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Research Article

Distinct Transcriptional and Anti-Mycobacterial Profiles of Peripheral Blood Monocytes Dependent on the Ratio of Monocytes: Lymphocytes

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ABSTRACT

The ratio of monocytes and lymphocytes (ML ratio) in peripheral blood is associated with tuberculosis and malaria disease risk and cancer and cardiovascular disease outcomes. We studied anti-mycobacterial function and the transcriptome of monocytes in relation to the ML ratio.

Mycobacterial growth inhibition assays of whole or sorted blood were performed and mycobacteria were enumerated by liquid culture. Transcriptomes of unstimulated CD14+ monocytes isolated by magnetic bead sorting were characterised by microarray. Transcript expression was tested for association with ML ratio calculated from leucocyte differential counts by linear regression.

The ML ratio was associated with mycobacterial growth in vitro ($\beta = 2.23$, SE 0.91, $p = 0.02$). Using sorted monocytes and lymphocytes, *in vivo* ML ratio (% variance explained $R^2 = 11\%$, $p = 0.02$) dominated over *in vitro* ratios ($R^2 = 5\%$, $p = 0.10$) in explaining mycobacterial growth. Expression of 906 genes was associated with the ML ratio and 53 with monocyte count alone. ML-ratio associated genes were enriched for type-I and -II interferon signalling ($p = 1.2 \times 10^{-8}$), and for genes under transcriptional control of *IRF1*, *IRF2*, *RUNX1*, *RELA* and *ESRRB*. The ML-ratio-associated gene set was enriched in TB disease (3.11-fold, 95% CI: 2.28–4.19, $p = 5.7 \times 10^{-12}$) and other inflammatory diseases including atopy, HIV, IBD and SLE.

The ML ratio is associated with distinct transcriptional and anti-mycobacterial profiles of monocytes that may explain the disease associations of the ML ratio.

1. Introduction

The diagnostic utility and prognostic value of leucocyte differential counts were appreciated contemporaneously with the development of microscopy methods to distinguish leucocyte subsets in 1880 (Ehrlich, 1880). A growing body of evidence suggests that the ratio of leucocyte subsets may be of similar, or greater, prognostic import than absolute counts. Although a full blood count is amongst the most frequently performed assays in clinical practice, the monocyte:lymphocyte (ML ratio) is not a widely-used parameter or biomarker in clinical care.

Florence Sabin and colleagues reported in the 1920s that the ML ratio was associated with progress and outcomes of mycobacterial infections in rabbits (Cunningham et al., 1925; Sabin et al., 1926; Doan and Sabin, 1930). Rediscovering this experimental work, we recently performed a series of prospective cohort studies of adults, infants and pregnant women in sub-Saharan Africa (Naranbhai et al., 2014a, 2014b, 2014c). In each study we observed that elevated ML ratio was associated with the risk of subsequent tuberculosis disease. We, and others, made similar observations for malaria (Warimwe et al., 2013a) and extended these to demonstrate that the ML ratio stratifies efficacy of the candidate anti-malaria RTS,S vaccine (Warimwe et al., 2013b). Other investigators have shown that an elevated ratio is associated with poor outcomes of nasopharyngeal carcinoma (Lin et al., 2014a; Li et al., 2013), diffuse large B-cell (Li et al., 2012, 2014a, 2014b; Rambaldi et al., 2013; Koh et al., 2014; Markovic et al., 2014; Porrata et al., 2014a, 2014b; Watanabe et al., 2014; Wei et al., 2014; Yan-Li et al., 2014) and Hodgkin's (Koh et al., 2012; Porrata et al., 2012, 2013a, 2013b; Romano et al., 2012) lymphomas, multiple myeloma (Shin et al., 2013), clear-cell renal carcinoma (Hutterer et al., 2014), non-small cell lung cancer (Lin et al., 2014b), soft tissue sarcoma (Szkandera et al., 2014) and colon cancer (Stotz et al., 2014).

The neutrophil:lymphocyte (NL) ratio, another measure of the myeloid:lymphoid cell proportion, has also been reported to be associated with cardiovascular and cancer outcomes (Guthrie et al., 2013;

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Templeton et al., 2014; X. Wang et al., 2014). Since the ML and NL ratios are strongly correlated it is unclear whether one is a better predictor than the other. The pathophysiologic basis for association of myeloid:lymphoid cell ratios across diseases remains unclear.

Here we studied pathophysiologic mechanisms for the association between the ML ratio and mycobacterial disease susceptibility. We studied anti-mycobacterial and transcriptomic profiles of monocytes from healthy adult donors and found that qualitative differences in monocyte function partially explain the ML ratio association with mycobacterial growth *ex vivo*. Whole-transcriptome analysis of monocytes suggests that the ML ratio (but not the monocyte count) is a marker of monocyte function and that an elevated ratio is associated with an enrichment of interferon-associated transcripts in monocytes. We show that the transcript signature of elevated ML ratio overlaps with mycobacterial and several other disease transcriptomic signatures such as atopy and inflammatory bowel disease. Taken together our data suggest that the ML ratio may be associated with disease by acting as a marker of monocyte function.

2. Materials and Methods

2.1. Study Participants

We recruited 144 healthy adult Caucasian volunteers in Oxford, United Kingdom (Fairfax et al., 2014). A healthcare professional (nurse or physician) conducted a verbal review of clinical history to determine eligibility based on the absence of any major chronic illness, current medication administration or symptoms of infection. The median age of the 144 recruits was 32 years (IQR 24–41) and 76 (53%) were female. For mycobacterial growth inhibition assays, we included individuals who had received BCG, were negative by interferon-gamma release assay (IGRA) and had no history of tuberculosis whereas for transcriptomic experiments, donors had no history of tuberculosis but their BCG history and IGRA status was not verified.

2.2. Ethics Statement

This study was approved by the Oxfordshire Research Ethics Committee (COREC reference 06/Q1605/55 & 10/H0505/3) and each individual gave written informed consent to participation.

2.3. Full Blood Counts

Leucocyte differential counts were performed at the Oxford Radcliffe Hospitals Pathology Laboratory, an accredited clinical laboratory, using standard procedures on a Sysmex automated haematology analyser. The ML ratio was calculated as the quotient of the absolute monocyte and lymphocyte counts.

2.4. Isolation of Monocytes

Whole blood was collected into sodium-heparin containing blood collection tubes (Becton Dickinson) and processed with 4–6 h after collection. The methods used in monocyte isolation have been previously described (Fairfax et al., 2014) but briefly, we isolated peripheral blood mononuclear cells by density gradient centrifugation of blood-diluted with Hanks Buffered Saline solution (HBSS, Life Technologies, UK) layered on Lymphoprep (Axis-Shield, Norway), and sorted CD14+ monocytes using magnetic-activated cell sorting (MACS, Miltenyi Biotech). The CD14- fraction was used in recreating ML ratios after counting cells by microscopy.

2.5. Mycobacterial Growth Inhibition Assays

Whole blood or mixed leucocyte growth inhibition assays were performed using the BACTEC mycobacteria growth indicator tube (MGIT) system (Becton Dickinson) as previously described (Wallis et al., 2001), with the exception that whole blood was incubated with BCG (Pasteur) for 96 h instead of 72 h and mixed leucocytes (monocytes + non-monocytes = total 1×10^6 live cells) incubated with BCG Pasteur for 72 h. Growth inhibition was determined by calculating time to positivity (TTP) in the sample and TTP in the control (cell free culture) and converting to colony-forming units using a standard curve.

2.6. Gene Expression

RNA from 5×10^5 sorted monocytes was extracted using the Qiagen RNAeasy kit (Qiagen, UK). RNA was quantified and integrity assessed using a Bioanalyser RNA 6000 Nano kit (Agilent, UK). Gene expression was quantified using the Illumina HumanHT-12 v4 BeadChip gene expression array platform with 47,231 probes according to the manufacturer's instructions. Samples were randomly placed across expression chips and run in a single batch. Gene expression data were normalized using random-spline normalization, transformed by variance-stabilising transformation and sample outliers were iteratively removed and normalization repeated. We excluded 8 individuals as sample outliers.

Probe sequences mapping to more than one genomic location or regions with underlying polymorphisms frequent in >1% of the population were excluded from analysis (n = 18,220 probes). Only probes that were expressed and detected (GenomeStudio probe detection $p < 0.01$) in monocytes were included in the analysis.

2.7. Transcription Factor Binding Site Analysis

We used oPOSSUM (Kwon et al., 2012) v3.0 to perform single-site analysis with the JASPAR core reference and the following search parameters: conservation cutoff = 0.4, matrix score threshold = 85% and upstream and downstream sequence lengths 10,000 bp. Of 906 genes submitted for analysis 821 matched genes in the database.

2.8. Statistical Methods

Analyses were conducted in *R*, using the packages: limma, lumi and 'vsn',. For data presentation the 'gridExtra', 'ggbio' and 'ggplot2' packages were used. Linear regression adjusting for age and sex was performed using the lm function in R. Enrichment analyses were performed using the fisher.test function in R comparing the observed vs. expected overlap in gene lists.

Ingenuity Pathway Analysis where used, was performed using a background set of all human genes on the Ilumina array. A listing of external files and their sources is given in Supplementary Table 14. FDR where shown denotes the false discovery rate calculated using the Benjamini Hochberg procedure using the p.adjust function.

2.9. Accession Numbers

Gene expression data is available through ArrayExpress (E-MTAB-2232). The accession numbers of studies accessed through the ExpressionAtlas are shown in Supplementary Table 4.

3. Results

3.1. Qualitative Differences in Monocytes Dependent on ML Ratio Dominate Over Quantitative Differences in Explaining the ML Ratio Association With Mycobacterial Growth

Recent epidemiological studies (Naranbhai et al., 2014b) suggest that elevated peripheral blood ML ratio is associated with risk of TB disease in adults, infants and pregnant women. To evaluate whether this finding is replicated *ex vivo*, we cultured whole blood from healthy adult donors with *BCG* (*Pasteur*) in a mycobacterial growth inhibition