinfection compared to other groups.

Conclusions: Assessment of Notch ligands in peripheral blood may enhance the diagnosis of individuals with active TB that are co-infected with HIV. The study will also need to be validated in in a larger cohort.

1. Introduction

The mammalian Notch system consists of 4 ligands and 5 receptors that dynamically interact to regulate cell function[1]. Notch ligands, expressed on many cell types throughout the course of development, transmit signals to nearby cells expressing Notch receptors that result in cellular differentiation and the expression of specific gene programs [2,3]. Within the hematopoietic system, Notch ligand expression is used to govern T cell education and differentiation in lymphocyte precursors that enter the thymus[1,4]. Mature T cells also encounter Notch ligands in the periphery on antigen-presenting cells (APCs) and stromal cells [5,6]. The Notch/Notch ligand interactions that occur between T cells and APCs are known to influence T cell survival, proliferation and cytokine secretion in viral infections, autoimmunity and chronic disease states[7–10].

The expression of Notch ligands on APCs is in part affected by host pathogen interactions. For example, the ligand DLL4 is upregulated as a result of MyD88 signaling in dendritic cells [11,12], and the ligand DLL1 is expressed on the surface of macrophages in response to high levels of type 1 interferon (T1IFN)[13]. As numerous studies have demonstrated that active TB carries a T1IFN gene signature[14–16], we sought to test for the presence of DLL1 and DLL4 in both latent and active TB. Although many diagnostics exist for TB, a rapid, inexpensive, easy-to-use, sensitive and specific diagnostic that works in low-resource

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settings remains elusive. We hypothesized that the presence of Notch ligands in peripheral blood of individuals with TB infection may contribute to the development of such a diagnostic. Although our hypothesis was incorrect, we observed that the measurement of Notch ligands may have some utility in the diagnosis of HIV/TB coinfection.

1.1. Materials and Methods

1.1.1. Participants

De-identified serum samples were obtained from the FIND diagnostics serum bank with the primary goal of determining if soluble Notch ligands were present in patients infected with tuberculosis. The research was classified as not regulated under University of Michigan IRB HUM00100328 and conforms to all guidelines set forth by this board. A summary of the covariables of the 78 samples is described in Table 1. All samples were from a cohort developed by FIND in South Africa (including 8 HIV+/SS- and 10 HIV+/SS+ individuals) or Viet Nam (30 HIV-/SS+ and 30 HIV-/SS-). Active pulmonary TB infection was determined by a sputum smear with acid-fast bacteria and confirmed by bacterial culture and HIV was diagnosed by PCR. No cases of extrapulmonary TB were used in this study.

2. ELISA

DLL1 was measured using a Quantikine ELISA kit from R&D (#DLL10) following manufacturer's instructions. DLL4 was measured using a matched antibody pair from Sino Biologicals (SEK10171) using tetramethylbenzidine (TMB) substrate (Millipore Sigma) for detection as recommended by the manufacturer and a standard laboratory protocol [17].

Both DLL4 and DLL1 antigens were detected when following these protocols. The blank-corrected absorbances of the standard curve measurements indicated that both DLL1 and DLL4 had a comparable sensitivity. We detected a minimal amount DLL4 in SS-/HIV- individuals (mean 112 pg/mL ± 140.7), whereas DLL1 was present at a higher level in the same group (mean 1743 pg/mL ± 398 in SS-/HIV-) (Fig. 1). The antibody pair used in the DLL4 assay did not detect DLL1, and the antibody pair used in the DLL1 assay did not detect DLL4, thus eliminating cross-reactivity as a source of signal in our assays.

3. Statistical methods

Linear regression was performed using SPSS (version 21) and used to determine overall significance. Receiver operator curves were generated in GraphPad Prism.

Table 1
Study patient characteristics.

	HIV+/SS+n = 10	HIV+/SS−n = 8	HIV-/SS+n = 30	HIV-/SS−n = 30	p-value
Age (years)	37 ± 8	33 ± 5	34 ± 12	60 ± 17	p < 0.0001
Sex (male)	8 (80%)	6 (75%)	19 (63%)	24 (80%)	p = 0.48
Former or current smoker	1 (10%)	6 (75%)	12 (40%)	23 (77%)	p = 0.0005
BCG vaccination	7 (70%)	7 (88%)	16 (53%)	1 (3%)	p < 0.0001
Previously received medication to treat TB	7 (70%)	3 (38%)	8 (27%)	24 (80%)	p = 0.0003
Country of origin	South Africa	South Africa	Viet Nam	Viet Nam	

3.1. Results

We measured both DLL1 and DLL4 in the serum of patients from South Africa and Viet Nam, as detailed in Table 1. The cohort included 18 individuals from South Africa infected with HIV, of which 8 had a confirmed case of TB (HIV+/SS+) and 10 were negative for TB (HIV+/SS-). The remainder of the samples consisted of 60 HIV- individuals, of which 30 were diagnosed with TB (HIV-/SS+) and 30 were negative for TB (HIV-/SS-). Of the 30 individuals that were negative for TB by sputum smear, 10 had a history of previous TB infection. Active infection with *M. tuberculosis* was determined by consecutive sputum smears and confirmed by both chest x-ray and mycobacterial culture in sputum smear positive (SS+) individuals. HIV status was determined by a standard PCR test for all individuals.

We observed that the levels of DLL1 and DLL4 were upregulated in the serum of patients that were diagnosed with HIV or HIV/TB co-infection (Fig. 1 A-B). Linear regression, using DLL1 or DLL4 as dependent variables and the type of infection (HIV, TB, or HIV/TB) as the independent variables indicated HIV infection accounted for 61% ($r^2 = 0.617$) of the variation in DLL1 levels and 83% (and $r^2 = 0.836$) of the variation in DLL4 levels compared to subjects not infected with HIV (Fig. 1 A-B). We observed the highest levels of Notch ligands in the serum of individuals with HIV/TB coinfection. ANOVA analysis indicates that both DLL1 and DLL4 levels were significantly increased in the serum of HIV+/SS+ individuals compared individuals in the HIV+/SS-, HIV-/SS+ or HIV-/SS- groups. To test the sensitivity and specificity of Notch ligands as a diagnostic for HIV/TB coinfection we generated receiver operator curves for each ligand (Fig. 1 C-F). Both ligands were sensitive and specific for HIV/TB coinfection when compared to individuals HIV+/SS- or HIV-/SS+ or uninfected individuals, with an area under the ROC of 0.96 for DLL1 and 0.97 for DLL4. We then generated ROC curves using only the HIV+/SS- individuals in the as the control population. In this scenario, the area under the ROC was 0.73 for DLL1 and 0.86 for DLL4. Receiver operator curve analysis for HIV/TB co-infection suggest that Notch ligands DLL1 and DLL4 are a sensitive and specific assay to distinguish individuals with HIV/TB co-infection in this pool of samples (Fig. 1 C-F).

3.2. Discussion

In the past decade, the number of HIV- TB cases has declined while the number of individuals with HIV/TB coinfection has remained consistent [18]. This persistence is in part due to difficulty diagnosing TB infection in people living with HIV (PLHIV), which often present as negative for TB by both the sputum smear and interferon gamma release assay test [19]. The most accurate test for detection of pulmonary TB infections in PLHIV is the Xpert MTB/RIF assay, with a sensitivity of 80% [20]. However, the Xpert test has variable sensitivity for the diagnosis of extrapulmonary TB [21] and may not be an effective tool for the diagnosis of extrapulmonary infection PLHIV. Detection of lipoarabinomannan (LAM) in urine comprises a second test that is commonly used to diagnose TB in PLHIV [22]. Lateral flow LAM tests can function as a point-of-care test and have the advantage of being inexpensive and easy to use. The tests also have a good specificity, ranging from 90 to 98 percent depending on the clinical setting. However, the sensitivity of the assay in inversely correlated with CD4+ T cell counts, with the highest sensitivity being in those individuals with a $CD4^+$ T cell count of less that 100 [23]. In this context the use of a blood-based marker to determine TB infection in an HIV+ individual may fill a niche in the suite of diagnostics needed to control TB, especially if it is independent of CD4 + T cell counts ($CD4^+$ T cell counts were not determined in the samples analyzed in this manuscript). Current blood-based diagnostic tests have shown promise in detecting TB infection in PLHIV [24]. Measurement of Notch ligands in may increase the accuracy of these diagnostics, functioning as a test to determine if PLHIV are infected with TB. This hypothesis is confirmed by a study that finds

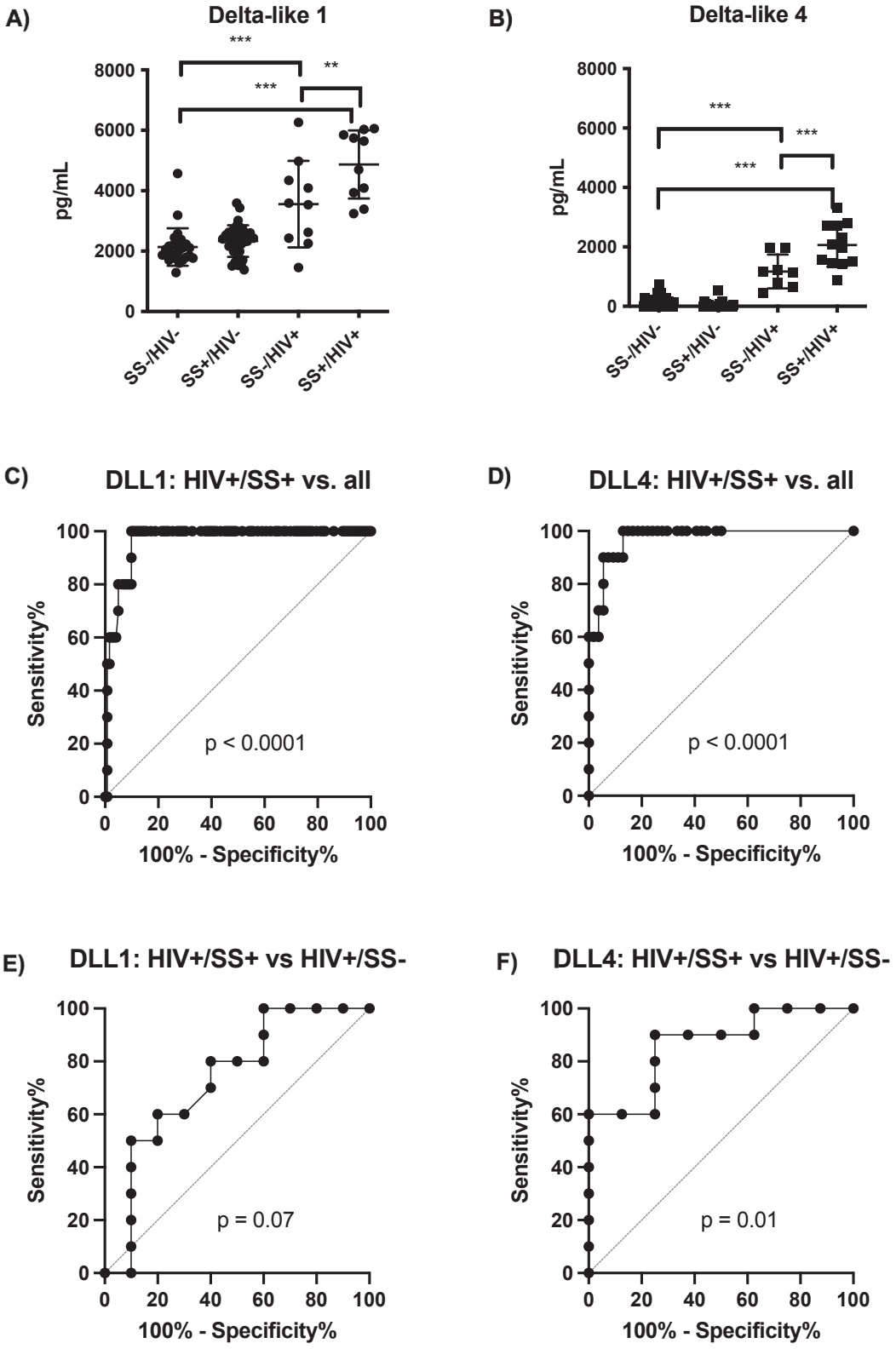

Fig. 1. Elevated levels of DLL1 and DLL4 in HIV and HIV/TB co-infection. A standard sandwich ELISA was run on serum from 78 patients. A) ANOVA analysis indicates that DLL1 levels are significantly upregulated in all individuals with HIV (***$p < 0.0001$) and individuals with HIV/TB co-infection (**$p = 0.0025$). B) ANOVA analysis indicates that DLL4 levels are significantly upregulated in all individuals with HIV and individuals with HIV/TB co-infection (***$p < 0.0001$). Gray dots are subcategories of the data depicted in the black dots and not a separate set of data. C&D) ROC curves to determine if Notch ligands are a sensitive and specific assay for HIV/TB co-infection when compared to all other samples in the cohort. The area under the curve is 0.96 for DLL1 and 0.97 for DLL4.

elevated levels of DLL1 in the spinal fluid of HIV+ patients diagnosed with TB meningitis [25]. Because Notch ligands are elevated as part of the host response to infection, this test lacks any information on the presence of genes related to antibiotic resistance. The use DLL1 as a blood-based marker also does not indicate the location of the infection. Given these limitations, a sensitive and specific measurement of Notch ligands in peripheral blood may prove useful as a point-of-care triage test to indicate referral for further testing or treatment. Such a test may have utility in unscreened individuals or in PLHIV. Because elevated Notch ligands are a host response to infection, establishing the specificity of this test would be a crucial first step which would involve screening blood from individuals with a variety of confirmed viral and bacterial infections endemic to the area where the test would be used. If the test was specific, the sensitivity could then be tested in PLHIV and stratified by CD4+ T cell counts.

DLL4 expression is upregulated on the surface of myeloid cells in both humans and mice as a result of mycobacterial infection [10] and DLL1 is upregulated on the surface of macrophages as a result of exposure to type 1 interferon [13]. Upon binding to a Notch receptor, it is accepted that the majority of Delta ligands are endocytosed and in some cases may be recycled back to the surface to promote future Notch interactions [26]. Several studies have indicated that Notch must be immobilized or on the cell surface in order to transmit a signal to the Notch receptor [27,28], which provokes the question of why cell-free Notch ligands are increased in HIV infected individuals. DLL4 has been found on exosomes generated by murine embryonic stem cells[29] and DLL4 containing exosomes have measurable biological effects on angiogenesis *in vitro* [30,31], suggesting that exosomal Notch ligands can alter Notch signaling. A second possibility is that the Notch ligands exist as soluble, non-membrane bound proteins. The role of increased soluble Notch ligands during HIV infection or other infections is unknown. However, soluble versions of DLL1 do not activate the Notch pathway in other systems [27,28,32]. The elevated levels of Notch ligands found in the serum of individuals with HIV suggest that these ligands may have an unknown function related to host defense. The significant increase in DLL1 and DLL4 serum levels that occurs in individuals with HIV/TB co-infection suggests that these two pathogens synergize to cause a host response that is more severe than that caused by either infection on its own.

Due to the myriad cell types that express Notch in the human body, it is difficult to speculate on the source of increased Notch ligand in the samples from HIV-infected individuals. A recent report suggests that $CD4^+$ T cells can express Notch ligands, including DLL1 and DLL4, when activated [33]. Therefore, it is possible that HIV infection of $CD4^+$ T cells increases the release of a soluble or exosome-bound form of Notch ligands through a $CD4^+$ T cell mediated mechanism. Further study will determine if the soluble versions of these ligands benefit the host or the pathogen during infection. Although we originally hypothesized that Notch ligands in the serum would be elevated in correlation with a diagnosis of TB, we determined that levels of both DLL1 and DLL4 were not correlated with TB alone. The increased levels of these ligands in HIV/TB coinfection may provide a rapid useful biomarker in resource poor areas.

There are several limitations to this study. First, these results are obtained from a small cohort and will need to be repeated in a larger cohort of individuals to confirm the findings. Second, the cohort is derived from two different continents, with all of the HIV+ individuals coming from South Africa and all of the HIV- individuals coming from Viet Nam. This two-gate design is known to lead to overestimates of accuracy when assessing the potential of a diagnostic [34]. Although HIV is likely the factor that causes increases in Notch ligand expression, the construction of the cohort does not rule out variables including genetics, diet and environment that may contribute to these findings. An additional limitation is the lack of individuals with extrapulmonary TB in our cohort, which limits the interpretation of the data to individuals that only have the pulmonary form of TB.

4. Ethics statement

De-identified serum samples were obtained from the FIND diagnostics serum bank with the primary goal of determining if soluble Notch ligands were present in patients infected with tuberculosis. The research was classified as not regulated under University of Michigan IRB HUM00100328 and conforms to all guidelines set forth by this board. Informed consent was obtained for all patients prior to blood draw.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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