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ANALYSIS OF EARLY STROKE-INDUCED CHANGES IN CIRCULATING LEUKOCYTE COUNTS USING TRANSCRIPTOMIC DECONVOLUTION

Grant C. O'Connell[*],
Julia H.C. Chang

*School of Nursing,
Case Western Reserve University,
Cleveland, OH*

Abstract

Growing evidence suggests that stroke alters the phenotype of the peripheral immune system; better characterization of this response could provide new insights into stroke pathophysiology. In this investigation, we employed a deconvolution approach to informatically infer the cellular composition of the circulating leukocyte pool at multiple timepoints following stroke onset based on whole blood mRNA expression. Microarray data generated from the peripheral blood of 23 cardiovascular disease controls and 23 ischemic stroke patients at 3, 5, and 24 hours post-symptom onset were obtained from a public repository. Transcriptomic deconvolution was used to estimate the relative counts of nine leukocyte populations based on the expression of cell-specific transcripts, and cell counts were compared between groups across timepoints. Inferred counts of lymphoid cell populations including B-cells, CD4+ T-cells, CD8+ T-cells, γδ T-cells, and NK-cells were significantly lower in stroke samples relative to control samples. With respect to myeloid cell populations, inferred counts of neutrophils and monocytes were significantly higher in stroke samples compared to control samples, however inferred counts of eosinophils and dendritic cells were significantly lower. These collective differences were most dramatic in samples collected at 5 and 24 hours post-symptom onset. Findings were subsequently confirmed in a second dataset generated from an independent population of 24 controls and 39 ischemic stroke patients. Collectively, these results offer a comprehensive picture of the early stroke-induced changes to the complexion of the circulating leukocyte pool, and provide some of the first evidence that stroke triggers an acute decrease in eosinophil counts.

Introduction

It is becoming increasingly evident that the peripheral immune system responds robustly to stroke, and that this response influences clinical outcome. For example, peripheral immune changes triggered by stroke are believed to contribute to the pathogenesis of adverse complications such as secondary tissue damage, hemorrhagic transformation, and post-stroke infection [1]. Thus, better characterization of the stroke-induced peripheral immune response could provide novel insights into stroke pathophysiology and open new avenues for immunotherapeutic intervention.

Many studies which have investigated the peripheral immune response to stroke in humans have done so using the standard white blood cell differential collected as part of routine clinical evaluation; unfortunately, the clinical white blood cell differential provides quantification of a limited number of cell populations, often only total neutrophils, monocytes, and lymphocytes, and thus provides a relatively low-detail picture regarding peripheral immune status. Multi-color flow cytometry experiments have been used to examine more discrete subpopulations of leukocytes, however they have often only focused on small numbers of cell types in a single analysis. Thus, more detailed characterization of the stroke-induced changes to the cellular complexion of the peripheral immune system could reveal nuanced alterations which are pathologically relevant.

Several prior studies have performed genome-wide transcriptomic profiling of peripheral whole blood with the goal of identifying clinically-useful stroke biomarkers [2–6]. Recent work by our group suggests that similar to other conditions [7], several of the gene expression changes observed between stroke patients and controls in these investigations were likely artifacts of underlying changes in leukocyte counts, and not true changes in transcription at the cellular level [8,9]. Transcriptomic deconvolution is a process which leverages such phenomena to informatically infer the cellular composition of complex biological samples based on aggregate gene expression through the analysis of cell-specific transcripts [10]. In this study, in an attempt to better characterize the stroke-induced peripheral immune response, we employed a deconvolution approach to infer the counts of nine major circulating leukocyte populations at multiple timepoints following stroke onset using publicly available human whole blood gene expression data.

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* E-mail: grant.oconnell@case.edu

Methods

Microarray data processing

Raw microarray data generated from the peripheral whole blood of 23 controls, as well as 23 ischemic stroke patients at 3, 5, and 24 hours post-symptom onset, were downloaded as .CEL files from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) via accession number GSE58294. Probe annotations were updated via the 'annotate' package for R (R project for Statistical Computing). Raw perfect match probe intensities were background corrected, quantile normalized, and summarized at the set level via robust multi-array averaging using the rma() function of the 'affy' package. Data were further summarized at the gene level via max intensity using the collapserows() function of the 'WGCNA' package.

Deconvolution

Estimated counts of B-cells, CD4+ T-cells, CD8+ T-cells, gamma delta (γδ) T-cells, natural killer (NK) cells, monocytes, neutrophils, eosinophils, and dendritic cells were generated from normalized expression data using a list of 226 cell-specific genes (Figure 1) aggregated from a compendium of immune cell microarray data compiled by Newman *et al.* [11]. Weighted correlation network analysis was used to produce a relative count for each cell population based on the expression levels of its associated genes using the collapserows() function of the 'WGCNA' package according to the method described by Miller *et al.* [12]. Relative counts of each cell population were arbitrarily scaled from zero to one using unity-based normalization.

Demographic information

Clinical and demographic information associated with samples was aggregated from the descriptors reported in Stamnova *et al.* [13].

Statistics

All statistics were performed using R 3.3. Fisher's exact test was used for comparison of dichotomous variables. T-test or one-way ANOVA was used for comparisons of continuous variables where appropriate. The null hypothesis was rejected when $p<0.05$.

Validation of results

Findings were subsequently confirmed via deconvolution of a second publically available microarray dataset generated from an independent population of 24 controls and 39 ischemic stroke patients (GEO accession number GSE16561) [14].

Results

Clinical and demographic characteristics

Stroke samples originated from patients which were significantly older than control counterparts, but were well matched in terms of sex and ethnicity. In terms of risk factors for cardiovascular disease, groups were well matched with regards to rates of hypertension and diabetes, however control subjects displayed a significantly higher prevalence of dyslipidemia relative to stroke patients. All stroke patients had received thrombolytic intervention via recombinant tissue plasminogen activator (rtPA) following 3 hour blood collection (Table 1).

Inferred leukocyte counts

Inferred counts of lymphoid populations including B-cells, CD4+ T-cells, CD8+ T-cells, γδ T-cell and NK-cells were all significantly reduced in stroke samples relative to controls (Figure 2A-E); this effect was most pronounced in samples collected at 5 and 24 hours following onset of symptoms. With regards to myeloid populations, inferred counts of neutrophils and monocytes were significantly higher in stroke samples in comparison to controls (Figure 2F-G), however, inferred counts of dendritic cells and eosinophils were significantly lower (Figure 2H-1). Once again, these differences were most pronounced at 3 and 5 hours post-onset. An identical overall pattern of changes was observed in a second microarray dataset generated from an independent patient population (Supplemental Figure 1).

Discussion

Better characterization of the peripheral immune response to stroke could provide novel insights into stroke pathophysiology and identify new targets for immunotherapeutic intervention. In this study, we employed a transcriptomic deconvolution approach to infer the relative counts of nine major circulating leukocyte populations in blood samples collected at multiple timepoints over the first 24 hours following stroke onset. With respect to several cell types, our results confirmed the findings of prior cytometric studies, however, our analysis also revealed changes in other leukocyte populations that have yet to be widely reported on in human stroke.

Our observations suggest that the circulating counts of lymphoid cell populations are ubiquitously suppressed in response to stroke. This is consistent with prior cytometric investigations which have reported stroke-induced decreases in total lymphocyte counts [15–18], as well as reductions in counts of more discrete lymphoid subpopulations such as B-cells, CD4+ T-cells, CD8+ T-cells, and NK-cells [15,16,18]. To our knowledge, only three other human studies have reported on circulating γδ T-cell counts in stroke; one reporting that γδ T-cell counts are elevated [19], one reporting that they are unaffected [20], and one reporting that they are decreased [21]. Our results provide additional evidence suggesting that stroke triggers an acute decrease in circulating γδ T-cell numbers similar to that which is observed in other lymphoid populations.

Our results are also consistent with those of several prior cytometry-based investigations reporting that counts of the two most abundant peripheral blood myeloid cell populations, neutrophils and monocytes, become robustly elevated in response to stroke [15,17,22,23]. However, our findings also suggest that two lesser-studied myeloid populations, dendritic cells and eosinophils, are significantly reduced. With respect to dendritic cells, our observations are consistent with those reported in the limited number of prior cytometric studies performed in humans [24]. With respect to eosinophils, this current study is one of the first case-control analyses to report evidence of an acute reduction in eosinophil counts in response to stroke; our observations align well with two recent associative studies reporting
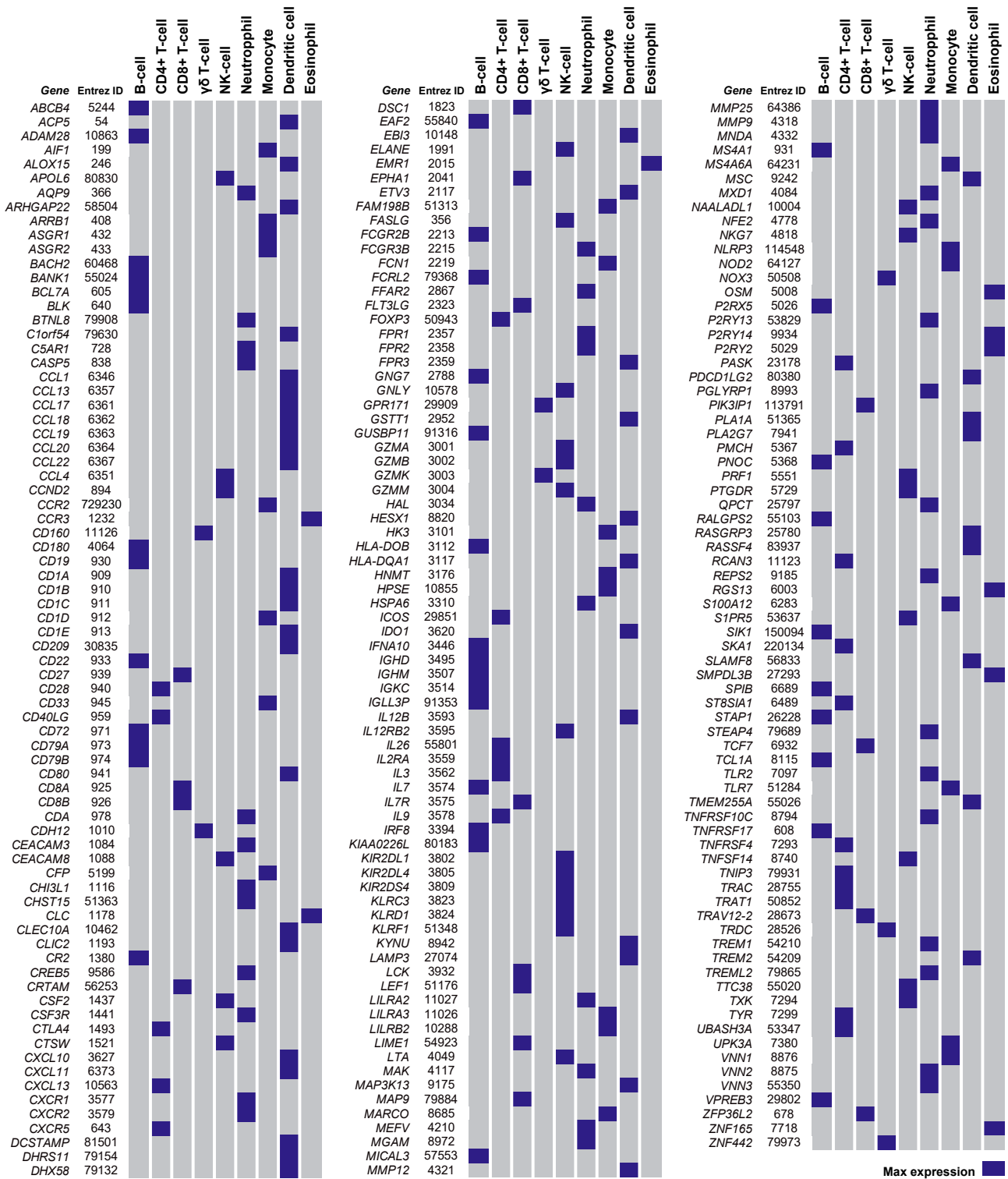
Figure 1. Cell-specific genes used for deconvolution. 226 cell-specific genes used for the deconvolution of whole blood microarray data along with their predominant leukocyte population of expression.

Table 1. Clinical and demographic characteristics.

	Cardiovascular Disease (n=23)	Ischemic Stroke (n=23)	p
[a]**Age** *mean±SD*	57.9 ± 3.3	71.7 ± 7.9	<0.001*
[b]**Female** *n(%)*	11 (47.8)	11 (47.8)	1.000
[b]**Non-caucasian** *n(%)*	4 (17.4)	8 (34.8)	0.314
[b]**Dyslipidemia** *n(%)*	16 (69.6)	6 (26.1)	0.007*
[b]**Hypertension** *n(%)*	16 (69.6)	16 (69.6)	1.000
[b]**Diabetes** *n(%)*	5 (21.7)	4 (17.4)	1.000
[a]**Baseline NIHSS** *mean±SD*	0.0 ± 0.0	15.4 ± 7.4	<0.001*
brtPA *n(%)*	0 (0.0)	23 (100.0)	<0.001*

[a]Compared via two-sample two-tailed t-test; [b]Compared via Fisher's exact test; *Statistically significant

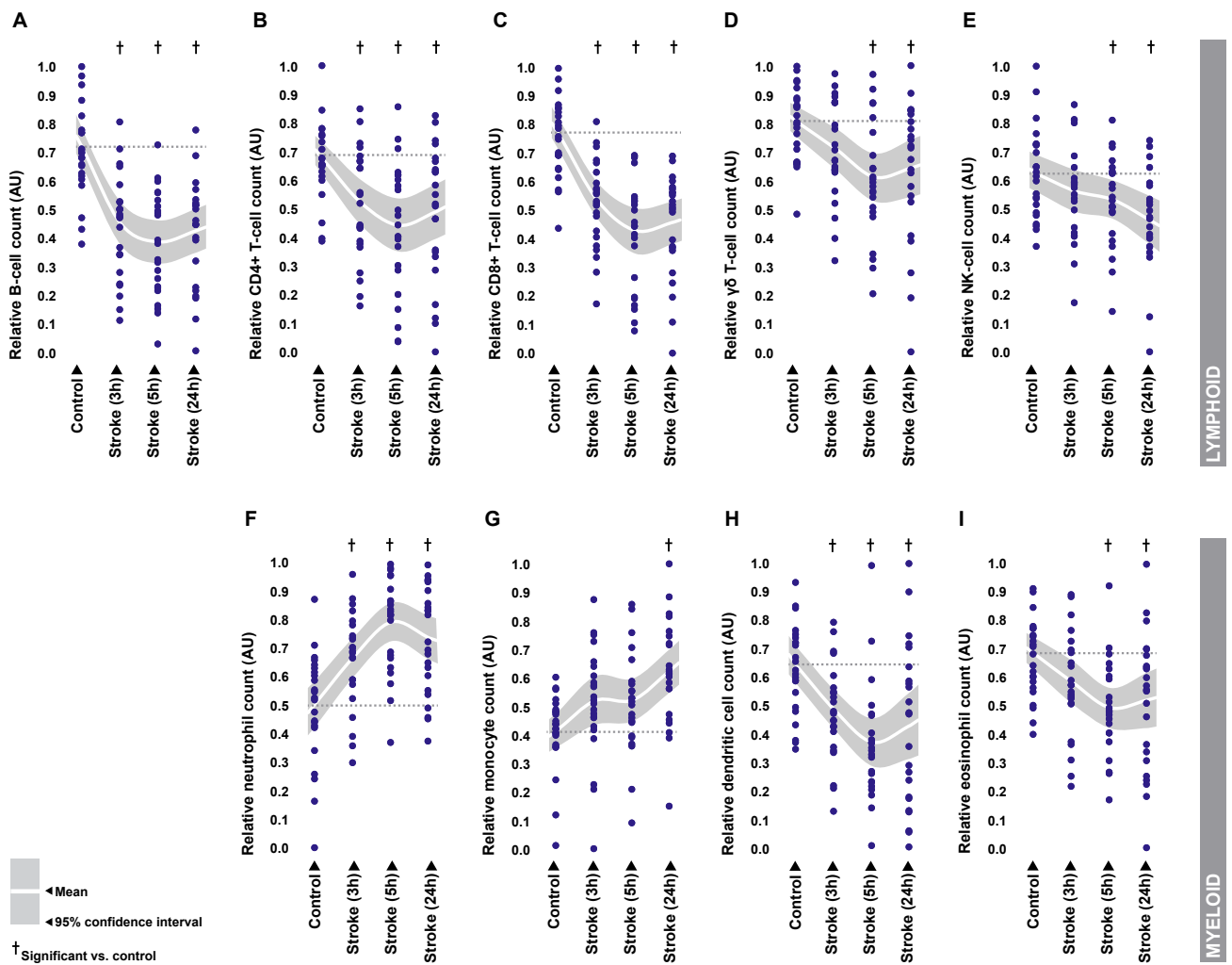**Figure 2.** Inferred counts of circulating leukocyte populations. (A-I) Estimated relative counts of B-cells, CD4+ T-cells, CD8+ T-cells, γδ T-cells, NK-cells, monocytes, neutrophils, eosinophils, and dendritic cells in blood sampled from controls and stroke patients at 3, 5, and 24 hours post symptom onset. Counts were statistically compared between stroke and control samples across time points using one-way ANOVA with subsequent planned comparisons via Bonferoni-corrected two-sample two-tailed t-test.

that post-stroke circulating eosinophil counts are negatively correlated with stroke severity and risk of mortality [25,26], suggesting that the pathophysiological relevance of a stroke-induced reduction in peripheral blood eosinophil counts warrants further investigation.

It should be stated that this study is not without limitations; most notable in this regard is the fact that we did not directly measure leukocyte counts, and instead informatically inferred them using gene expression data. While transcriptional deconvolution approaches have been shown to accurately enumerate cell counts in numerous benchmarking studies [10–12], the fact that they rely on reference expression signatures generated from isolated healthy cells has the potential to introduce confounds. The handling and manipulation of reference cells during isolation could potentially alter gene expression and introduce artifacts. Furthermore, because reference signatures are generated from the cells of healthy donors, disease-specific differential regulation of reference signature genes could reduce the accuracy of analyses. However, the aforementioned limitations are most likely to introduce confounds when trying to discriminate between highly similar cell populations or in disease states which introduce dramatic alterations in transcription; the cell types which were enumerated in our analysis are relatively distinct at the molecular level,

and the magnitude of transcriptional changes observed in stroke at the level of whole blood are of a relatively small [2,4,6]. Furthermore, the fact that our findings align well with those of previous cytometry-based investigations suggests that our deconvolution analysis was well implemented. Nonetheless, our findings, in particular those regarding stroke-induced changes in eosinophil counts, should be confirmed in future work using direct cytometric analysis.

It is also important to note that because samples originated from patients who received thrombolytic treatment following the initial 3 hour blood collection, it is possible that some of the differences in inferred leukocyte counts observed between stroke patients and controls at the 5 and 24 hour timepoints were driven by effects of rtPA. However, we find this scenario unlikely due to the fact we observed a similar pattern of changes in a second dataset originating from blood samples which were collected prior to administration of thrombolytics.

Collectively, our results offer a comprehensive picture of the early stroke-induced changes to the complexion of the circulating leukocyte pool. Our findings confirm the results of prior cytometric investigations, and additionally, provide some of the first human evidence that stroke triggers an acute decrease in circulating eosinophil counts.

Conflict of interest

GCO has a patent pending re: genomic patterns of expression for stroke diagnosis. GCO has received consulting fees from Valtari Bio incorporated. The remaining authors report no potential conflicts of interest.

Compliance with ethical standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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