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14. ABSTRACT Ongoing complement activation is a feature of patients with systemic lupus erythematosus. Complement activation products deposit on the surface of cells and alter their function. During the course of this project we established that: 1) There is a significant difference in the distribution of complement proteins on T cells from SLE patients compared to other autoimmune diseases and normal individuals (C3d, C4d, CD59). 2) Complement deposition (C4d and C3d) is associated with SLE disease activity. 3) Complement deposition affect T cells function and decrease (or goes along with) Ca flux, IL- 2 production and cytotoxic activity. 4) C3d decorated T cells are prone to produce the proinflammatory cytokine IL-17 which may contribute to the inflammation-initiated tissue damage in SLE patients.					
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Introduction:

The complement system plays an important role in protection against pathogenic organisms and modulates several aspects of the humoral and cellular immune response. Complement is invariably activated in patients with infectious and autoimmune diseases including systemic lupus erythematosus (SLE). Complement activation is linked to the expression of tissue pathology through either the formation of immune complexes or its direct deposition on tissues and cell surface membranes. The complement membrane attack complex C5b-9 binds to cell membranes, independent of any receptor, and it may activate multiple signaling pathways. Complement is known to alter B and T cell function. In B cells, it lowers the activation threshold and supports the development of B-cell memory and immune tolerance. Complement modulate antigen- presenting cell (APCs) and indirectly T cell function. The complement system and its regulatory proteins have an important role in the determination of T cell responses by modulating T cell apoptosis and inducing regulatory T cells. Recent studies have shown that complement can initiate or modulate a surface membrane signaling response in T cells. However, the underlying signaling pathways involved in these effects are still obscure. Ahearn and colleagues have found that complement activation fragments C3d and C4d are deposited on the surface membrane of erythrocytes and lymphocytes in patients with SLE. The deposition of C4d on the surface of red cells may represent a clinically useful disease biomarker. These observations lead to the hypothesis that lymphocyte-bound C3d and C4d may also serve as biomarkers for lupus diagnosis. In addition, deposition of the complement fragments C4d and C3d on the surface of T cells has unknown as of yet

functional repercussions. T cells are major contributors to SLE pathogenesis. SLE T cells display aberrant cell surface mediated signaling responses, and they produce increased amounts of IL-17 although the production of IL-2 is compromised. Interestingly, lipid rafts, cholesterol rich domains on the surface of cells harboring signaling molecules aggregate on the surface membrane of SLE T cells and contribute to disease pathology in lupus-prone mice. We assumed that the complement activation product C3d might alter the functional activity of these T cells.

The work supported by this grant represents the first report of that lymphocytes (Both B cells and T cells) from SLE patients are highly deposited with C3d and C4d (complement activation products), results suggest strong potential for the use of these as diagnostic biomarkers for SLE (Paper1: **“Lymphocyte-Bound Complement Activation Products**

as Biomarkers for Diagnosis of Systemic Lupus Erythematosus”). In addition, we

report here that C3d fragments localized within the lipid rafts of SLE T cells and

contribute to abnormal T cell function (Paper2: **“Complement fragment C3d is localized within the lipid raftsof T cells and promotes cytokine production”**).

Moreover, when C4d bound to critical surface membrane proteins of SLE T cells may lead to aberrant signal transduction and additional downstream effects, which in turn may contribute to T cell dysfunction and overall abnormalities of the immune system in SLE patients (Paper3: **“Cell-Bound Complement Activation Products and T Cell Dysfunction in Systemic Lupus Erythematosus”**).

Body:

In this report we will present the data gathered in the last year of the grant. In addition, see attached three manuscripts that present the data collected by the grant support.

Localization of C4d on the surface of SLE T cells

An interesting observation reported in **paper1** is that C4d is significantly highly deposited over the surface of SLE T cells, as demonstrated by uniformly shift-to-the-right fluorescent intensity histograms (Fig. 1A). Initial characterization of T cells from SLE patients, patients with other diseases, and healthy controls failed to detect surface-expression of complement receptor (CR) 1, CR2, and Fc receptors (FcR) (data shown in the 2009 report), suggesting that C4d is unlikely to bind through CR and FcR to the surface of SLE T cells. We speculate that, if C4d depositions are deposited at or near lipid rafts, they may gain close access to the TCR/CD3 complexes and consequently alter downstream signaling processes. To investigate the localization of C4d on the surface of SLE T cells confocal microscopy was performed. As shown in Figure 1B, C4d appeared to be present on SLE cells in various patterns, ranging from a punctuate pattern with a concentrated “cap” (upper panels) to a diffuse distribution (middle panels). Interestingly, C4d appeared to co-localize with CD3 (upper panels) and lipid rafts (middle panels) on SLE T cells. Because lipid rafts are known to be enriched in TCR/CD3 complexes and other signaling molecules, SLE T cells were also stained with CT-B (cholera toxin B which is known lipid raft marker) and anti-CD3 to ensure the authenticity of the staining patterns

observed (lower panels). Together, these results suggested that C4d is associated with signaling molecules in lipid rafts of SLE T cells.

To further verify the possibility that C4d may covalently bind to surface molecules that are essential for T cell signaling (e.g., the TCR/CD3 complex), co-immunoprecipitation experiments were performed. As revealed by the representative results shown in Fig. 2, an anti-C4-reactive protein ($M_r \sim 65\text{-}70\text{ kD}$) was present in the eluate of anti-CD3-coated beads, but not in that of mouse IgG-coated beads. The apparent molecular mass of this molecule suggests that it is a complex of C4d ($M_r 45\text{ kD}$) and a subunit of the TCR/CD3 complex (e.g., CD3 γ , CD3 ϵ , or CD3 δ ; M_r ranging from 20-23 kD). This result implied that C4-derived products are associated with the TCR/CD3 complexes in SLE T cells and thus may play a role in perturbing T cell signaling and functioning.

Abnormal signaling processes in C4d-bearing SLE T cells

The myriad abnormalities reported for SLE T cells may, at least partially, stem from dysregulated signaling processes in these cells. Therefore, it is important to determine if the intracellular signaling pathways are abnormal in SLE T cells and whether abnormal signaling processes may be associated with C4d deposition on T cells. Measurement of phosphorylation of signaling proteins has commonly been performed to investigate signaling pathway. Thus, intracellular phosphoprotein staining studies were performed to examine the Jak-Stat pathway and MAPK/ERK pathway, two important families of signaling proteins that regulate immune response, in T cells. Specific responses of these signaling pathways to different stimuli occurred in an expected manner. However, erratic

responses were detected in T cells from SLE patients (Fig. 3). The signaling responses to cytokines and mitogens appeared to remain relatively normal in SLE T cells bearing none or low levels of C4d. However, these signaling proteins appeared to respond hyperactively or non-specifically in T cells bearing high levels of C4d. These results not only demonstrated irregular signaling processes in SLE T cells, but also implied a causative relationship between the binding of C4d to T cell signaling molecules and aberrant signaling events.

Increased cytokine production by C4d-bearing SLE T cells

It is known that the immune abnormalities in SLE include skewed production of cytokines by T cells and other immune effector cells. We therefore speculated that the binding of C4d to peripheral blood cells of SLE patients may trigger specific intracellular signals that ultimately influence the production of cytokines. To explore this possibility, antibody array experiments were performed to determine the global profile of cytokine production by peripheral blood mononuclear cells derived from SLE patients and healthy individuals. Cells derived from SLE patients with the T-C4d phenotype appeared to produce higher levels of IL-8, IL-4, IL-12, and IL-17 than did cells derived from healthy controls (Fig. 4A). The increase in cytokine production by C4d-bearing cells derived from SLE patients was further corroborated by real-time RT-PCR analyses of transcripts encoding various cytokines (Fig. 4B). IL-17, a pro-inflammatory cytokine, is produced predominantly by Th17 cells. Elevated levels of IL-17 have also been detected in the sera of patients with SLE. . In this context, the observed increase in IL-17 proteins and transcripts in C4d-bearing SLE T cells warranted further investigation. Thus, intracellular cytokine staining

was performed to analyze the production of IL-17 by SLE T cells at the single-cell level. As shown in Fig. 4C, significantly higher frequencies of IL-17-producing CD4 T cells were detected in SLE patients, as compared to patients with other autoimmune diseases and healthy controls. This result provides additional support for the hypothesis that Th17 cells are involved in the pathogenesis of SLE.

Dysregulated proliferative responses of C4d-bearing SLE T cells

It is conceivable that binding of C4d to targets on T cells may directly influence a variety of cellular activities that culminate in cell proliferation. Since it has been reported that T cells from lupus-prone mice and SLE patients exhibited heightened proliferation responses upon stimulation *in vitro*, we postulated that deposition of C4d on T cells may, in part, be responsible for altering the reactivity and proliferation of T cells in SLE patients. As shown in Fig. 5, T cells bearing high levels of C4d (SLE patients) proliferated more readily and profoundly than T cells bearing low levels of T-C4d (patient #1096). These data suggested that deposition of C4d on the surface of SLE T cells may prime them and consequently heighten their responses to antigenic or inflammatory stimuli.

Cytokine production by C3d+ T cells is increased

To assess the functional importance of C3d fragments we compared the profile of cytokine production between the C3d+ T cell population and the C3d- T cell population and between the different study groups. We used conjugated antibody against C3d combined with intracellular staining for different cytokines and recorded the data using flow cytometry studies. The production (by the whole T cell population) of IL-2 and IFN-

gamma was decreased in the SLE T cells when compared with T cells from normal ($p < 0.05$) and OAD patients ($P < 0.05$). However, the subpopulation of C3d⁺ T cells produced significantly more IL-2, IFN- γ , IL-4 and IL-17 (Figure 6 A-D) when compared to the C3d⁻ T cells. The increased production of various cytokines by the C3d⁺ T cell population was not limited to T cells from SLE patients and a similar pattern was observed in T cells from patients with OAD and normal subjects.

C3d partially co-localized within aggregated lipid rafts on a subpopulation of SLE T cells.

To investigate this possibility that C3d deposit into the lipid rafts, we used wide-field fluorescence microscopy (Figure 7A), followed on some cases by de-convolution (Figure 7D) to analyze the cell surface distribution of the cholera toxin B (CTB) conjugated to AF- 488 (green) and an anti-C3d antibody conjugated to AF- 568 (red).. Even though the SLE T cells were not stimulated, in a subpopulation of T cells the lipid rafts were clustered, as reported previously. By microscopical analysis, the percentage of double positive T cells (for both C3d and CTB) represented about 10% of all the cells investigated. Figure 7A shows a low magnification of several T cells stained for both lipid rafts (green) and complement fragment C3d (red), imaged at their largest diameter. The region of interest on the plasma membrane of one of the T cells is shown in yellow in Figure 7B. Our results (Figure 7C) show that only certain C3d molecules were associated with lipid rafts (peaks 1 and 4) whereas others (peaks 2, 3 and 5) showed minimal or no co-localization. To further analyze the extent of co-localization between C3d and lipid rafts, we performed three-dimensional reconstitution of T cells, and the collective signal

from the entire membrane surface was analyzed (Figure 7D). Our results show that on certain areas of T cell surface, C3d fragments were adjacent to lipid rafts (Figure 7D, top insert), fully co-localized (Figure 7D, middle insert) or were separate (Figure 7D, bottom insert). Twenty one percent of the lipid raft molecules co-localized with C3d fragments, whereas 39% of the total C3d signal co-localized with clustered lipid rafts. Taken together, these results suggest that in a subpopulation of SLE T cells, about 40% of the cell surface C3d fragments bind to lipid rafts.

Key research accomplishment:

1. C4d bound to critical surface membrane proteins of SLE T cells – Lipid rafts and CD3.
2. C4d is associated with aberrant signal transduction and additional downstream effects, which in turn may contribute to T cell dysfunction and overall abnormalities of the immune system in SLE patients.
3. There is increased cytokine production by C4d + SLE T cells.
4. T cells bearing high levels of C4d (SLE patients) proliferated more readily and profoundly than T cells bearing low levels of T-C4d.
5. These and previous studies of T-C4d suggest that these lymphocytes, tagged with ligands generated during complement activation, may serve as lupus biomarkers and also identify potential targets for therapeutic intervention.
6. There is increased cytokine production by C3d+ T cells.
7. C3d is partially co-localized within aggregated lipid rafts on a subpopulation of SLE T cells.

Reportable outcome: ATTACHED PDFs

1. Liu CC, Kao AH, Hawkins DM, Manzi S, Sattar A, Wilson N, Ahearn JM.
Lymphocyte-bound complement activation products as biomarkers for diagnosis of systemic lupus erythematosus. *Clin Transl Sci*. 2009 Aug;2(4):300-8.
2. Olga Borschukova, Ziv Paz, Ionita C. Ghiran, Chau-Ching Liu, Amy H Kao, Susan Manzi, Joseph M Ahearn. and George C Tsokos. Complement fragment C3d is localized within the lipid rafts T cells and promotes cytokine production. *Lupus*. In revision.
3. Chau-Ching Liu, George C. Tsokos , Amy H. Kao, Susan Manzi, and Joseph M Ahearn Cell-Bound Complement Activation Products and T Cell Dysfunction in Systemic Lupus Erythematosus. In press.

Conclusion:

C4d bounds to critical surface membrane proteins of SLE T cells may lead to aberrant signal transduction and additional downstream effects, which in turn may contribute to T cell dysfunction and overall abnormalities of the immune system in SLE patients. These and previous studies of T-C4d suggest that these lymphocytes, tagged with ligands generated during complement activation, may serve as lupus biomarkers and also identify potential targets for therapeutic intervention.

C3d fragments are localized in the lipid rafts of SLE T cells and contribute to abnormal T cell function by increasing cytokine production. The propensity of C3d decorated T cells to produce more cytokines is not limited to SLE T cells and it appears to represent a general phenomenon. Probably, complement activation that occurs during the response to

any pathogen may facilitate cytokine production to further improve that ability of the host organism to eliminate intruding pathogens. The increased numbers of CD4⁺ T cells in patients with SLE may though contribute to the immunopathogenesis of the disease because increased production of IL-4, and IL17 may further advance the inflammatory response and tissue damage.

References

None

Appendices:

1. Three manuscripts are attached as PDF files
2. Figures and figures legends are attached as word file

Supporting data:

None

Figure Legends

Figure 1. C4d is present on the surface of the entire population of SLE T cells and is colocalized with CD3 in lipid rafts within T cell membranes. (A) Representative histograms of flow cytometric analyses of C4d present on the surface of SLE T cells. Note the significant and complete shift-to-the-right of SLE T cells stained with anti-C4d mAb. Closed peak: isotype control mouse IgG1; open peak: anti-C4d. **(B)** Representative confocal microscopic images of C4d-positive SLE T cells. PBMC isolated from an SLE patient with the positive T-C4d phenotype were stained with an anti-C4d labeled with Alexa Fluor 488 (green) and an anti-CD3 mAb labeled with Alexa Fluor 647 (deep red) (upper panels) or with FITC-conjugated cholera toxin beta subunit (CT-B) (green) and Alexa Fluor 647-labeled anti-C4d antibody (deep red) (middle panels) or Alexa Fluor 647-labeled anti-CD3 antibody (deep red) (lower panels). 400X.

Figure 2. C4d is associated with TCR/CD3 complexes within the surface membrane of SLE T cells. Total lysates of PBMC derived from SLE patients with the positive T-C4d phenotype were subjected to co-immunoprecipitation analysis as described in the text. Shown in the figure is a representative blot developed using the ECL method. Lane 1: 2nd eluate of anti-CD3 co-IP; lane 2: 2nd eluate of mIgG1 co-IP; lane 3: 3rd eluate of anti-CD3 co-IP; lane 4: 3rd eluate of mIgG1 co-IP; lane 5: purified human C4b.

Figure 3. Aberrant intracellular signaling in SLE T cells bearing high levels of T-C4d. PBMC isolated from study participants were briefly stimulated and subjected to intracellular phosphoprotein staining. Results shown were derived from T cells gated on positive staining with anti-CD3 mAb. **(A)** Histograms of intracellular phosphoprotein flow cytometric analysis of

unstimulated or stimulated cells derived from representative study subjects (3 healthy controls, 3 patients with other diseases, 3 SLE patients with the negative T-C4d⁺ phenotype, and 3 SLE patients with the positive T-C4d phenotype). Arrowheads point to abnormal responses of protein phosphorylation that occurred spontaneously or inappropriately in response to T cell stimuli. Phosphorylation of appropriate signaling molecules (“normal” responses) in response to respective stimuli is indicated by arrows. **(B)** Bar graph summary of the flow cytometric analysis shown in (A). Arrows point to abnormal patterns of phosphorylation that occurred spontaneously or inappropriately in response to T cell stimuli. Y axis: specific median fluorescence intensity of phosphorylated signaling molecules stained with antibodies specific for the respective phosphorylated form.

Figure 4. C4d-positive SLE T cells produce more cytokines than C4d-negative T cells. (A) Lysates of PBMC prepared from a healthy control and 2 representative SLE patients with the T-C4d-positive phenotype were analyzed for cytokine production. The Antibody Array assay was performed using Version 1.0 of TransSignal Human Cytokine Antibody Array, following the manufacturer’s instruction. The Antibody Array membrane is coated with 18 cytokine antibodies in duplicate. The identities of the cytokines are outlined in the grid in the right lower panel. The cytokines produced by PBMC appear as dark spots and were demarcated by rectangular boxes. **(B)** PBMC were stimulated with PMA and ionomycin in the presence of brefeldin A for 4 hours prior to the procedure of intracellular cytokine staining and flow cytometric analysis. CD4 T cells and CD8 T cells were electronically gated separately for analysis. Results shown are representative dot plots of the staining for IFN γ (x axis) and IL-17A (y axis) in CD4 T cells derived from SLE patients with the positive T-C4d phenotype, a patient with rheumatoid

arthritis, and a healthy control. **(C)** IL-17A production in peripheral blood lymphocytes prepared from patients with SLE (n=81), patients with RA (n=28), patients with other diseases (n=22), and healthy controls (n=14) were analyzed using the intracellular staining/flow cytometry technique. Comparison of differences in the frequencies of IL-17A-producing cells between each paired group was performed using student's *t* test.

Figure 5. Differential proliferative responses of T-C4d-high cells and T-C4d-low cells to TCR/CD3-triggered stimulation. PBMC isolated from 3 representative SLE patients were cultured in medium alone or in the presence of anti-CD3 and/or anti-CD28 antibodies for 2-6 days. Cells stimulated with anti-IgD/IgM were included as a background control for the proliferation of B cells. Each experimental condition was tested in triplicates. T cell proliferation was measured by incorporation of ³H-thymidine and presented as proliferation Index.

Figure 6. C3d+ T cells produce increased amounts of cytokines.

(A-D) Three groups of peripheral T cells (SLE, OAD, N) were stimulated with anti-CD3/anti-CD28. Harvested T cells were stained for C3d and thereafter for intracellular cytokines (IL2, IL4, IL17, IFN γ). The cytokine production was significantly increased in the C3d+ T cell population within all the groups studied (SLE, OAD, N). Every dot in the figures (A-D) represents one patient. The y- axis represents percentage of T cells producing the indicated cytokine.

OAD- other autoimmune diseases; N- normal; IL – interleukin; IFN- interferon

Figure 7. C4 complement fragments partially co- localize with lipid rafts.

Lymphocytes were stained as above and fixed in suspension with 3.8% paraformaldehyde for 5 min. Cells were then washed and mounted in anti-fading media on slides. A) Low magnification image of a group of T cells. B) Overlay of the region of interest (yellow circle) where intensity analysis of the signals originating from the lipid rafts and the C3d fragments was performed. C) Histogram of the intensity profile of the lipid raft marker (*green*) and C3d fragments (*red*) starting counter-clockwise. The numbers associated with each peak corresponds to the numbers adjacent to the cell in “B”. D) Serial sections were acquired using a wide-field microscope and used to reconstitute the maximum intensity projection (MIP) of the whole T cell following de-convolution. T cells displayed a strong surface staining for lipid raft marker (*green*) and C3d fragments (*red*) with no detectable intracellular staining. There was adjacent staining of the two markers on the top section (magnified top inset), significant co-localization detailed in the middle inset as well as separate locations (bottom inset). The bar represents 8 μm in A and B and 2.8 μm in D.

Figure 1

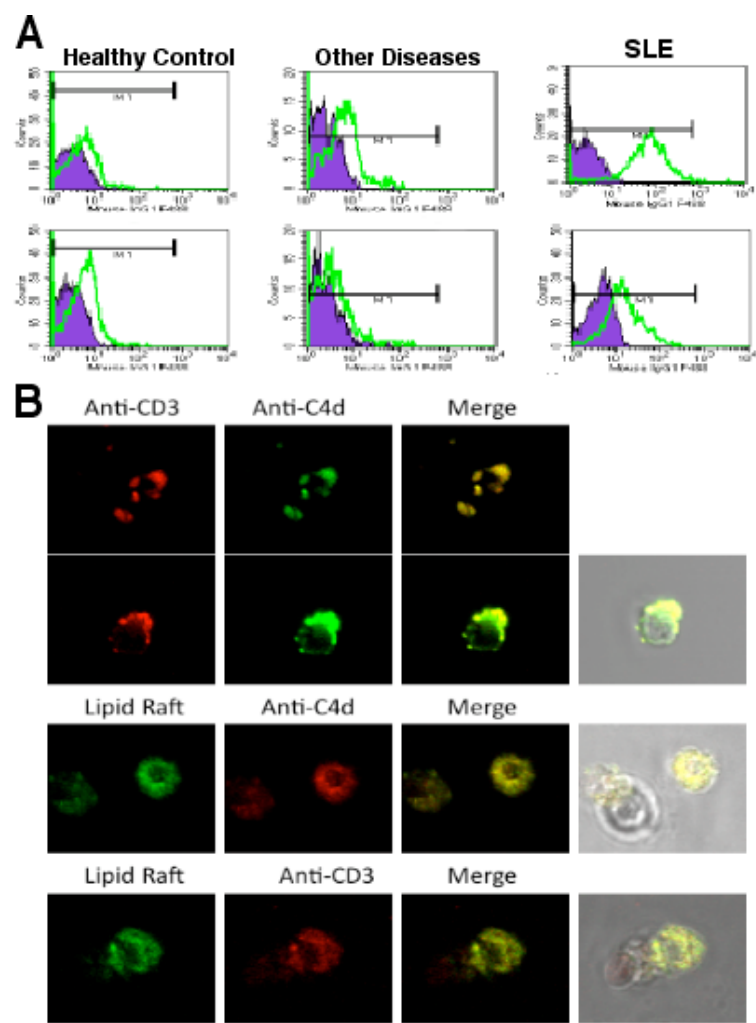


Figure 2

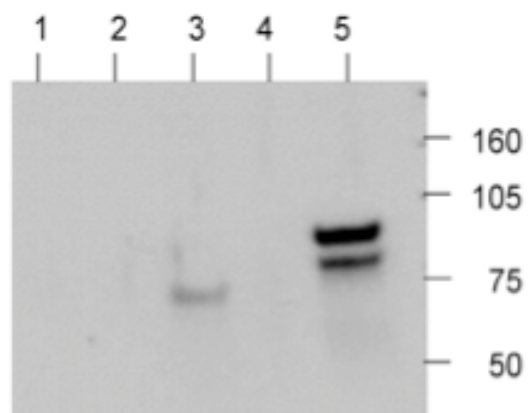


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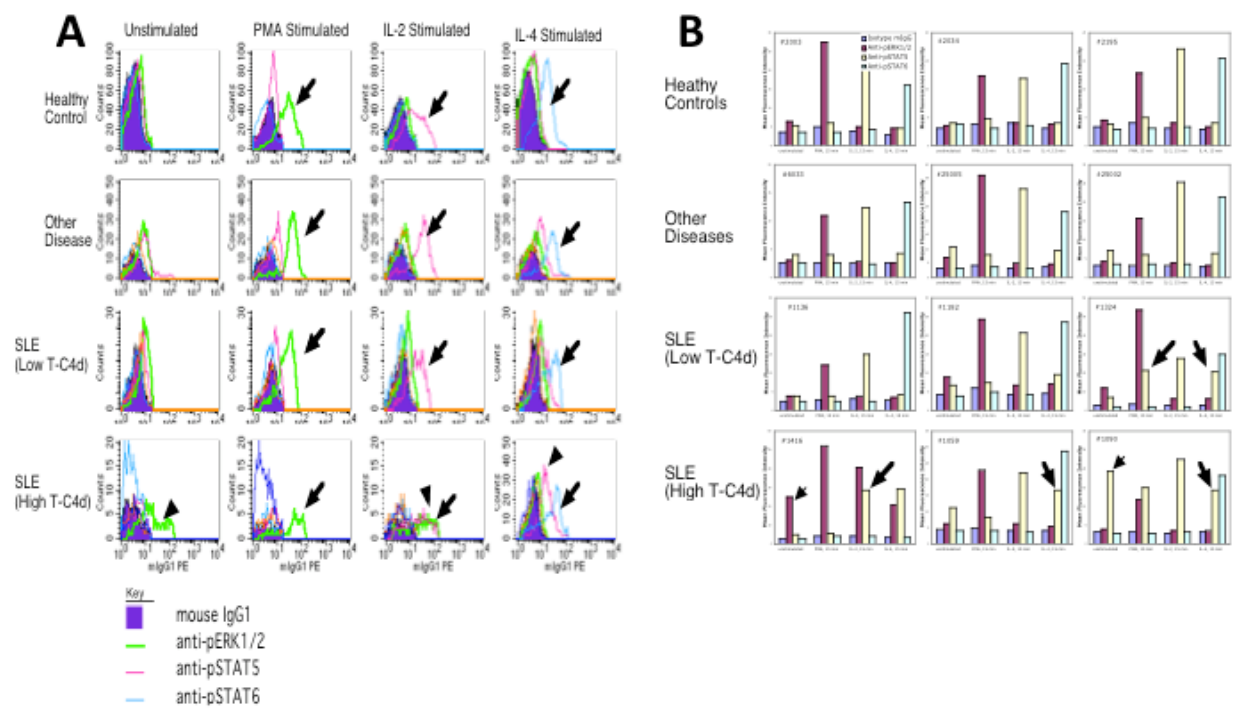


Figure 4

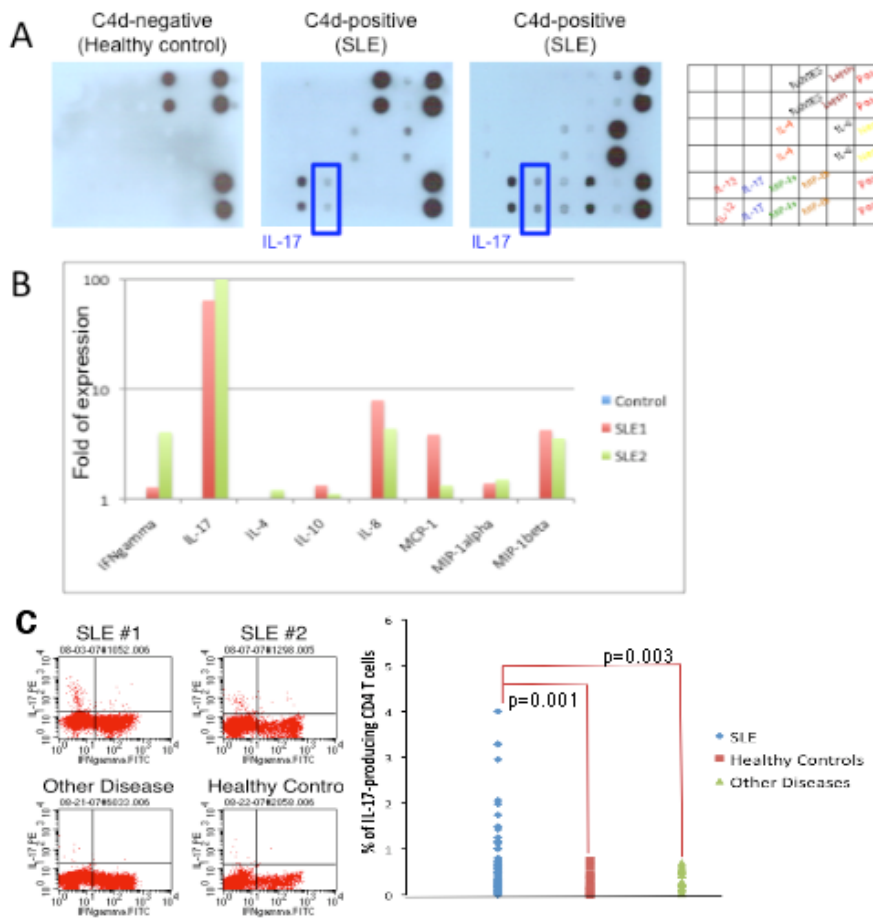


Figure 5

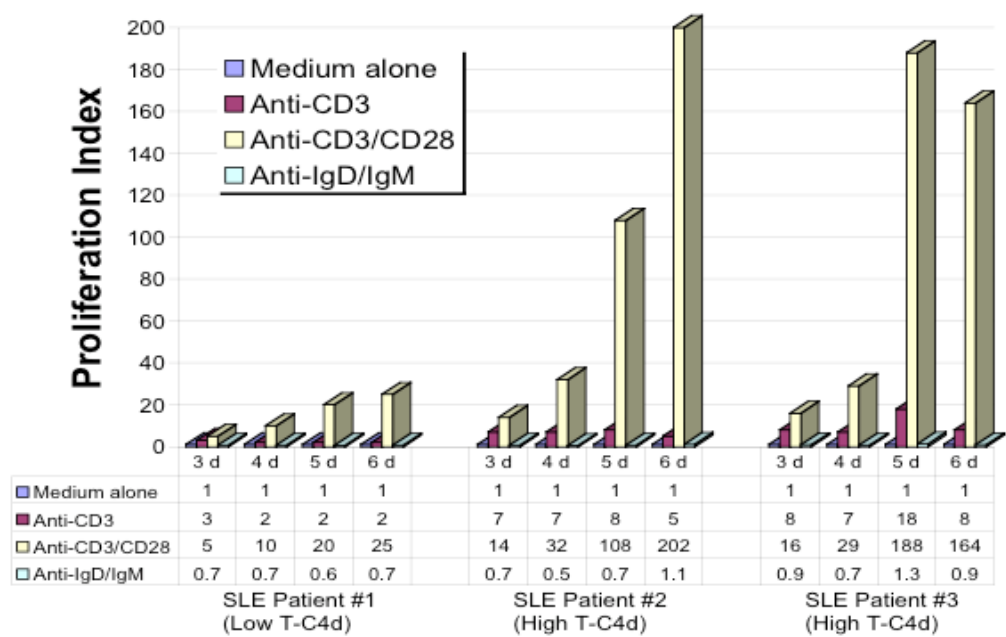


Figure 6

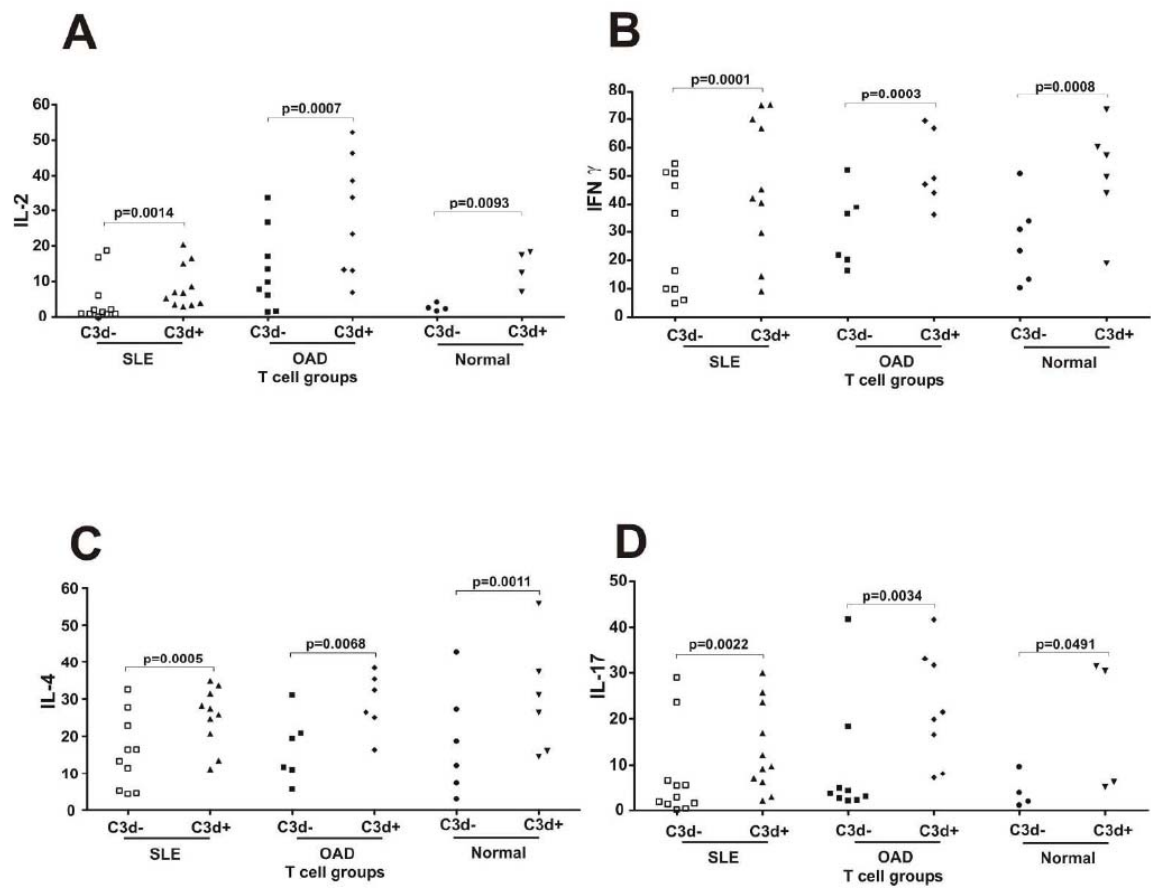
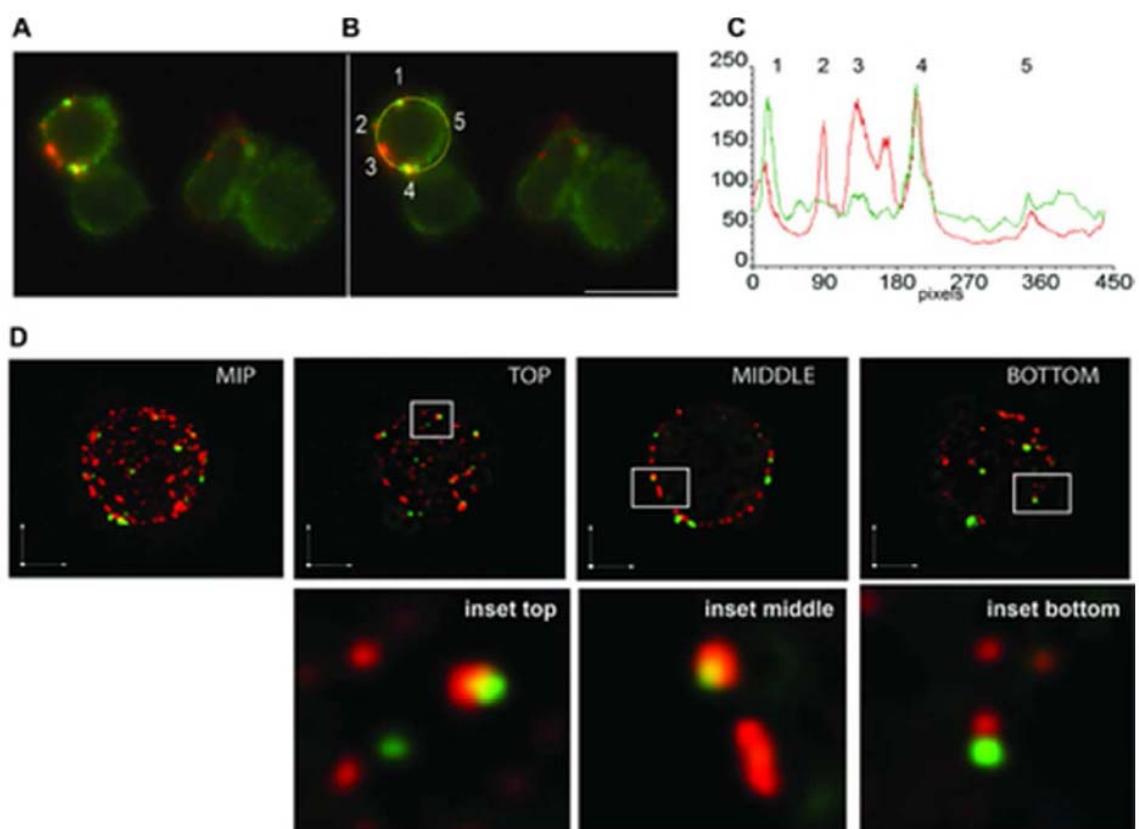


Figure 7



Lymphocyte-Bound Complement Activation Products as Biomarkers for Diagnosis of Systemic Lupus Erythematosus

Chau-Ching Liu¹, Amy H. Kao¹, Douglas M. Hawkins³, Susan Manzi^{1,2}, Abdus Sattar², Nicole Wilson¹, and Joseph M. Ahearn¹

Abstract

Systemic lupus erythematosus (SLE) is frequently misdiagnosed due to the lack of definitive diagnostic tests. The purpose of this study was to determine specifically whether complement activation products (CAP) are deposited on lymphocytes of SLE patients and whether lymphocyte-bound CAP (LB-CAP) may serve as novel biomarkers for the diagnosis of SLE. We conducted a cross-sectional study of 224 patients with SLE, 179 patients with other diseases, and 114 healthy controls. LB-CAP on peripheral blood lymphocytes was measured by flow cytometry. Diagnostic utility of LB-CAP was determined by receiver operating characteristic (ROC) analysis. Significantly elevated levels of C4d and C3d were detected specifically on T and B lymphocytes (designated T-C4d, T-C3d, B-C4d, and B-C3d) of SLE patients. As diagnostic tools, T-C4d and B-C4d, respectively, were 56% sensitive/80% specific and 60% sensitive/82% specific in differentiating SLE from other diseases. Moreover, compared with measurement of anti-dsDNA, serum C3, or serum C4, measurement of T-C4d/B-C4d was significantly more sensitive in identifying SLE patients during a single clinic visit. This is the first investigation of lymphocytes bearing complement activation products in human disease. T-C4d and B-C4d have high diagnostic sensitivity and specificity for SLE and may have added value to current laboratory tests for SLE diagnosis.

Keywords: systemic lupus erythematosus, SLE, lupus, complement, biomarker

Introduction

Systemic lupus erythematosus (SLE) is arguably the most clinically and serologically diverse autoimmune disease, with more than 100 autoantibodies found in patients and disease spectra ranging from subtle symptoms to life-threatening multi-organ failure.¹⁻³ Owing to its complex etiopathogenesis, heterogeneous presentation, and unpredictable course, SLE remains one of the greatest diagnostic challenges to physicians, including rheumatologists.^{4,5} Currently, the diagnosis of SLE is primarily based upon American College of Rheumatology (ACR) criteria,^{6,7} many of which are subject to interpretation and may require years to evolve. The lack of specific, reliable, and validated biomarkers for SLE not only leads to misdiagnosis and misguided therapy, but also may result in flawed clinical trials if patients in “lupus” treatment arms include false-positive diagnosis.

Serum C3 and C4 levels have been measured for decades in attempts to monitor disease activity in patients with SLE; however, these complement assays are not considered useful for the diagnosis of SLE.⁸⁻¹³ We have previously revisited the complement system as a source of SLE biomarkers and discovered that cell-bound complement activation products (CB-CAP) hold significant promise as diagnostic biomarkers for SLE. Specifically, erythrocyte-bound C4d (E-C4d), erythrocyte CR1 (E-CR1), and platelet-bound C4d (P-C4d) are highly sensitive and specific biomarkers for lupus diagnosis.^{14,15} Initial studies indicate that these CB-CAP provide significant added value to current diagnostic tests for SLE, and are capable of capturing the majority of patients who test negative for anti-double stranded DNA (dsDNA).¹⁴ During these investigations, we found that patients with abnormal levels of E-C4d would not necessarily have abnormal levels of P-C4d and vice versa, suggesting a surprising and intriguing hematopoietic lineage specificity. These observations lead to the hypothesis that lymphocyte-bound complement activation products (LB-CAP) may also serve as biomarkers for lupus diagnosis. This hypothesis was addressed

in a cross-sectional study to determine and compare levels of LB-CAP in patients with SLE, patients with other diseases and healthy individuals.

Patients and Methods

Study participants

All study participants were 18 years of age or older and provided written informed consent. No one was excluded based on gender or ethnicity. Ethnicity was self-reported by study participants. The University of Pittsburgh Institutional Review Board approved this study.

Patients with SLE who met the ACR 1982⁶ or 1997⁷ revised classification criteria were recruited for this study during routine visits to the University of Pittsburgh Lupus Patient Care and Translational Research Center. A total of 224 patients were studied from June 2004 through August 2007. As part of their routine care, all patients with SLE underwent routine blood work including complete blood count, erythrocyte sedimentation rate, serum levels of C3 and C4, antinuclear autoantibodies (ANA), and anti-dsDNA level. Tests for ANA (fluorescent assay) and anti-dsDNA (fluorescent assay using *Crithidia lucillae* or enzyme-linked immunosorbent assay) were performed by certified clinical pathology laboratories. In addition, each of these patients underwent a history and physical examination by a physician (AHK or SM), who was blinded to the LB-CAP results. Disease activity was assessed at the time of the visit using the Safety of Estrogens in Lupus Erythematosus: National Assessment (SELENA) version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)¹⁶ and the Systemic Lupus Activity Measure (SLAM).¹⁷

One hundred seventy-nine patients with non-SLE autoimmune or inflammatory diseases, including scleroderma, idiopathic inflammatory myositis, Sjögren's syndrome,

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rheumatoid arthritis, Wegener's granulomatosis, hepatitis C, vasculitis, primary Raynaud's phenomenon, psoriatic arthritis, osteoarthritis, antiphospholipid syndrome, cutaneous lupus, and undifferentiated connective tissue diseases, were recruited during the period from June 2004 through August 2007. The diagnosis was confirmed by their treating specialist physicians from various outpatient facilities at the University of Pittsburgh Medical Center. A total of 114 healthy individuals were recruited through local advertisements posted around the University of Pittsburgh campus. To confirm their healthy status, participants completed a brief questionnaire regarding existing medical conditions.

Flow cytometric assay for measurements of lymphocyte-bound complement activation products (LB-CAP)

At the time of each participant's visit, a 3 mL sample of blood was collected into a Vacutainer® tube containing EDTA as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA). Blood samples were stored at 4°C and analyzed within 24 hours after collection. Peripheral blood leukocytes were isolated using the following protocol. Briefly, the blood sample was centrifuged at $200 \times g$; the buffy coat was carefully transferred into a fresh tube and contaminating erythrocytes were hypotonically lysed. The leukocyte suspension was washed extensively with phosphate buffered saline (PBS) to remove lysed erythrocytes, resuspended, and divided into equal-volume portions and stained for different cell surface markers and CAP. T and B lymphocytes, monocytes, and granulocytes within isolated leukocytes were distinguished based on their unique features of forward/side scattering and expression of characteristic surface molecules. Cells isolated using this protocol exhibited characteristics similar to those isolated using the conventional Ficoll gradient centrifugation method (not shown).

The levels of LB-CAP, specifically T cell-bound C4d and C3d (T-C4d and T-C3d) and B cell-bound C4d and C3d (B-C4d and B-C3d), were measured using a three-color flow cytometric assay. Briefly, phycoerythrin- or phycoerythrin Cy5-conjugated mouse monoclonal antibodies (mAb) reactive with lineage-specific cell surface markers (CD3, CD4, and CD8 for T lymphocytes; CD19 for B lymphocytes; BD Biosciences, San Diego, CA) were used in conjunction with either anti-human C4d mAb (mouse IgG1; reactive with C4d-containing fragments of C4; Quidel, San Diego, CA, USA) or anti-human C3d mAb (mouse IgG1; reactive with C3d-containing fragments of C3; Quidel) that had been labeled with Alexa Fluor 488 using the Zenon antibody labeling kit (Invitrogen, Carlsbad, CA, USA). After staining, cells were analyzed using a FACS Calibur™ flow cytometer and Cell Quest software (Becton Dickinson Immunocytometry Systems). To ensure the specificity of the antibody staining detected, leukocyte aliquots from each patient stained with mouse IgG of appropriate isotypes were routinely included in all experiments. All mAb were used at a concentration of 5 μ g/mL. Levels of C3d or C4d on the surface of lymphocytes were expressed as specific median fluorescence intensity (SMFI), which was calculated as the C4d (or C3d)-specific median fluorescence intensity minus the isotype control median fluorescence intensity. To ensure the day-to-day reliability of LB-CAP measurements, the FACS Calibur flow cytometer was calibrated daily using CaliBrite 3 beads and FACSComp software (Becton Dickinson Immunocytometry Systems). The instrument settings were also calibrated daily using PBL stained with an isotype control IgG labeled with Alexa

Fluor 488 to ensure that the background fluorescence intensity remained constantly less than 3.5.

Statistical analysis

Descriptive statistics, including means, standard deviations, medians, and interquartile range (IQR: 25th to 75th percentile), were computed for continuous data. Normality of data distribution was checked using Shapiro–Wilk test. Differences in the levels of T-C4d, T-C3d, B-C4d, and B-C3d among the three study groups were compared by Kruskal–Wallis test. Two-sample Wilcoxon rank-sum (Mann–Whitney) test was performed to determine the statistical significance of the differences between each of the paired study groups. Correlations between C4d levels on T cells and B cells as well as between levels of C4d on CD4⁺ and CD8⁺ T cells within individual SLE patients were determined using the Spearman's rank correlation technique. Utility of T-C4d and B-C4d as diagnostic tests for SLE was assessed using the receiver-operating characteristic (ROC) analysis.^{18–20}

Results

Characteristics of the study participants

The study population consisted of 224 patients with SLE, 179 patients with other autoimmune or inflammatory diseases, and 114 healthy controls. Demographic and clinical characteristics of the patients with SLE are summarized in *Table 1*. Unless otherwise specified, data shown were ACR criteria-defined clinical manifestations that had been present at any time point during the course of disease. This cohort included patients with new-onset as well as long-standing disease, representing a broad range of disease activity with a wide spectrum of organ involvement. The group of patients with other diseases had a mean age of 46.1 ± 14.9 years (range 18–83), and were 92.2% Caucasian and 86.6% female. The healthy control group had a mean age of 45.5 ± 13.7 years (range 18–80), and were 84.2% Caucasian and 87.7% female.

Specific presence of CAP on lymphocytes of SLE patients

To investigate the possibility that CAP are specifically present on the surface of lymphocytes in SLE patients, we conducted a cross-sectional study utilizing a three-color flow cytometric assay to measure and compare the levels of CAP deposited on the surface of peripheral blood T and B lymphocytes of SLE patients, patients with other inflammatory/autoimmune diseases, and healthy individuals. Initial studies revealed that C4d and C3d were routinely detectable on the surface of T and B lymphocytes (*Figure 1A*), but C4b, C3b, iC3b, and C_{5b-9} were not detectable (data not shown). Therefore, the present study of lymphocyte-bound CAP (LB-CAP) was focused on C4d and C3d.

The experimental results obtained from the three study groups demonstrated that significantly elevated levels of C4d and C3d were detected on both T lymphocytes (designated T-C4d and T-C3d) and B lymphocytes (designated B-C4d and B-C3d) in a significant fraction of SLE patients (*Figure 1B*). In contrast, variable, yet generally low, levels of C4d and C3d were detected on T and B lymphocytes prepared from healthy controls and patients with other diseases. When the LB-CAP levels were compiled for the entire study population of SLE, other diseases and healthy subjects, the mean $\pm$ SD levels of T-C4d (12.1 ± 20.5 ; 2.5 ± 3.0 ; 1.7 ± 1.0), T-C3d (2.8 ± 3.4 ; 1.5 ± 1.5 ; 1.0 ± 0.7), B-C4d

Characteristic		Patients with SLE (<i>n</i> = 224)	
Age (year) mean ± SD; range		43.4 ± 12.6; 18–78	
Race (% Caucasian)		83.5	
Sex (% female)		92.8	
Disease duration (year) mean ± SD; range		10.6 ± 8.7; 0–40	
Malar rash* (%)		42.0	
Discoid rash* (%)		3.1	
Photosensitivity* (%)		53.1	
Oral ulcers* (%)		49.6	
Arthritis* (%)		88.0	
Serositis* (%)		39.3	
	Pleuritis (%)		30.8
	Pericarditis (%)		18.3
Renal disorder* (%)		32.6	
Neurological disorder* (%)		12.5	
	Seizure (%)		10.8
	Psychosis (%)		4.9
Hematological disorder* (%)		67.9	
	Anemia (%)		7.6
	Leukopenia (%)		31.7
	Lymphopenia (%)		48.7
	Thrombocytopenia (%)		21.4
Immunological disorder* (%)		79.5	
	Anti-Smith (%)		16.1
	Anti-phospholipid (%)		42.4
	Anti-dsDNA*† (%)		60.3*; 29.2†
	Anti-SSA, anti-SSB, rheumatoid factor, etc. (%)		42.4
Antinuclear antibodies (%)		99.6	
Raynaud's phenomenon* (%)		53.1	
SLAM ^c mean ± SD; median; range		5.1 ± 3.2; 5; 0–21	
SELENA-SLEDAI [‡] mean ± SD; median; range		3.2 ± 2.8; 2; 0–14	
mSELENA-SLEDAI [§] mean ± SD; median; range		1.7 ± 2.2; 2; 0–10	
Serum C3 (% below normal; <i>n</i> = 221) [†]		30.3	
Serum C4 (% below normal; <i>n</i> = 221) [†]		37.1	

*A clinical manifestation is recorded positive if ever present in a patient; % of patients with a positive history of the indicated manifestation, unless otherwise specified.
[†]% of patients with positive anti-dsDNA, or below normal levels of serum C3 or C4 at the time of the study visit.
[‡]Disease activity score at the time of the study visit.
[§]Modified SELENA-SLEDAI: SELENA-SLEDAI excluding the scores for low complement and positive dsDNA.

Table 1. Demographic and clinical characteristics of the SLE patient cohort.

(49.0 ± 73.2; 14.7 ± 26.8; 8.1 ± 5.8), and B-C3d (17.1 ± 12.4; 13.7 ± 11.7; 8.8 ± 4.8) in patients with SLE were significantly higher than those in healthy controls (all *p* < 0.0001) as well as those in patients with other diseases (*p* < 0.0001) (Table 2A). Notably, levels of B-C4d and B-C3d were considerably higher than those of T-C4d and T-C3d in all three study groups. Even though T-C3d and B-C3d levels (2.8 ± 3.4 and 17.1 ± 12.4, respectively) were significantly elevated in patients with SLE, they were noticeably lower than those of T-C4d and B-C4d (12.1 ± 20.5 and 49.0 ± 73.2,

respectively). Subsequent studies of the utility of LB-CAP as SLE biomarkers, therefore, were concentrated on T-C4d and B-C4d.

Initial studies showed that lymphocyte-bound C4d and C3d measures were not influenced by lymphocyte count in SLE patients (data not shown). Initial studies also indicated that the binding of C4d and C3d to lymphocytes was stable, as repeated measurements of the same blood sample stored at 4 °C showed insignificant variation over a 3-day period (data not shown). This was supported by the observation that exposure to acidic or

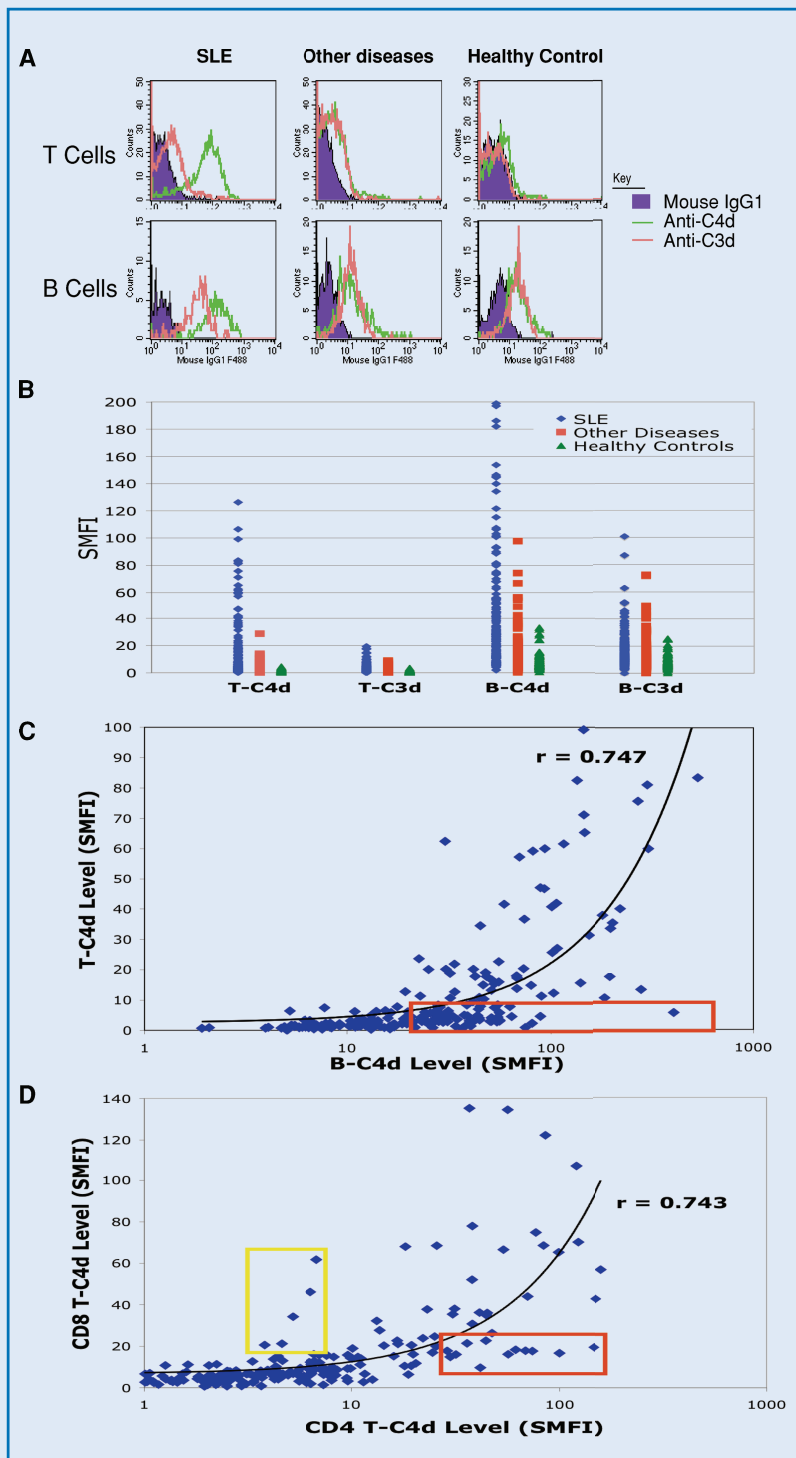


Figure 1. Specific deposition of C4d and C3d on the surface of lymphocytes in patients with SLE. The presence of C4d and C3d on the surface of peripheral blood lymphocytes in patients with SLE, patients with other diseases, and healthy controls was examined using flow cytometry. (A) Representative histograms of flow cytometric analysis of T cells and B cells from one SLE patient, one patient with rheumatoid arthritis, and one healthy control are shown. Note the shift-to-the-right of the entire C4d-specific and C3d specific peaks. (B) Abnormally high levels of surface-bound C4d and C3d were detected on T cells (T-C4d and T-C3d) and B cells (B-C4d and B-C3d) in a significant fraction of SLE patients. (C) Levels of T-C4d and B-C4d were significantly correlated within most SLE patients, but were discordant in some patients (red-boxed). (D) Levels of CD4 T-C4d and CD8 T-C4d were significantly correlated within most SLE patients, but were discordant in some patients (red-boxed and yellow-boxed). Surface-bound C4d and C3d levels are shown as specific median fluorescence intensity (SMFI) (see text).

low-ionic strength buffers did not influence LB-CAP levels (data not shown).

Comparison of the LB-CAP phenotypes of SLE lymphocytes

We next examined the relationships among the LB-CAP phenotypes of different lymphocyte populations within each blood sample. Several observations were made. First, within each of the four specific assays, the phenotype of the T and/or B cells was homogeneous among all cells in a given patient. Histograms rarely contained shoulders or multiple peaks (representing subsets of cells with distinctly different levels of C4d and/or C3d), but rather were uniformly positive as demonstrated by complete shift of the fluorescent intensity histograms (Figure 1A). Second, in general, levels of T-C4d and B-C4d within a given patient were significantly correlated (Spearman's rank coefficient (r_s) = 0.747, $p < 0.0001$) (Figure 1C). However, discordant levels were observed in some patients, who had elevated B-C4d levels but low or even normal T-C4d levels (Figure 1C, red-box).

This cellular specificity for LB-CAP phenotype was further investigated with studies of CD4 and CD8 T lymphocyte subsets. As shown in Table 2B, C4d levels of CD4 T cells (CD4 T-C4d) were significantly elevated in patients with SLE, compared to those of other disease, and healthy subjects (both $p < 0.0001$). Likewise, C4d levels of CD8 T cells (CD8 T-C4d) were also significantly elevated in the SLE group compared to those of the other two groups (both $p < 0.0001$). Levels of C3d on CD4 T cells (CD4 T-C3d) and CD8 T cells (CD8 T-C3d) were lower when compared to CD4 T-C4d and CD8 T-C4d, but still differed significantly between SLE patients and patients with other diseases ($p = 0.0006$; $p = 0.002$) and between SLE patients and healthy individuals ($p < 0.0001$; $p = 0.02$), respectively. In general, CD4 T-C4d and CD8 T-C4d were significantly correlated within a given patient ($r_s = 0.743$, $p < 0.0001$) (Figure 1D). Although in most SLE patients, similar levels of C4d and C3d were detected simultaneously on CD4 and CD8 T cells, we were able to identify a fraction of patients in which distinct levels of C4d and C3d were present on CD4 versus CD8 T cells (Figure 1D; data shown only for C4d).

Sensitivity and specificity of T-C4d and B-C4d measures for SLE diagnosis

The statistically significant and specific elevation of LB-CAP, particularly T-C4d and B-C4d, in patients with SLE suggests that these measures may serve as unique biomarkers for SLE and may be of added value to current tests for earlier and more accurate diagnosis of SLE. To explore this possibility, diagnostic sensitivity and specificity of T-C4d and B-C4d were first calculated at different cutoff levels using patients with SLE as

A. CAP levels on CD3 ⁺ T cells and CD19 ⁺ B cells				
LB-CAP level*	T-C4d	T-C3d	B-C4d	B-C3d
SLE	12.1 ± 20.5	2.8 ± 3.4	49.0 ± 73.2	17.1 ± 12.4
(n = 224)	(3.7; 1.8–11.3)	(1.6; 0.8–3.2)	(26.7; 11.6–52.3)	(14.0; 9.5–20.7)
Other diseases	2.5 ± 3.0	1.5 ± 1.5	14.7 ± 26.8	13.7 ± 11.7
(OD) (n = 179)	(1.7; 1.1–2.9)	(1.1; 0.6–1.7)	(8.8; 5.9–14.3)	(9.2; 6.6–16.7)
Healthy controls	1.7 ± 1.0	1.1 ± 0.7	8.1 ± 5.8	8.8 ± 4.8
(HC) (n = 114)	(1.6; 1.0–2.4)	(1.0; 0.5–1.4)	(6.4; 4.8–9.4)	(7.3; 5.6–11.3)
p value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
(SLE vs. OD)				
p value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
(SLE vs. HC)				
B. CAP levels on T-cell subsets				
LB-CAP level*	CD4 T-C4d	CD4 T-C3d	CD8 T-C4d	CD8 T-C3d
SLE	15.6 ± 27.6	2.5 ± 2.6	15.7 ± 24.2	5.6 ± 6.3
(n = 224)	(5.0; 2.2–11.6)	(2.3; 0.7–3.2)	(7.2; 4.2–15.8)	(3.5; 2.1–6.7)
Other diseases	3.2 ± 3.7	1.6 ± 1.6	6.4 ± 5.6	4.0 ± 4.4
(OD) (n = 179)	(2.3; 1.3–3.8)	(1.3; 0.6–2.1)	(5.1; 3.4–8.1)	(2.8; 1.4–4.9)
Healthy controls	2.2 ± 1.2	1.2 ± 0.8	4.8 ± 3.0	3.6 ± 3.2
(HC) (n = 114)	(1.8; 1.3–2.7)	(1.0; 0.6–1.8)	(4.6; 2.8–6.2)	(3.0; 1.2–5.4)
p value	< 0.0001	0.0006	< 0.0001	0.002
(SLE vs. OD)				
p value	< 0.0001	< 0.0001	< 0.0001	0.02
(SLE vs. HC)				

*Data shown are: mean ± SD (median; IQR) of the specific median fluorescence intensity (SMFI) of the indicated lymphocyte-bound CAP measure.

Table 2. Comparison of lymphocyte-bound CAP levels in patients with SLE, patients with other diseases, and healthy controls.

the diagnostic group and patients with other diseases as the reference group. Diagnostic performance of T-C4d and B-C4d in distinguishing patients with SLE from patients with other inflammatory or autoimmune diseases was further assessed using the ROC analysis.¹⁸ The ROC curve was constructed by measuring the sensitivity (true positive) and 1-specificity (false positive) across various cutoff values that define the positivity of T-C4d or B-C4d. The differentiating power of T-C4d and B-C4d was then estimated using the area under the ROC curve (AUC).^{19,20} As shown in *Figure 2*, the AUC for the T-C4d assay and the B-C4d assay were 0.727 and 0.770, respectively. Based on these data, it was estimated that T-C4d and B-C4d are 56% sensitive/80% specific and 60% sensitive/82% specific in differentiating SLE from other diseases, respectively.

Comparison of T-C4d and B-C4d with other laboratory tests for SLE diagnosis

We next sought to determine whether T-C4d and B-C4d would provide added value to ANA and anti-dsDNA, laboratory tests conventionally used for SLE diagnosis. Our SLE study cohort consisted of patients with a broad range of disease activity (SLAM and SLEDAI scores ranging from 0 to 21 and 0 to 14, respectively) and disease duration (0–40 years). Given the insidious and heterogeneous course of SLE, a laboratory test that is capable of facilitating the diagnosis during a single clinic visit would be extremely useful. The capability of T-C4d, B-C4d, and anti-dsDNA

measures, when used separately or in combination, for identifying SLE patients at the time of the study visit was investigated and compared using this study cohort (*Figure 3*). At the time of the study visit, 223 of 224 SLE patients (99.6%) were positive for ANA. The analysis was performed with two different decision trees. First, ANA test results were followed by T-C4d and B-C4d assays and then with the anti-dsDNA test (*Figure 3A*). Of the 223 ANA-positive patients, 141 (63%) patients had abnormally elevated levels of T-C4d and/or B-C4d (cut points defined as: the mean level + 2 SD of the healthy control group; 2.71 for T-C4d and 19.6 for B-C4d; ref. *Table 2*) (*Figure 3A*). Moreover, the anti-dsDNA test captured only 9 of the 82 ANA positive/T-C4d/B-C4d-negative patients. The lone ANA-negative patient tested negative for both anti-dsDNA and T-C4d/B-C4d.

In the second decision tree, ANA-tested patients were followed by the anti-dsDNA test and then with T-C4d and B-C4d assays (*Figure 3B*). Of the 223 ANA-positive patients, anti-dsDNA test was performed on 209. Only 59 of these 209 ANA-positive patients (28%) exhibited positive anti-dsDNA at the study visit (compared with 63% positive for T-C4d and/or B-C4d). Most patients who were anti-dsDNA-positive also tested positive for T-C4d and/or B-C4d (50/59; 85%). Of the 150 patients who were ANA-positive but anti-dsDNA-negative, the majority (83; 55%) were positive for T-C4d/B-C4d.

In the third and fourth decision trees (*Figure 3C* and *D*), we compared T-C4d and B-C4d with the traditional measures of

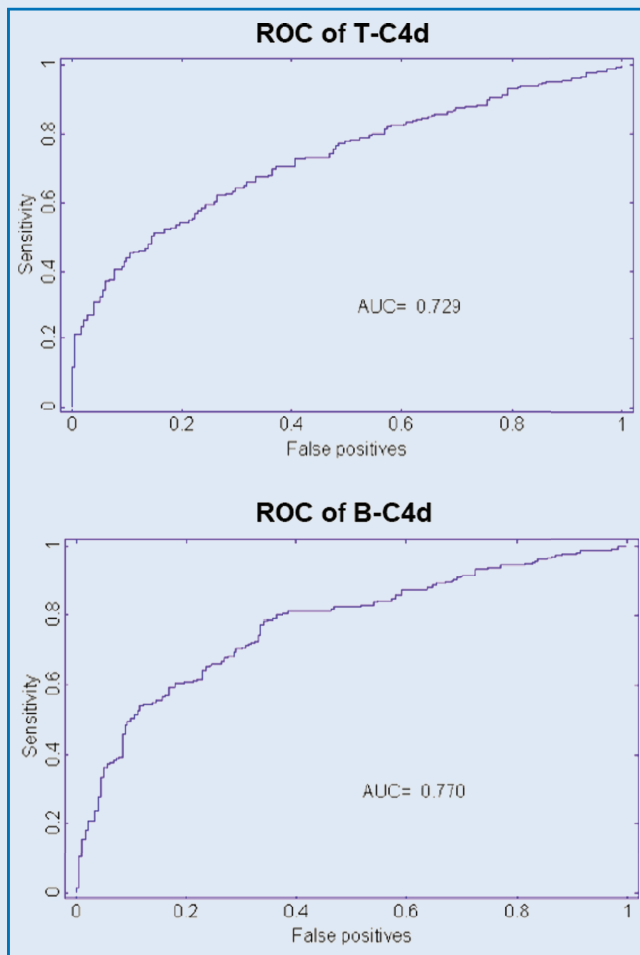


Figure 2. ROC-AUC analysis of the performance of T-C4d and B-C4d levels as diagnostic markers for SLE.

complement activation—serum C3 and C4. The results indicated that of 221 patients who were tested for serum C3 and C4, only 67 (30%) and 82 (37%) had abnormally low levels of serum C3 and C4, respectively. Most patients with abnormal levels of serum C3 and C4 also tested positive for T-C4d/B-C4d (56/67; 84% and 67/82; 82%, respectively). In addition, the majority (82/154; 53%) of patients who had normal serum C3 levels were positive for T-C4d/B-C4d, and the majority (71/139; 51%) of patients with normal serum C4 levels were positive for T-C4d/B-C4d. All three patients who were not tested for serum C3 and C4 were also positive for T-C4d/B-C4d.

Collectively, these results obtained using the four different decision trees indicate that: (1) when used independently, the T-C4d/B-C4d assay alone is more sensitive than the anti-dsDNA test alone, serum C3 measure alone, or serum C4 measure alone in confirming a diagnosis of SLE, and (2) by combining these assays, the T-C4d/B-C4d assays identify SLE patients who are negative for anti-dsDNA and/or have normal serum C3/C4 levels at a single visit.

Discussion

The current study was undertaken to investigate the potential for LB-CAP to serve as biomarkers for SLE diagnosis. The results indicate that T-C4d and B-C4d are highly sensitive and specific biomarkers for diagnoses of SLE, T-C4d and B-C4d may be more

sensitive biomarkers for SLE diagnosis as compared with anti-dsDNA at one clinic visit, and simultaneous determination of anti-dsDNA and T-C4d/B-C4d may provide the highest combined sensitivity-specificity for SLE diagnosis reported to date.

There are several strengths of this study design. First, it is prospective, dictated by the need for fresh blood samples to perform LB-CAP assays. Second, the SLE patient cohort studied spanned a broad range of ages, disease activity and clinical manifestations, as the design was to enroll consecutive patients simply based upon willingness to participate. Third, all patients were evaluated at the same site by the same lupologists, ensuring that the diagnosis was as accurate as possible. Fourth, assays were performed on the same flow cytometer by the same operator, and calibrations and standards were established prior to the study. Fifth, the other disease control group was enriched for rheumatologic and autoimmune diseases that are frequently misdiagnosed as SLE, to ensure a most stringent test and the worst-case scenario for ROC analysis. Sixth, we performed a head-to-head comparison between our candidate biomarker(s) and the current gold standards for SLE, ANA, anti-dsDNA and serum C3/C4, at a single visit to emulate a real practice scenario.

The results of this study demonstrate significant potential for LB-CAP as diagnostic biomarkers for SLE. T-C4d and B-C4d were 56% sensitive/80% specific and 60% sensitive/82% specific for the diagnosis of SLE, respectively. The sensitivity of ANA in this study was 99.6%. However, it is generally held that ANA-positivity is highly nonspecific for SLE with a positive-predictive value as low as 11% in some studies.^{21,22} Conversely, anti-dsDNA testing has been shown to be highly specific for confirming a diagnosis of SLE, but the mean sensitivity of anti-dsDNA testing for SLE among published studies is only 57%.²² Although 60% of the patients with SLE in this study were anti-dsDNA-positive at some point during the course of their disease, the sensitivity of the test at one visit in the current study was only 29% (Table 1). Another laboratory measure commonly used by physicians to aid in diagnosing SLE, despite not being among the ACR classification criteria, is abnormally low levels of serum C3 and/or C4. In the current study, the sensitivity of serum C3 and C4 measurements at one visit was only 30% (67 of 221 patients tested) and 37% (82 of 221 patients), respectively (Table 1). Thus, based upon evaluation at a single visit in this study, T-C4d and B-C4d can be considered more specific than ANA and more sensitive than anti-dsDNA, serum C3, and serum C4.

Most importantly, LB-CAP testing was diagnostic for SLE in 55% (83/150) of patients who were negative for anti-dsDNA (Figure 3B), 53% (82/154) of patients who had normal serum C3 levels (Figure 3C), and 51% (71/139) of patients who had normal serum C4 levels (Figure 3D). It is also important to note that the sensitivity and specificity of the LB-CAP assay are determined by the points on the ROC that are selected. Thus, if LB-CAP are included in a diagnostic panel with other biomarkers, the cutoff values can be selected based upon the sensitivity and specificity of the other components in the panel and the value desired from the LB-CAP assays. For example, a B-C4d cutoff value could be selected to provide nearly 100% specificity with 20% sensitivity based upon the ROC analysis.

The multi-ethnic cohort of SLE patients studied in the current study consisted predominantly of Caucasians (83.5%; 13.8% Africa Americans; 2.7% Others; Table 1). However, preliminary analyses have indicated that a higher frequency of non-Caucasian SLE patients (28/37; 77%) tested positive for T-C4d/B-C4d than did Caucasian SLE patients (113/187; 60%) at a single study visit. This

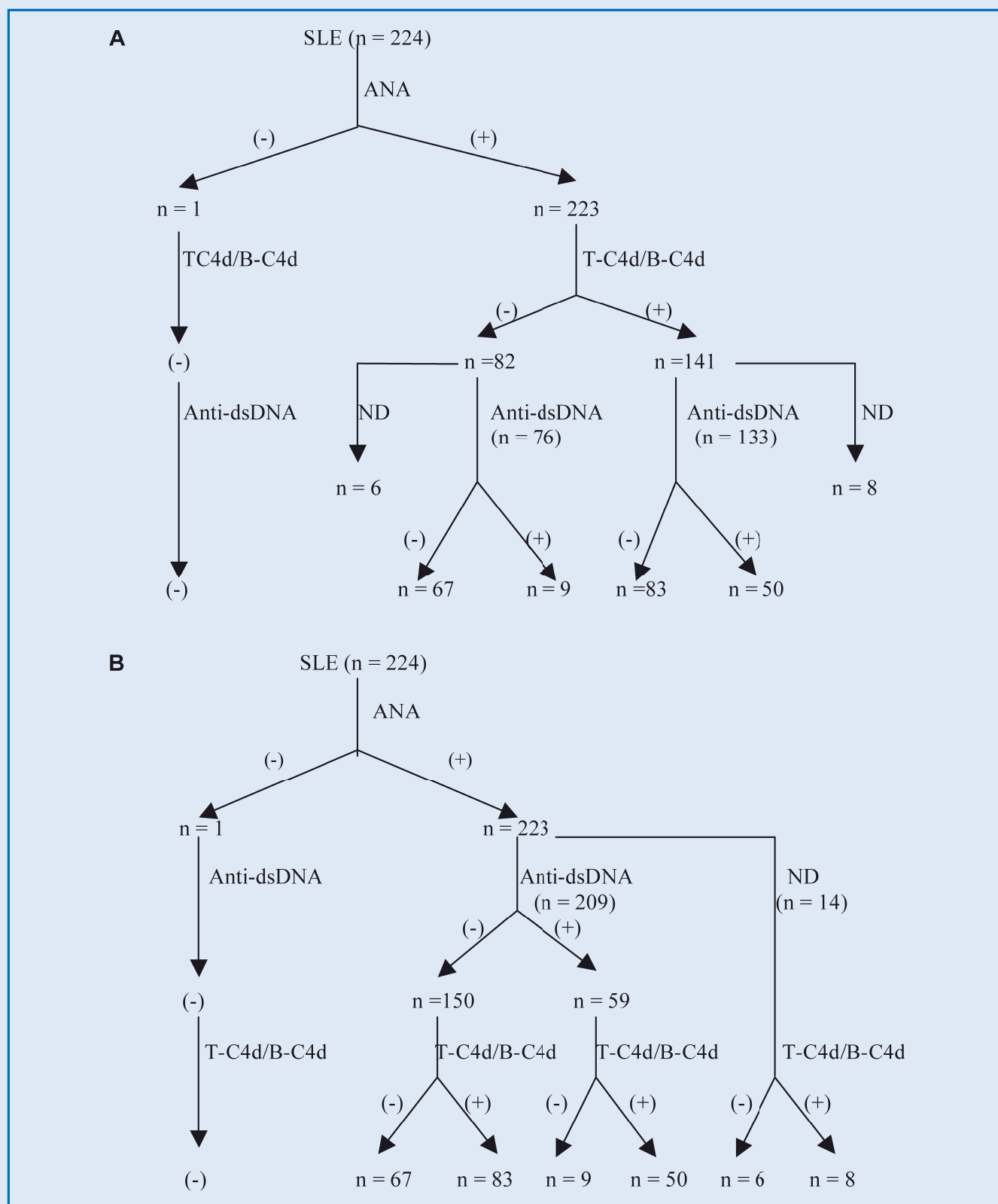
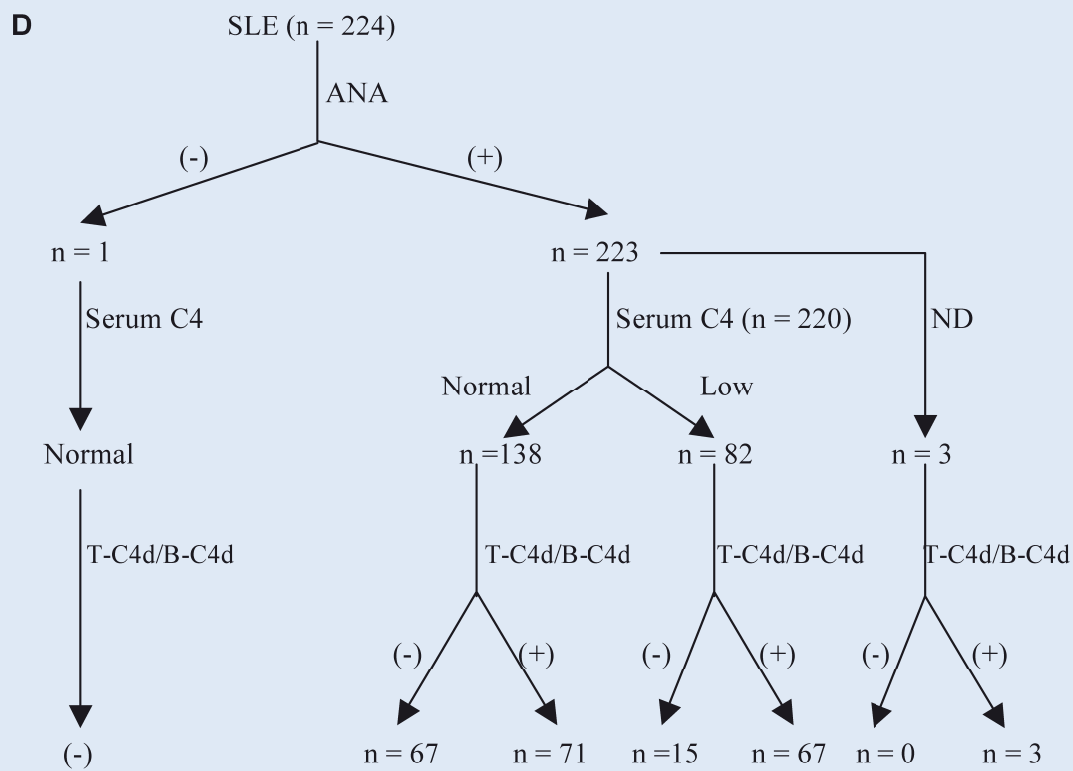
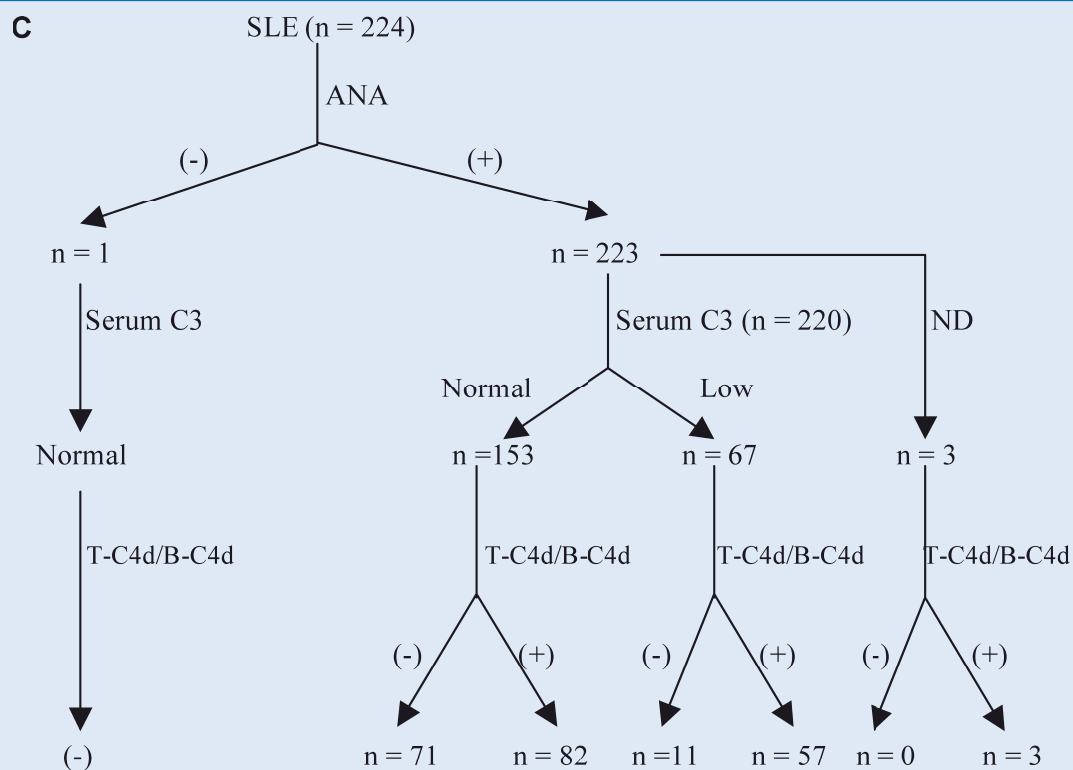


Figure 3. Comparison of the capacity of T-C4d/B-C4d, anti-dsDNA, serum C3, and serum C4 measures for SLE diagnosis. Tests for T-C4d/B-C4d, anti-dsDNA, serum C3, and serum C4 were performed on samples obtained on the day of the study visit. Of the 224 SLE patients in the study cohort, 223 were positive for antinuclear autoantibodies (ANA). Both ANA-positive and ANA-negative patients were further tested using the T-C4d/B-C4d measure (**panel A**), anti-dsDNA measure (**panel B**), serum C3 measure (**panel C**), or serum C4 measure (**panel D**) as the first confirming test. Fourteen patients were not tested for anti-dsDNA on the day of the study visit, but were tested for T-C4d/B-C4d; eight of them were positive for T-C4d/B-C4d. Three patients were not tested for serum C3 and C4; each of these was positive for T-C4d/B-C4d.



*ND: Not done

Figure 3. Continued.

result suggests that T-C4d/B-C4d as a diagnostic biomarker may be as sensitive, if not more sensitive, in non-Caucasian patients as in Caucasian patients. The impact of ethnicity on T-C4d/B-C4d as diagnostic lupus biomarkers warrants further investigation.

In addition to demonstrating utility as diagnostic biomarkers, this study generated data that may suggest clues to the mechanisms responsible for generation of LB-CAP. Although we had previously noted that E-C4d,¹⁴ R-C4d,²³ and P-C4d¹⁵ phenotypes do not necessarily correlate within a given patient, it was still surprising to discover that different LB-CAP assays such as T-C4d and B-C4d levels do not correlate in the same blood sample prepared from some SLE patients (Figure 1C). Even more surprising was the observation that levels of C4d and C3d on T-cell subsets do not necessarily correlate in a given patient at one point in time (Figure 1D). These observations further support the hypothesis that there is a tissue-specific mechanism responsible for generation of cell-bound complement activation products (CB-CAP), and elevations of CB-CAP are not simply the result of systemic activation of the complement system with nonspecific deposition on circulating cells. The findings also suggest that a panel composed of multiple CB-CAP assays will have the greatest diagnostic value, with a combined sensitivity and specificity greater than any single assay can provide. Further investigation may also produce insight into whether patterns of CAP deposition have a clinical subsetting potential.

Another potential insight into the mechanism of LB-CAP generation can be gleaned by comparison of the relative levels of C4d and C3d observed on each cell and cell type. Although C4d and C3d were generally detected simultaneously on lymphocyte surfaces (Figure 1A and B), the levels of C4d were generally higher than those of C3d (Figure 1B and Table 2). Because C3 activation is more distal in the enzymatic amplification cascade of complement activation, we initially considered that C3d would be a more sensitive LB-CAP biomarker. However, the unexpected “C4d > C3d” phenomenon was consistently observed for both T and B lymphocytes in study participants from all three study groups (Table 2). Moreover, in spite of high T-C4d and B-C4d levels detected in SLE patients, CAP of the terminal step of complement activation, such as the C_{5b-9} membrane attack complex, were not detectable (data not shown). These results not only indicate that CAP present on the surface of lymphocytes of SLE patients are products of the classical, and possibly the lectin, pathway of complement activation, but also suggest that specific regulatory mechanisms are in place to harness the activation cascade to prevent generation of C3- and C5-convertases and overt lysis of lymphocytes. The attachment of nonlytic forms/levels of CAP to surface molecules of lymphocytes may potentially alter the physiological functions of lymphocytes and lead to significant consequences in (auto) immune responses in SLE patients.

In summary, this is the first report of lymphocytes bearing complement activation products in human health and disease. The results suggest strong potential for B-C4d and T-C4d as diagnostic biomarkers for SLE and provide clues to potential pathogenic mechanisms. Future investigations including multicenter validation are warranted.

Acknowledgments

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Complement fragment C3d is localized within the lipid rafts of T cells and promotes cytokine production

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Abstract:	The complement system plays an important role in tissue inflammation and damage in SLE patients. High levels of C3d were detected on the surface of erythrocytes and lymphocytes of SLE patients. The objective of this study was to assess the functional consequences of C3d fragments deposited on the surface membrane of SLE T cells. Methods: 46 SLE patients, 43 patients with other autoimmune diseases (OAD) and 33 healthy individuals (N) were enrolled in this study. T cells were isolated from peripheral blood and flow cytometry studies were conducted to assess the levels of C3d fragments, Ca⁺⁺ influx responses and cytokine production. Confocal microscopy was used to study co-localized molecules. Student's t-test was performed to determine statistical significance among study groups. Results: A significant percentage of the SLE T cells were found to be positive for C3d (13.58 +/- 3.92%) when compared with normal T cells (4.52 +/- 2.92%) ($P \leq 0.0000547$) and T cells from patients with other autoimmune diseases (6.31 +/- 4.57%) ($P \leq 0.00513$). Peak Ca⁺⁺ influx responses were significantly higher in C3d- SLE T cells compared with the C3d+ SLE T cells ($p < 0.011$). C3d+ T cells produced significantly more IL-2, IFN-gamma, IL-4 and IL-17. C3d fragments were found to be localized within the lipid rafts. Conclusion: C3d fragments are localized in the lipid rafts of SLE T cells and contribute to abnormal T cell function by modulating Ca⁺⁺ influx responses and increased cytokine production.

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Complement fragment C3d is co-localized within the lipid rafts of T cells and promotes cytokine production

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Keywords: T cells, C3d, lipid rafts, systemic lupus erythematosus

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Abstract

The complement system plays an important role in tissue inflammation and damage in SLE patients. High levels of C3d were detected on the surface of erythrocytes and lymphocytes of SLE patients. The objective of this study was to assess the functional consequences of C3d fragments deposited on the surface membrane of SLE T cells.

Methods: 46 SLE patients, 43 patients with other autoimmune diseases (OAD) and 33 healthy individuals (N) were enrolled in this study. T cells were isolated from peripheral blood and flow cytometry studies were conducted to assess the levels of C3d fragments, Ca^{++} influx responses and cytokine production. Confocal microscopy was used to study co-localized molecules. Student's t-test was performed to determine statistical significance among study groups.

Results: A significant percentage of the SLE T cells were found to be positive for C3d (13.58 $\pm$ 3.92%) when compared with normal T cells (4.52 $\pm$ 2.92%) ($P \leq 0.0000547$) and T cells from patients with other autoimmune diseases (6.31 $\pm$ 4.57%) ($P \leq 0.00513$). Peak Ca^{++} influx responses were significantly higher in C3d- SLE T cells compared with the C3d+ SLE T cells ($p < 0.011$). C3d+ T cells produced significantly more IL-2, IFN-gamma, IL-4 and IL-17. C3d fragments were found to be localized within the lipid rafts.

Conclusion: C3d fragments are localized in the lipid rafts of SLE T cells and contribute to abnormal T cell function by modulating Ca^{++} influx responses and increased cytokine production.

Introduction

The complement system plays an important role in protection against pathogenic organisms and modulates several aspects of the humoral and cellular immune response. Complement is invariably activated in patients with infectious and autoimmune diseases including systemic lupus erythematosus (SLE). Complement activation is linked to the expression of tissue pathology through either the formation of immune complexes or its direct deposition on tissues and cell surface membranes [1]. The complement membrane attack complex C5b-9 binds to cell membranes, independent of any receptor, and it may activate multiple signaling pathways [2] Complement is known to alter B and T cell function. In B cells, it lowers the activation threshold [3] and supports the development of B-cell memory and immune tolerance [4]. Complement modulate antigen- presenting cell (APCs) [5] and indirectly T cell function [6-7]. The complement system and its regulatory proteins have an important role in the determination of T cell responses by modulating T cell apoptosis [8-9] and inducing regulatory T cells [10-12]. Recent studies have shown that complement can initiate or modulate a surface membrane signaling response in T cells [1,5-6]. However, the underlying signaling pathways involved in these effects are still obscure.

Ahearn and colleagues have found that complement activation fragments C3d and C4d are deposited on the surface membrane of erythrocytes and lymphocytes in patients with SLE [13-14]. The deposition of C4d on the surface of red cells may represent a clinically useful disease biomarker [15]. Deposition of the complement fragment C3d on the

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3 surface of T cells has unknown as of yet functional repercussions. T cells are major
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5 contributors to SLE pathogenesis [16]. SLE T cells display aberrant cell surface
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7 mediated signaling responses, and they produce increased amounts of IL-17 although the
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9 production of IL-2 is compromised [17-20]. Interestingly, lipid rafts, cholesterol rich
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11 domains on the surface of cells harboring signaling molecules aggregate on the surface
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13 membrane of SLE T cells [19] and contribute to disease pathology in lupus-prone mice
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15 [20]. We assumed that the complement activation product C3d might alter the functional
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17 activity of these T cells. We report here that C3d fragments localized within the lipid rafts
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19 of SLE T cells and contribute to abnormal T cell function.
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Patients and methods

Research subjects

This study was conducted in collaboration with University of Pittsburgh Lupus Patient Care and Translational Research Center. Patient selection and data collection were described in detail recently [14]. Briefly, all study participants were 18 years of age or older and provided written informed consent. All subjects met the 1982 or 1997 ACR revised classification criteria [21-22] for definite SLE and were consecutively recruited. The University of Pittsburgh Institutional Review Board approved this study. A total of 122 subjects were studied from February 2008 through November 2009. This cohort included three groups: SLE patients (n=46), patients with other autoimmune diseases (n=43) and healthy individuals (n=33). The group of other autoimmune diseases (OAD) included mainly patients with Sjögren’s syndrome (n=24), rheumatoid arthritis (n=11), dermatomyositis (n=5), scleroderma (n=1) and primary Raynaud’s syndrome (n=1). As part of their routine care, all patients with SLE underwent routine blood work including complete blood count (CBC), erythrocyte sedimentation rate, serum levels of C3 and C4, antinuclear autoantibody (ANA) and anti-dsDNA antibody levels. In addition, each patient underwent history and physical examination. Disease activity was assessed using the Safety of Estrogens in Lupus Erythematosus: National Assessment (SELENA) version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) Systemic Lupus Activity Measure (SLAM) [23-24].

T cell purification

T cells were isolated from peripheral blood samples by negative selection using the RosetteSep T cell purification kit following the instructions of the manufacturer (Stem Cell Technologies, Vancouver, Canada). Flow cytometric analysis was performed using a LSRII flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey), to assess the level of purity, and $\geq 95\%$ of the purified cell population were positive for CD3 the T cell specific cell surface marker. After purification, T cells were stored at -70°C or used immediately. Stored and fresh T cells have been shown in our laboratory to function similarly.

T cell culture and stimulation: cytokines production experiments

T cells (2.5×10^5 to 1×10^6) were cultured in RPMI 1640 medium (Cellgro Mediatech, Manassas, VA) which included: 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. The cells were cultured at 37°C , with CO_2 levels of 5% for 3-5 days (dependent on the experiment). T cells were incubated in plates pre-coated with anti-CD3 (clone AKT3) Ab (5 $\mu\text{g/ml}$) and anti-CD28 Ab (1 $\mu\text{g/ml}$). On the last day of the incubation, three hours prior data acquisition, phorbol myristate acetate (PMA) (10 ng/ml) (Sigma-Aldrich, St. Louis, MO) and calcium ionophore A23187 (0.5 $\mu\text{g/ml}$) (Sigma- Aldrich, St. Louis, MO) were added along with 1 $\mu\text{l/ml}$ brefeldin A (Sigma-Aldrich, St. Louis, MO). To measure the levels of IL4 and $\text{IFN}\gamma$, brefeldin A (1 $\mu\text{l/ml}$) was added five hours before data acquisition.

Antibodies

The following mouse anti-human Abs were used: anti-CD4-Pacific blue, anti-CD8-allophycocyanin-Cy7, anti-CD3-FITC, anti-CD4-PerCP, anti-IL2-PE and anti-IL17A-AF488, anti-IL4-AF488 and anti-IFN γ -PE (Biolegend, San Diego, CA, USA), anti-C3d (Quidel, San Diego, CA, USA), anti C3-PE (Santa Cruz Biotechnology, Inc., CA, USA). Cholera Toxin Subunit B (CT-B) Alexa Fluor 488 conjugate (Invitrogen, CA, USA). The anti-C3d Antibody (Quidel, San Diego, CA, USA) was labeled either with Alexa Fluor 647 or with Alexa Fluor 568 dye (Monoclonal Antibody Labeling Kit, Invitrogen, CA, USA) according to the instructions provided by the manufacturer.

Confocal microscopy

Freshly purified T cells were stained with conjugated cholera toxin subunit B for 45 min at 4°C, washed with PBS three times, fixed with 3% paraformaldehyde in PBS, washed again and incubated with anti-C3d-AF-568 for 1h at room temperature. After three more washes, the nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) for 5 min. After staining, cells were washed three times, mounted in fluorescence mounting media (DakoCytomation, Carpinteria, CA), and imaged using an Olympus BX62 motorized microscope fitted with a cooled Hamamatsu Orca AG CCD camera. The microscope, filters, and camera were controlled by iVision v. 4.0.9 (BiovisionTechnoogies, Exton, PA). For colocalization studies, the microscope was fitted with Zero-Shift DAPI, Texas Red and GFP-green filters (Semrock, Rochester, NY) that provided exact registration between pixels following channel overlap. Acquired images were further processed using the colocalization module of Volocity 4.2 (Improvision, Waltham, MA). For analysis of

the intensity profiles on fixed lymphocytes, both the green and the red channel of each image were analyzed with iVision 4.0.9 and the values generated exported to Prism 4.0 (GraphPad, La Jolla, CA).

Flow cytometry

Multi-color immunofluorescent staining was performed. Briefly, 50 µl of each sample (1-3 million cells) was stained using mouse anti-human monoclonal antibodies conjugated to different fluorochromes. We used antibodies against T cell surface markers (CD3, CD4 and CD8), and complement fragment C3d. The gate for analysis was set using isotype control monoclonal IgG1-allophycocyanin or monoclonal IgG1-PE, (Biolegend, San Diego, CA, USA). Positive populations were quantified as percent of positive cells within each subset. Samples were acquired using a LSRII flow cytometer. Data analysis was performed using with FlowJo v.7.5.5. (Tree Star Inc.). Intracellular staining was performed with the BD Cytofix/Cytoperm kit, according to instructions of the manufacturer. The surface staining with anti C3d, anti CD4 and anti CD8 antibodies, was done before the specific intracellular staining. All monoclonal antibodies were used at a concentration of 5 µg/ml.

Ca⁺⁺ influx analysis

These assays were performed as previously described [27]. Briefly, 5x10⁶ non-stimulated T cells were treated with 2 mM R406 (Sigma-Aldrich, St. Louis, MO) or equal volumes of DMSO (Sigma-Aldrich, St. Louis, MO) for 1 hr at 37°C and incubated with 1 mg/ml

Indo-AM (Molecular Probes Inc., Eugene, OR) for 30 min at 37°C. After staining with anti-C3d-AF647 (Quidel, San Diego, CA, USA), samples were run and after 30 seconds, either anti-CD3 (10 mg/ml) or the isotype control mIgG2a were added followed by goat anti-mouse cross-linker at 1 min and the ratio of the fluorescence (of violet to blue emission) which is directly proportional to free cytosolic Ca^{++} , was recorded for 400 seconds using a LSRII flow cytometer. The results were analyzed using FlowJo (v.7.5.5) software package (Tree Star Inc.).

Statistical analysis

Descriptive statistics were computed as the mean $\pm$ standard deviation. Student’s t-test was performed to determine statistical significance among the three study groups (we compared SLE with each of the other 2 groups): SLE, OAD and normal controls (N). A p-value of ≤ 0.05 was considered significant.

Results

Population demographics

Study subjects (n=122) were separated into three different groups: SLE patients, patients with OAD and healthy controls. The demographic characteristics of these groups are summarized in Table 1. Briefly, the majority of the patients were Caucasian women ($\geq 80\%$). As expected, SLE patients were younger by an average of twenty years when compared to the other groups (29 ± 10 years). Major clinical manifestations of the SLE patients are summarized in Table 2. Mean disease duration of the SLE group was 14.4 ± 9.2 (range 1-41) years. The majority of the SLE patients had arthritis or arthralgia (91.3%), 95.6% were ANA positive and 80.4% had autoantibodies other than ANA. One third of the SLE patients had decreased complement levels. Mean disease activity, as defined by SELENA-SLEDAI and SLAM, was 3.1 ± 4.2 and 6 ± 3.9 , respectively. The patients had been treated with multiple (one to 5, average: 2.7) medications. The most prevalent medications were: Prednisone (78%), hydroxychloroquine (63%) and methotrexate (27%). Only few patients were treated with mycophenolate mofetil (18%) and azathioprine (12%). Cyclophosphamide and colchicine were used only in one patient each. Significant portions of the patients were treated with non-steroidal anti-inflammatory drugs (57%). No correlation was found between the type, combination and number of medications used to treat patients and the levels of the C3d fragments on the surface of T cells (data not shown).

SLE T cells are highly decorated with C3d

Using flow cytometry, we determined the levels of C3d fragments on the surface membrane of non-stimulated T cells from SLE patients, patients with OAD and healthy individuals. Significantly higher proportion of the SLE T cells were found to be positive for C3d (Figure 1A-B) ($13.58 \pm 3.92\%$) when compared with normal subjects ($4.52 \pm 2.925\%$) ($P \leq 0.00005$) and OAD patients ($6.31 \pm 4.57\%$) ($P \leq 0.005$). In addition, SLE CD4+, CD8+ and double negative (DN) subsets were found to be highly decorated with C3d compared with the same subpopulations in the other study groups (Figure 1C). Interestingly, the C3d fragments were more prominent on the CD8 subpopulation.

We next searched for possible correlation between the levels of C3d fragments on the surface of T cells and various clinical parameters. We failed to detect any correlation between the levels of C3d fragments on T cell surface and various clinical parameters including disease activity, as defined by SLAM and SLEDAI scores, serum C3 and C4 levels, anti dsDNA titers, ESR and CRP levels.

C3 and C3 fragments are prevalent on the surface of SLE T cells

In SLE literature, the distinction between C3d and C3 end fragments is not always made, and therefore fragments identified on T cells membranes are called C3d irrespective of their actual size. The monoclonal anti-C3d antibody that we used is specific for an antigen expressed on the C3d domain of C3 and therefore potentially identifies C3 and all C3d-containing fragments of C3. Using flow cytometry we failed to detect cells that stained positive with anti-C3b or anti-iC3b mAb (data not shown).

Ca⁺⁺ influx response is decreased in C3d+ SLE T cells

T cells from SLE patients have been shown to display multiple abnormalities including hyperactive TCR/CD3-triggered responses [27]. In T cells, ligation of the TCR/CD3 complex leads to a rapid increase in the intracellular Ca⁺⁺ concentration. We considered that the presence of C3d fragments on the surface of T cells might be responsible for the altered T cell responses to TCR/CD3 engagement. We compared the anti-CD3-induced changes in Ca⁺⁺ influx in C3d+ T cells and in C3d- T cells from SLE patients. The baseline recordings were similar for both C3d+ and C3d- SLE T cells (Figure 2A-2B). Peak Ca⁺⁺ flux responses were significantly higher in the C3d- as compared with the C3d+ (p < 0.011) cells (Figure 2A, 2C). The time to peak (the time difference between the cross linker application and the Ca⁺⁺ influx peak) appeared to be shorter in the C3d+ group (p < 0.101) (Figure 2D).

Cytokine production by C3d+ T cells is increased

To assess the functional importance of C3d fragments we compared the profile of cytokine production between the C3d+ T cell population and the C3d- T cell population and between the different study groups. We used conjugated antibody against C3d combined with intracellular staining for different cytokines and recorded the data using flow cytometry studies. The production (by the whole T cell population) of IL-2 and IFN- γ was decreased in the SLE T cells when compared with T cells from normal (p<0.05) and OAD patients (P<0.05). However, the subpopulation of C3d+ T cells produced significantly more IL-2, IFN- γ , IL-4 and IL-17 (Figure 3 A-D) when compared to the C3d- T cells. The increased production of various cytokines by the C3d+ T cell

population was not limited to T cells from SLE patients and a similar pattern was observed in T cells from patients with OAD and normal subjects.

C3d partially co-localized within aggregated lipid rafts on a subpopulation of SLE T cells.

The natural receptor of C3d is CR2 (CD21), normally found on the surface of B cells. Fifteen percent of human T cells [28] and thymocytes [29] have been reported to express CR2 on the surface membrane. We considered that C3d may bind to CR2 expressed on the surface of T cells and by ligand- receptor interaction mediated the effects we observed. When SLE and normal T cells were stained with an anti-CR2 Ab, we failed to detect measurable amounts of CR2 (data not shown). Therefore, we considered that C3d deposits could directly into the lipid rafts on the surface of T cells. To investigate this possibility, we used wide-field fluorescence microscopy (Figure 4A), followed on some cases by deconvolution (Figure 4D) to analyze the cell surface distribution of the cholera toxin B (CTB) conjugated to AF- 488 (green) and an anti-C3d antibody conjugated to AF- 568 (red). Cholera toxin B (CTB) is a known lipid raft marker as it attaches to ganglioside GM1, which is a specific lipid raft glycosphingolipid. Even though the SLE T cells were not stimulated, in a subpopulation of T cells the lipid rafts were clustered, as reported previously [25-26]. By microscopical analysis, the percentage of double positive T cells (for both C3d and CTB) represented about 10% of all the cells investigated. Figure 4 A shows a low magnification of several T cells stained for both lipid rafts (green) and complement fragment C3d (red), imaged at their largest diameter. The region of interest on the plasma membrane of one of the T cells is shown in yellow in Figure

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3 4B. Our results (Figure 4C) show that only certain C3d molecules were associated with
4 lipid rafts (peaks 1 and 4) whereas others (peaks 2, 3 and 5) showed minimal or no co-
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6 localization. To further analyze the extent of co-localization between C3d and lipid rafts,
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8 we performed three-dimensional reconstitution of T cells, and the collective signal from
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10 the entire membrane surface was analyzed (Figure 4D). Our results show that on certain
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12 areas of T cell surface, C3d fragments were adjacent to lipid rafts (Figure 4D, top insert),
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14 fully co-localized (Figure 4D, middle insert) or were separate (Figure 4D, bottom insert).
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16 Twenty one percent of the lipid raft molecules co-localized with C3d fragments, whereas
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18 39% of the total C3d signal co-localized with clustered lipid rafts. Taken together, these
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20 results suggest that in a subpopulation of SLE T cells, about 40% of the cell surface
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22 C3d fragments bind to lipid rafts.
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Discussion

Complement activation occurs at a low grade under physiological conditions and is necessary to fend off infections, eliminate tumor cell growth and participate in the homeostasis of the immune system [30-32]. Excessive activation of the complement cascade, as seen in patients with SLE, contributes to tissue injury through the formation of immune complexes or by binding to cell surface membranes and altering cell function. We report here increased C3d fragments deposition on the surface membrane of CD4+ CD8+ and DN cells from patients with SLE compared to T cells from normal individuals or other autoimmune diseases. C3d was found deposited within the lipid rafts and more was associated with significantly increased production of: IL-2, 4, 17 and IFN-g.

The frequency of C3d+ T cells is remarkably variable among SLE patients, ranging from barely detectable to 25%. This variation probably represents the fact that we included patients with variable degrees of disease activity presenting with a wide range of complement activation. Yet, cross-sectional analysis of our data failed to reveal any correlation between the percentage of C3d+ T cells and various disease activity parameters including serum levels of C3 or C4. Failure to detect an association between the serum complement levels and C3d+ T cells may be because the rate of generation of C3d fragments may reflect the degree of complement activation rather than the absolute values of C3. Alternatively, the presence of C3d fragments on the surface of T cells may be off phase with the measured clinical parameters probably preceding them by an unknown Δt [15]. In that respect, the presence of C3d fragments on the surface of T cells

may represent a marker of disease activity as claimed previously for complement deposition on the surface of erythrocytes [13].

The source of these observed C3 fragments on the surface of T cells could be either the circulating C3 or C3 that is produced by T cells upon activation, as demonstrated during allograft rejection [33] and autoimmune encephalomyelitis [34]. When the complement system is activated, C3 is cleaved to C3b, which in turn amplifies the complement cascade and deposits on the cell surface and in turn degrades to C3dg and its product C3d. As the half-life of the thioester bonds in C3b molecules is around sixty milliseconds, C3d fragments identified on T cells can have two possible origins: newly generated following interaction of anti-T cell auto-antibodies with T cells or, generated in solution by preexistent immune-complexes and covalently deposited on proteins on T cell membranes.

In light of the possibility that autoantibody-triggered complement activation may contribute to increases in SLE disease activity, it is reasonable to postulate that autoantibodies that recognize and bind to T cell surface antigens are, at least in part, responsible for mediating the observed C3d fragments (and C4d) on SLE T cells. Anti-lymphocyte autoantibodies (ALAs) have previously been reported in patients with SLE and other autoimmune diseases [35] and have been implicated in functional alternation and complement-mediated lysis of target lymphocytes [35]

To explore this possibility, we performed a pilot study using a large cohort of SLE patients (inclusive of the patients reported in the present study) to investigate the involvement of ALAs in mediating complement activation on the surface of SLE T cells.

Preliminary data showed that immunoglobulins could be detected concomitantly with C3d (and C4d) on the surface of T cells in approximately 30% of SLE patients (Liu et al, unpublished data). This finding implies a possible mechanism whereby ALAs bind to the surface of SLE T cells and trigger in situ the activation of complement proteins present in the plasma, resulting in deposition of C3d (and C4d) on T cells. However, considering that immunoglobulins was not always detectable on T cells in the SLE patients investigated in our pilot study, the possibility that other mechanisms are involved in mediating surface deposition of C3d (and C4d) cannot be ruled out.

Having previously established that CD3-mediated cell signaling is aberrant in SLE [18-20], we asked whether C3d+ T cells define a T cell subset with altered early signaling response. The observation that C3d+ T cells display a rather dampened Ca^{++} influx response following the engagement of CD3 suggests that C3d fragments may represent an effort to suppress the increased response of T cells to autoantigen. Alternatively, C3d decorated T cells may represent a final effector cell subset unable to respond further to antigenic stimulation. Yet, the C3d+ cells are not undergoing apoptosis because they did not express annexin V on the surface membrane (not shown). It should be noted that Ca^{++} influx is only a general marker of cell activity and it does not always correlate with other functional studies and cytokine production, for example in SLE T cells Ca^{++} influx is increased [27] but IL-2 production is decreased [20].

The subpopulation of C3d+ T cells produced significantly more IL-2, IFN- γ , IL-4 and IL-17 (Figure 3 A-D). This observation suggests that deposition of C3d on the surface of

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3 SLE T cells and more specifically, within the aggregated lipid rafts, occurs after the cells
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5 have been routed to produce a cytokine typifying Th1, Th2 or Th17. The fact that the
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7 cytokine production is modified in all C3d+ groups (SLE, OAD and N) may implicate
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9 that the complement fragments themselves modify the T cell function and the observed
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11 phenotype is not related to SLE T cells intrinsic abnormalities rather to a general
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13 immunologic interaction between C3d and T cells. However, we assume that SLE T cells
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15 abnormalities combined with the prolonged and persistent activation of the complement
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17 system in these patients make SLE T cells more susceptible to increased deposition of
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19 complement activation products and their effects. Nevertheless, C3d+ T cells represent a
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21 hyperactive subpopulation that may contribute to the pathogenesis of SLE by enhanced
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23 interaction with different components of the immune system mediated by the increased
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25 cytokine production.
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34 Lipid rafts are highly ordered cholesterol and ganglioside (GM1)-rich platforms that can
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36 facilitate and coordinate close interactions between critical signaling molecules to
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38 amplify signals downstream to TCR/CD3 complexes [36-37]. SLE T cells have a
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40 significantly larger intracellular pool of GM1 than normal T cells [38-39]. In addition, the
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42 lipid rafts are aggregated on the surface membrane of SLE T cells even before
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44 stimulation with unique distribution of crucial signaling molecules when compared with
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46 normal T cells with exclusive expression of FcR γ chain, decrease expression of CD3 ζ
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48 and detectable levels of active Syk kinase (phosphorylated Syk) [25-26]. We were able to
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50 demonstrate a localization of the C3d fragments within the aggregated lipid rafts (Figure
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55 5 A-B). We suggest that C3d becomes part of the lipid rafts and modulates TCR/CD3-
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mediated signaling process as manifested by the increased cytokine production. Moreover, calcium influx has been shown to be lipid raft-dependent [40-41] and we assume that it could be modified by the C3d fragments being in close vicinity to the TCR and in the lipid rafts.

Conclusion

This study has identified increased numbers of C3d+ T cells in patients with SLE but not with other autoimmune diseases and that these cells produce increased amounts of cytokines. The propensity of C3d decorated T cells to produce more cytokines is not limited to SLE T cells and it appears to represent a general phenomenon. Probably, complement activation that occurs during the response to any pathogen may facilitate cytokine production to further improve that ability of the host organism to eliminate intruding pathogens. The increased numbers of C3d+ T cells in patients with SLE may though contribute to the immunopathogenesis of the disease because increased production of IL-4, and IL17 may further advance the inflammatory response and tissue damage. A prospective study is needed to determine the value of determining the numbers of C3d+ T cells in predicting disease activity. Although complement inhibition has been shown to mitigate disease in lupus-prone mice, studies are needed to determine how complement activation advances organ damage by involving T cells.

List of abbreviations

SLE- systemic lupus erythematosus; OAD- other autoimmune disease; N- normal; IL – interleukin; IFN- interferon; AF- Alexa Fluor

Authors' contributions

OB performed the majority of the scientific experiments and part of the statistical analysis and helped to draft the manuscript. ZP participated in the design of the study, performed part of the scientific experiments, performed part of statistical analysis and drafted the manuscript. IG was instrumental to the gathering and the analysis of the microscope data. CCL, AK, SM, JA recruited the patients, analyzed t clinical data and helped to coordinate the work. GT designed the study, helped to coordinate the work and to draft the manuscript. All authors read and approved the final manuscript.

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Figure 1 - Increased C3d+ T population in SLE patients

(A) Representative histogram of an SLE and a normal sample. (B) The average percentage of C3d+ T cells in SLE (n=8), OAD (n=6) and normal (N) (n=9) samples. (C) C3d+ CD4+, CD8+ and double negative (DN) cells in SLE, OAD and N samples. OAD- other autoimmune diseases; N- normal

Figure 2 – C3d+ SLE T cells display decreased anti-CD3-induced free intracytoplasmic Ca^{++} .

(A) Representative histogram of the free Ca^{++} concentration as recorded in C3d- and C3d+ SLE T cell subpopulations. C3d+ SLE T cells are less excitable when compared with C3d- SLE T cells. (B) Representative diagram demonstrating the gating used to define the C3d- and the C3d+ subpopulations. (C) The peak of the Ca^{++} influx (ratio of indo-violet to indo-blue) was calculated by subtracting baseline ratio of indo-violet to indo-blue from the peak values. The Ca^{++} levels recorded from the C3d+ SLE T cell population were significantly decreased when compared with the C3d- subpopulation ($p \leq 0.011$). The standard error for the C3d- population is 1.846 and for the C3d+ is 1.104. (D) The Ca^{++} influx peak times (the time between the point of adding cross-linker and the

Ca⁺⁺ influx peak) tended to be shorter in the C3d+ T cell population (p≤ 0.101). Every dot in the figures (C-D) represents one patient.

Figure 3 - C3d+ T cells produce increased amounts of cytokines.

(A-D) Three groups of peripheral T cells (SLE, OAD, N) were stimulated with anti-CD3/anti-CD28. Harvested T cells were stained for C3d and thereafter for intracellular cytokines (IL2, IL4, IL17, IFN γ). The cytokine production was significantly increased in the C3d+ T cell population within all the groups studied (SLE, OAD, N). Every dot in the figures (A-D) represents one patient. The y- axis represents percentage of T cells producing the indicated cytokine.

OAD- other autoimmune diseases; N- normal; IL – interleukin; IFN- interferon

Figure 4- C4 complement fragments partially co- localize with lipid rafts.

Lymphocytes were stained as above and fixed in suspension with 3.8% paraformaldehyde for 5 min. Cells were then washed and mounted in anti-fading media on slides. A) Low magnification image of a group of T cells. B) Overlay of the region of interest (yellow circle) where intensity analysis of the signals originating from the lipid rafts and the C3d fragments was performed. C) Histogram of the intensity profile of the lipid raft marker (*green*) and C3d fragments (*red*) starting counter-clockwise. The numbers associated with each peak corresponds to the numbers adjacent to the cell in “B”. D) Serial sections were acquired using a wide-field microscope and used to reconstitute the maximum intensity projection (MIP) of the whole T cell following de-convolution. T cells displayed a strong surface staining for lipid raft marker (*green*) and C3d fragments (*red*) with no

detectable intracellular staining. There was adjacent staining of the two markers on the top section (magnified top inset), significant co-localization detailed in the middle inset as well as separate locations (bottom inset). The bar represents 8 μm in A and B and 2.8 μm in D.

Table 1: Demographic features of study groups

Diagnosis	SLE	Other autoimmune diseases	Healthy individuals
Gender (% of female)	91.3%	96%	84.8%
Race (% of Caucasian)	82.6%	90%	90.9%
Age (year) mean $\pm$ SD; range	28.85 $\pm$ 10.2; 11- 58	52.86 $\pm$ 12.5; 26-74	43.64 $\pm$ 15.7; 19- 73

Table 2: Clinical manifestations of SLE patients.

CLINICAL MANIFESTATION AND DISEASE ACTIVITY SCORES	TOTAL NUMBER OF PATIENTS WITH SLE, N=46
Positive clinical manifestation - if ever present:	

Arthritis/ Arthralgia	91.3%
Malar rash	47.8%
Discoid rash	13.0%
Photosensitivity	52.1%
Serositis ^a	34.7%
Oral ulcers	63.0%
Neurological complications ^b	10.8%
Renal involvement	39.1%
Hematological abnormalities ^c	67.3%
Serological findings	80.4%
Anti- Smith	15.2%
Anti- Phospholipid	2.1%
Anti- dsDNA	52.1%
Antinuclear antibodies	95.6%
Positive clinical manifestation during the cohort:	
Serum C3 (%below normal; N=46	15.2%
Serum C4 (%below normal; N= 46)	34.7%
SLAM mean ± SD; range	6 ± 3.9; 0-25
SLEDAI mean ± SD; range	3.1 ± 4.2; 0- 24
^a Pleuritis (23.9%) and pericarditis (21.7%)	
^b Psychosis (4.3%) and seizure (6.5%)	
^c Anemia (6.5%), leukopenia (34.7%), lymphopenia (50.0%) and thrombocytopenia	

(30.4%)

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Figure 1

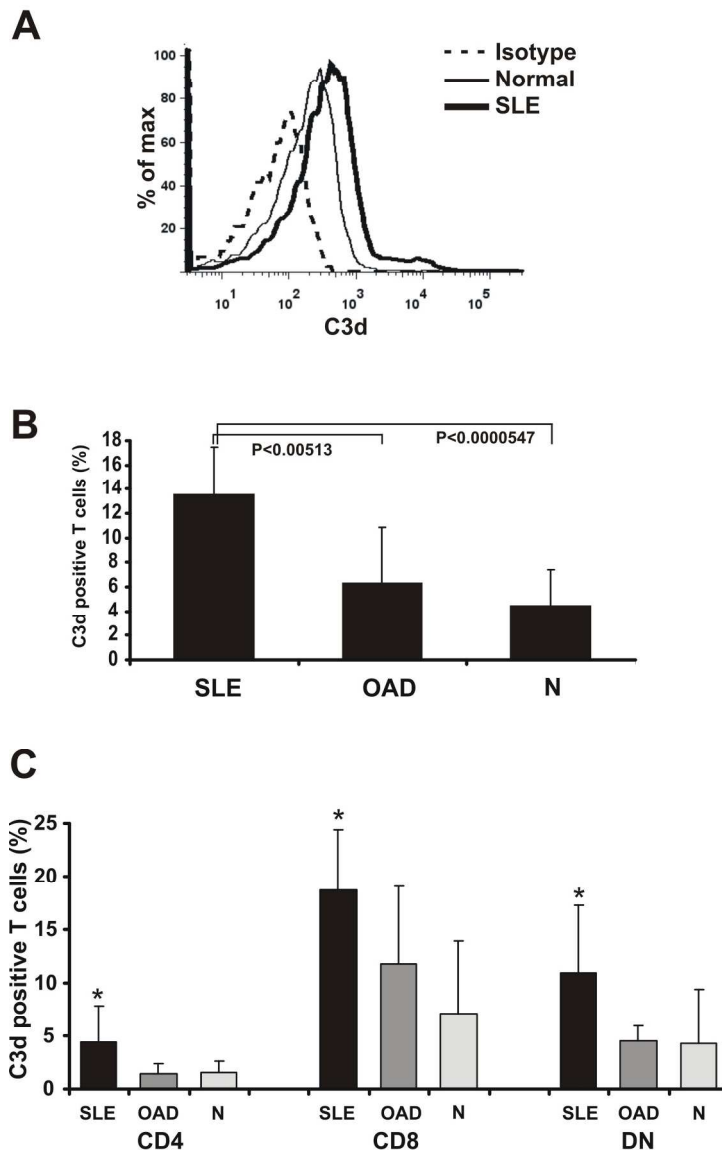


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 OAD- other autoimmune diseases; N- normal

159x254mm (300 x 300 DPI)

Figure 2

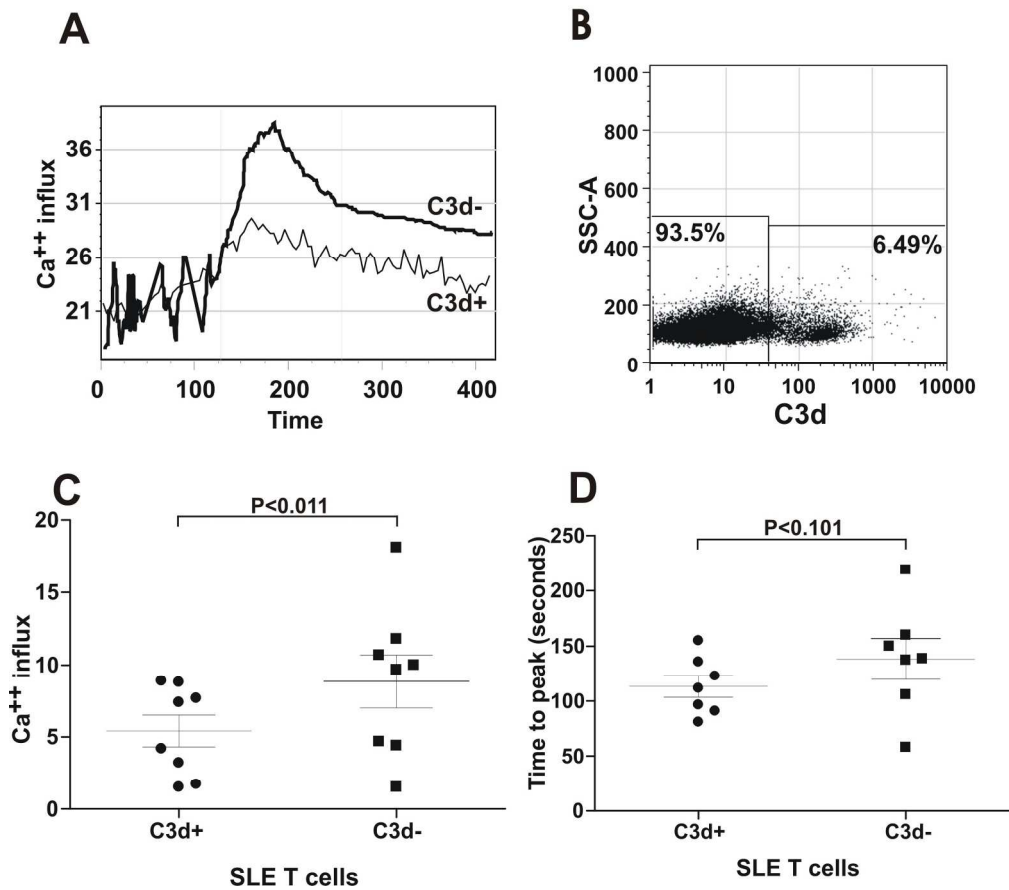


Figure 2 – C3d+ SLE T cells display decreased anti-CD3-induced free intracytoplasmic Ca^{++} .

(A) Representative histogram of the free Ca^{++} concentration as recorded in C3d- and C3d+ SLE T cell subpopulations. C3d+ SLE T cells are less excitable when compared with C3d- SLE T cells. (B) Representative diagram demonstrating the gating used to define the C3d- and the C3d+ subpopulations. (C) The peak of the Ca^{++} influx (ratio of indo-violet to indo-blue) was calculated by subtracting baseline ratio of indo-violet to indo-blue from the peak values. The Ca^{++} levels recorded from the C3d+ SLE T cell population were significantly decreased when compared with the C3d- subpopulation ($p \leq 0.011$). The standard error for the C3d- population is 1.846 and for the C3d+ is 1.104. (D) The Ca^{++} influx peak times (the time between the point of adding cross-linker and the Ca^{++} influx peak) tended to be shorter in the C3d+ T cell population ($p \leq 0.101$). Every dot in the figures (C-D) represents one patient.

199x187mm (300 x 300 DPI)

Figure 3

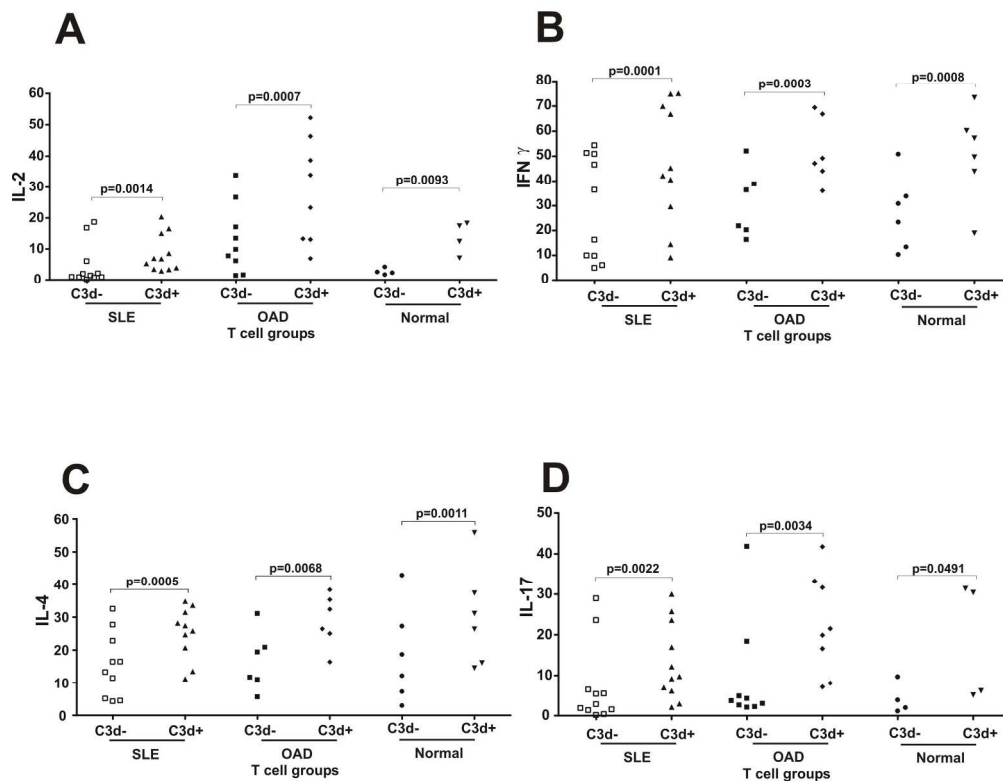


Figure 3 - C3d+ T cells produce increased amounts of cytokines.

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OAD- other autoimmune diseases; N- normal; IL – interleukin; IFN- interferon

199x198mm (300 x 300 DPI)

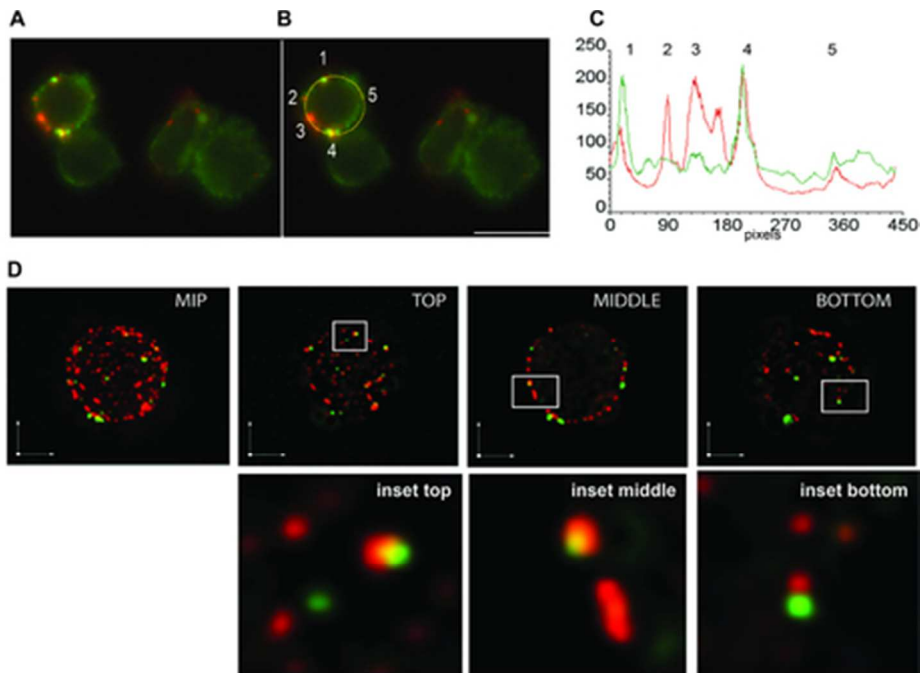


Figure 4- C4 complement fragments partially co- localize with lipid rafts. Lymphocytes were stained as above and fixed in suspension with 3.8% paraformaldehyde for 5 min. Cells were then washed and mounted in anti-fading media on slides. A) Low magnification image of a group of T cells. B) Overlay of the region of interest (yellow circle) where intensity analysis of the signals originating from the lipid rafts and the C3d fragments was performed. C) Histogram of the intensity profile of the lipid raft marker (green) and C3d fragments (red) starting counter-clockwise. The numbers associated with each peak corresponds to the numbers adjacent to the cell in "B". D) Serial sections were acquired using a wide-field microscope and used to reconstitute the maximum intensity projection (MIP) of the whole T cell following de-convolution. T cells displayed a strong surface staining for lipid raft marker (green) and C3d fragments (red) with no detectable intracellular staining. There was adjacent staining of the two markers on the top section (magnified top inset), significant co-localization detailed in the middle inset as well as separate locations (bottom inset). The bar represents 8 μ m in A and B and 2.8 μ m in D. 38x28mm (300 x 300 DPI)

**Cell-Bound Complement Activation Products and T Cell Dysfunction
in Human Patients with Systemic Lupus Erythematosus**

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Conflict of Interest statement: Dr.Ahearn and Dr. Manzi are paid consultants of Exagen

Diagnostics. Dr. Liu, Dr. Ahearn, and Dr. Manzi are co-inventors of the Cell-Bound Complement Activation Products (CB-CAP) intellectual property.

Abstract

Several decades of investigation have intimately linked systemic lupus erythematosus (SLE) with the complement system. However, the consequences of complement activation with regard to T cell function in SLE are largely unexplored. It has recently been reported that significant levels of C4d, a complement activation product (CAP), are present specifically on the surface of T cells of patients with SLE. This observation led to the hypothesis that CAPs such as C4d may bind to SLE T cells and cause functional dysregulation of these cells, thereby initiating and/or augmenting immunopathogenic mechanisms in SLE. The current study was performed to investigate the localization and functional consequences of C4d deposition on SLE T cells. Confocal microscopy and co-immunoprecipitation studies showed that C4d was associated with TCR/CD3 complexes in membrane lipid rafts of SLE T cells. Intracellular phosphoprotein analysis showed that C4d-positive SLE T cells exhibited abnormalities in signal transduction as evidenced by erratic phosphorylation of signaling proteins upon stimulation. Antibody array and RT-PCR studies showed that SLE cells bearing high levels of C4d produced increased levels of cytokines including IL-17A. Intracellular cytokine staining study confirmed that significantly higher frequency of IL-17A-producing T cells was detected in SLE patients than in patients with other diseases or healthy controls. Moreover, C4d-positive SLE T cells, as compared to C4d-negative T cells, proliferated more significantly in response to polyclonal T cell stimulation *in vitro*. Together, these results demonstrate that C4d bound to critical surface membrane proteins of SLE T cells can lead to aberrant signal transduction and other downstream effects, which may contribute to T cell dysfunction and SLE pathogenesis.

Introduction

Systemic lupus erythematosus (SLE) is arguably the most clinically and serologically diverse autoimmune disease, with more than 100 autoantibodies found in patients (1) and disease spectra ranging from subtle symptoms to life-threatening multi-organ failure. The hallmark changes in SLE, including production of autoantibodies, deposition of immune complexes in tissues, and excessive complement activation, are generally thought to be consequences of immune dysregulation (2, 3).

Several lines of evidence support an essential role for T cells in the development and full disease penetrance of SLE (4, 5). First, the presence of high-affinity, isotype-switched antibodies against autoantigens (e.g., anti-double stranded DNA (dsDNA) IgG) in the sera of SLE patients suggests a T cell-dependent humoral immune response (6). Second, T cells derived from patients with SLE in general exhibit phenotypic features of activated cells (e.g., CD25⁺, CD69⁺, CD40L⁺, HLA-DR⁺) (7). Third, disease activity-correlated clonal expansion of selected populations of autoreactive T cells has been reported in some patients with SLE (8). Fourth, autoreactive T cells isolated from patients with SLE have been shown to drive the production of potentially pathogenic anti-dsDNA antibodies by autoreactive B cells (9). Fifth, T cells have been shown to infiltrate the inflamed kidneys of SLE patients (10). Lastly, a recent study has demonstrated that T cells are capable of promoting/sustaining SLE-like disease in lupus-prone mice that lack the ability to produce secreted antibodies (11).

Autoreactive T cells have been reported to exist in healthy individuals as well as patients with SLE (12, 13). Whereas such autoreactive T cells apparently remain quiescent in healthy individuals, they seem to thrive and mediate, directly or indirectly, a cascade of aberrant immune events in patients with SLE. It is possible that T cells from patients with SLE carry certain inherent defects that can promote their survival and (hyper)active state and consequently contribute to the generalized dysregulation of the immune system characteristic of SLE (14). Recent studies have demonstrated that such defects include decreased production of interleukin-2 (15), reduced expression of the ζ chain of the T cell receptor/CD3 complex (16), increased and accelerated TCR-mediated signaling responses (17), hyperreactivity to antigenic stimulation (18), and resistance to anergy induction (19). Moreover, it has recently been shown that some of these abnormalities may result from alterations in the composition and distribution of lipid rafts (20, 21). This latter finding is of particular interest, because lipid rafts, highly ordered cholesterol and ganglioside-rich platforms in the surface membrane of T cells, are capable of facilitating and coordinating close interactions between critical signaling molecules involved in downstream responses (22). The observed hyperactivation and dysregulation of T cells may also result from interplay between these cells and diverse extrinsic signals that are unique to SLE-susceptible individuals. Recently, several laboratories have reported elevated IFN α levels in the serum of patients with SLE, distinct patterns of dysregulated expression of type 1 IFN-inducible genes by peripheral blood mononuclear cells of SLE patients, and a correlation between IFN α levels and SLE disease severity (23, 24). These observations suggest an important role for IFN α in the immunopathogenesis of SLE. However, given the complex nature of SLE, additional extrinsic triggers are likely to exist.

Because of the well-established role of complement in SLE (25, 26), it is conceivable that complement proteins, especially their proteolytic fragments generated during activation (herein referred to as complement activation products (CAPs)), may play a crucial role in regulating T cells in SLE. Since complement proteins are abundant in the circulation and in tissues, they can readily interact with cells circulating in the blood (e.g., erythrocytes and lymphocytes) or infiltrating tissues. We recently confirmed this possibility by detecting significant amounts of CAPs, particularly C4d, present specifically on the surface of erythrocytes, reticulocytes, platelets, and lymphocytes from SLE patients (27-30). Accordingly, we hypothesized that CAPs, upon binding to immune effector cells such as T cells, may lead to dysfunction of these cells and in turn initiate/perpetuate autoimmune responses contributing to the immunopathogenesis of SLE. Based upon this rationale, the present study was conducted to investigate the functional changes associated with CAP-bearing T cells derived from patients with SLE.

Results and Discussion

It has recently been reported that complement activation products, particularly C4d, can bind to circulating blood cells including erythrocytes (E-C4d), platelets (P-C4d), and lymphocytes (T-C4d) (27-30). Such cell-bound complement activation product (CB-CAP) phenotypes have been shown to be specifically associated with SLE and may serve as lupus biomarkers for diagnosis and disease activity monitoring (27, 29-31). For example, T-C4d levels of patients with SLE (mean $\pm$ SD: 12.1 ± 20.5) were significantly higher than those of patients with non-SLE autoimmune diseases (2.5 ± 3.0) and healthy controls (1.7 ± 1.0) (30); T-C4d was 56% sensitive and 80% specific in differentiating SLE from other diseases (30). Given the importance of T cells in the pathogenesis of SLE (4, 5), it is reasonable to postulate that the binding of significant amounts of C4d to T cells would have substantial functional impact on these cells.

An interesting observation reported in a recent study is that the T-C4d phenotype apparently prevailed upon all T cells in a given SLE patient at a given time, as demonstrated by uniformly shift-to-the-right fluorescent intensity histograms (30) (Fig. 1A). This latter finding suggests that the functional impact of C4d binding, if any, is likely to be on the entire population of T cells instead of a subpopulation of T cells. Therefore, it is feasible to study functional alteration of SLE T cells using the unfractionated peripheral blood cell population with the aid of T cell-specific antibodies and stimulants. In the present study, we attempted to investigate the role of C4d in the dysregulation of SLE T cells by comparing the functional responses of cells derived from SLE patients with a positive T-C4d phenotype, SLE patients with a negative T-C4d phenotype, patients with non-SLE autoimmune diseases, and healthy individuals. Representative

patients were randomly selected from the large cohort of patients reported in our recent study (30).

Localization of C4d on the surface of SLE T cells

A crucial question concerning the role of CAP binding in T cell functioning is how CAP-mediated signals are integrated and transduced into cellular responses. In this regard, the location of these CAPs and the nature of their “receptor/acceptor” molecules undoubtedly may play important role in influencing a variety of cellular activities of T cells. Initial characterization of T cells from SLE patients, patients with other diseases, and healthy controls failed to detect surface-expression of complement receptor (CR) 1, CR2, and Fc receptors (FcR) (data not shown), suggesting that CAPs are unlikely to bind through CR and FcR to the surface of SLE T cells. Recent studies have shown that lipid rafts, highly organized cholesterol and ganglioside-rich platforms within the plasma membrane, can facilitate and coordinate close interactions between critical signaling molecules such as TCR/CD3 complexes (22, 32, 33). Based on those reports, we speculate that, if CAPs are deposited at or near lipid rafts, they may gain close access to the TCR/CD3 complexes and consequently alter downstream signaling processes.

To investigate the localization of C4d on the surface of SLE T cells and to explore the possibility that C4d may bind to molecules/micro-structures crucial for T cell signaling/functioning (e.g., TCR/CD3 complexes and lipid rafts), confocal microscopy was performed. As shown in Figure 1B, C4d appeared to be present on SLE cells in various patterns, ranging from a punctuate pattern with a concentrated “cap” (upper panels) to a diffuse distribution (middle panels). Interestingly, C4d appeared to co-localize with CD3 (upper panels) and lipid rafts (middle

panels) on SLE T cells. Because lipid rafts are known to be enriched in TCR/CD3 complexes and other signaling molecules, SLE T cells were also stained with CT-B and anti-CD3 to ensure the authenticity of the staining patterns observed (lower panels). Together, these results suggested that C4d is associated with signaling molecules in lipid rafts of SLE T cells.

To further verify the possibility that C4d may covalently bind to surface molecules that are essential for T cell signaling (e.g., the TCR/CD3 complex), co-immunoprecipitation experiments were performed. The lysates of PBMC isolated from SLE patients were first incubated with agarose beads coated with a mouse anti-CD3 mAb or with isotype control mouse IgG, and the proteins eluted were analyzed for the presence of CAPs such as C4d. As revealed by the representative results shown in Fig. 2, an anti-C4-reactive protein (M_r ~65-70 kD) was present in the eluate of anti-CD3-coated beads, but not in that of mouse IgG-coated beads. The apparent molecular mass of this molecule suggests that it is a complex of C4d (M_r 45 kD) and a subunit of the TCR/CD3 complex (e.g., CD3 γ , CD3 ϵ , or CD3 δ ; M_r ranging from 20-23 kD). This result implied that C4-derived products are associated with the TCR/CD3 complexes in SLE T cells and thus may play a role in perturbing T cell signaling and functioning. Interestingly, in a recently established series of *in vitro* studies that generated CAP-bearing T cells, it was observed that TCR/CD3-negative variants of human Jurkat T cells, as compared to TCR/CD3-positive Jurkat cells, were significantly less susceptible to C4d binding (Liu et al, unpublished data). This finding lent additional support to the possibility that TCR/CD3 complexes are important "acceptor" molecules for CAPs on T cell surfaces.

Abnormal signaling processes in C4d-bearing SLE T cells

The myriad abnormalities reported for SLE T cells may, at least partially, stem from dysregulated signaling processes in these cells. Therefore, it is important to determine if the intracellular signaling pathways are abnormal in SLE T cells and whether abnormal signaling processes may be associated with CAP deposition on T cells. Measurement of phosphorylation of signaling proteins has commonly been performed to investigate signaling pathways (34-36). Thus, intracellular phosphoprotein staining studies were performed to examine the Jak-Stat pathway and MAPK/ERK pathway, two important families of signaling proteins that regulate immune responses (37, 38), in T cells (39). Peripheral blood cells isolated from patients with SLE, patients with other diseases, or healthy controls were stimulated with IL-2, IL-4, or PMA, and subsequently stained with antibodies specific for CD3 and phosphorylated STAT5 (triggered by IL-2), STAT6 (triggered by IL-4), and ERK1/2 (triggered by PMA). In T cells of healthy controls and patients with other diseases, specific responses of these signaling pathways to different stimuli occurred in an expected manner. However, erratic responses were detected in T cells from SLE patients (Fig. 3). The signaling responses to cytokines and mitogens appeared to remain relatively normal in SLE T cells bearing none or low levels of C4d. However, these signaling proteins appeared to respond hyperactively or non-specifically in T cells bearing high levels of C4d. This observation is in agreement with previously reported increased cytosolic protein tyrosine phosphorylation responses of SLE T cells (40). Interestingly, increased signaling responses to mitogens were similarly observed in human Jurkat T cells that had been rendered C4d-positive *in vitro* (data not shown). These results not only demonstrated irregular

signaling processes in SLE T cells, but also implied a causative relationship between the binding of C4d to T cell signaling molecules and aberrant signaling events.

Increased cytokine production by C4d-bearing SLE T cells

It is known that the immune abnormalities in SLE include skewed production of cytokines by T cells and other immune effector cells. We therefore speculated that the binding of CAPs to peripheral blood cells of SLE patients may trigger specific intracellular signals that ultimately influence the production of cytokines. To explore this possibility, antibody array experiments were performed to determine the global profile of cytokine production by peripheral blood mononuclear cells derived from SLE patients and healthy individuals. Cells derived from SLE patients with the T-C4d phenotype appeared to produce higher levels of IL-8, IL-4, IL-12, and IL-17 than did cells derived from healthy controls (Fig. 4A). The increase in cytokine production by C4d-bearing cells derived from SLE patients was further corroborated by real-time RT-PCR analyses of transcripts encoding various cytokines (Fig. 4B).

IL-17, a pro-inflammatory cytokine, is produced predominantly by Th17 cells (41, 42). Elevated levels of IL-17 have also been detected in the sera of patients with systemic sclerosis (43), inflammatory bowel disease (44), and SLE (45). These findings, combined with studies demonstrating increased infiltration of IL-17/Th17 within inflamed kidneys of SLE patients (10), suggest that dysregulated activation of Th17 cells and elevated levels of IL-17 are likely to contribute to the pathogenesis of various human autoimmune diseases including SLE (46). In this context, the observed increase in IL-17 proteins and transcripts in C4d-bearing SLE T cells warranted further investigation. Thus, intracellular cytokine staining was performed to analyze

the production of IL-17 by SLE T cells at the single-cell level. As shown in Fig. 4C, significantly higher frequencies of IL-17-producing CD4 T cells were detected in SLE patients, as compared to patients with other autoimmune diseases and healthy controls. In contrast, comparable frequencies of IFN γ -producing CD4 T cells and TNF α -producing CD4 T cells were found in SLE patients, patients with other diseases, and healthy controls (data not shown). This result provides additional support for the hypothesis that Th17 cells are involved in the pathogenesis of SLE.

Collectively, these results suggest that: 1) CAP-bearing immune effector cells of SLE patients preferentially produce a panel of cytokines different from that of normal cells, and 2) changes in cytokine production may result, at least in part, from stimulatory signals triggered by the binding of CAPs to signaling molecules within cell surface membrane.

Dysregulated proliferative responses of C4d-bearing SLE T cells

It is conceivable that binding of CAPs to targets on T cells may directly influence a variety of cellular activities that culminate in cell proliferation. Alternatively, such signals triggered via CAPs may indirectly augment and/or suppress other signals mediated through the TCR/CD3 complexes and co-stimulatory molecules such as CD28. Since it has been reported that T cells from lupus-prone mice and SLE patients exhibited heightened proliferation responses upon stimulation *in vitro* (18, 47), we postulated that deposition of CAPs on T cells may, in part, be responsible for altering the reactivity and proliferation of T cells in SLE patients. To investigate this possibility, peripheral blood cells isolated from SLE patients, patients with other diseases, or healthy controls were cultured in the presence or absence of various T cell stimulants, and cell proliferation was measured using the ^3H -thymidine incorporation assay. As shown in Fig. 5, T cells bearing high

levels of C4d proliferated more readily and profoundly than T cells bearing low levels of T-C4d . These data suggested that deposition of C4d on the surface of SLE T cells may prime them and consequently heighten their responses to antigenic or inflammatory stimuli.

Implications and perspective

The present study demonstrates that C4d bound to critical surface membrane proteins of SLE T cells may lead to aberrant signal transduction and additional downstream effects, which in turn may contribute to T cell dysfunction and overall abnormalities of the immune system in SLE patients. These and previous studies of T-C4d suggest that these lymphocytes, tagged with ligands generated during complement activation, may serve as lupus biomarkers and also identify potential targets for therapeutic intervention.

Methods

Study participants

SLE patients who met the ACR 1982 (48) or 1997 (49) revised classification criteria were recruited for this study during routine visits to the University of Pittsburgh Lupus Patient Care and Translational Research Center. Patients with non-SLE autoimmune or inflammatory diseases were recruited at various outpatient facilities at the University of Pittsburgh Medical Center. Healthy individuals were recruited through local advertisements posted around the University of Pittsburgh campus. Characteristics of these study participants have been described in detail recently (30). All study participants were 18 years of age or older and provided written informed consent. The University of Pittsburgh Institutional Review Board approved this study.

A blood sample was obtained from each study participant at the time of the study visit. An aliquot of each blood sample was immediately analyzed by flow cytometry to determine the levels of T cell-bound C4d and C3d (T-C4d and T-C3d) as described in a recent report that demonstrated significantly elevated levels of T-C4d and T-C3d on T cells of SLE patients (30). Representative SLE patients who were determined to have highly elevated T-C4d levels (referred to hereafter as “positive T-C4d phenotype”), SLE patients who had minimally elevated T-C4d (negative T-C4d phenotype), patients with other diseases, and healthy controls were selected for further studies described below.

Antibodies and reagents

Monoclonal antibodies (mAb) specific for human C4d and C3d (both mouse IgG1) were obtained from Quidel (San Diego, CA). Purified or fluorochrome-conjugated anti-human CD3 mAb, anti-

human CD4 mAb, anti-human CD8 mAb, anti-human CD28 mAb, anti-phosphorylated ERK1/2 mAb, anti-phosphorylated STAT5 mAb, anti-phosphorylated STAT6 mAb, anti-phosphorylated STAT6 mAb (all mouse IgG1), and mouse IgG1 isotype control were obtained from BD Biosciences (San Jose, CA). Reagent kits for intracellular cytokine staining (BD Cytofix/CytopermTM fixation/permeabilization solution kit) and intracellular phosphoprotein staining (BD PhosFlow starter kit), and Brefeldin A were also obtained from BD Biosciences. Phycoerythrin (PE)-conjugated mAb specific for human IL-17A, human IFN γ , and human TNF α were purchased from eBioscience (San Diego, CA). Recombinant human IL-2 and IL-4 were purchased from R&D Systems (Minneapolis, MN). Ionomycin, phorbol myristate ester (PMA), cholera toxin beta subunit, and a rabbit-anti-human C4 polyclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO). Antibody-labeling reagents (Alexa Fluor[®] dyes and Zenon[®] labeling kits) were purchased from Invitrogen (Carisbad, CA), Ficoll-Paque Plus, HRP-conjugated anti-rabbit Ig, and ECL Western Blotting Detection kit were purchased from GE Healthcare (Piscataway, NJ).

Cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated from SLE patients with a positive T-C4d phenotype, SLE patients with a negative T-C4d phenotype, patients with other diseases, and healthy controls using Ficoll density gradient centrifugation. One half of the isolated cells were immediately divided and used for culture, flow cytometry, and imaging studies. The other half of the isolated cells were washed and stored at -80°C for subsequent preparation of cell lysates and total RNA.

Confocal microscopy

PBMC freshly isolated from the selected patients were stained with an anti-C4d mAb labeled with Alexa Fluor 488[®] (green) and an anti-CD3 mAb labeled with Alexa Fluor 647 (deep red) (using Zenon[®] Tricolor mouse IgG1 Labeling kit). In parallel, an aliquot of isolated PBMC were stained with fluorescein isothiocyanate (FITC)-conjugated cholera toxin beta subunit (CT-B) (a ligand for GM1 ganglioside - a lipid raft marker (32)), and Alexa Fluor 647-labeled anti-C4d antibody. After washing, cells were adhered to poly-L-lysine-coated glass microslides, fixed with 0.75% paraformaldehyde, air-dried, and mounted using Permafluor (Shandon Lipshaw, Pittsburgh, PA) with coverslips. The slides were then analyzed using an upright confocal laser scanning microscope (Olympus FluoView 500).

Co-immunoprecipitation study

PBMC isolated from the selected SLE patients were lysed with a lysis buffer (phosphate-buffered saline (PBS) supplemented with 1% Triton-X100 and protease inhibitors (Pierce Halt Protease inhibitor cocktail; Thermo Scientific, Rockford, IL), and centrifuged to remove insoluble debris. The resulting cell lysates were incubated with gel beads that had been conjugated anti-CD3 mAb or isotype control mouse IgG1 following the manufacturer's instruction (Pierce Co-Immunoprecipitation kit; Thermo Scientific). After extensive washes, proteins bound on the beads were eluted using an elution buffer provided by the manufacturer. The eluted proteins were electrophoresed under reducing condition on 10% polyacrylamide gels containing SDS, electrotransferred onto Immobilon-P membranes (Millipore, Billerica, MA), and incubated with a polyclonal rabbit-anti-human C4 antibody followed by an HRP-conjugated secondary antibody. The immunoblots were visualized using ECL detection reagents.

Intracellular phosphoprotein staining

PBMC freshly isolated from selected study participants were resuspended in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin), aliquoted into 6-well culture plates (5×10^5 cells/well), and incubated at 37°C for 2 hours to overcome cellular agitation potentially induced during the isolation procedure. At the end of the 2-hr incubation, cells in individual wells were stimulated with, respectively, IL-2 (300U/ml), IL-4 (100 ng/ml), and PMA (40 nM) at 37°C for 15 min. Immediately following the stimulation, cells were fixed with paraformaldehyde (final concentration 1%) for 10 min, washed two times with PBS, and stained with a fluorescein isocyanate (FITC)-conjugated anti-CD3 mAb at 4°C for 20 min. After the surface staining procedure, cells were permeabilized with 75% methanol at -20°C overnight. After two washes with PBS, permeabilized cells were divided into 4 aliquotes and incubated with PE-conjugated mAb specific for phosphorylated STAT5 (pSTAT5), pSTAT6, pERK1/2, and isotype control mouse IgG, respectively. After a 20-min incubation, cells were washed with PBS and analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). T cells were specifically gated for analysis based on their expression of CD3 and forward/side scattering characteristics.

Antibody array analysis

Lysates of PBMC isolated from selected study participants were prepared as described above in the co-immunoprecipitation study. The cell lysate derived from each study participant was incubated with a TranSignal™ RayBio Human Cytokine Array membrane that is spotted in duplicate with antibodies specific for 18 cytokines and chemokines (Panomics, Redwood City, CA). After a 2-hr

incubation, the membrane was washed, incubated sequentially with the primary detecting antibody, the secondary detecting antibody, and chemiluminescence detection reagents, following the manufacturer's instruction.

Intracellular cytokine staining

PBMC freshly isolated from selected study participants were resuspended in RPMI 1640 culture medium at 1×10^6 cells/ml and stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (1 mg/ml) in the presence of Brefeldin A (10 mg/ml) at 37°C. After a 4-hour stimulation, cells were collected, washed, resuspended in FACS buffer (PBS with 1% calf serum), and stained for T cell surface markers using FITC-conjugated anti-CD8 and phycoerythrin-cyanine 5 (PECy5)-conjugated anti-CD4. Following the staining of surface markers, cells were washed, fixed, and permeabilized using the BD Cytofix/Cytoperm fixation/permeabilization kit following the manufacturer's instruction. The fixed/permeabilized cells were aliquoted and stained with PE-conjugated mAb specific for human IL-17A, human IFN γ , and human TNF α , respectively, for 20 min at room temperature. After washing, cells were subjected to flow cytometry analysis. CD4 T cells and CD8 T cells were gated separately for analysis based on the forward/side scattering characteristics and expression of CD4 and CD8.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from PBMC isolated from selected study participants using Trizol reagent (Invitrogen) and quantified by OD₂₆₀ measurement using spectrophotometry. One μ g of RNA derived from each study participant was reverse-transcribed using oligo(dT) primers and Reverse Transcription System (Promega, Madison, WI) following the manufacturer's

instructions. Real time PCR for cytokines was performed using a 7300 Real Time PCR System (Applied Biosystems). All reactions were performed using TaqMan® Gene Expression Master Mix and primers/probes purchased from Applied Biosystems. The expression level of a specific cytokine was calculated relative to the CD3ε amplicons amplified from the same sample using the comparative threshold cycle (Ct) method (also referred to as the $2^{-\Delta\Delta C_t}$ method) (50, 51). The cytokine transcripts analyzed included IL-2, IFN γ , IL-4, IL-6, IL-8, IL-10, IL-12p35, IL-12p40, IL-17A, MIP-1 α , and MIP-1 β .

Cell proliferation assay

PBMC isolated from study participants were plated into a 96-well culture plate in which each well had been coated with an anti-CD3 and/or anti-CD28 antibody at different concentrations (ranging from 0.1 to 10 mg/ml) or medium alone. Each experimental condition was tested in triplicate. Cells were cultured for different periods of time ranging from 2 to 6 days. During the final 18 hrs of the culture period, cells were labeled with ^3H -thymidine and harvested using a semiautomatic cell harvester (Tomtec Harvester) onto glass fiber filters. ^3H -thymidine incorporated by proliferating T cells was measured using a 1205 Betaplate liquid scintillation counter (LKB Wallac). The extent of cell proliferation was expressed as: proliferation index = counts per minute (cpm) of cells cultured in the presence of antibodies/cpm of cells cultured in medium alone.

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Figure 1. C4d is present on the surface of the entire population of SLE T cells and is colocalized with CD3 in lipid rafts within T cell membranes. (A) Representative histograms of flow cytometric analyses of C4d present on the surface of SLE T cells. Note the significant and complete shift-to-the-right of SLE T cells stained with anti-C4d mAb. Closed peak: isotype control mouse IgG1; open peak: anti-C4d. (B) Representative confocal microscopic images of C4d-positive SLE T cells. PBMC isolated from an SLE patient with the positive T-C4d phenotype were stained with an anti-C4d labeled with Alexa Fluor 488 (green) and an anti-CD3 mAb labeled with Alexa Fluor 647 (deep red) (upper panels) or with FITC-conjugated cholera toxin beta subunit (CT-B) (green) and Alexa Fluor 647-labeled anti-C4d antibody (deep red) (middle panels) or Alexa Fluor 647-labeled anti-CD3 antibody (deep red) (lower panels). 400X.

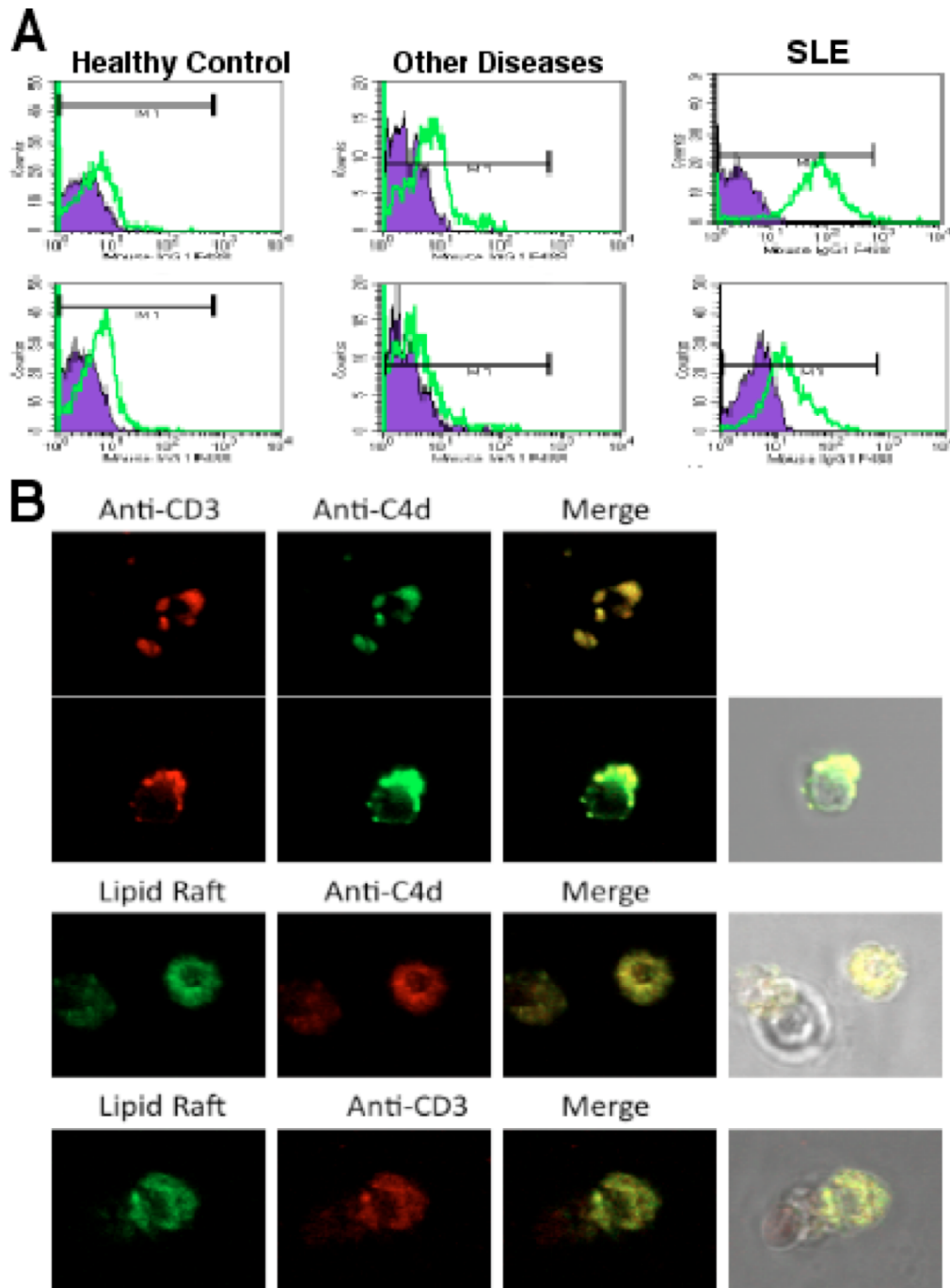


Figure 2. C4d is associated with TCR/CD3 complexes within the surface membrane of SLE T cells. Total lysates of PBMC derived from SLE patients with the positive T-C4d phenotype were subjected to co-immunoprecipitation analysis as described in the text. Shown in the figure is a representative blot developed using the ECL method. Lane 1: 2nd eluate of anti-CD3 co-IP; lane 2: 2nd eluate of mIgG1 co-IP; lane 3: 3rd eluate of anti-CD3 co-IP; lane 4: 3rd eluate of mIgG1 co-IP; lane 5: purified human C4b.

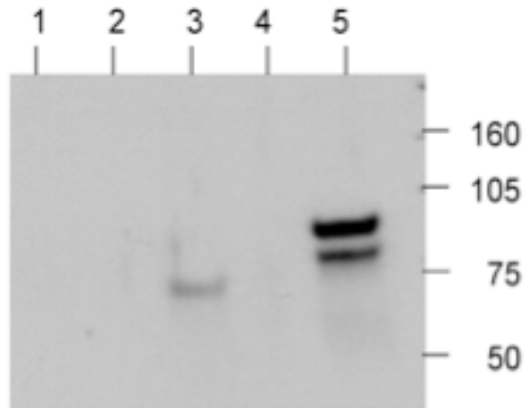


Figure 3. Aberrant intracellular signaling in SLE T cells bearing high levels of T-C4d.

PBMC isolated from study participants were briefly stimulated and subjected to intracellular phosphoprotein staining, as described in the text. Results shown were derived from T cells gated on positive staining with anti-CD3 mAb. **(A)** Histograms of intracellular phosphoprotein flow cytometric analysis of unstimulated or stimulated cells derived from representative study subjects (3 healthy controls, 3 patients with other diseases, 3 SLE patients with the negative T-C4d⁺ phenotype, and 3 SLE patients with the positive T-C4d phenotype). Arrowheads point to abnormal responses of protein phosphorylation that occurred spontaneously or inappropriately in response to T cell stimuli. Phosphorylation of appropriate signaling molecules (“normal” responses) in response to respective stimuli is indicated by arrows. **(B)** Bar graph summary of the flow cytometric analysis shown in (A). Arrows point to abnormal patterns of phosphorylation that occurred spontaneously or inappropriately in response to T cell stimuli. Y axis: specific median fluorescence intensity of phosphorylated signaling molecules stained with antibodies specific for the respective phosphorylated form.

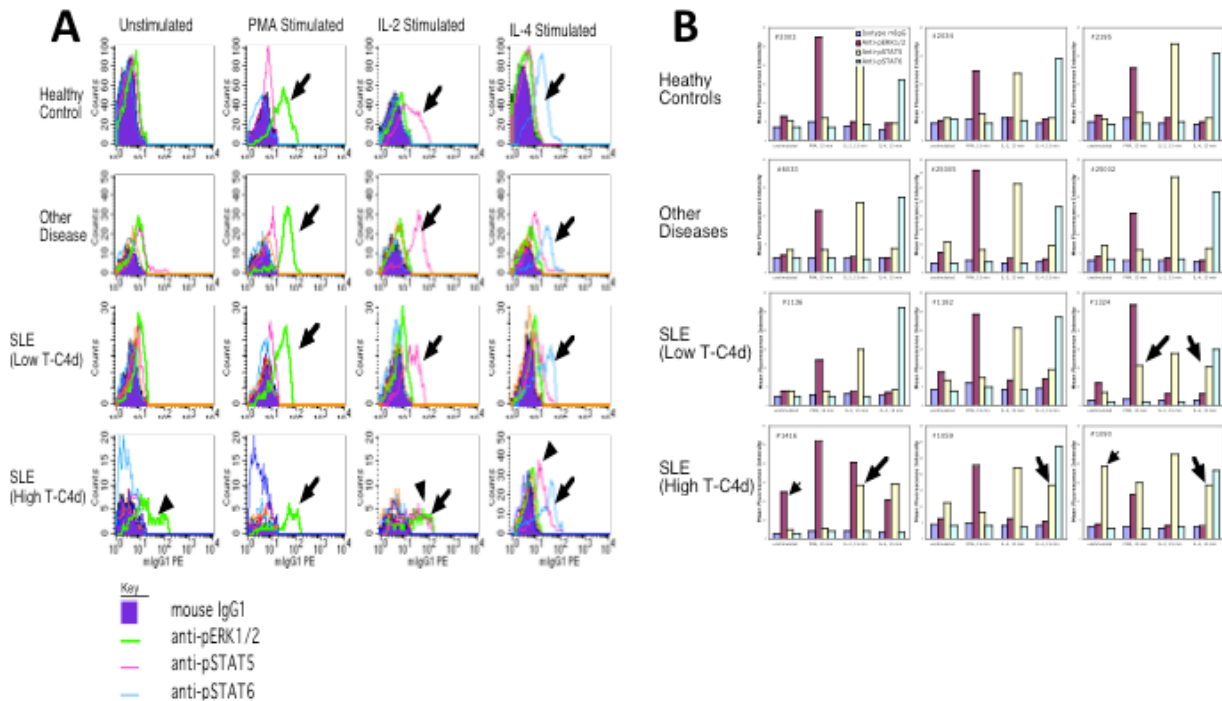


Figure 4. C4d-positive SLE T cells produce more cytokines than C4d-negative T cells. (A)

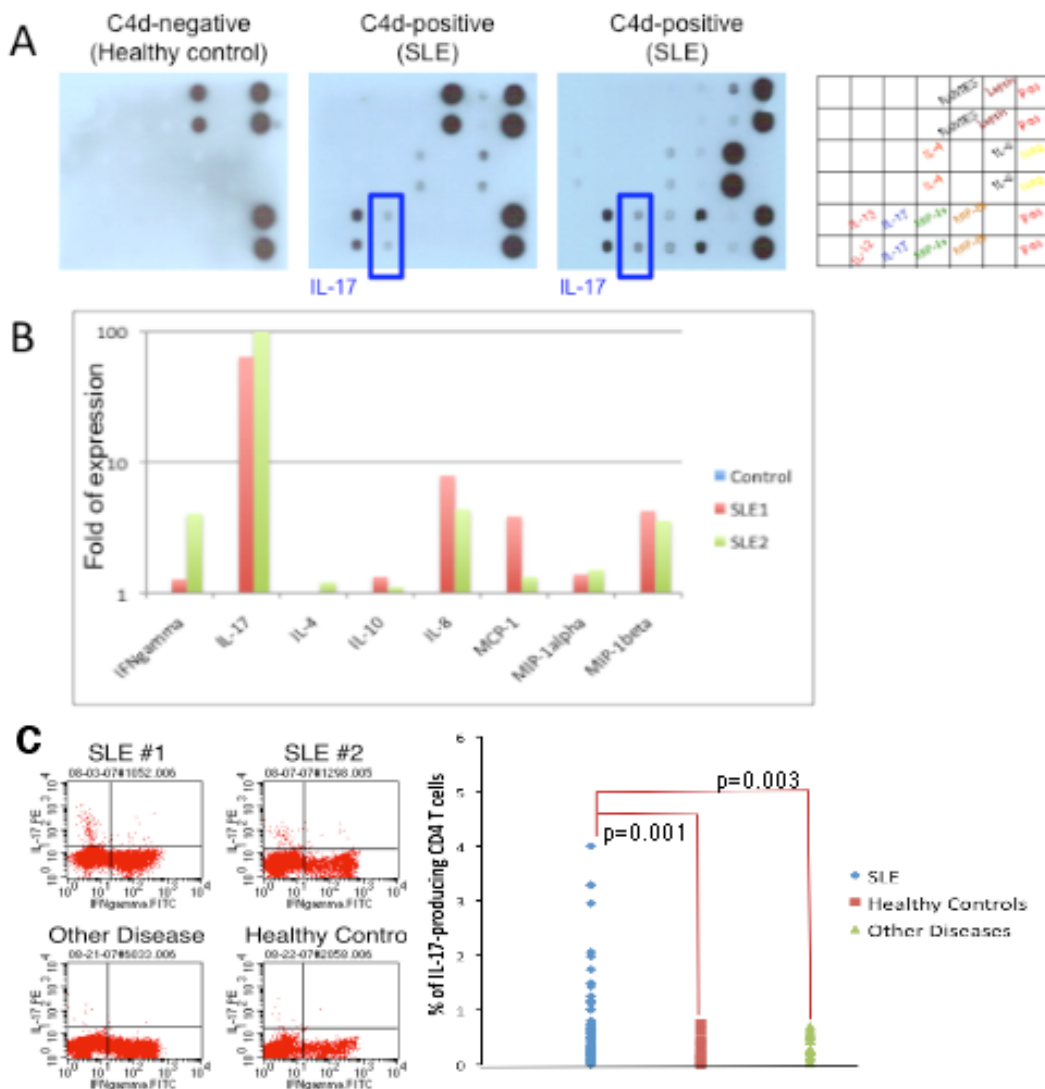


Figure 5. Differential proliferative responses of T-C4d-high cells and T-C4d-low cells to TCR/CD3-triggered stimulation. PBMC isolated from 3 representative SLE patients were cultured in medium alone or in the presence of anti-CD3 and/or anti-CD28 antibodies for 2-6 days. Cells stimulated with anti-IgD/IgM were included as a background control for the proliferation of B cells. Each experimental condition was tested in triplicates. T cell proliferation was measured by incorporation of ³H-thymidine and presented as proliferation Index (see text).

