

Chapter 2

The Lupus Biomarker Odyssey: One Experience

Joseph M. Ahearn, Susan Manzi, and Chau-Ching Liu

Abstract

The last decade has witnessed an explosion in efforts to discover and validate lupus biomarkers. The currently steep trajectory of this progress is unprecedented. However, advances in the lupus biomarker field remain fewer and slower than physicians, patients, and pharmaceutical companies have hoped for. This chapter will review the challenges confronted by physicians and scientists in pursuit of lupus biomarkers and will present our experience on this path and specific efforts to surmount some of the obstacles in this endeavor. A comprehensive review of the current landscape in lupus biomarker research has recently been published elsewhere (Ahearn et al. *Transl Res* 159:326–342, 2012; Liu et al. *Ther Adv Musculoskelet Dis* 5:210–233, 2013; Liu and Ahearn *Best Pract Res Clin Rheumatol* 23:507–523, 2009; Liu et al. *Curr Opin Rheumatol* 17:543–549, 2005).

Key words Systemic lupus erythematosus, Biomarkers, Complement, Flow cytometry, CB-CAPs

1 Introduction

There is an urgent need for lupus biomarkers for several reasons. First, SLE is commonly misdiagnosed, even by experienced rheumatologists. The diagnosis requires interpretation of complex criteria developed by the American College of Rheumatology, as no single test is sufficiently sensitive and specific to be diagnostic. It has been generally held that many individuals with a positive test for ANA alone are misdiagnosed as lupus, despite many patients with other diseases and even healthy individuals also testing positive for ANA. Although anti-dsDNA is highly specific for lupus and a positive test essentially guarantees an accurate lupus diagnosis, the majority of patients with lupus test negative for anti-dsDNA at a given point in time. Published studies document that even experienced rheumatologists misdiagnose lupus. In one report, of 263 patients referred with a presumptive diagnosis of lupus, 125 were found to be misdiagnosed and of these 76 were ANA-positive but did not have an autoimmune disease [5]. Second, the course of SLE in a given patient is characterized by unpredictable flares and

remissions. Again, there is no laboratory test with reliable capacity to identify or predict a disease flare. Third, the lack of biomarkers has impeded efforts to evaluate new SLE therapeutics in clinical trials. Pharmaceutical companies, with potential therapies in the pipeline and in hand, may be reluctant to invest in clinical trials because response to therapy cannot be determined with confidence or trials that are completed may fail for the same reason. Fourth, biomarker discovery and development are a critical link between pathogenic mechanisms and therapeutics. In some cases, discovery of molecular and cellular mechanisms (e.g., those involving interferon) may lead to identification of candidate biomarkers that may in turn suggest potential therapeutic targets and provide direction for drug development. The capacity of the innovative therapeutics to interfere with the targeted pathogenic mechanisms can then be evaluated with those same biomarkers that led to the drug discovery.

2 Challenges to Lupus Biomarker Discovery and Validation

The disappointing pace of lupus biomarker development, even compared with similar efforts in other diseases, is at least partly due to challenges unique to SLE. First, the extraordinary clinical heterogeneity of lupus, most likely due to distinct and overlapping pathogenic mechanisms, requires precision medicine to pair the patient not only with the right therapeutic but also with the biomarker(s) that will accurately reflect the disease process in that same patient. If all patients with lupus are evaluated as a single group for efficacy of a single biomarker, no difference might be observed compared with the control group(s) although the biomarker might be highly valuable among a subset of patients for diagnosis, monitoring or predicting response to a specific therapeutic intervention. Second, because lupus is frequently misdiagnosed, it is possible that the “lupus” subjects in a particular biomarker investigation may not actually have the disease. Third, studies of lupus biomarkers for disease activity are extremely challenging because there is no clear “gold standard” by which the potential biomarkers can be evaluated. If a study is designed to demonstrate an impact on clinical care of lupus patients, it must be superior to what is currently in use. The numerous complex disease activity indices used by lupus aficionados are not employed in routine clinical practice yet these may be the standards to which novel biomarkers for monitoring patients are held for validation and acceptance. Fourth, laboratory assays for lupus diagnosis and monitoring that have been used for decades, such as anti-dsDNA, serum C3/C4, and ANA, have never been standardized or validated themselves, yet these are the standards to which emerging biomarkers are typically compared. This dilemma regarding the utility of ANA testing has recently been thoughtfully

considered [6]. Frequent false-positive results with the traditional indirect immunofluorescence ANA assay and recent false-negative results with higher-throughput ANA technologies further emphasize the need for advances and standardization in lupus diagnostics and beyond. In addition to these obstacles that may be particularly relevant to the development of lupus biomarkers, another challenge common to many biomarker discovery strategies includes the reality that humans are not rodents and many advances made with animal models of human disease cannot be directly translated to humans because of different genetics, biology, pathophysiology, or other interspecies differences. Additional challenges to biomarker validation in general include considerations of time and temperature, shipping, freeze-thawing, and other quality control issues that are essential but not always rigorously evaluated in development.

3 Biomarker Discovery Strategies

The two general strategies employed in biomarker development are the technology-driven versus the candidate biomarker approaches. Technology-driven approaches capitalize on the power of recent and rapidly developing genomic, proteomic, metabolomic, peptidomic, and microarray technologies to compare various sets of biologic samples to identify meaningful differences. Many such efforts are not necessarily driven by specific hypotheses beyond predicting that differences will be identified when comparing serum, plasma, urine, DNA, RNA, etc. from two or more groups of study subjects. The alternate strategy is to focus on specific molecular and/or cellular pathways, usually because prior discoveries have suggested them as fertile sources to mine for biomarkers. Of course, these two approaches are not mutually exclusive. For example, a microarray study might identify a promising signature that is then specifically mined for candidate molecular biomarkers. Alternatively, the knowledge that a specific molecular and cellular pathway is involved in a disease might lead to targeted proteomic characterization of these pathways in serum or plasma from patients versus control subjects. Regardless of which general approach is taken, the biomarker discovery journey typically progresses through sequential stages of conceptualization, identification, development, and validation. The remainder of this chapter will describe our efforts for the past decade during which we took a candidate-driven approach from conceptualization to validation focused on cell-bound complement activation products (CB-CAPs) as lupus biomarkers for diagnosis, monitoring, stratification, and precision (personalized) medicine. Hopefully, lessons learned from our experience may help guide future efforts in the field as the lupus biomarker odyssey has just begun.

4 CB-CAP Conceptualization

The compelling rationale for mining the complement system for lupus biomarkers has been thoroughly described previously [7–11] and will be recapitulated here. The complement system is a highly complex group of plasma and membrane-bound proteins that form three distinct pathways (classical, alternative, and lectin-dependent) evolved primarily to protect against invasion of foreign pathogens. The classical pathway is the major effector mechanism for antibody-mediated immune responses. Because antibody/immune complex-triggered activation of the complement system is believed to play an important role in the pathogenesis of SLE, measurement of serum C3 and C4 has traditionally been the “gold standard” for monitoring disease activity in SLE patients. Decreased C3 and C4 levels are considered to be markers of inflammation and increased SLE disease activity. However, there are several drawbacks in this approach. First, there is a wide range of variation in serum C3 and C4 levels among healthy individuals, and this range overlaps with the range observed in SLE patients. Second, standard laboratory tests measure the concentration of parental C3 and C4 molecules rather than products of activation. Third, acute phase response during inflammation may lead to an increase in C4 and C3 synthesis, which can balance the activation and increased consumption of these proteins. Fourth, partial deficiencies of C4, which are commonly present in the general population and in SLE patients, may result in lower than normal serum C4 levels because of decreased synthesis rather than increased complement activation and/or active SLE. As a result of these complexities, there have been conflicting conclusions regarding the value of serial measurement of serum C3 and C4 in monitoring disease activity in SLE patients. Some studies have found these assays valuable in this regard, while others have found C3 and C4 levels to remain normal during SLE flares. These conflicting results suggest that current standard tests, based on serum levels of the native form of complement proteins, are inadequate to accurately and promptly detect SLE disease flares. During the past several years, other investigators have explored the potential for measurement of soluble complement activation products such as C3a and C4d to serve as biomarkers in SLE. Despite some intriguing observations, serum levels of complement activation products have not replaced measurement of native C3 and C4 as gold standards.

Together, these prior observations led to the hypothesis that complement activation products (CAPs) during the course of lupus pathogenesis may covalently bind to surfaces of circulating cells, potentially rendering the cells dysfunctional. As such, CB-CAPs might serve as lupus biomarkers to guide clinical care of patients while also participating directly in lupus pathogenesis through a novel molecular-cellular pathway.

5 CB-CAP Identification

Efforts during the past decade have led to the CB-CAP technology platform, which currently consists of a panel of assays designed to identify C4d and C3d complement activation products that have been deposited on essentially any circulating cell type. Cell types and cell subsets are identified by routine flow cytometric gating practices and the use of cell-specific phenotypic surface markers, while CB-CAPs are simultaneously identified with anti-C4d and anti-C3d monoclonal antibodies (Fig. 1).

5.1 E-C4d: The First CB-CAP

Initial efforts to prove the CB-CAP hypothesis were focused on the erythrocyte [12] because normal erythrocytes are known to bear constitutively low levels of C4d and are unique in this regard among circulating human cells. An initial study showed that abnormally high levels of C4d are deposited on the surface of erythrocytes (E-C4d) in patients with SLE as compared with healthy

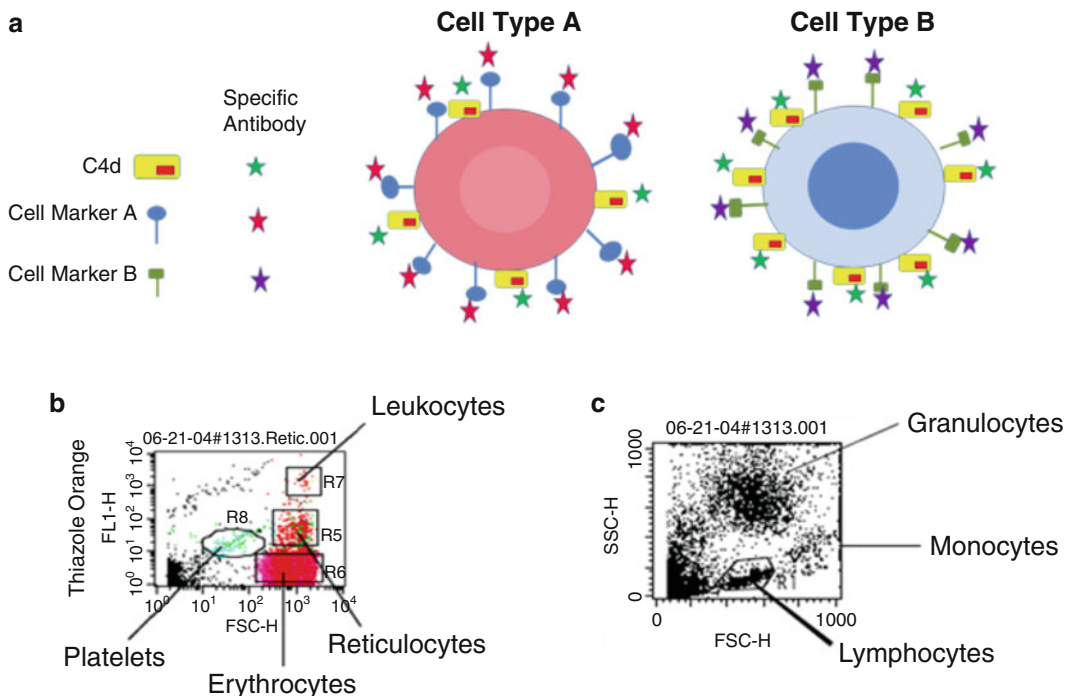


Fig. 1 Flow cytometric assay of CB-CAPs. (a) Schematic illustration of the multicolor staining of circulating cells for cell type-specific surface markers and surface-bound CAPs (e.g., C4d). (b) A representative dot plot shows the identification of reticulocytes (gate R5) and erythrocytes (gate R6) by thiazole orange staining (Y-axis). Contaminating leukocytes and platelets present in the samples can be differentiated from reticulocytes and erythrocytes based on the thiazole orange staining intensity and forward scattering property. (c) A representative dot plot demonstrates the identification of lymphocytes, monocytes, and granulocytes. These cell types are also differentiated by using mAbs specific for different cell lineages added to the cell suspension (e.g., anti-CD3, anti-CD19, etc.). The latter staining pattern is not shown here

subjects and patients with other diseases. As a diagnostic marker for SLE, E-C4d was 72 % sensitive and 79 % specific in differentiating SLE from other inflammatory diseases.

It was also noted during these studies of erythrocytes that E-C4d levels in the same SLE patient examined on different days varied considerably, suggesting that changes in E-C4d levels in SLE patients might reflect fluctuation in disease activity. We pursued this hypothesis through both cross-sectional and longitudinal studies [13]. In the initial longitudinal study, we analyzed the erythrocyte-based markers as disease activity biomarkers in patients with SLE using a regression formulation in which each patient's evolving clinical status was regressed on each of the biomarkers. The Systemic Lupus Activity Measure (SLAM), and the SLE Disease Activity Index (SLEDAI), were used as the clinical measures to assess disease activity in these patients. In addition to the CB-CAP biomarkers, we also tested the more traditional markers of disease activity for SLE, serum C3 and C4, and anti-dsDNA. Briefly, 156 patients with SLE, 290 patients with other diseases, and 256 healthy individuals were followed prospectively over a 5-year period (2001–2005), encompassing 1,005 patient-visits (SLE patients), 660 patient-visits (patients with other diseases), and 395 subject-visits (healthy individuals). All of these 156 patients met at least four American College of Rheumatology criteria to be considered definitive SLE, and all had a minimum of three study visits. Study participants were closely followed for clinical disease activity (SLE patients, using SLAM and SLEDAI), clinic laboratory measures (serum C3, C4, anti-dsDNA, and ESR), and erythrocyte-based biomarker measures (E-C3d and E-C4d).

As we had previously observed, SLE patients had higher levels of E-C4d than did the healthy controls and patients with other diseases. Levels of E-C3d were also higher in the SLE group than in the other two groups. The variances within patient and between patients for E-C3d and E-C4d were high, while the variability for E-CRI was low in the SLE patients as compared to the other two groups. The high variability of E-C3d and E-C4d in SLE patients suggested that levels of these biomarkers vary not only between different SLE patients but also within the same SLE patient over time. This notion was further verified by univariate and multivariable analysis of covariance. While the univariate analysis demonstrated that E-C4d, E-C3d, and serum C3 levels were significantly associated with SLAM and SLEDAI (all $p < 0.001$), the multivariate analysis showed that only E-C4d remained significant predictors of SLE disease activity even after adjusting for serum C3, C4, and anti-dsDNA antibody.

5.2 E-C4d as a Time Capsule of Lupus Disease Activity

These E-C4d data strongly supported the possibility that E-C4d levels may reflect disease activity in SLE patients, leading to the E-C4d “time capsule” hypothesis as follows. Human erythrocytes

survive in the circulation for approximately 120 days. While erythrocytes circulating during a disease flare (i.e., increased complement activation) may have an increased amount of C4d deposited on their surface, erythrocytes emerging from the bone marrow after the flare has subsided (i.e., diminished complement activation) may have a low (“remission”) level of surface C4d. Considering that erythrocytes of different ages ranging from 1 day old to 120 days old are present in the circulation at any given time, we postulated that detection of erythrocyte subpopulations expressing distinct levels of C4d at a specific time point should theoretically reveal, much like time capsules, SLE disease activity during the preceding 120 days.

To verify this hypothesis, we performed experiments examining the C4d levels on age-fractionated erythrocytes. The most well-established methodology for separating human erythrocytes of different ages is density gradient fractionation. This technique is based on the concept that the buoyant density of erythrocytes increases with cell age. Briefly, erythrocytes derived from 0.25 ml of freshly drawn blood were washed in PBS containing EDTA (to avoid cell clumping), resuspended in 0.5 ml isotonic buffer, and centrifuged through a continuous Percoll gradient (0–50 % Percoll; 1–1.15 g/ml density; GE Healthcare Biosciences). Erythrocyte fractions were sequentially collected from the bottom of the gradient and washed two times with PBS to remove residual Percoll. Levels of C4-derived products on age-fractionated erythrocytes and unfractionated erythrocytes from the same individual (SLE patient or healthy control) were assayed by flow cytometry and quantitated as SMFI. SMFI data of the whole set of fractions derived from each patient and control were analyzed collectively to obtain the overall pattern of the differential E-C4d levels on old versus young erythrocytes. Three general patterns of E-C4d levels among age-fractionated erythrocytes were identified. Figure 2 shows the data obtained from three representative SLE patients respectively. The first pattern demonstrates high E-C4d levels on the older erythrocytes (Fig. 2, left upper panel patient #1043); the second pattern demonstrates constant E-C4d levels on all fractions regardless of the age of erythrocytes (Fig. 2, left middle panel patient #1014); the third pattern showed demonstrates high E-C4d levels on the youngest erythrocytes (Fig. 2, left lower panel patient #1066). We speculated that these different E-C4d patterns may represent, respectively, a previously active, a stable (or chronically active), and a recently activated disease state. Collectively, these data provide support for the “time capsule” hypothesis indicating that the levels of C4d on erythrocytes of SLE patients may contribute informative clues to remote, current, and imminent disease activity.

5.2.1 Method: *Erythrocyte CB-CAP Assays*

Blood was collected in Vacutainer™ tubes containing EDTA as an anticoagulant (Becton Dickinson) and used for experiments within 24 h after collection. After partitioning by centrifugation at $800 \times g$,

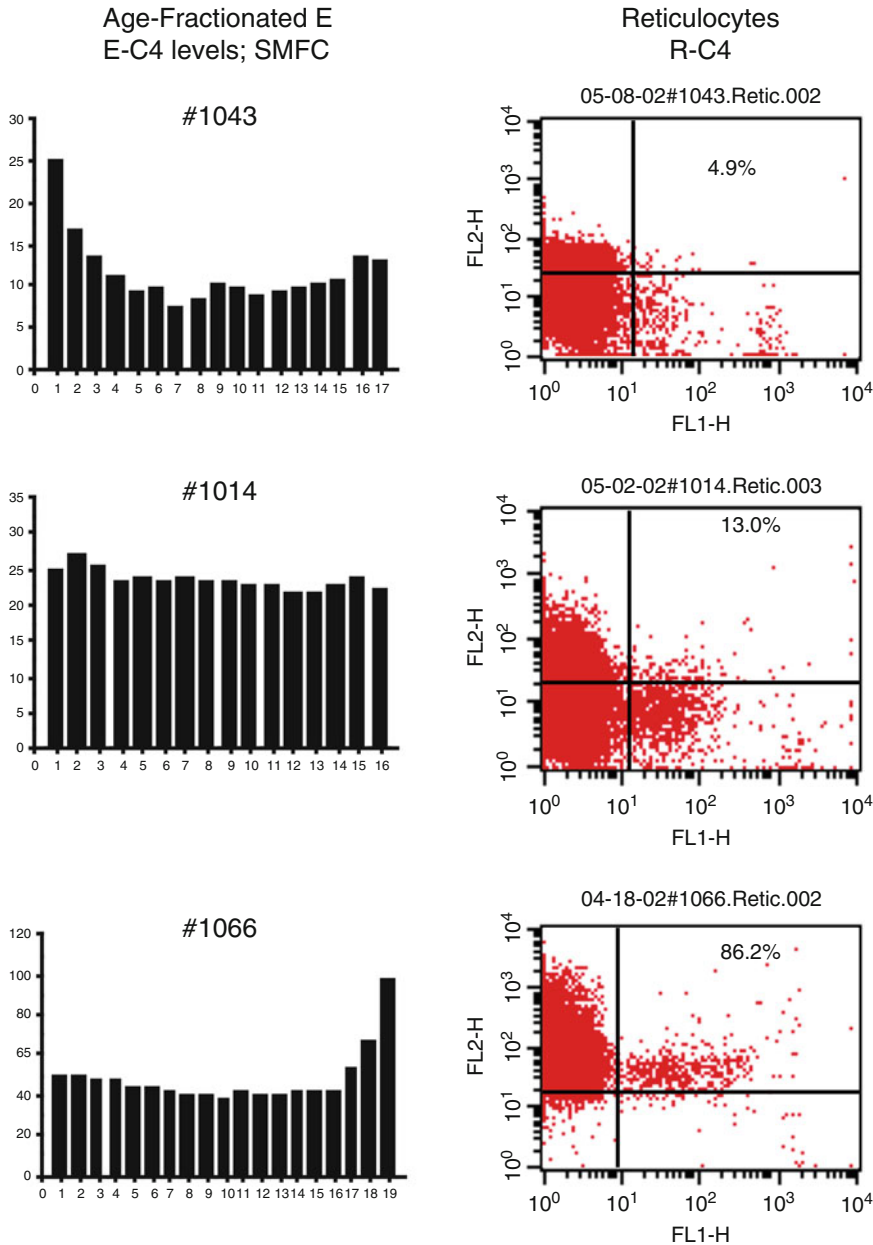


Fig. 2 Three general patterns of E-C4d deposition on age-fractionated erythrocytes. The EC4d levels (SMFI) of all fractions of erythrocytes of each of three patients are presented as a bar graph on the *left*. The X-axes are the numbered fractions with 1 being the oldest fraction. The Y-axes are the MFI of C4d determined by flow cytometry. C4d deposition on reticulocytes of the same patient analyzed on the same day is shown as a dot blot on the *right*. *Numbers* in the dot blots represent the percentage of C4d+ reticulocytes

the plasma was removed for storage and the blood cell portion diluted in phosphate buffered saline (PBS). Erythrocytes in the diluted blood were washed with PBS, resuspended in PBS, and aliquotted for antibody staining using mouse anti-human C4d

monoclonal antibodies (mAb) (reactive with native C4, C4b, iC4b, and C4d; from Quidel), mouse anti-human C3d mAb (reactive with native C3, C3b and C3d), or the isotype-matched mouse IgG control. Additional studies have demonstrated that the erythrocyte surface antigens reactive with these two mAbs are indeed C4d and C3d, not the native molecules or other activation products. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG F(ab')₂ (Jackson ImmunoResearch Laboratories, Inc.) at a concentration of 10 µg/ml was used as the secondary antibody. Stained cells were analyzed using a FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems) in conjunction with CellQuest Software. Erythrocytes were electronically gated based on forward and side scatter properties to include only single cells. Levels of surface-bound C4d and C3d on gated cells were expressed as specific mean fluorescence intensity: C4d- or C3d-specific mean fluorescence minus the isotype control mean fluorescence.

5.3 Reticulocyte-Bound C4d as a Biomarker for Ongoing SLE Disease Activity

These “time capsule” studies led to a focus on the reticulocyte as a potential “instant messenger” of lupus disease activity [14]. As mentioned above, erythrocytes ranging from 1 day old to 120 days old are present in the circulation at any given time point. The E-C4d levels on such a heterogeneous population may theoretically represent a cumulative result of complement activation/binding during the preceding 120 days, confounding the differential diagnosis of ongoing disease activation versus an earlier SLE flare. To overcome this problem, we established another assay examining the level of complement C4-derived activation products bound to reticulocytes (R-C4d). These cells are the youngest erythrocytes that emerge from the bone marrow, and they circulate in the blood for approximately 1 day before fully maturing into erythrocytes. We speculated that a high level of C4d identified on reticulocytes may indicate ongoing complement activation and reflect the degree of SLE disease activity on that day. Indeed, upon examining the presence of C4-derived products on reticulocytes of SLE patients, a wide spectrum of R-C4d ranging from undetectable to high levels was detected (Figs. 2 and 3). As noted above, during our studies of age-fractionated erythrocytes of SLE patients, high E-C4d levels were detected on younger erythrocytes, but not older erythrocytes, in some patients (Fig. 2, left lower panel patient #1066). Interestingly, reticulocytes of these same patients also exhibited high R-C4d levels (Fig. 2, right lower panel patient #1066). Moreover, preliminary inspection suggested a correlation between the R-C4d levels and clinical disease activity. These initial studies supported our hypothesis that reticulocytes may serve as “instant messengers” by carrying products of complement activation to reflect ongoing disease activity in SLE patients.

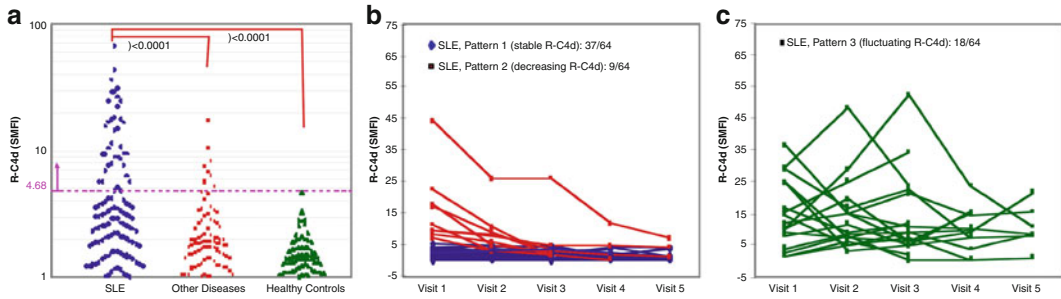


Fig. 3 Reticulocyte-C4d levels are significantly elevated in patients with SLE and fluctuate over time. (a) Reticulocytes from patients with SLE have significantly higher levels of C4d than those from patients with other diseases or healthy controls. Shown on the Y-axis is the C4d-specific median fluorescence intensity for reticulocytes from 156 patients with SLE, 140 patients with other diseases, and 159 healthy controls. The *pink line* represents an empirically determined cutoff point. Numbers of SLE patients, patients with other diseases, and healthy controls with R-C4d higher than this point are shown in *pink* in the X-axis legend (see text for details). (b and c) R-C4d levels fluctuate in a significant fraction of patients with SLE. Shown are R-C4d levels of 64 patients with SLE examined at three to five different study visits. In 37 patients, R-C4d remained stably low. In nine patients, R-C4d was elevated at the first visit but decreased in subsequent visits. Significant fluctuation of R-C4d was observed in 18 patients

To date, we have analyzed, in both cross-sectional and prospective fashions, R-C4d levels in 156 patients with SLE, 140 patients with other autoimmune diseases, and 159 healthy individuals. The R-C4d levels were found to be significantly higher in SLE patients than in patients with other autoimmune diseases or healthy controls. Moreover, during longitudinal observation, the R-C4d levels in a significant fraction of SLE patients varied considerably over time (Fig. 3), suggesting that fluctuations in R-C4d levels coincide with changes in disease activity. In addition, within the SLE patient population, the level of R-C4d appears to be proportionate to the clinical disease activity in a given SLE patient, i.e., patients with higher R-C4d levels have higher disease activity scores. These results strongly suggest that R-C4d levels, in contrast to E-C4 levels, reflect more precisely and promptly ongoing disease activity in an SLE patient, supporting the role for reticulocytes as “instant messengers” of SLE disease activity.

5.3.1 Method:

Reticulocyte

CB-CAP Assays

A two-color immunofluorescence staining method was employed to examine the levels of C4- and C3-derived CAPs on reticulocytes. Briefly, 10 μ l of the erythrocyte suspension (containing reticulocytes), was incubated sequentially with (1) anti-human C4d or anti-C3d mAb or isotype-matched mouse IgG control followed by a phycoerythrin (PE)-conjugated anti-mouse IgG antibody and (2) thiazole orange (a supravital dye emitting green fluorescence; Retic-Count™ reagent; Becton Dickinson). All mAbs were used at a concentration of 10 μ g/ml. After staining, cells were analyzed

using a FACSCalibur™ cytometer and CellQuest Software. Reticulocytes were identified by thiazole orange staining as well as forward and side scatter properties.

5.4 Platelet-Bound C4d as a Biomarker for Stratification in SLE

The erythrocyte studies, as mentioned above, were initially conducted because normal red blood cells are known to be unique in their C4d-positive phenotype, despite not bearing any receptors for C4d. Demonstration of the utility of E-C4d and RC4d as lupus biomarkers suggested that cells other than those in the erythroid lineage might also carry the CB-CAP phenotype. Therefore, our attention turned to the platelet [15].

In view of the biological role of platelets in hemostasis, coagulation, and thrombosis, we postulated that abnormal CB-CAPs on platelets may serve as a useful biomarker for SLE patients who are at increased risk of cardiovascular and cerebrovascular events. Using flow cytometric analysis, P-C4d measure was shown to be a specific (98 %) diagnostic assay for SLE. Moreover, this study showed that P-C4d correlated with a history of neurological event ($p=0.006$) and positive antiphospholipid antibody tests ($p=0.013$), a clinical manifestation, and a known risk factor for thrombotic complications of SLE, respectively. This observation suggested that P-C4d may represent a stratification biomarker capable of identifying a unique set of SLE patients with increased risk for developing cerebrovascular and neurologic complications.

Most recently, PC4d was also shown to be associated with stroke and with all-cause mortality in SLE patients [16]. In this study, a cohort of 356 consecutive patients with SLE was followed from 2001 to 2009. Seventy SLE patients (19.7 %) were positive for P-C4d. P-C4d was associated with all-cause mortality (hazard ratio 7.52, 95 % confidence interval/CI 2.14–26.45, $p=0.002$) after adjusting for age, ethnicity, sex, cancer, and anticoagulant use. Patients with positive P-C4d were also more likely to have had vascular events compared to those who were P-C4d negative (35.7 % versus 18.2 %, $p=0.001$). Specifically, P-C4d was associated with ischemic stroke (odds ratio 4.54, 95 % CI 1.63–12.69, $p=0.004$) after adjusting for age, ethnicity, and antiphospholipid antibodies. These observations suggest that PC4d may be a clue to a unique relationship shared by complement activation, the thrombocyte, cerebrovascular disease, and perhaps worse outcome for patients with SLE.

5.4.1 Method: Platelet CB-CAP Assays

Immediately after collection, an aliquot of the blood was diluted using $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and divided into equal-volume portions and stained for different molecules. Platelets were distinguished from other blood cells based on the expression of CD42b (GPIb) using a PE-conjugated anti-CD42b antibody (BD Biosciences). The presence of platelet-associated CAPs was examined by a

dual-color staining procedure using, in conjunction with PE anti-CD42b, a mouse anti-human C4d mAb (or anti-C3d mAb, or isotype-matched mouse IgG control) labeled with Alexa Fluor 488 (Invitrogen/Molecular Probes).

5.5 Lymphocyte CB-CAPs

Extending the panel of CB-CAPs from the erythroid to the megakaryocyte lineage suggested that all circulating cells might potentially have the capacity to carry CB-CAPs, and the myeloid cells were characterized as such [17]. Flow cytometric analysis of C4d on (T-C4d) and (B-C4d) cells demonstrated that both T-C4d and B-C4d levels are significantly and specifically elevated in SLE patients, as compared with healthy controls and patients with other diseases. Initial studies demonstrated that T-C4d and B-C4d are, respectively, 56 % sensitive and 80 % specific and 60 % sensitive and 82 % specific in differentiating SLE patients from patients with other diseases reflecting potential value as lupus diagnostic biomarkers.

In addition, levels of C4d bound to T and B cells of the same patients were strongly correlated ($r=0.708$; data not shown). Based on T-C4d and B-C4d levels, with those greater than the “mean plus 2 SD of the levels in healthy controls” defined as the “high” phenotype, the SLE patients could be classified into four subgroups: T-C4d^{Low}/B-C4d^{Low} (37.0 %), T-C4d^{High}/B-C4d^{Low} (5.8 %), T-C4d^{Low}/B-C4d^{High} (14.3 %), and T-C4d^{High}/B-C4d^{High} (42.9 %). Interestingly, we noticed that SLE patients with the T-C4d^{High}/B-C4d^{High} phenotypes were younger but had longer disease duration than did patients with the other phenotypes such that the T-C4d^{High}/B-C4d^{High} phenotype of a given SLE patient may be suggestive of earlier onset disease.

We also observed that, in contrast to the constant T-C4d levels in healthy individuals, T-C4d levels in a given SLE patient examined on different days vary significantly (Fig. 4), suggesting a potential for T-C4d as a biomarker for tracking SLE disease activity over time.

5.5.1 Method: Lymphocyte, Monocyte, and Granulocyte CB-CAP Assays

To simultaneously enrich mononuclear cells (lymphocytes and monocytes) and polynucleated granulocytes, we developed a simple method as an alternative to the conventional isolation of peripheral blood mononuclear cells by Ficoll-Paque gradient centrifugation. Briefly, 5 ml of blood was collected into an EDTA-containing Vacutainer™ tube (Becton Dickinson). After low-speed centrifugation, the buffy coat consisting of leukocytes was carefully transferred into a fresh tube and contaminating erythrocytes were hypotonically lysed. The leukocyte suspension was washed extensively with PBS to remove lysed erythrocytes, resuspended, divided into equal-volume portions, and stained for different cell surface markers and C4- or C3-derived CAPs. Lymphocytes, monocytes, and granulocytes were distinguished based on their unique features of forward (size)/side (granularity) scattering and expression of

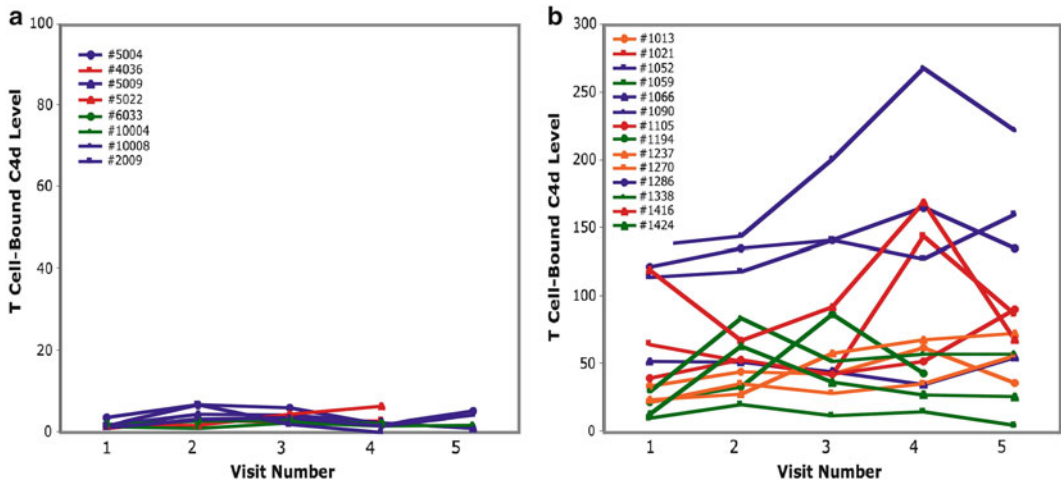


Fig. 4 T cell-bound C4d levels in SLE patients fluctuate over time but remain stable in patients with other diseases and healthy controls. Shown in (a) are T-C4d levels of patients with Sjogren's syndrome, rheumatoid arthritis, or inflammatory myopathy or of healthy controls examined at four or five different visits. Shown in (b) are T-C4d levels of 14 SLE patients examined at five visits. Y-axis: specific median fluorescence intensity

characteristic surface molecules. Granulocytes were differentiated from mononuclear cells by forward/side scatter properties through electronic gating. The presence of CAPs on cells was examined by three-color or four-color flow cytometric analysis, in which mAbs reactive with lineage-specific cell surface markers (e.g., CD3, CD4, CD8 for T cells, CD19 for B cells, and CD14 for monocytes; from BD Biosciences) were used in conjunction with either an anti-human C4d mAb (Quidel) or an anti-human C3d mAb (Quidel). All mAbs were used at a concentration of 10 $\mu\text{g}/\text{ml}$. After staining, cells were analyzed using a FACSCalibur™ cytometer and CellQuest Software. To ensure the specificity of the primary antibody, leukocyte aliquots from each patient stained with mouse IgG of appropriate isotypes were routinely included in all experiments.

5.6 Validation of the CB-CAP Technology Platform

A biomarker platform can be defined as a group of selected biomarkers, the assays to detect them, and algorithms to interpret and translate the results. Prior to being implemented for clinical decision-making and patient care, a biomarker platform requires carefully conducted validation studies in well-characterized patient cohorts. The validated biomarker platform should be accurate, stable, reliable, and sensitive to measure its intended application such as disease presence (diagnosis), activity (monitoring) subsets (stratification), and/or theranostics (precision or personalized medicine).

Single-center validation of CB-CAPs as lupus biomarkers for both diagnosis and monitoring has been reported by Yang et al. who demonstrated significant elevation of EC4d levels in patients

with SLE compared to healthy subjects and patients with other diseases [18]. In addition, EC4d levels were correlated with the SLEDAI and inversely correlated with serum C3 and C4 levels.

In addition to these independent single-center validation studies, a multicenter validation effort was conducted independent of our patient cohort and laboratories where the assays were discovered. This effort required transfer of the technology to a central laboratory at Exagen Diagnostics, San Diego. Prior to the technology transfer, all CB-CAP studies had been performed in our laboratory on patient samples that had been obtained in a clinic proximate to the laboratory, without the need for commercial shipping. Since CB-CAP assays require fresh cells for the flow cytometric assays, the influence of time and temperature during shipping was essential to determine as the first step in the transfer of the CB-CAP technology platform. Samples were obtained from study subjects on day 0, split into two aliquots, and assayed immediately for levels of E-C4d, P-C4d, B-C4d, R-C4d, and T-C4d. The second aliquot of each sample was shipped overnight at ambient temperature to our own laboratory and assayed on day 1. The results of the paired assays performed on each sample were compared and shown to generate nearly identical results, indicating that overnight shipping of whole blood samples at ambient temperature would not compromise the integrity of the CB-CAP assays (Fig. 5). Following this quality control study, Kalunian et al. completed the CAPITAL study, a multicenter validation of CB-CAPs as diagnostic lupus biomarkers [19]. This study was conducted at 14 sites in the United States by investigators with lupus expertise. An assay panel consisting of E-C4d, B-C4d, anti-mutated citrullinated vimentin antibody (anti-MCV), ANA, and anti-dsDNA was evaluated in a cross-sectional study of 593 well-characterized subjects (210 SLE patients, 178 patients with other rheumatic diseases, and 205 healthy subjects). An algorithm was generated to calculate an index score, which was 80 % sensitive for SLE and 87 % specific versus other rheumatic diseases. An ongoing companion study focused on validation of CB-CAPs as biomarkers for monitoring disease activity in patients with SLE was launched in 2012. The success of the multicenter CAPITAL validation study coincided with the commercial launch of the Avise SLE test (Exagen Diagnostics, San Diego) that contains the same assay panel, including EC4d and BC4d, as that validated in the CAPITAL study.

6 The CB-CAP Signature

Collectively, these data clearly demonstrated that CAPs generated during systemic or local activation of the complement system in patients with SLE are capable of binding to essentially all circulating blood cells. However, it remained to be determined whether CAP

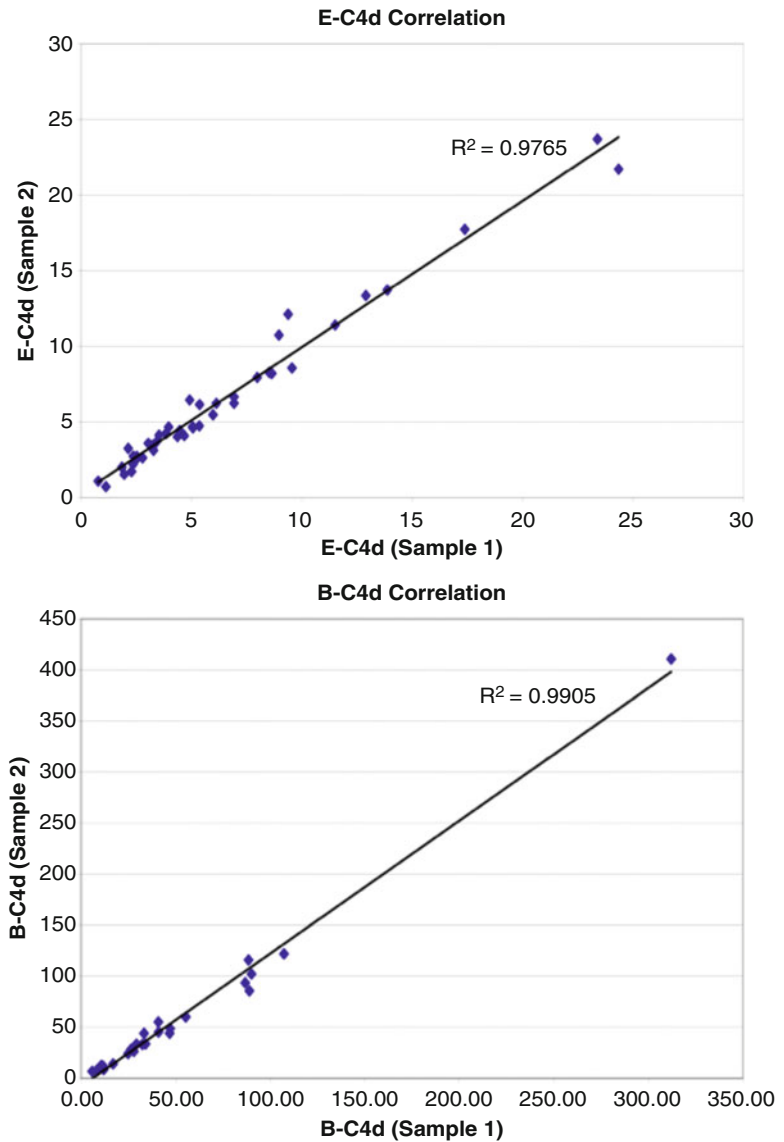


Fig. 5 Shipping stability of CB-CAPs. CB-CAP levels remain stable after overnight shipment of blood samples. Aliquots of the blood sample derived from the same patient were analyzed for CB-CAPs on the day of blood drawn (sample 1) or the next day after overnight shipment (sample 2). Shown are the comparisons of E-C4d and B-C4d levels on the blood sample pairs prepared from 53 patients. The correlation between the datasets of sample 1 and sample 2 was evaluated using the Pearson correlation coefficient calculation. Note the excellent correlation coefficients for both the E-C4d and B-C4d comparison

deposition occurs nonspecifically and indiscriminately on all cells in a given patient as a result of systemic complement activation or whether specific cell types are targeted and these targets differ among individual patients. Therefore, we conducted a pilot study

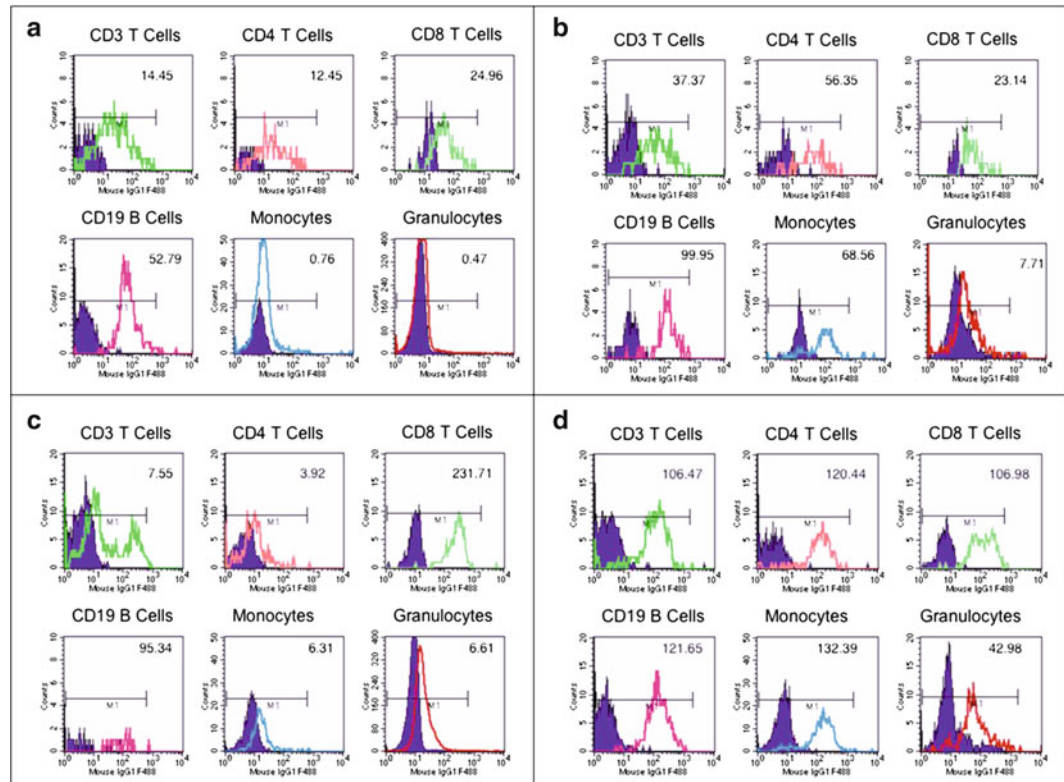


Fig. 6 CAPs bind to circulating blood cells in cell type-specific patterns in individual SLE patients. Shown are the CB-C4d histograms of the differential CB-CAP signatures of four representative SLE patients. The levels of erythrocyte-bound C4d (E-C4d), reticulocyte-bound C4d (R-C4d), and platelet-bound C4d (P-C4d) of these patients are, respectively, 4.46, 1.10, 0.00 (patient A; all within normal range), 12.16, 5.08, 9.23 (patient B; all in abnormal range), 7.59, 2.36, 3.96 (patient C; R-C4d moderately elevated, P-C4d elevated), and 6.57, 1.30, 30.10 (patient D; P-C4d highly abnormal)

to characterize simultaneously C4d deposition on a panel of circulating blood cell types in patients with SLE. The results demonstrated that high levels of C4d were not necessarily present concurrently on erythrocytes, platelets, lymphocytes, monocytes, and granulocytes of a given SLE patient on a particular day (Fig. 6). Moreover, the cell type-specific pattern/signature (not absolute level) of C4d deposition appeared to remain stable in a given patient over time. Together, these observations indicate that a patient-dependent, cell lineage-specific mechanism is responsible for an individual's CB-CAP signature. It is not simply due to indiscriminate complement activation that affects all cells in the circulation simultaneously and equally. These observations led us to conclude that CB-CAP signatures are highly characteristic of lupus patients and to propose the model shown in Fig. 7. This model is based upon the hypothesis that CB-CAP signatures may provide value as lupus biomarkers beyond the value contributed by each

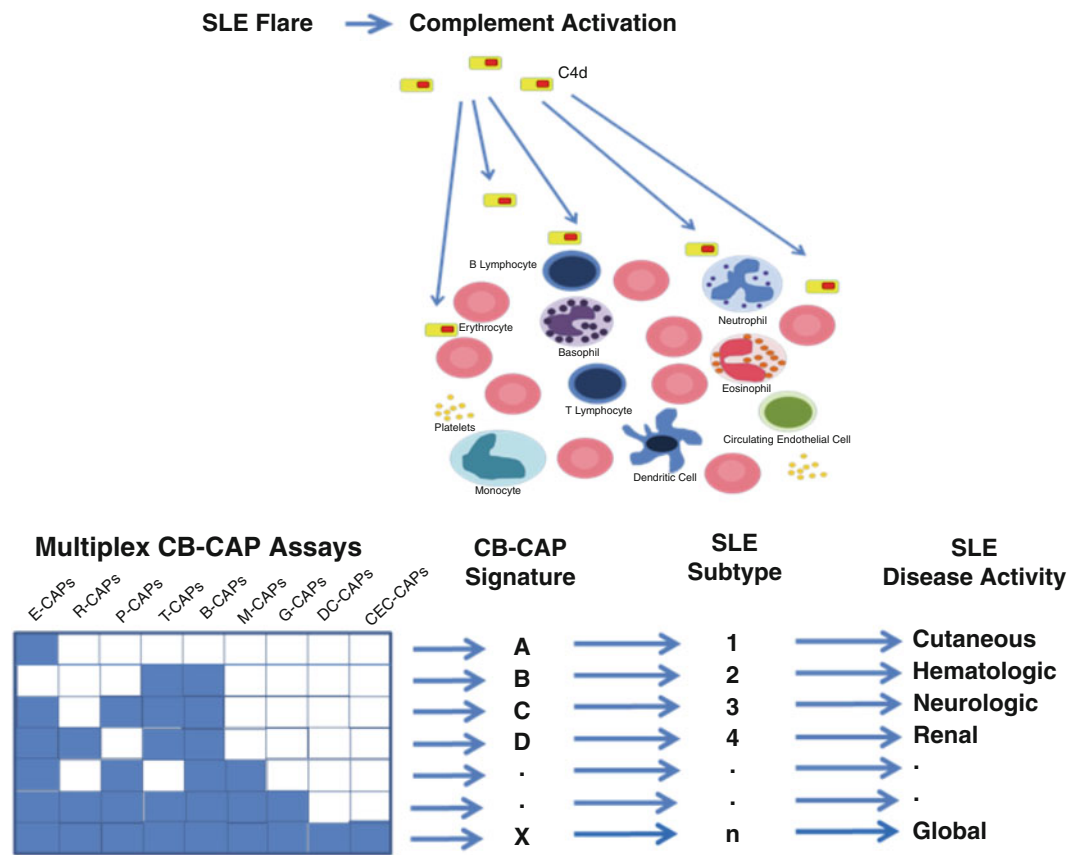


Fig. 7 The CB-CAP signature. Patient-specific patterns of CB-CAP deposition on distinct lineages and subsets of circulating cells may ultimately be proven to correlate with and/or predict clinical stratification, autoantibody profiles, or response to specific therapies

individual CB-CAP. Studies are in progress to determine if CB-CAP signatures might serve as biomarkers for lupus diagnosis, monitoring, prediction of flare, stratification, and/or response to specific therapies.

7 CB-CAPs in Lupus Pathogenesis

The collective findings described above also led to the hypothesis that CB-CAPs, upon binding to circulating cells, may lead to dysfunction of these cells and in turn contribute to a wide range of pathophysiologic mechanisms in patients with SLE. CB-CAP deposition may not only serve as lupus biomarkers but they may also contribute to tissue damage and the disease process. This hypothesis has been confirmed by two studies to date. First, Kyttaris and colleagues have shown that deposition of E-C4d leads to calcium-dependent cytoskeletal changes in RBC that render them less

deformable, possibly impairing their capacity to flow through capillaries and deliver oxygen to tissues [20]. The second demonstration that CB-CAP deposition leads to cellular dysfunction was reported by Tsokos and colleagues who demonstrated that C3d fragments are localized to lipid rafts in T cells of patients with SLE and contribute to cellular dysfunction by modulating calcium influx responses and significantly increasing production of IL-2, IL-4, IL-17, and IFN-gamma [21].

8 Conclusion

There is an urgent need for lupus biomarkers to diagnose, monitor, stratify, and predict patient flares and response to therapy. The steep trajectory of advance in this field is unprecedented yet still impeded by obstacles such as the complexities of disease heterogeneity, difficulties in accurate diagnosis even by experienced rheumatologists, and required comparisons of potential value of new biomarkers to gold standards that have never themselves been validated and standardized despite decades of use by clinicians. We have undertaken a candidate approach to lupus biomarker discovery based upon long-standing recognition of the uniquely intimate link between lupus and the complement system. This approach has led to the discovery of CB-CAPs, which now include E-C4d, E-C3d, P-C4d, P-C3d, T-C4d, T-C3d, B-C4d, B-C3d, and E-CR1, among others. E-C4d and B-C4d, as part of the Avise SLE panel, are the first lupus biomarkers to be successfully validated in a multicenter study, and other CB-CAPs have shown promise as biomarkers for diagnosis, stratification, monitoring, and precision medicine. In addition to their value as lupus biomarkers, deposition of CB-CAPs may also contribute to lupus pathogenesis by rendering cells dysfunctional and may thereby represent a potential target for therapeutic intervention.

References

1. Ahearn JM, Liu CC, Kao AH, Manzi S (2012) Biomarkers for systemic lupus erythematosus. *Transl Res* 159:326–342
2. Liu CC, Kao AH, Manzi S, Ahearn J (2013) Biomarkers in systemic lupus erythematosus: challenges and prospects for the future. *Ther Adv Musculoskelet Dis* 5:210–233
3. Liu CC, Ahearn JM (2009) The search for lupus biomarkers. *Best Pract Res Clin Rheumatol* 23:507–523
4. Liu CC, Manzi S, Ahearn JM (2005) Biomarkers for systemic lupus erythematosus: a review and perspective. *Curr Opin Rheumatol* 17:543–549
5. Narain S, Richards HB, Satoh M, Sarmiento M, Davidson R, Shuster J (2004) Diagnostic accuracy for lupus and other autoimmune diseases in the community setting. *Arch Intern Med* 164:2435–2441
6. Fritzler M (2011) The antinuclear antibody test: last or lasting gasp? *Arthritis Rheum* 63:19–22
7. Liu CC, Manzi S, Kao AH, Navratil JS, Ahearn JM (2010) Cell-bound complement biomarkers for systemic lupus erythematosus: from benchtop to bedside. *Rheum Dis Clin North Am* 36:161–172

8. Calano SJ, Shih PB, Liu CC, Kao AH, Navratil JS, Manzi S et al (2006) Cell-bound complement activation products (CB-CAPs) as a source of lupus biomarkers. *Adv Exp Med Biol* 586:381–390
9. Liu CC, Danchenko N, Navratil JS, Nilson SE, Manzi S, Ahearn JM (2005) Mining the complement system for lupus biomarkers. *Clin Appl Immunol Rev* 5:185–206
10. Liu CC, Manzi S, Danchenko N, Ahearn JM (2004) New advances in measurement of complement activation: lessons of systemic lupus erythematosus. *Curr Rheumatol Rep* 6:375–381
11. Liu CC, Ahearn JM, Manzi S (2004) Complement as a source of biomarkers in systemic lupus erythematosus: past, present and future. *Curr Rheumatol Rep* 6:85–88
12. Manzi S, Navratil JS, Ruffing MJ, Liu CC, Danchenko N, Nilson SE et al (2004) Measurement of erythrocyte C4d and complement receptor 1 in systemic lupus erythematosus. *Arthritis Rheum* 50:3596–3604
13. Kao AH, Navratil JS, Ruffing MJ, Liu CC, Hawkins D, McKinnon KM et al (2010) Erythrocyte C3d and C4d for monitoring disease activity in systemic lupus erythematosus. *Arthritis Rheum* 62:837–844
14. Liu CC, Manzi S, Kao AH, Navratil JS, Ruffing MJ, Ahearn JM (2005) Reticulocytes bearing C4d as biomarkers of disease activity for systemic lupus erythematosus. *Arthritis Rheum* 52:3087–3099
15. Navratil JS, Manzi S, Kao AH, Krishnaswami S, Liu CC, Ruffing MJ et al (2006) Platelet C4d is highly specific for systemic lupus erythematosus. *Arthritis Rheum* 54:670–674
16. Kao AH, McBurney CA, Sattar A, Lertratanakul A, Wilson NL, Rutman S et al (2013) Relation of platelet C4d with all-cause mortality and ischemic stroke in patients with systemic lupus erythematosus. *Transl Stroke Res* (in press)
17. Liu CC, Kao AH, Hawkins DM, Manzi S, Sattar A, Wilson N (2009) Lymphocyte-bound complement activation products as biomarkers for diagnosis of systemic lupus erythematosus. *Clin Transl Sci* 2:300–308
18. Yang DH, Chang DM, Lai JH, Lin FH, Chen CH (2009) Usefulness of erythrocyte-bound C4d as a biomarker to predict disease activity in patients with systemic lupus erythematosus. *Rheumatology* 48:1083–1087
19. Kalunian KC, Chatham WW, Massarotti EM, Reyes-Thomas J, Harris C, Furie RA et al (2012) Measurement of cell-bound complement activation products enhances diagnostic performance in systemic lupus erythematosus. *Arthritis Rheum* 64:4040–4047
20. Ghiran IC, Zeidel ML, Shevkoplyas SS, Burns JM, Tsokos GC, Kyttaris VC (2011) SLE serum deposits C4d on red blood cells, decreases red blood cell membrane deformability, and promotes nitric oxide production. *Arthritis Rheum* 63:503–512
21. Borschukova O, Paz Z, Ghiran IC, Liu CC, Kao AH, Manzi S et al (2012) Complement fragment C3d is colocalized within lipid rafts of T cells and promotes cytokine production. *Lupus* 21:1294–1304

Systemic Lupus Erythematosus

Methods and Protocols

Eggleton, P.; Ward, F.J. (Eds.)

2014, XVII, 263 p. 35 illus., 22 illus. in color., Hardcover

ISBN: 978-1-4939-0325-2

A product of Humana Press