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14. ABSTRACT

Two single-nucleotide polymorphisms (SNPs) in *ITGAM*, the gene encoding CD11b, associate with the risk of having lupus. One of these SNPs causes a ^{77}Arg→^{77}His amino acid change in the extracellular domain of CD11b and the other SNP causes a^{1146}Pro→^{1146}Ser change in its cytoplasmic domain. The goal of this project is to understand how these SNP-associated amino acid changes might affect the function of CD11b and explain their association with lupus. Using Epstein-Barr virus (EBV) transformed B cells and B cells from SLE patients, we will test the hypothesis that the ^{77}His and ^{1146}Ser variants alter CD11b mediated intracellular signaling in B cells, thereby change CD11b membrane mobility, clustering, and cytoskeletal association in ways that impact B cell biology and foster the development of lupus. To date we have made 2 major and new findings: First, we found that the SNP variant ^{1146}Ser residue is a target for phosphorylation by at least two kinases (ERK and GSK3β). Second, the presence of the SNP variant ^{1146}Ser residue is associated with increased surface expression of CD11b by EBV-B cells. We expect these changes in CD11b will have a major impact on CD11b signaling in EBV-transformed and primary B cells, helping understand why this SNP is associated with the risk of developing lupus.

15. SUBJECT TERMS
Mac-1, CR3, β2-integrin, B cell, lymphocyte, autoimmunity, SLE, lupus erythematosus, polymorphism

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1. INTRODUCTION:

In different racial and ethnic groups two single-nucleotide polymorphisms (SNPs) in *ITGAM*, the gene that encodes the α_M chain (CD11b) of the $\alpha_M\beta_2$ integrin receptor called Mac-1 (CD11b/CD18; **Fig. 1**), have consistently been shown to be associated with systemic lupus erythematosus (SLE). One of these (SNP rs1143679) leads to a ^{77}Arg→^{77}His amino acid exchange in the extracellular ligand-binding domain of CD11b while the other (SNP rs1143678) causes a^{1146}Pro→^{1146}Ser exchange in its cytoplasmic signaling domain (**Fig. 1**). The goal of this project is to understand how these SNP-associated changes in CD11b might affect the function of CD11b and the Mac-1 receptor on B cells, thereby altering B cell biology in a way that drives autoimmunity and SLE risk. To pursue this goal we are probing the impact of the ^{77}His and ^{1146}Ser variants on CD11b/Mac-1 function using Epstein-Barr virus (EBV) transformed B cells and B cells isolated from SLE patients. We hypothesize that ^{77}His and ^{1146}Ser will alter CD11b-mediated intracellular signaling and change Mac-1 membrane mobility, clustering, and cytoskeletal association, thereby altering B cell biology and fostering SLE development. To test these hypotheses we are pursuing three specific aims:

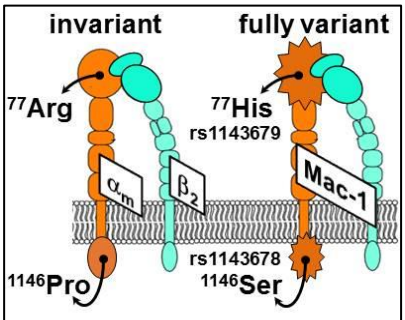

Figure 1. *ITGAM* encodes the α_M chain of Mac-1. We will study the impact on B cell functions of the SLE-associated extracellular ^{77}Arg→^{77}His exchange and the cytoplasmic ^{1146}Pro→^{1146}Ser exchange.

- Define the impact of the *ITGAM* ^{77}His and ^{1146}Ser variants on Mac-1 dependent signaling in B cells: Working hypothesis: ^{1146}Ser will change the extent and pattern of phosphorylation of the CD11b cytoplasmic tail and thus have a major impact on Mac-1 inside-out signaling (*i.e.* cell signaling needed to activate the Mac-1 receptor's ligand binding activity), whereas ^{77}His will affect outside-in signaling (*i.e.* cell signaling triggered by Mac-1 binding to ligand) and the downstream phosphorylation of signaling molecules. Approach: use EBV-transformed B cells from *ITGAM* genotyped healthy donors to assess the impact of ^{77}His and ^{1146}Ser on CD11b phosphorylation and downstream cell signaling after direct ligation of Mac-1 with ligands versus after indirect activation of Mac-1 via Toll-like receptor 4 (TLR4) and the B-cell receptor (BCR).
- Assess the impact of ^{77}His and ^{1146}Ser on Mac-1 cytoskeletal association, membrane mobility, and clustering on B cells: Working hypothesis: both SNP variants will perturb Mac-1 cytoskeletal associations, mobility, and clustering following Mac-1, TLR4, and BCR activation. Approach: by multispectral imaging flow cytometry (MIFC) we will compare the impact of *ITGAM* variants on Mac-1 cytoskeletal association, mobility, and clustering on EBV-B cells from *ITGAM* genotyped healthy donors.
- Confirm that *ITGAM* variation impacts Mac-1 biology in B cells from SLE patients: Working hypothesis: Mac-1 mediated functions of CD11b$^+$ B cells will be altered by the *ITGAM* variants in a manner consistent with their association to SLE. Approach: study peripheral blood B cells freshly isolated from healthy versus SLE affected donors with known *ITGAM* genotypes.

2. KEYWORDS:

Mac-1, CR3, β2-integrin, B cell, lymphocyte, autoimmunity, SLE, lupus erythematosus, polymorphism.

3. ACCOMPLISHMENTS:

The major goals of our project are listed in **Table 1**. Progress towards these goals is described below, with the major new findings of our project indicated by **red bold font**.

- Subtask 1A: Completed. Final IRB approval was for June 9, 2021 to June 8, 2022.
- Subtask 1B: 50% complete. For our proposed studies EBV-B cells from donors with *ITGAM* invariant and 4 different *ITGAM* variant genotypes were needed (10 de-identified female healthy donors for each of the 5 genotypes). Although we have identified these EBV-B cells in our biobank we list this subtask as only 40% complete, since the viability of the frozen EBV-B cells can only be ascertained once they are retrieved, thawed, and expanded in cultures. Ultimately we tested specimens from 20 separate donors (10 invariant and 10 ^{1146}Ser variant) and all but 2 were expanded successfully. To expand B cells from frozen samples we thaw them quickly and propagate them for 7 days in cell culture medium (RPMI 1640 + 10-15% FBS). Note that our effort under this subtask and all other subtasks was greatly disrupted by the COVID-19 pandemic (see part 5. CHANGES/PROBLEMS). Under this subtask because of COVID-19 pandemic-related supply-chain disruptions, the availability of the required cell culture medium was sporadic and hampered. Nevertheless, by flow cytometry we confirmed that (routinely) the majority of cells (~90%) remained viable

(live/dead staining shown in **Fig. 2A**). Findings from two ^{1146}Pro and two ^{1146}Ser donors showed that CD11b expression (total surface CD11b detected using antibody M1/70) is higher on EBV-B cells from patients with the ^{1146}Ser genotype (**Fig. 2, B and C**). Notably, for donors with the ^{1146}Ser genotype CD11b expression is heightened across all subpopulations of B cells (**Fig. 2D**). The data suggest that **SNP rs1143678 alters Mac-1 expression on B cells**.

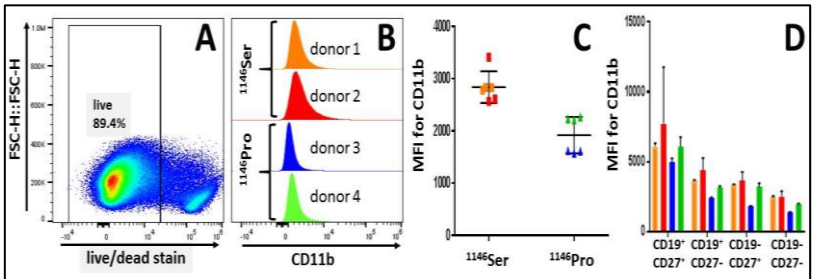

Figure 2. Viability of EBV-B cells is high (**A**) and they express different amounts of CD11b depending on their SNP rs1143678 genotype (**B-D** showing expression of CD11b for two 1146Pro (invariant) and two 1146Ser (variant) donors.

- We studied additional specimens and validated that expression of CD11b is heightened on ^{1146}Ser variant CD19$^+$ EBV B-cells. Importantly, we discovered that the elevation of CD11b associated with ^{1146}Ser is accompanied by a ~15% increase in expression of CD24 (**Fig. 3A**). CD24 is expressed at high levels by B-cell progenitors and mature resting B cells, but is not expressed on plasma cells, so we sought to identify the B cell subpopulation(s) that might account for the overall increase in abundance of CD24 on CD19$^+$ EBV B-cells carrying SNP rs1143678. We found that the increased abundance of CD24 on ^{1146}Ser variant EBV B-cells is attributable to a significant increase in the number of CD19$^+$CD27$^+$CD24$^+$ memory B cells (**Fig. 3B**). **These new findings are the first to connect the SLE-linked SNP rs1143678 to a distortion in the proportions of B cell subsets;** an increased number of memory B cells might explain why SNP rs1143678 is associated with SLE.

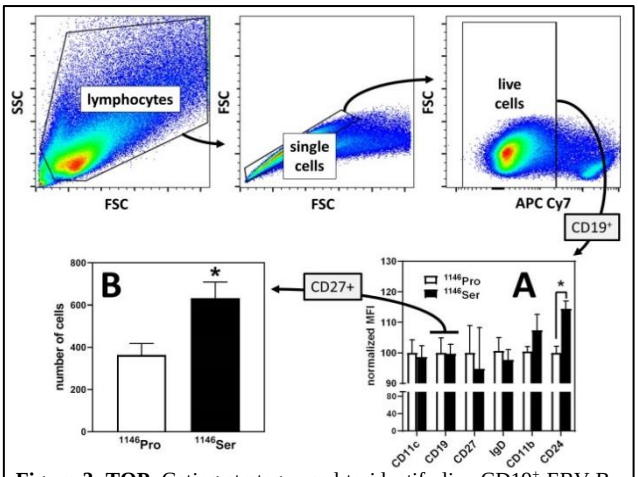

Figure 3. TOP. Gating strategy used to identify live CD19$^+$ EBV B-cells. **A.** Expression of B cell markers on CD19$^+$ EBV B cells from ^{1146}Pro (n=6) versus ^{1146}Ser donors (n=4). The mean + sem for mean fluorescence intensity (MFI) is normalized to ^{1146}Pro and the axis is broken. Asterisk indicates P=0.0109 for a 2-tailed 1-sample *t*-test. **B.** The number of CD19$^+$CD27$^+$CD24$^+$ memory B cells present in a sample of 10,000 CD19$^+$ B cells from the donors shown in panel **A**. The asterisk indicates P<0.05 for a 2-tailed 2-sample *t*-test.

- Subtask 1C: Completed.
- Subtask 1D: 40% complete. We tested combinations of various commercially available antibodies and found that (i) the anti-CD11b antibody M1/70 that we use for flow cytometry (**Figs. 2 and 3**) could be used to capture and immune-precipitate Mac-1 from B cell lysates, and (ii) the anti-CD11b antibody 2LPM19C could subsequently be used to probe Western blots to detect the immune-precipitated CD11b. For immunoprecipitation 1 x 10^7 EBV-B cells from an *ITGAM* homozygous (^{77}Pro/ ^{1146}Ser) donor were lysed in 500 µL of RIPA buffer containing protease and phosphatase inhibitors. Total protein in the resulting lysate was quantitated (BCA Protein Assay; Pierce) and 1 mg was pre-cleared by incubation overnight at room temperature with 20 µL of Protein A/G Magnetic Beads (Pierce). The beads were then removed by magnetic selection and the supernatant retained. The pre-cleared supernatant was then incubated with 10 µg of anti-CD11b clone M1/70 and 20 µL of fresh Protein A/G Magnetic Beads, each for 1 hr at room temperature. The beads were then removed by magnetic selection, gently washed, and the proteins retained by the beads eluted by exposure to low pH (Classic Magnetic IP/Co-Ip protocol; Pierce). Samples of the eluted proteins (30 µl) were then subjected to non-reducing SDS-PAGE and electro-transfer to PVDF membranes, and the membranes probed using 2LPM19C, anti-phospho-^{1142}Ser, and anti-phospho-^{1146}Ser. The results established that probing with 2LPM19C detects both CD11b (band at ~175 *k*Da) and the intact Mac-1 heterodimer (CD11b/CD18 at ~220 *k*Da) (**Fig. 4;** leftmost strip). Utilizing our phospho-specific antibodies we confirmed previous reports that the invariant ^{1142}Ser is phosphorylated (**Fig. 4;** middle strip) and most importantly **we showed for the first time that the SLE-associated ^{1146}Ser residue is phosphorylated** (**Fig. 4;** rightmost membrane).

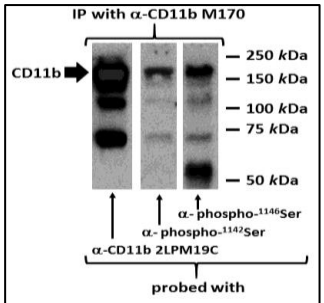

Figure 4. Western blots of EBV-B cell lysates from a donor expressing ^{1146}Ser. Immunoprecipitation (IP) was with antibody M1/70 and probing was with antibody 2LPM19C (detects CD11b), anti- phosphorylated ^{1142}Ser, or anti-phosphorylated ^{1146}Ser.

- Subtask 1E: 60% complete. We subjected synthetic CD11b peptides to tandem liquid chromatography–mass spectrometry (LC-MS) and confirmed we could detect the phosphorylated versus non-phosphorylated ^{1142}Ser and ^{1146}Ser residues (**Fig. 5**). Next, we excised from SDS-PAGE gels the ~150-250 kDa range bands containing CD11b/Mac-1 (**Fig. 4**) and subjected them to trypsin digestion and LC-MS. In this manner we had hoped to confirm that ^{1142}Ser and ^{1146}Ser in *bona fide* CD11b were phosphorylated, but unfortunately we were unable to detect phosphorylated ^{1142}Ser or ^{1146}Ser in EBV-B cell lysates. Likely this was because the amount of CD11b in the protein lysates was insufficient for its detection by LC-MS. Ultimately, we were unable to overcome this problem. However, **we were able to detect many cytoskeletal/focal adhesion proteins whose abundance associates with *ITGAM* genotype** (see subtasks 1G and 2J).

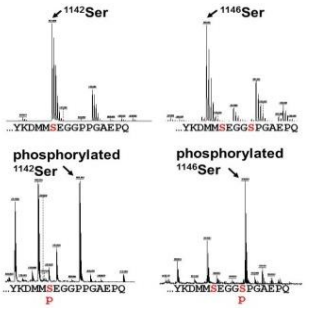

Figure 5. MS traces for peptides encoding the CD11b invariant ^{1142}Ser and the SNP variant ^{1146}Ser (phosphorylated or not).

- Subtask 1F: 60% complete. We used our synthetic CD11b cytoplasmic tail peptides as substrates for immunoblot and *in vitro* kinase assays. For immunoblottimg (**Fig. 6**) each peptide was co-incubated (24 hours at 37°C) with kinases (GSK-3β, Erk2, and CDK5/P25) in ATP containing buffer provided in the ADP-Glo kinase assay kit (Promega). After stopping the reaction and washing out excess substrates, the peptides were blotted onto nitrocellulose membrane and allowed to air dry. Each membrane was probed with either anti-phosphorylated ^{1142}Ser or anti-phosphorylated ^{1146}Ser for 2 hours, rinsed, and then incubated with secondary antibody for 1 hour. Immuno-reactive peptides were illuminated using Pico West reagents and images captured and analyzed on a ChemiDoc Gel Imaging System (Bio-Rad). The results suggested that the **^{1146}Ser residue introduced by SNP rs1143678 can be phosphorylated (by ERK2 and GSK3β) but only when the invariant ^{1142}Ser is phosphorylated** (**Fig. 6**; red circles). For the *in vitro* kinase assays (**Fig. 7**) the peptides were incubated with GSK-3β, Erk2, and CDK5/P25 for up to 2 hours at room temperature in ATP containing buffer (as described for the immunoblots), and then luminescence (the reporter of kinase activity) was measured. The results overall were not highly accurate, but we did reproducibly observed that GSK-3β phosphorylated the ^{1142}Ser/^{1146}p-Ser peptide (**Fig. 7**; blue bars) to a degree greater than it phosphorylated the ^{1142}Ser/^{1146}Ser peptide (**Fig. 7**; black bars). All kinases had greatly increased activity compared to the negative control. **The combined results establish that GSK-3β can phosphorylate both the ^{1142}Ser and ^{1146}Ser residues.**

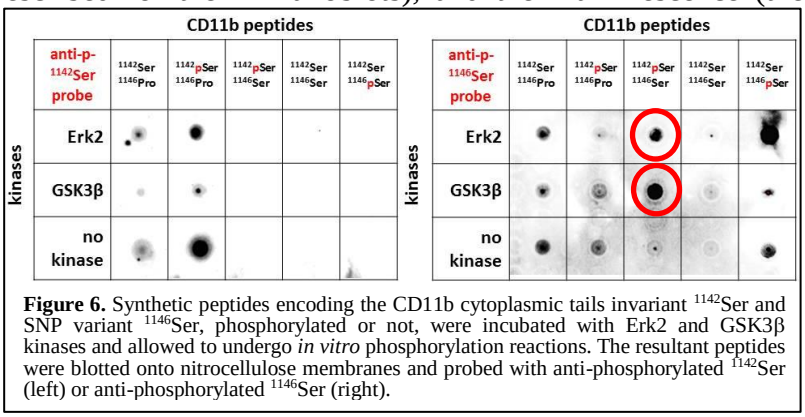

Figure 6. Synthetic peptides encoding the CD11b cytoplasmic tails invariant ^{1142}Ser and SNP variant ^{1146}Ser, phosphorylated or not, were incubated with Erk2 and GSK3β kinases and allowed to undergo *in vitro* phosphorylation reactions. The resultant peptides were blotted onto nitrocellulose membranes and probed with anti-phosphorylated ^{1142}Ser (left) or anti-phosphorylated ^{1146}Ser (right).

- Subtask 1G: 75% complete. The Comprehensive Flow Cytometry Facility (CFCC) houses the cytometry equipment we needed to perform our proposed SCNP studies, but the CFCC stopped access to this equipment in order to comply with the 'social distancing' requirements put in place by our institute in response to the COVID-19 pandemic (see section 5. CHANGES/PROBLEMS). In addition, the CFCC (now called the Flow Cytometry and Cell Sorting Core, FCSC) moved its physical location. To sidestep these problems we used an alternative LC-MS-based proteomics approach and made substantial progress on this subtask. Briefly, we immune-precipitated Mac-1 from EBV-B cell lysates, isolated the CD11b/Mac-1 proteins by excising from SDS-PAGE gels the bands that contained them, subjected these to trypsin digestion, and performed LC-MS as described under subtasks 1D and 1E. The raw data obtained were subjected to analysis with *Metacore* (Clarivate Analytics) to identify and quantify peptides/proteins and with *GeneGo* (Clarivate Analytics) to perform systems biology and pathway analyses. Thereby we were able to identify biological pathways/signaling networks that were over- or underrepresented in cells obtained from donors expressing the invariant ^{1146}Pro versus SNP variant

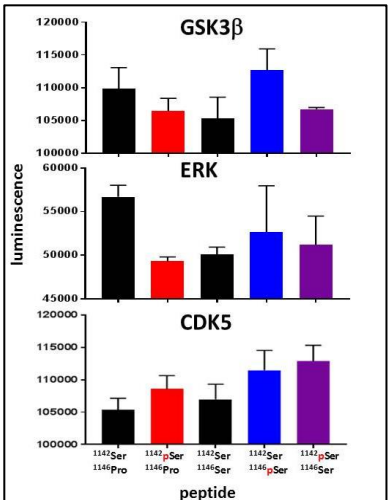

Figure 7. In vitro kinase assays measuring the ability of GSK3b, ERK, and CDK5 to phosphorylate peptides encoding the CD11b cytoplasmic tails invariant ^{1142}Ser and SNP variant ^{1146}Ser.

^{1146}Ser residues. This unbiased approach **provided new and compelling evidence that an interferon→ JAK/STAT → antigen presentation pathway was over-represented in EBV B-cells from ^{1146}Ser donors (Fig. 8; top).** Since distortions in B-cell subsets and an 'interferon signature' have been observed in SLE patients, and since IFN-γ is a primary driver of the identified interferon → JAK/STAT → antigen presentation pathway, we tested whether ^{1146}Ser altered the expression of IFN-γ by EBV B-cells or changed their responsiveness to IFN-γ. We found that **EBV B-cells from donors expressing ^{1146}Ser have increased spontaneous secretion of IFNγ and increased expression of the IFNγR2 receptor (Fig. 8; bottom)**. Since both B cells and IFNγ are known to play an important role in the pathogenesis of SLE, **amplification of the interferon → JAK/STAT → antigen presentation pathway in individuals expressing ^{1146}Ser could explain their increased risk for progression to SLE.** In mouse models B cell-intrinsic IFNγ/STAT1 signaling has been shown to promote the formation of spontaneous autoimmune germinal centers, and antigen presentation by B cells has been shown to promote the development of SLE. Taken together our new findings indicate that the SLE-linked SNP rs1143678 (and the consequent ^{1146}Pro→^{1146}Ser exchange it causes in the cytoplasmic domain of CD11b) may lead to an increase in the antigen presenting capacity of B cells. This would drive both germinal center formation and autoantibody production and thereby increase the risk of lupus.

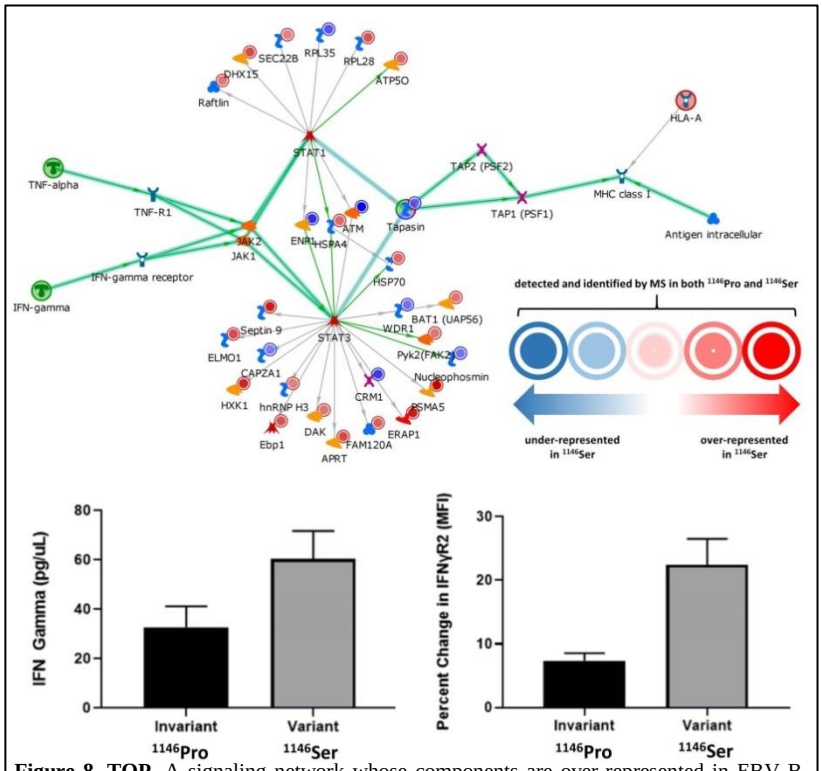

Figure 8. TOP. A signaling network whose components are over-represented in EBV B-cells from donors expressing the SNP variant ^{1146}Ser residue shows (from left to right) IFN-γ induced JAK/STAT activation culminating in antigen presentation. **BOTTOM.** EBV B-cells from donors expressing ^{1146}Ser have increased spontaneous secretion of IFNγ (**LEFT**) and increased expression of the IFNγR2 receptor after being placed into fresh medium and stimulated with 10 pg/ml exogenously administered IFNγ (**RIGHT**).

- Subtask 1H: 30% complete. In response to the COVID-19 pandemic the FCSC also limited our access to the imaging analyzer we need to perform multispectral imaging flow cytometry (MIFC) (see section 5. CHANGES/PROBLEMS for additional details). However as under subtask 1G, we used an alternative LC-MS-based proteomics approach and still made substantial progress on this subtask. Notably, our proteomics approach points to an over representation of cytoskeletal proteins participating in the actin remodeling complex in EBV B-cells expressing the ^{1146}Ser variant (data not shown). **This network is putatively involved in the formation of focal adhesions**, which indirectly supports our hypothesis that ^{1146}Ser alters CD11b-mediated changes in Mac-1 membrane mobility, clustering, and cytoskeletal association. Interestingly, the ^{1146}Ser-associated changes in the cytoskeletal network predicted by our pathway analysis are quite similar to those seen in the autoimmune disease Wiskott-Aldrich syndrome (WAS), suggesting that a WAS-like pathway might be engaged in EBV-B cells with an ^{1146}Ser genotype. The implications of this finding are discussed further under Part 4.
- Subtask 1I: 95% complete. We have completed a full-length manuscript summarizing the main findings of our project and plan to submit it for review to *Frontiers in Immunology*. A draft of the manuscript is included in the Appendix.
- Subtask 2J and 2K: 20% complete. See Subtask 1H and see section 5. CHANGES/PROBLEMS for additional details.
- Subtask 3L, 3M, 3N, and 3O: 0% complete. This subtasks was not pursued.

- **This project provided excellent opportunities for training and professional development.** Joseph Blake (the PhD student on this project) was involved in all of the studies described above and he generated the bulk of the data, including the proteomics analyses. Unfortunately, his progress was significantly impacted by the COVID-19 pandemic. See section 5. CHANGES/PROBLEMS for additional details.
- **How were the results disseminated to communities of interest?** Joseph Blake presented an abstract describing the results shown in **Figures 1-8** at *ACR Convergence 2020*, the annual meeting of the American College of Rheumatology. Joseph's abstract was selected by the Editors of ACRConvergenceToday.org as one of the 2,064 accepted abstracts to be highlighted on the website of the *ACR Convergence 2020* annual meeting. See the appendix for a copy of the abstract and a copy of the accompanying Editorial.
- **What do you plan to do during the next reporting period to accomplish the goals?** A 12-month No Cost Extension for this project expired on 9/14/2022. Nevertheless our priority remains publishing a full-length manuscript describing our findings.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
- The major new discovery we made is that a single amino acid change in a protein, encoded by a gene that is known to be associated with the risk of systemic lupus erythematosus, causes changes in the biological function of the receptor. For the antibody producing B cells in your body, this amino acid change increases the amount of receptor expressed and alters how the receptor signals the antibody producing cells when they are activated. Our most recent findings suggest the overarching effect of the amino acid change is to shift B cells towards heightened IFNγ responsiveness and antigen presentation. This brings up the distinct possibility that **the reason SNP rs1143678 is associated with lupus is because the resultant ^{1146}Ser change directly impacts CD11b structure and its ability to transmit cell signals, and thereby directly impacts the biology and function of B cells**. **A novel observation is that it is the antigen-presenting function of B cells, and *not* their antibody producing ability, that seems altered by SNP rs1143678.** This discovery is likely to make a major impact on our understanding of lupus and possibly point to new ways to treat this disease.

- **What was the impact on other disciplines?**
- CD11b is widely distributed on myeloid cells and B cell dysregulation is a characteristic of multiple autoimmune diseases, so our findings will likely have an impact also on infectious disease and other autoimmune diseases. Perhaps the best example of this is Wiskott-Aldrich syndrome (WAS), a disease characterized by immune deficiency, eczema, and microthrombocytopenia leading to hypocoagulation. There is also an increased risk of inflammatory disorders in people with WAS, and autoimmune diseases have been reported in 26 to 70 percent of WAS patients. These conditions have overlapping signs and symptoms with lupus; indeed, WAS is used as an animal model for lupus in mice. The chance of developing lymphoma is also increased in people with WAS. Our MS analyses indicate a WAS-like pathway might be engaged in EBV-B cells with an ^{1146}Ser genotype (for additional details see the appended manuscript).

- **What was the impact on technology transfer?**
- Nothing to Report.

- **What was the impact on society beyond science and technology?**
- Nothing to Report.

5. CHANGES/PROBLEMS:

- The biggest problem we faced during the project period was the COVID-19 pandemic. Social distancing policies of the University of Alabama at Birmingham led to our laboratory being shut down for many months, our access to needed shared facilities was restricted, the amount of time students and staff could spend on campus was limited, and supply-chain problems emerged. Unfortunately, as a direct consequence of the impact of the COVID-19 pandemic Joseph Blake (the student on this project) withdrew from graduate school and did not complete his dissertation.

TABLE 1. Approved SOW

Task 1: Define the impact of the *ITGAM* ^{77}His and ^{1146}Ser variants on Mac-1 dependent signaling in B cells		
Subtask	**Details**	**Projected Timeline**
A: local IRB approval	Alexander J. Szalai, PhD and Jeffrey Edberg, PhD will ensure that all IRB requirements are met	achieved by month 3
B: identify EBV B cells from donors with different *ITGAM* genotypes	Jeffrey Edberg, PhD will identify EBV B cells representing the *ITGAM* invariant plus 4 *ITGAM* variant genotypes for study. Cells from 10 (de-identified) female healthy donors each.	identified by month 3
C: recruit a graduate student for this project	Alexander J. Szalai, PhD is part of the Immunology Admissions Committee and will thereby identify and mentor this student	identified by month 3
D: Western blots to detect phosphorylation of *ITGAM* residues ^{1142}Ser and ^{1146}Ser	Guided by Alexander J. Szalai, PhD the student will perform this task. Phosphorylated residues will be detected in EBV B cell lysates from *ITGAM* invariant and ^{1146}Ser (homozygous) donors. Cells from 3 donors of each genotype will be used	months 1-6
E: mass spectrometry to detect phosphorylation of *ITGAM* residues ^{1142}Ser and ^{1146}Ser	Guided by Alexander J. Szalai, PhD the student will generate B cell lysates as above and provide these to the Targeted Proteomic and Metabolomics Laboratory for trypsin digestion and mass spectrometry	months 1-6
F: *in vitro* phosphorylation studies	Guided by Chander Raman, PhD the student will use synthetic CD11b cytoplasmic tail peptides as substrates to test if purified GSK-3β (Abcam) phosphorylates ^{1142}Ser or ^{1146}Ser *in vitro* and if this is blocked by the selective GSK-3 inhibitor CHIR-98014 (Selleck)	months 3-9
G: signaling network analysis	Guided by Chander Raman, PhD the student will perform SCNP on EBV B cells as described in the Research Strategy	months 6-12
H: multispectral imaging flow cytometry of nuclear translocation and Mac-1 co-localization	Guided by Daniel Bullard, PhD the student will perform MIFC on EBV B cells as described in the Research Strategy	months 9-15
I: preparation of manuscript		months 9-12
Task 2: Assess the impact of ^{77}His and ^{1146}Ser on Mac-1 cytoskeletal association, membrane mobility, and clustering on B cells		
J: Mac-1 cytoskeletal association and clustering studies	Guided by Daniel Bullard, PhD the student will perform MIFC on EBV B cells as described in the Research Strategy	months 9-24
K: preparation of manuscript		months 18-24
Task 3: Confirm that *ITGAM* variation impacts Mac-1 biology in B cells from SLE patients		
L: identify donors with different *ITGAM* genotypes that are available for recall	Jeffrey Edberg, PhD will direct the UAB Participant & Clinical Interactions Resource of the UAB Center for Clinical and Translational Sciences to identify donors representing the *ITGAM* invariant plus 4 *ITGAM* variant genotypes for study.	months 18-35
M: recall donors with different *ITGAM* genotypes for phlebotomy	Jeffrey Edberg, PhD will direct the UAB Participant & Clinical Interactions Resource of the UAB Center for Clinical and Translational Sciences to recall donors for phlebotomy	months 18-35
N: isolation of primary B cells and performance of experiments	Guided by Alexander J. Szalai, PhD the student will isolate B cells and perform analysis as described in the Research Strategy	months 18-35
O: preparation of manuscript		months 30-36

6. PRODUCTS:

- **Publications, conference papers, and presentations.**

Journal publications.

Effects of *ITGAM* Genetic Variation on CD11b structure and Mac-1 Mediated Signaling in B cells
Joseph L. Blake, Rachel V. Jimenez, Jeffrey C. Edberg, James Mobley, Daniel C. Bullard, Alexander J. Szalai. *Frontiers in Immunology*. In progress (A copy of the draft manuscript is included in the Appendix)

Other publications, conference papers, and presentations.

An SLE-linked ITGAM Gene Variant Changes Mac-1 Structure, Signaling, and Surface Expression and Enhances IFNγ Production and Antigen Presentation by B Cells. Joseph Blake, Jeffery Edberg, Alexander J Szalai, and James Mobley. American College of Rheumatology Annual Meeting, ACR Convergence 2020. November 5 – 9, 2020. A copy of the abstract is included in the Appendix.

- **Website(s) or other Internet site(s)**. Nothing to Report
- **Technologies or techniques**. Nothing to Report
- **Inventions, patent applications, and/or licenses**. Nothing to Report
- **Other Products.** Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	**Joseph L. Blake**
Project Role:	Graduate Student
Researcher Identifier	ORCID ID: 0000-0003-2298-2407
Nearest person month worked:	3 (includes remote work due to COVID-19)
Contribution to Project:	Performed experiments that generated all of the data shown.
Funding Support:	This award

Name:	**Alexander Szalai**
Project Role:	PI
Researcher Identifier	ORCID ID: 0000-0001-6638-579X
Nearest person month worked:	1.8
Contribution to Project:	Provides scientific direction and oversight for all studies.
Funding Support:	NIH/NIAMS P50 AR060772 (PD/PI: Saag) *Investigations in Gout, Hyperuricemia, and ComorbidiTies (INSIGHT) Center of Research Translation* 09/20/2017 – 08/31/2022 (Co-Investigator: 0.6 CM)

Name:	**Jeff Edberg**
Project Role:	Co-Investigator
Researcher Identifier	ORCID ID: 0000-0002-0248-7647
Nearest person month worked:	1
Contribution to Project:	identified EBV-B cells from *ITGAM* genotyped donors
Funding Support:	NIH/NCATS UL1 TR003096 (Kimberly) Center for Clinical and Translational Science (CCTS) 05/06/19 – 04/30/24 (Co-Investigator: 3.6 CM)

Name:	**Dan Bullard**
Project Role:	Co-Investigator
Researcher Identifier	ORCID ID: 0000-0003-3500-1057
Nearest person month worked:	1
Contribution to Project:	His experience with the analysis of Mac-1 and its ligands was utilized by Joseph Blake during performance of his experiments
Funding Support:	NIH/NIGMS 2 R25 GM086256 (Co-PIs: Bullard and Gavin) *UAB PREP Scholars Program* 04/1/18 - 03/31/23 (0.6 CM)

Name:	**Chander Raman**
Project Role:	Co-Investigator
Researcher Identifier	ORCID ID: 0000-0001-7775-9988
Nearest person month worked:	1
Contribution to Project:	His flow cytometry and kinase knowledge was ligands was utilized by Joseph Blake during performance of his experiments
Funding Support:	NIH/NIAMS R01 AR071157 (PI: Yusuf): *Mechanisms Elicited by Type I Interferons in Cutaneous Photocarcinogenesis* 09/01/16 – 08/31/21 (Co-Investigator: 1.2 CM)

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - For **Dr. Szalai:** The award W81XWH1810631 is now in no cost extension until 2022/09/14
  - For **Dr. Bullard:** Virginia Commonwealth University named Dr. Bullard their new dean of the Graduate School effective October 20, 2020. Dr. Bullard moved to Virginia later that year and has been employed by VCU since that time. Other than his

contribution to our manuscript in progress, he will no longer participate on grant NIH/NIGMS 2 R25 GM08625 and W81XWH1810631

- For **Dr. Edberg:** Dr. Edberg is MPI (2 CM) on the following new grant:
  R01-AR077927 NIH/NIAMS (Edberg, Reynolds; MPI)
  Functional and Integrative Omics of Recurrent Gout Flares
  2021/09/25 – 2024/07/31

  Dr. Edberg is a Co-Investigator (2 CM) on the following new grant:
  1U01 AI148108, NIH (Kimberly, PI)
  Host Factors in Response to Therapeutic Monoclonal Antibodies and Vaccination
  2020/02/01 – 2025/01/31

- For **Dr. Raman** award W81XWH-16-1-0537 has ended.

  Dr. Raman is PI (4 CM each) on the following new grants:

  5R21MD015319 (Raman, Meador; MPI)
  Molecular Mechanisms of Multiple Sclerosis Disease Severity In African Americans
  2020/08/24 – 2022/04/30

- **What other organizations were involved as partners?** Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

- **QUAD CHARTS:** A copy of the Quad Chart is included in the APPENDICES.

9. APPENDICES:

- Copy of abstract presented at *ACR Convergence 2020*

  An SLE-linked ITGAM Gene Variant Changes Mac-1 Structure, Signaling, and Surface Expression and Enhances IFNg Production and Antigen Presentation by B Cells. Joseph Blake, Alexander J Szalai, Jeffery Edberg, and James Mobley. American College of Rheumatology Meeting *ACR Convergence 2020*. November 5 – 9, 2020. Abstract number 0286

- Copy of interview with Joseph Blake published by *ACR TODAY*

- Draft of manuscript in progress

  Effects of *ITGAM* Genetic Variation on CD11b structure and Mac-1 Mediated Signaling in B cells
  Joseph L. Blake, Rachel V. Jimenez, Jeffrey C. Edberg, James Mobley, Daniel C. Bullard, Alexander J. Szalai. *Frontiers in Immunology*.

- Copy of QUAD CHART

ABSTRACT NUMBER: 0286

An SLE-linked ITGAM Gene Variant Changes Mac-1 Structure, Signaling, and Surface Expression and Enhances IFNg Production and Antigen Presentation by B Cells

Joseph Blake[1], Alexander Szalai[2], Jeffrey Edberg[3] and James Mobley[3], [1]UAB, Birmingham, [2]University of Alabama at Birmingham, birmingham, AL, [3]UAB, Birmingham, AL

Meeting: ACR Convergence 2020

Keywords: Adhesion, Adhesion molecules, autoimmune diseases, B-Lymphocyte, interferon, Systemic lupus erythematosus (SLE)

SESSION INFORMATION

Date: Friday, November 6, 2020

Session Type: Poster Session A

Session Title: SLE – Etiology & Pathogenesis Poster

Session Time: 9:00AM-11:00AM

Background/Purpose: SLE is a chronic and debilitating disease; in the USA with an estimated incidence of 3-10 per 100,000 people and currently affecting an estimated 300,000 people. The exact pathogenesis of SLE remains unknown, but there is a growing body of evidence that various genetic, hormonal, and environmental factors contribute to its occurrence and severity. Each is thought to modulate the activation and regulation of innate and adaptive immunity in ways that drive autoimmunity, the underlying cause of SLE. The hallmark of autoimmunity in SLE is B cell hyperactivity leading to the generation of autoantibodies, which together drives the deposition of deleterious immune complexes. SLE has strong familial clustering, high heritability, and high concordance rates between monozygotic versus dizygotic twins, suggesting it's a polygenic disease. Among the many different gene variants reportedly associated with SLE risk or severity are single nucleotide polymorphisms (SNPs) in the integrin alpha-M (*ITGAM*) gene, which are present in more than 1/3 of SLE patients. *ITGAM* encodes the protein CD11b, which pairs with CD18 and forms the Mac-1 receptor. Among the *ITGAM* SNPs associated to SLE is SNP rs1143678, encoding a P1146S amino acid substitution in the cytoplasmic tail of CD11b. Mac-1 is widely expressed by myeloid cells, and it is reported that SNP rs1143678 alters myeloid cell functions in ways that could propel SLE. Mac-1 is also expressed on lymphocytes, but comparatively little is known about its role in B cells or whether SNP rs1143678 has any impact on B cell biology. To fill this gap in knowledge we are investigating the impact of *ITGAM* SNP rs1143678 on Epstein-Barr Virus (EBV) transformed B cells from healthy human donors.

Methods: N/A

Results: We report here three of our main findings. First, juxtaposition of the cytoplasmic tails of CD11b and CD18 – and their activation induced separation – are thought to be required for Mac-1-mediated cell signaling. Our *in silico* structural modelling (Fig. 1) predicts that the SLE-associated P1146S substitution significantly changes the conformation of CD11b's cytoplasmic tail. This shape-shift should allow CD11b to accommodate additional or new cytoskeletal/cytoplasmic binding partners, and thereby could influence Mac-1 signaling. Second, phosphorylation of 1142Ser (an invariant residue in CD11b) is thought to be required for Mac-1 signaling, but using *in vitro* kinase assays and Western blots we provide the first direct evidence that the SLE-associated 1146Ser residue is also phosphorylated (data not shown). Third, by mass-spectrometry combined with proteomics and network analyses, we obtained evidence that both IFNγ and antigen presentation pathways are enriched in B cells from donors expressing the *ITGAM* SNP variant (Fig 2). Indeed, compared to B cells from *ITGAM* invariant donors, B-cells from donors carrying the *ITGAM* SNP showed increased expression of CD11b, increased spontaneous secretion of IFNγ, and increased expression of the IFNγR2 receptor following stimulation (Fig 3). This trio of new findings together suggests that the *ITGAM* SNP rs1143678 significantly alters the biology of B cells, which might explain the strong association rs1143678 to SLE.v

Conclusion: N/A

ACR TODAY

Joseph Blake: SNP rs1143678 and B cell biology in SLE

Joseph Blake

Poster presenter: Joseph Blake, graduate student, University of Alabama, Birmingham

Poster title: An SLE-linked ITGAM Gene Variant Changes Mac-1 Structure, Signaling, and Surface Expression and Enhances IFNg Production and Antigen Presentation by B Cells

Scheduled poster session day and time: Friday, Nov. 6, 9 – 11 a.m. EST

Visit the Poster Hall

What is your poster about?

We found that the systemic lupus erythematosus (SLE)-associated SNP rs1143678 might affect the structure and function of the associated protein CD11b and looked into how this might impact B cell biology and, ultimately, SLE. Rs1143678 encodes the CD11b amino acid substitution Pro1146Ser. This is interesting because it provides a phosphorylation site four amino acids downstream from the canonical CD11b phosphorylation site at 1142Ser that is known to control Mac-1 activation. In B cells we have the first evidence that both serines appear to be constitutively phosphorylated, and that this changes the cells' signaling equilibrium. Specifically, by proteomics analysis we inferred that B cells that are homozygous for the SNP over-express proteins associated with response to INF-γ, with antigen processing and presentation, and with the formation of focal adhesions.

Why did you decide to investigate this topic?

I'm a soon-to-graduate student in the laboratory of senior author Alexander J. Szalai, PhD. Dr. Szalai and his group began to pursue this project based on the results of genome-wide-association studies published around 2008 that identified that a series of single nucleotide polymorphisms (SNPs) in the Integrin Alpha M (ITGAM) gene are associated with systemic lupus erythematosus (SLE). One of my mentors on this project, Dan Bullard, PhD, is an expert on ITGAM biology, and he, Jeff Edberg, PhD, and Dr. Szalai combined their skills and ideas to pursue this question. Integrin Alpha M encodes the protein CD11b, which pairs with CD18 to form the heterodimeric receptor called Mac-1. This integrin is primarily studied on myeloid cells, and its expression is reportedly approximately tenfold higher than on B cells. But it's B cells that produce the autoantibodies associated with SLE. Preliminary experiments we did using Epstein Barr Virus-transformed B cells (from donors homozygous for the SLE-associated SNP) led to the initial observations that led to my project.

What excites you most about your work?

I'm most excited about how the project has expanded as we get more data. What started as a methodical measurement of protein structure and function has exploded into something that may affect our understanding of SLE pathogenesis.

What are you working on next related to this poster?

Although the story has not yet completely unfolded (thus the abstract), we can say with confidence that the data we have so far supports our claims; of course, we still need to expand on these data for publication of our manuscript.

Effects of *ITGAM* Genetic Variation on CD11b structure and Mac-1 Mediated Signaling in B cells

Joseph L. Blake[1*], Rachel V. Jimenez[1,2], Jeffrey C. Edberg[1], James Mobley[1], Daniel C. Bullard[1,3], Alexander J. Szalai[1]

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Keywords: SLE, lupus, IFN-γ, DN1, antigen presentation (Min.5-Max. 8)

Abstract

Systemic Lupus Erythematosus (SLE) is a chronic and debilitating disease; in the USA currently, estimates suggest it has an incidence of 3-10 per 100,000 people and affects approximately 300,000 people. The exact pathogenesis of SLE remains unknown, but a growing body of evidence indicates that various genetic, hormonal, and environmental factors contribute to its occurrence and severity. Each of the latter are thought to modulate the activation and regulation of innate and adaptive immunity in ways that drive autoimmunity, the underlying cause of SLE. The hallmark of autoimmunity in SLE is B cell hyperactivity and the generation of autoantibodies, which together drives the deposition of deleterious immune complexes. SLE has strong familial clustering, high heritability, and high concordance rates between monozygotic versus dizygotic twins, suggesting it is a polygenic disease. Among the many different gene variants reportedly associated with SLE risk or severity are single nucleotide polymorphisms (SNPs) in the integrin alpha-M (*ITGAM*) gene, which are present in more than 1/3 of SLE patients. Herein we report on the *ITGAM* SNP rs1143678, which encodes the substitution P1146S in the cytoplasmic tail of CD11b. Little information is available on the substitution's potential effects on CD11b function, and the majority of available data is in myeloid cells. We have shown that P1146S changes the structure of the cytoplasmic tail of CD11b, enabling it to utilize novel transcription factors and signaling machinery. Furthermore, in Epstein-Barr Virus (EBV) transformed B-cells *ITGAM* SNP rs1143678 is associated with an increased ability to present antigen and a heightened interferon and inflammatory phenotype that resembles DN1 cells.

Introduction

SLE is a difficult to diagnose, systemic, chronic, life-changing disease with an unpredictable course that disproportionately affects women and people of African American, Asian, and Hispanic descent [1-6]. There is no consensus about SLE's exact pathogenesis but there is growing appreciation that various genetic, hormonal, and environmental triggers can perturb the activation and regulation of innate immunity in a way that drives the breakdown of adaptive immunity that is characteristic of SLE [3, 4, 6]. SLE etiology also remains murky [7] but strong familial clustering, high heritability, and high concordance rates for the disease between monozygotic versus dizygotic twins suggests it is polygenic [8, 9]. Indeed, population-based studies have now linked the risk and severity of SLE and lupus nephritis to >80 different gene variants [6, 9, 10]. Two genome wide association studies in

2008 identified SNPs in the *ITGAM* gene as associated with SLE occurrence and severity [11, 12]. Of particular interest is the SNP rs1143687 which encodes the P1146S substitution in the cytoplasmic tail of CD11b. This is complemented by molecular studies on the contribution of autoantibodies and B cell subpopulations to SLE pathogenesis. Anti-DNA autoantibodies contribute to kidney and brain disease and are found in 50-70% of lupus patients. Anti-β2 glycoprotein I and anti-cardiolipin contribute to thrombosis, and anti-Ro antibodies cause fetal heart block in the offspring of women with lupus [13]. The origin of these antibodies and the associated B cells has been extensively studied. For example anti-DNA autoantibodies are frequently produced in germinal center reactions [14]. Other B cells secrete proinflammatory cytokines such as IFN-γ, IL-6, and lymphotoxin-α to form tertiary lymphoid tissue in organs affected in SLE [15]. Another B cell subpopulation associated with SLE is the $CD19^{+}IgD\text{-}CD27^{-}$ 'double negative' (DN) cells. Work by Ignacio Sanz et al has helped to elucidate that DN1 cells may function as memory precursor and further differentiate into $IgD^{-}CD27^{-}CD11c^{+}CXCR5^{-}$ 'double negative 2' (DN2) cells which may function as precursors to extrafollicular antigen presenting (ASC) B cells. These DN cells are thought to be responsive to IFN-γ and contribute to autoantibody pathogenesis [16]. There is extensive evidence of B cell pathology in SLE, but what is interesting to consider is whether *ITGAM* variation can impact B cells, thus explaining the association of the SNP with SLE.

ITGAM is translated to the protein CD11b which, together with CD18, forms the heterodimeric β2 integrin MAC-1/CR3. This integrin has a poly-specificity to at least 30 ligands including fibrinogen, iC3b, and bacterial patterns and MAC-1 can induce a variety of responses depending on ligand binding. For example, foreign or damaged cells opsonized with iC3b are phagocytosed by mononuclear phagocytes while the binding of fibrinogen induces Nf-Kb signaling and inflammatory cytokine secretion. While the cellular response is complex, two general signaling schema have been utilized to describe the direct actions of MAC-1. The process by which other stimuli result in the phosphorylation of CD11b at Ser1142 (and the resulting activation of MAC-1) is termed 'inside-out' while the subsequent process of ligand binding to MAC-1 and transducing a signal inside the cell is termed 'outside-in' [17-19]. Structurally, this is accomplished by three main regions (ligand binding, folding region, cytoplasmic tail) and a cycle of activation. The ligand binding region is kept sterically hindered near the plasma membrane by the folding region, but upon activation this fold is straightened and extends the ligand binding region into the extracellular space increasing ligand affinity by approximately 5-fold. The cytoplasmic tail and transmembrane region anchors Mac-1 to the cytoskeleton, controls unfolding and activation by phosphorylation at Ser1142, and induces signal transduction through binding partners. When studied on myeloid cells, it has been shown that 'outside-in' signaling also propels the cell effector response and recruits additional MAC-1 receptors. The latter process, called 'clustering', involves the coordinated release of Mac-1 from cytoskeletal tethers like kindlin and talin, its passive diffusion to the sites of initial ligand engagement, and then re-establishment of cytoskeletal tethers [17, 18]. The capacity to signal in both directions allows Mac-1 to regulate many myeloid cell functions that participate in SLE such as cell activation and recruitment, phagocytosis, netosis, apoptosis, autophagy, and immune tolerance [10, 20-23].

We have previously shown that in a strain of mice known to spontaneously developing lupus, lymphadenopathy increased and glomerulonephritis worsened when the *Itgam* gene encoding mouse CD11b was deleted [24]. Four years later an association of SLE and *ITGAM* SNPs was reported in humans [12]. Amongst the ITGAM SNPS associated with SLE three non-synonymous ones have received the most attention (rs1143678, rs1143679, and rs1143683); each of these encodes a missense mutation that result in amino acid exchanges (Pro1146Ser in the cytoplasmic tail, Arg77His near the ligand binding site, and Ala858Val in the folding region, respectively) in the CD11b protein [25-32].

Several groups have shown that the Arg77His variant modulates Mac-1 adhesion and phagocytosis by primary human leukocytes and that the 1146Ser variant impact phagocytosis and the kinetics of extracellular signal-regulated kinase (Erk) activation in transfected human and mouse myeloid cells [25-28, 30-35]. In addition to myeloid cells Mac-1 is also expressed on lymphocytes and is widely used as a lymphocyte lineage marker. Typically, about 1/3 of all peripheral blood B cells express Mac-1 [36, 37] and this can be used to define a sub-population of $CD11b^+$ B cells that are expanded ~3-fold in SLE [38]. The $CD11b^+$ B cell sub-population that is expanded in SLE was shown to secrete IL-10 spontaneously and thereby to regulate T cells (29-30). Other evidence suggests Mac-1 participates in B cell adhesion and migration [36, 37], the negative regulation of B cell antigen receptor (BCR) signaling [35], and antibody class switching [39]. As for the effect of ITGAM SNPs on B cells, we are aware of a single study that reported the Arg77His variant impacted cell signaling in transfected mouse B cells [35], but ours is the first study to report the effects of the 1146Ser variant on human B cells. Our modeling data suggests that this variant changes the structure of the CD11b cytoplasmic tail, and we show directly and for the first time that the ^{1146}Ser variant is phosphorylated. Since the ^{1146}Ser variant is proximal to the canonical phosphorylation site ^{1142}Ser, this is predicted to alter Mac-1 cytoplasmic tail regulation. Indeed, this impairs the clustering process by preventing the disassembly of Mac-1 and causing increased focal adhesions. This, in combination with an altered signaling equilibrium analyzed by proteomics contribute to spontaneous secretion of IFN-γ, increased response to IFN-γ, and increased antigen processing and presentation.

Materials and Methods

Epstein Barr Virus Transformed B cells:

EBV transformed B cells were obtained from donors in PROFILE, a US multi-ethnic, multi-regional, multi-institution assemblage of controls and patients affected with SLE [40]. At the time of their recruitment into PROFILE all SLE patients were ≥16 years old and fulfilled the revised American College of Rheumatology criteria for SLE [41] for ≤10 years. PBMCs were isolated from heparinized peripheral blood by Ficoll-Hypaque density gradient, the B cells enriched by removal of adherent cells (incubation in a plastic tissue-culture flask for 1 hour at 37°C) and removal of T cells (using anti-CD3 Dynabeads), and then the B cells were immortalized with EBV, expanded, and cryopreserved as described elsewhere [42].

In silico Models:

Cytoplasmic tail structures were predicted with MUSTER (MUlti-Sources ThreadER) using a CD11c template in the PDB library. 3D models were rendered using the COACH meta-server for protein-ligand binding site prediction. Then models were aligned using the DeepView/Swiss PDBViewer v4.1.0

Synthetic Peptides and Antibodies:

Peptides encoding the amino acid sequences of the invariant (^{1146}Pro) and variant (^{1146}Ser) CD11b cytoplasmic tail and their single phosphorylated states were generated as follows:

Peptide	Sequence
S1142 P1146	YKDMMSEGGPPGAEP
pS1142 P1146	YKDMMpSEGGPPGAEP
S1142 S1146	YKDMMSEGGSPGAEP
pS1142 S1146	YKDMMpSEGGSPGAEP
S1142 pS1146	YKDMMSEGGpSPGAEP

The peptide that results from the common allele with phosphorylation at 1142Ser and the peptide that results from the SNP rs1143678 with phosphorylation at 1146Ser were used to generate two different rabbit polyclonal phospho-specific antibodies.

Kinase Assay and Dot Blot:

Probable phosphorylation sites and their corresponding kinases were identified through Phosphomotif Finder (www.hprd.org/PhosphoMotif_finder). An aliquot of 10 µg of synthetic peptide were incubated with sufficient kinase for 900 nmol/min/mg activity for 24 hours at 36º C. Samples were then blotted onto nitrocellulose membranes using a vacuum apparatus until dry. The membranes were probed with phospho-specific antibodies using standard Western blot procedures.

IP and Western Blot:

EBV B cells were lysed with 70µL RIPA buffer per 1x106 cells. A Peirce Classic Immunoprecipitation kit was used to obtain CD11b. Lysates were pre-cleared with agarose resin and protein A/G. CD11b was precipitated with antibody clone 2LPM19c. Western blot analyses were performed with antibody clone M170.

Mass Spectrometry and Pathway Analysis

EBV B cells were lysed and 1mg total protein was used for liquid chromatography and tandem mass spectrometry (LC MS/MS). The results were assessed for quality control and statistical significance to generate hits. The hits were anlyzed by Clarivate Analytics pathway analysis software to generate pathway maps.

Results

The cytoplasmic tails of CD11b and CD18 are juxtaposed in Mac-1 and their physical separation and release from cytoskeletal constraints is a prerequisite for Mac-1 mobility, signaling, and clustering [17, 43]. A conserved 1142Ser residue (Fig. 1a) - normally the only serine in the CD11b cytoplasmic tail – was shown to be constitutively phosphorylated, and there is consensus that conversion of 1142Ser to phospho-1142Ser (p-1142Ser) is essential for Mac-1 signaling [44-47]. Also, Chua et al. provided evidence that phosphorylation of 1142Ser alters the structure of the CD11b cytoplasmic tail [45]. We propose 3 ways by which the 1146Ser variant too might influence Mac-1 signaling and function in ways that could account for its association to SLE. First, the 1146Pro→1146Ser change per se should significantly alter the structure of the CD11b cytoplasmic tail and thereby its interaction with CD18

and the cytoskeleton (Fig. 1c). Second, by generating a solvent accessible alcohol group, 1146Ser should add a new phosphorylation site to the CD11b cytoplasmic tail (Fig. 1b). The conserved 1142Ser could be phosphorylated by protein kinase A (PKA), casein kinase 1 (CK1), and G protein-coupled receptor kinase 1 (GRK1) (Fig. 1a), whereas the variant 1146Ser could be phosphorylated by mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), extracellular-signal-regulated kinase 1/2 (ERK1/2), and cyclin-dependent kinase 5 (CDK5). Third, 1146Ser completes the consensus sequence Ser/Thr-x-x-x-Ser/Thr-Pro recognized by glycogen synthase kinase 3 (GSK-3), a kinase known to be involved in a wide range of immune and inflammatory responses and for which FDA approved drugs are already available [48]. GSK-3 has a preference for primed substrates, i.e. ones pre-phosphorylated at a residue C-terminal to the site of GSK-3 phosphorylation [48]; accordingly, the conserved 1142Ser is the potential target residue whereas the variant 1146Ser is the potential priming residue. Thus in people expressing SNP rs1143678, p-1146Ser could act as a priming residue for the GSK-3 mediated hyper-phosphorylation of 1142Ser, thereby potentially enhancing Mac-1 mediated functions that might contribute to SLE. In favor of this prediction GSK-3β (one of two GSK-3 isoforms in mammals) is known to promote LN in mice and is abnormally activated in SLE [49]. Of direct relevance to our proposed study, GSK-3β is a metabolic checkpoint regulator for B cells [50, 51].

In order to test this hypothesis, 20 amino acid peptides mimicking the cytoplasmic tail of CD11b derived from the SNP allele, the common allele, and the phosphorylated versions of those peptides were synthesized. To analyze the phosphorylation state of these peptides, phospho-specific antibodies to phospho-serine 1142 and phosphor-serine 1146 were generated (Fig. 2a). The peptides were incubated in a kinase reaction and the resulting mixture was used in a dot blot to detect phosphorylation. The results show for the first time that the 1146Ser residue introduced into the CD11b cytoplasmic tail by *ITGAM* SNP rs1143678 is phosphorylated, and that this can be achieved by ERK1/2 and GSK3β (Fig. 2b). It is noteworthy that GSK3β requires a pre-activated CD11b at 1142Ser in order to phosphorylate the mutation at 1146ser. The loss of signal compared to synthetically phosphorylated controls may indicate that in these *in vitro* conditions the kinases act as phosphatases.

To determine if these findings were recapitulated in vivo, PBMCs were sourced from ITGAM genotyped donors and assessed for CD11b phosphorylation status by flow cytometry with cell permeabilization. The phospho-specific antibodies were functional in flow cytometry and showed the first evidence of phosphorylation of 1146ser *in vivo* (Fig. 3a). This was confirmed by Western blot analysis which showed bands at 222kD (MAC-1), 127kD (CD11b), and smaller cleavage fragments (Fig. 3b). Additionally, dot blot analysis of immunoprecipitated lysates showed increased staining for kinases specific to 1146ser, namely GSK3β (Fig. 3c). This data together confirm the novel post-translational modification to CD11b in the activation controlling region of the molecule as well as an increased concentration of a contributing kinase.

To further study the effects of the 1146ser mutation on cell signaling and to link it to SLE pathophysiology, B cells were harvested from ITGAM genotyped donors and transformed with Epstein Barr Virus (EBV). In addition to being a robust technique for B cell immortalization, EBV has been suspected of being an environmental contributor to SLE pathogenesis and severity with SLE patients having abnormally large viral loads, decreased EBV-specific T cell responses, and increased EBV-antibody titers. Additionally, the EBV transcription factor EBNA2 is known to bind to the ITGAM promoter and increase transcription which may exacerbate any ITGAM SNP driven phenotype.

The analysis of EBV B cell surface markers conforms to this hypothesis with an ITGAM genotype specific increase in CD11b expression. Furthermore, an ITGAM genotype specific change in multiple

cell surface markers indicates a change in B cell subpopulations (Fig. 4a). These changes reflect the DN1 subpopulation studied by Ignacio Sanz et al that has been linked to SLE pathology. The ITGAM SNP EBV B cells show a marked decrease in IgD expression as well as a decrease in CD27 that drives increased amounts of the DN1 subpopulation; however, while there was a significant difference in CXCR5 and CD11b within the B cell population, this did not continue into the DN1 subpopulation rendering no difference in the DN2 subpopulation between genotypes (Fig. 4b). In addition to the DN1 phenotype, ITGAM SNP EBV B cells show robustly increased expression in CD24. This drives an increased $CD24^{+}/CD27^{+}$ memory cell population (Fig. 4c). Taken together, these findings indicate that the ITGAM SNP EBV B cells show a distribution of extrafollicular B cells with an increased history of activation.

To investigate the effect of the ITGAM mutation on cell signaling instead of simply cell surface markers, we used liquid chromatography tandem mass spectrometry (LC MS/MS) to analyze the whole proteome of ITGAM genotyped EBV B cells in the presence and absence of stimulation. The effect of the ITGAM SNP in the absence of stimulation showed a robust change in intracellular signaling dynamics (Fig. 5a). These changes reflect an increased response to IFN-γ, antigen processing, and presentation (Fig. 5b), increased response to GSK3β (Fig 5c.), as well as decreased cytoskeletal remodeling and increased focal adhesions (Fig. 5d). In addition, general inflammation pathways involving IL-6 were upregulated in the variant (Fig. 5e). To explore the IFN-γ pathway present on the SNP carrying B cells we used an ELISA to detect spontaneous secretion of IFN-γ. We found that these cells spontaneously secrete 60 ng/ul of IFN-γ on average which is nearly 3-fold higher than the control B cells (Fig. 6a). It is important to note EBV transformed B cells are known to spontaneously secrete IFNγ, but this is independent of the genotype effect as all cell lines were similarly transformed. When stimulated with exogenous IFN-γ, the ITGAM SNP B cells increase the surface expression of Interferon Gamma Receptor 2 (IFNγR2) by 20% more than compared to 10% for the control cells (Fig. 6b). Taken together these findings suggest that B cells carrying the ITGAM SNP are primed to act as antigen presentation cells.

We then stimulated three key pathways related to the previous findings and submitted the lysates for LC MS/MS. We stimulated with fibrinogen (a known MAC-1 outside-in ligand), LPS (a TLR4 agonist that will induce kinase cascades that causes inside-out signaling for MAC-1), and IFN-γ to expand on the earlier seen effects that the ITGAM SNP B cell exhibited. The resulting data shows a feed-forward loop between CD11b stimulation and interferon signaling that results in increased antigen processing and presentation, increased inflammatory signaling, and increased clustering at the cell surface due to a breakdown in the actin remodeling complex (Fig. 7)

Discussion

Together these results tell an interesting story. The GWAS determined a link between the ITGAM SNP rs1143678 and SLE risk and severity. When assessed in B cells, the SNP was associated with novel cellular physiology such as increased CD11b and a shift in population distributions that indicate an increased propensity to form memory cells as well as increased DN1 cells which have previously been associated with SLE. When tested by whole proteome analysis, B cells containing the SNP also showed increased pathways associated with response to inflammatory stimuli, antigen presentation, and response to Type I and II interferons. Furthermore, SNP containing B cells spontaneously secrete higher levels of IFNγ and have a higher capacity to respond to it.

When constructed into a contiguous story, this data elucidates a novel pathway for potential pathogenesis in SLE. People with the ITGAM SNP may have B cells that are primed to over-respond to inflammatory signal, particularly through CD11b ligands. Furthermore, this could lead to a feed forward loop whereby CD11b stimulation leads to increased kinase cascade signals, particularly through GSK3β and ERK1/2, and subsequently causes increased phosphorylation at S1142 and thereby the high sensitivity state of CD11b. This loop is also intricately linked to other pathways that could contribute to autoimmunity. The Jak/Stat pathway is also over-expressed by the genotype effect alone as well as when cells were stimulated with CD11b ligand fibrinogen. This leads to over-representation of pathways associated with antigen presentation such as tapasin, ERAP, endoplasmin, and MHC I. This antigen presenting phenotype may be further exacerbated by malfunctions in the actin remodeling complex (ARP). It has previously been shown in myeloid cells that the CD11b snp rs1143678 is associated with increased clustering at the cell membrane. The decreased expression of ARP proteins combined with increased expression of cytoskeletal protein, increased surface expression of integrins and diverse cell surface receptors such as IFNγR2 and SLC25A6 could indicate that signaling clusters are becoming immobilized at the cell surface by a non-responsive ARP. This is supported by previously published data that the ITGAM SNP is associated with increased clustering (Li-Teng Ong JI 2016, find other). This could exacerbate a primed antigen presentation response. Other pathways present in the proteomics dataset likewise contribute to a pro-inflammatory, antigen presentation phenotype. While the ITGAM SNP independently causes IFNγ secretion and response, stimulation with exogenous IFNγ caused an IL-6 response. This is further exacerbated by fibrinogen stimulation generating IFNγ responses. Taken altogether, the pathway data indicates that CD11b stimulation and interferon signaling create a widespread and intricate proinflammatory/antigen presentation feed-forward cycle.

One of the primary areas of future research in this project is to move into primary B cells from ITGAM genotyped donors; however, the use of immortalized cells also provides an interesting data point in the context of research of SLE at large. It has long been suspected that the pathogenic cause of SLE will have both a genetic and environmental contribution. Many candidates for viral cause of SLE have been proposed but thus far none have proven conclusive. The Epstein Barr Virus was one such candidate and has interesting new potential given the context of the data presented here. For example, EBV transcription factor EBNA1 is known to increase the transcription of ITGAM (site). Furthermore, αEBNA1 cross-reacts with double stranded DNA, one of the hallmarks of SLE and autoimmunity. It is possible that B cells primed to present antigen through the effects of the ITGAM SNP have this pathway further exacerbated by infection by EBV and a subsequently increased ITGAM transcription and then present fragments of EBNA1 to generate antibodies that react on double stranded DNA. The results of experiments exploring this idea are yet to be seen, but this remains an exciting potential at this juncture.

1 Figure Captions

Figure 1. The P1146S substitution in the cytoplasmic tail changes its structure and signaling properties. (A) The structure resulting from the common allele folds up on itself and has a single established phosphorylation site at 1142Ser. (B) The introduction of P1146S by the ITGAM SNP creates a second potential phosphorylation site with additional kinase consensus sequences for ERK 1/2, GSK3β, and others. (C) The substitution of proline for a serine at position 1146 opens the cytoplasmic tail and creates an approximately 30Å pocket.

Figure 2. The serine introduced by the *ITGAM* SNP can be phosphorylated by ERK 1/2 and GSK3β. (A) Peptides mimicking the phosphorylated forms of the cytoplasmic tail of CD11b were used to

generate phosphospecific antibodies that have low cross-reactivity. (B) Incubation of CD11b cytoplasmic tail mimicking peptides with ERK 1/2 and GSK3β indicate that they both phosphorylate 1146Ser.

Figure 3. The cytoplasmic tail of mutant CD11b is phosphorylated at 1146Ser *in vivo.* (A) Flow cytometry analysis of *ITGAM* genotyped donor PBMCs with phosphospecific antibodies shows that CD11b phosphorylation can be detected in primary cells. (B)

Figure 4.

Figure 5.

Figure 6.

Figure 7.

2 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3 Author Contributions

The majority of experimental procedures, data interpretation, and experimental design were conducted by JLB.

Funding was obtained by AS, and the work was conducted in his lab.

RJ conducted preliminary work, experimental and intellectual, that led to this project.

JE, JM, and DB contributed significantly intellectually to the founding and continuation of this project.

JM conducted preliminary proteomics analysis as well as mentored JLB in further analysis.

JE maintained access to a library of donor cells.

4 Funding

Department of Defense Grant #

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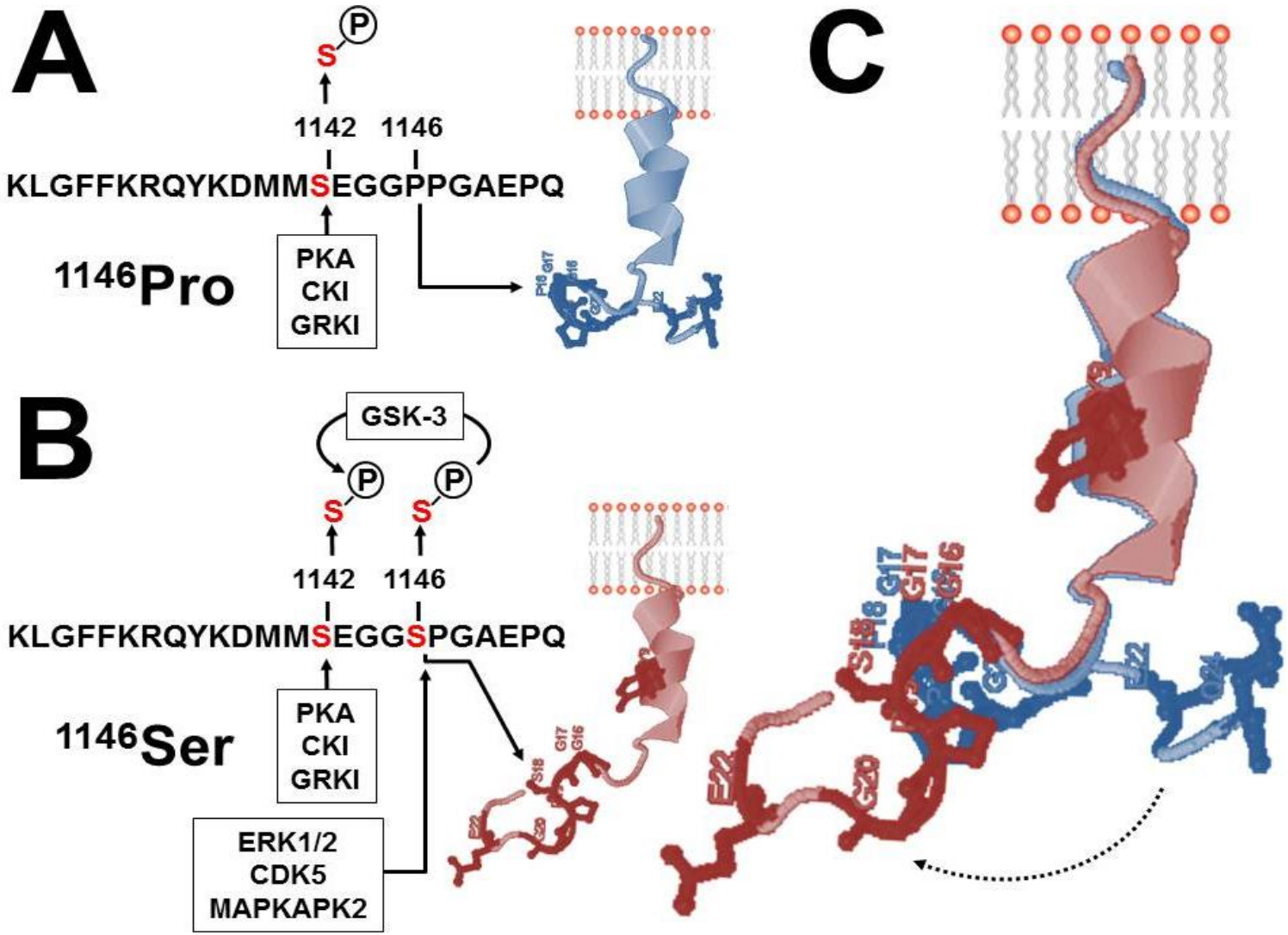
Fig 1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P
S
S
1142
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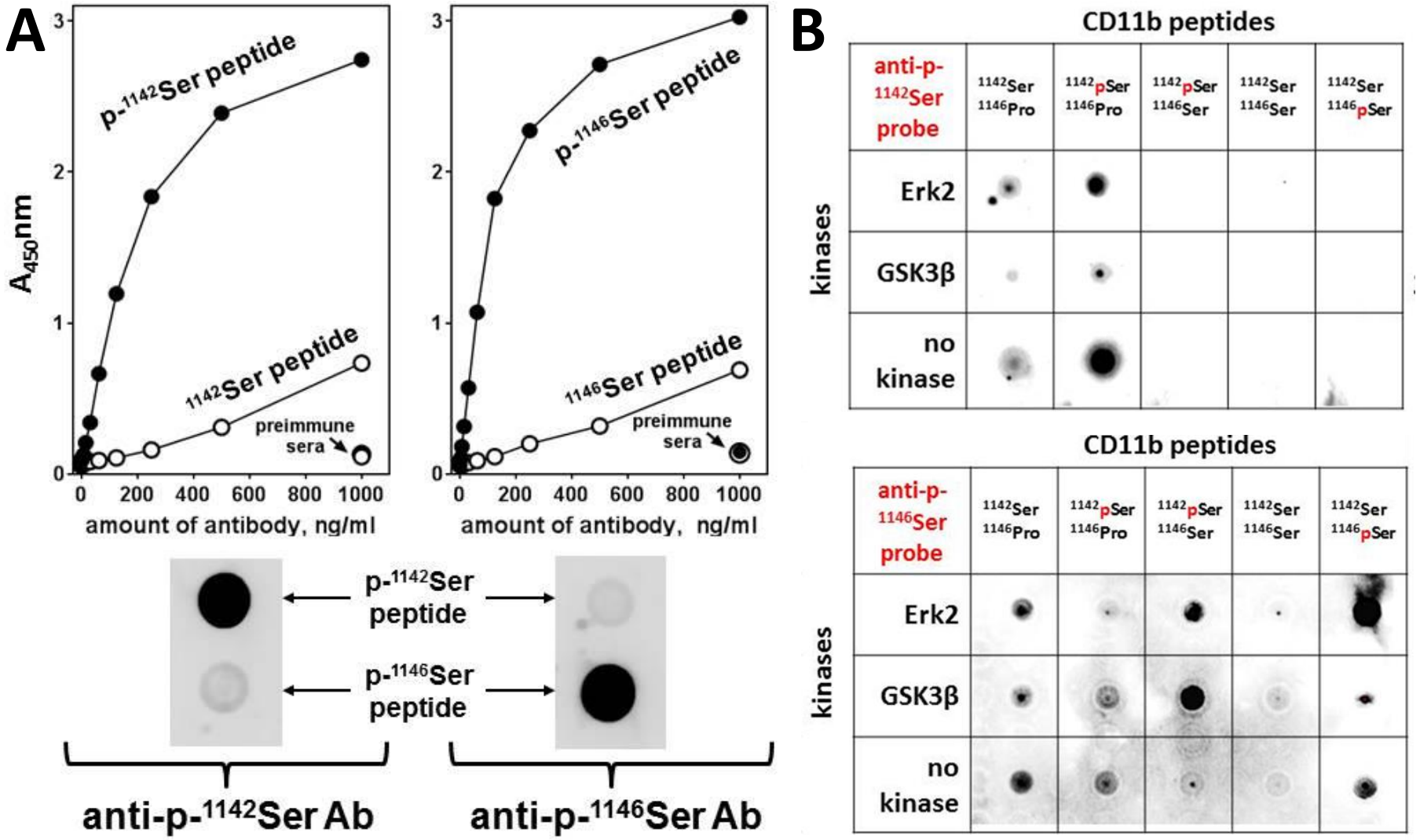
ERK1/2
CDK5
MAPKAPK2
C

Fig 2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p-1142Ser peptide
p-1146Ser peptide
anti-p-1142Ser Ab
anti-p-1146Ser Ab
B
CD11b peptides
anti-p-1142Ser probe
1142Ser 1146Pro
1142pSer 1146Pro
1142pSer 1146Ser
1142Ser 1146Ser
1142Ser 1146pSer
kinases
Erk2
GSK3β
no kinase
CD11b peptides
anti-p-1146Ser probe
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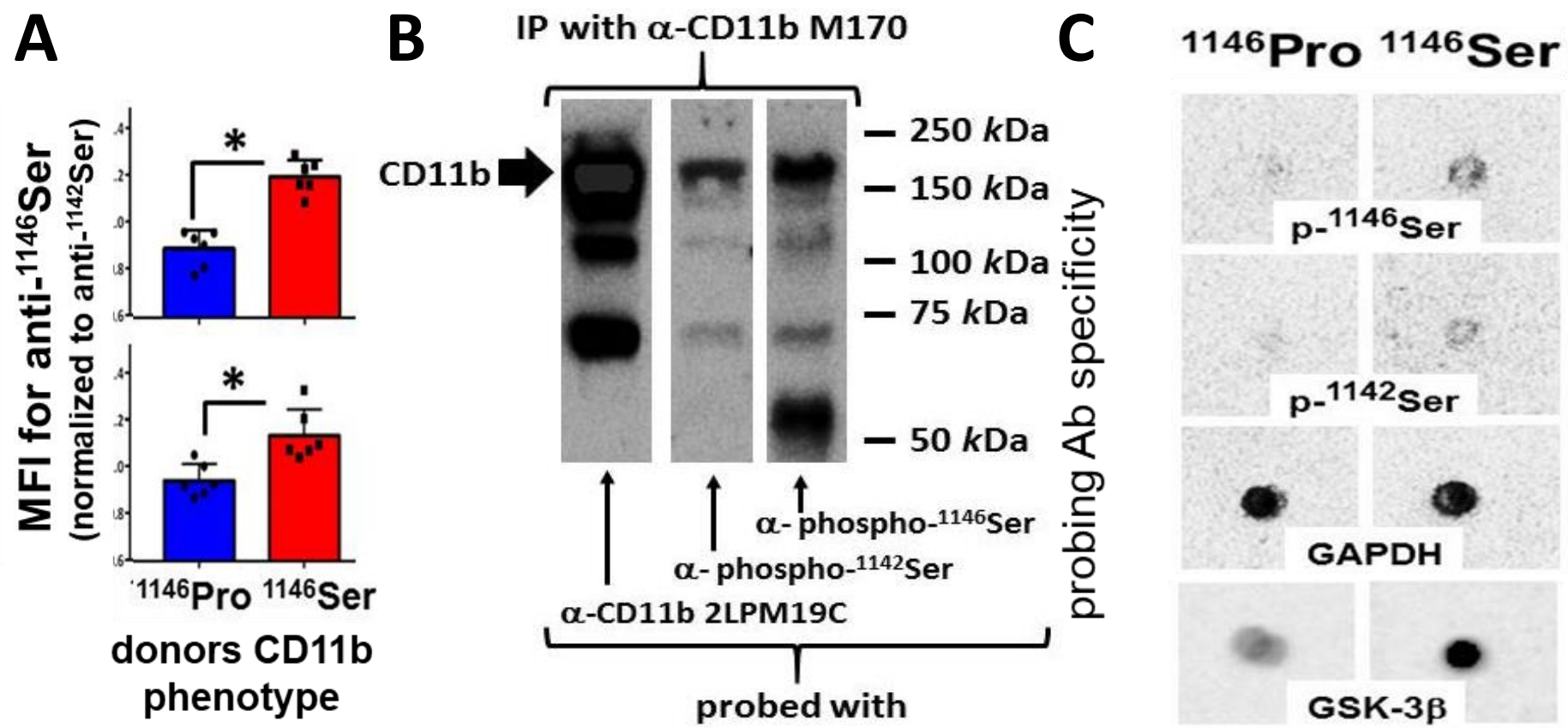
Fig 3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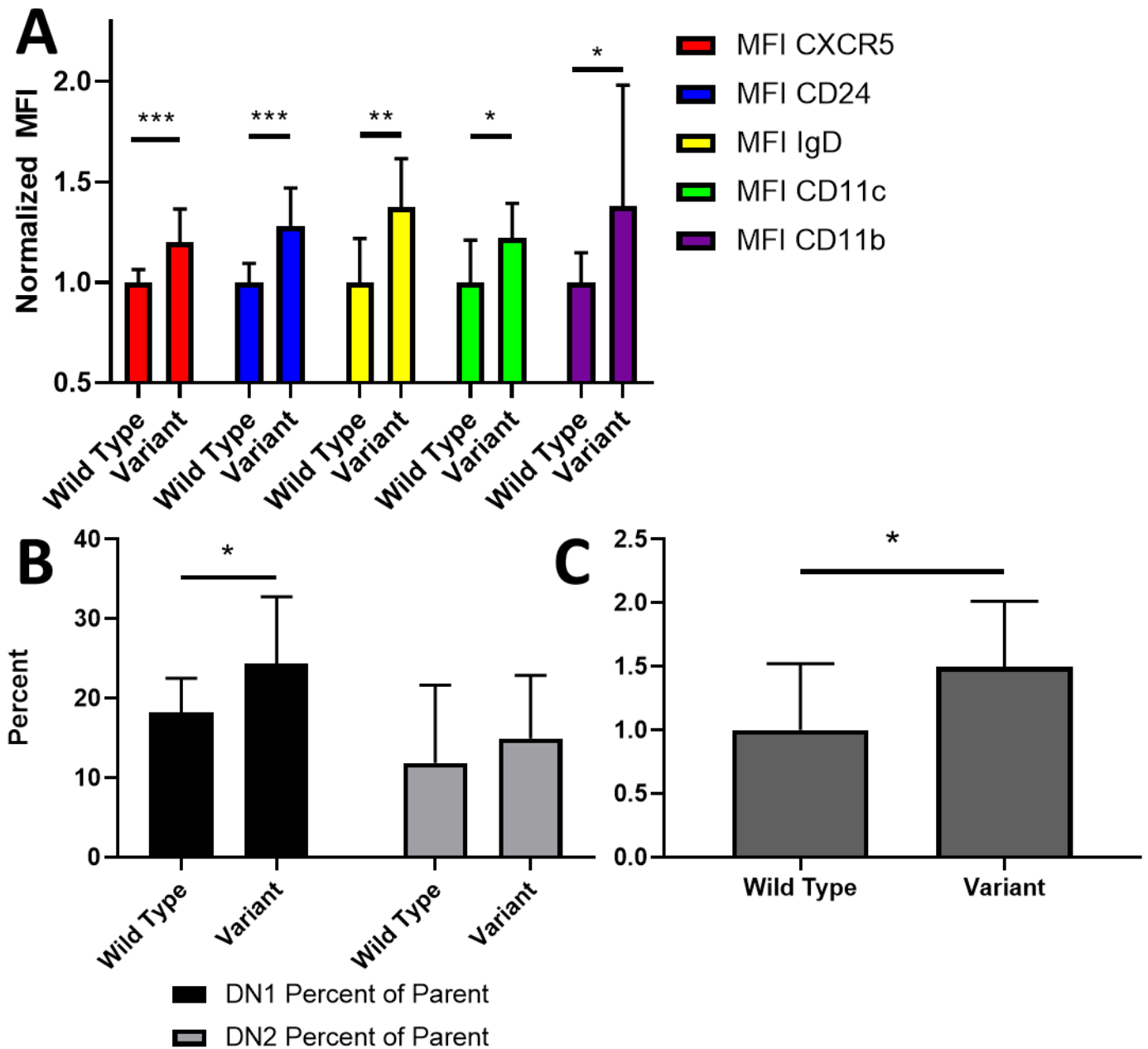
MFI for anti-1146Ser
(normalized to anti-1142Ser)
*
*
1146Pro
1146Ser
donors CD11b
phenotype
B
IP with α-CD11b M170
CD11b
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
α- phospho-1146Ser
α- phospho-1142Ser
α-CD11b 2LPM19C
probed with
C
1146Pro
1146Ser
probing Ab specificity
p-1146Ser
p-1142Ser
GAPDH
GSK-3β

Fig 4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Normalized MFI
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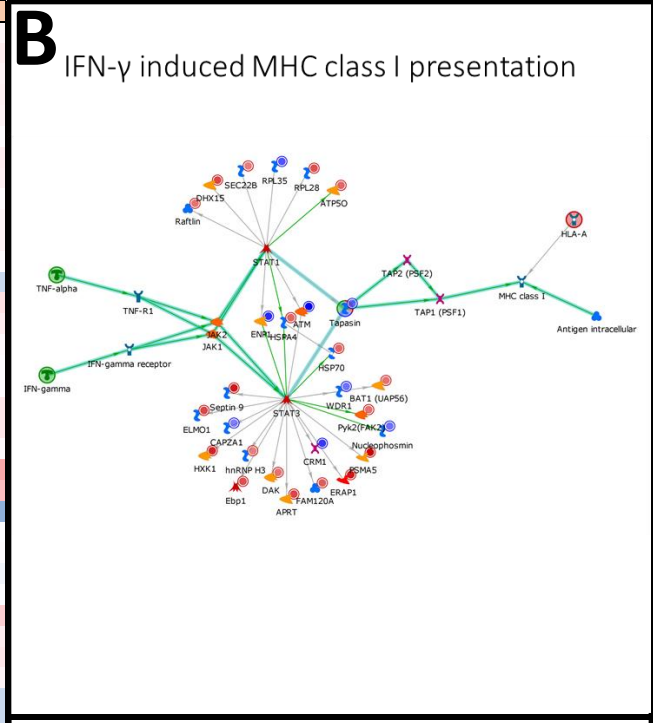
1.5
1.0
0.5
***
***
**
*
*
Wild Type
Variant
Wild Type
Variant
Wild Type
Variant
Wild Type
Variant
Wild Type
Variant
MFI CXCR5
MFI CD24
MFI IgD
MFI CD11c
MFI CD11b
B
Percent
40
30
20
10
0
*
Wild Type
Variant
Wild Type
Variant
DN1 Percent of Parent
DN2 Percent of Parent
C
2.5
2.0
1.5
1.0
0.5
0.0
*
Wild Type
Variant

A

Fig 5

UniProt Name	Common	Minor
Golgin subfamily B member 1		
Epididymis luminal protein 214		
Heterogeneous nuclear ribonucleoprotein H		
Serine/threonine-protein kinase mTOR		
Septin-9		
Endoplasmic reticulum aminopeptidase 1		
Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15		
Phenylalanine--tRNA ligase beta subunit		
PH-interacting protein		
Proliferation-associated protein 2G4		
E3 ubiquitin-protein ligase MYCBP2		
Kinesin-1 heavy chain		
Protein disulfide-isomerase		
Ribosomal protein L19		
Proteasome subunit alpha type-5		
RPL26 protein		
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1		
Adenine phosphoribosyltransferase		
Elongation factor Tu, mitochondrial		
Hexokinase-1 n=3 Tax=Homo sapiens		
MICOS complex subunit MIC60		
EIF4G1 protein		
Heat shock 70 kDa protein 4		
YLP motif-containing protein 1		
Adenylate kinase 2, mitochondrial		
60S ribosomal protein L18a		
Eukaryotic translation initiation factor 1A, X-chromosomal		
Protein disulfide-isomerase A6		
Inosine-5'-monophosphate dehydrogenase 2		
Formin-binding protein 1		
Baculoviral IAP repeat-containing protein 6		
E3 ubiquitin-protein ligase MYCBP2		
Eukaryotic translation initiation factor 1A, Y-chromosomal		
Glyoxylate reductase/hydroxypyruvate reductase		
40S ribosomal protein S27		
60S ribosomal protein L35a		
Capping protein (Actin filament) muscle Z-line, beta, isoform CRA_d		
Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1		
Probable ATP-dependent RNA helicase DDX5		
Glutathione S-transferase P		
Alpha-enolase		
Apoptosis-inducing factor 1, mitochondrial		
Engulfment and cell motility protein 1		
60S ribosomal protein L18		
HLA class II histocompatibility antigen, DRB1-11 beta chain		
WD repeat-containing protein 1		
Histone H2AX		
Nucleolin		
Nucleophosmin		
Tapasin		
Transcription elongation factor A protein 1		
ATP synthase subunit alpha, mitochondrial		
Myosin-9		
60S ribosomal protein L9		
Small nuclear ribonucleoprotein component		
Lactoferrin		
60S ribosomal protein L35		
Glyceraldehyde-3-phosphate dehydrogenase		
CAD protein		
Cleavage and polyadenylation specificity factor subunit 1		
Serine/arginine repetitive matrix protein 2		
Exportin-1		
Ectonucleoside triphosphate diphosphohydrolase 1		
ERO1-like protein alpha		
Receptor-type tyrosine-protein phosphatase C		
Serine/arginine-rich splicing factor 1		
Methionine--tRNA ligase, cytoplasmic		
Serine-protein kinase ATM		

B



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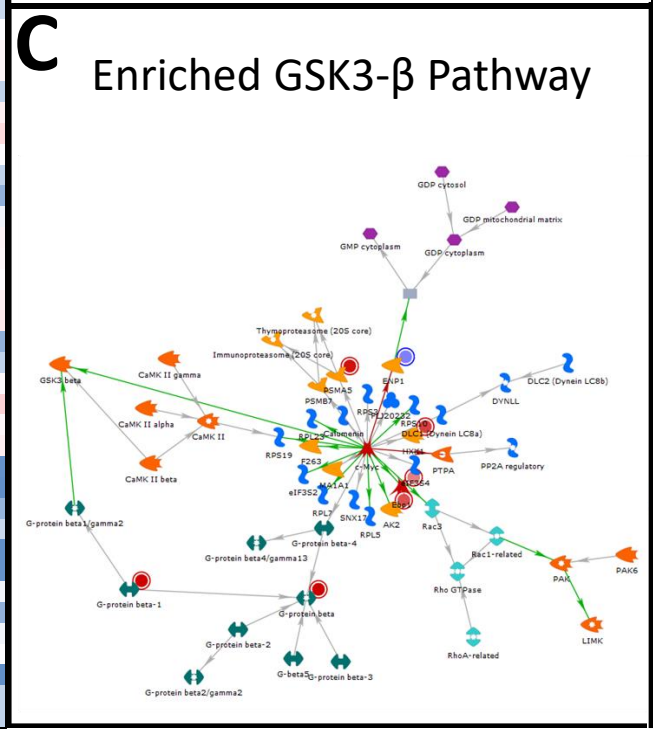

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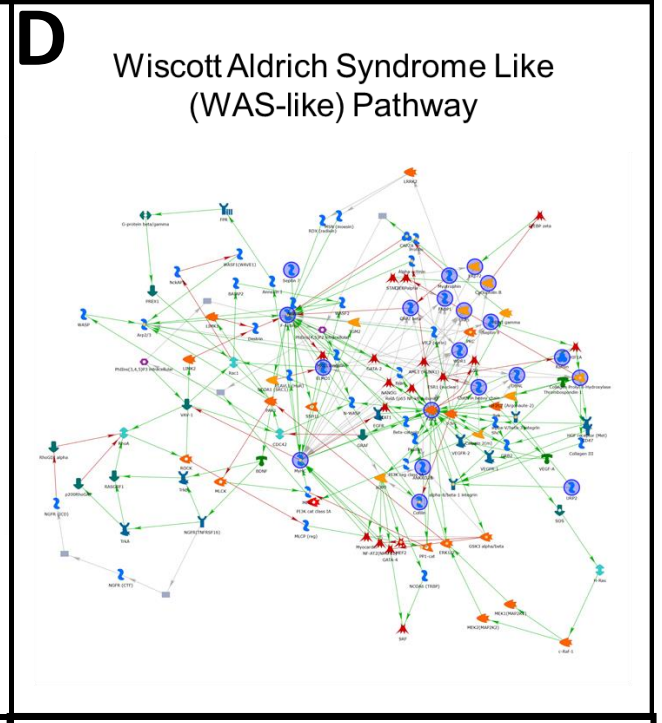

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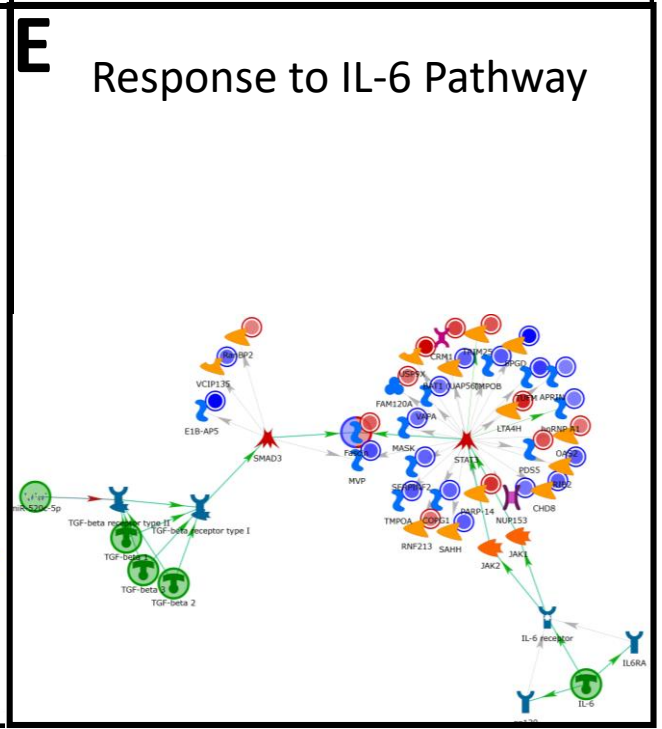
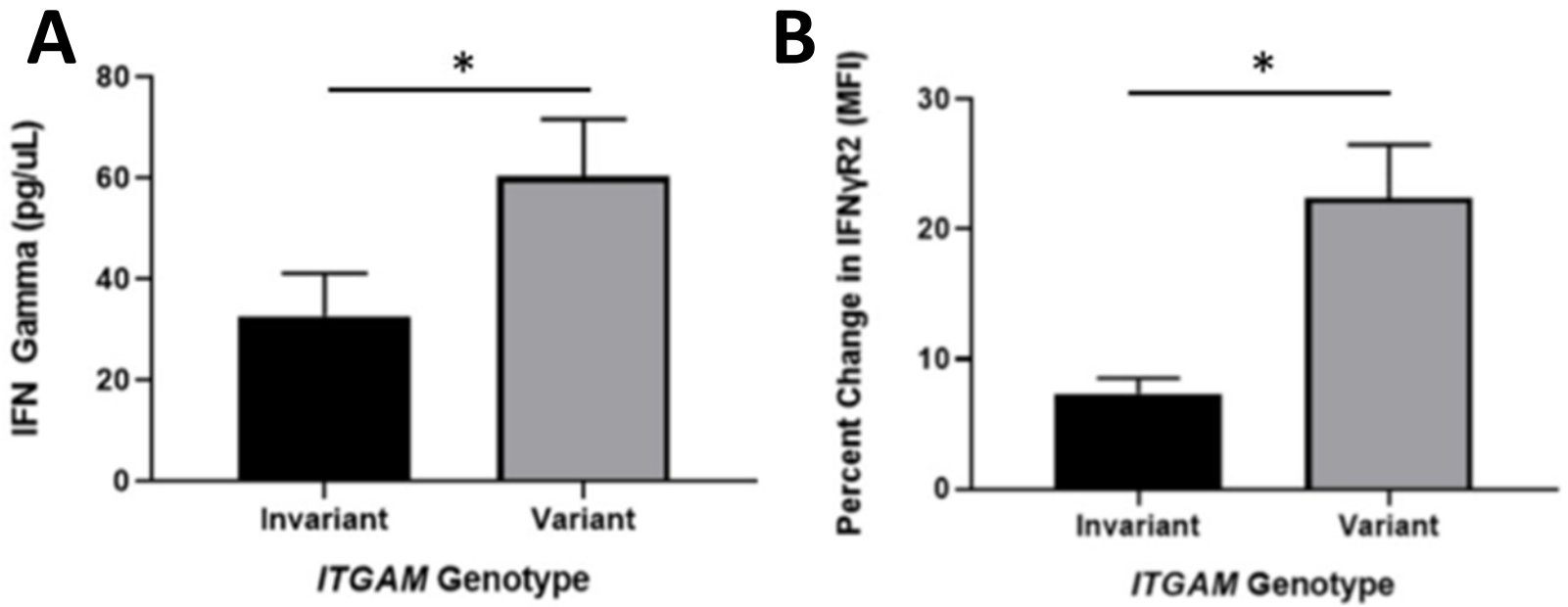


Fig 6


A
*
80
60
40
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IFN Gamma (pg/uL)
Invariant
Variant
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20
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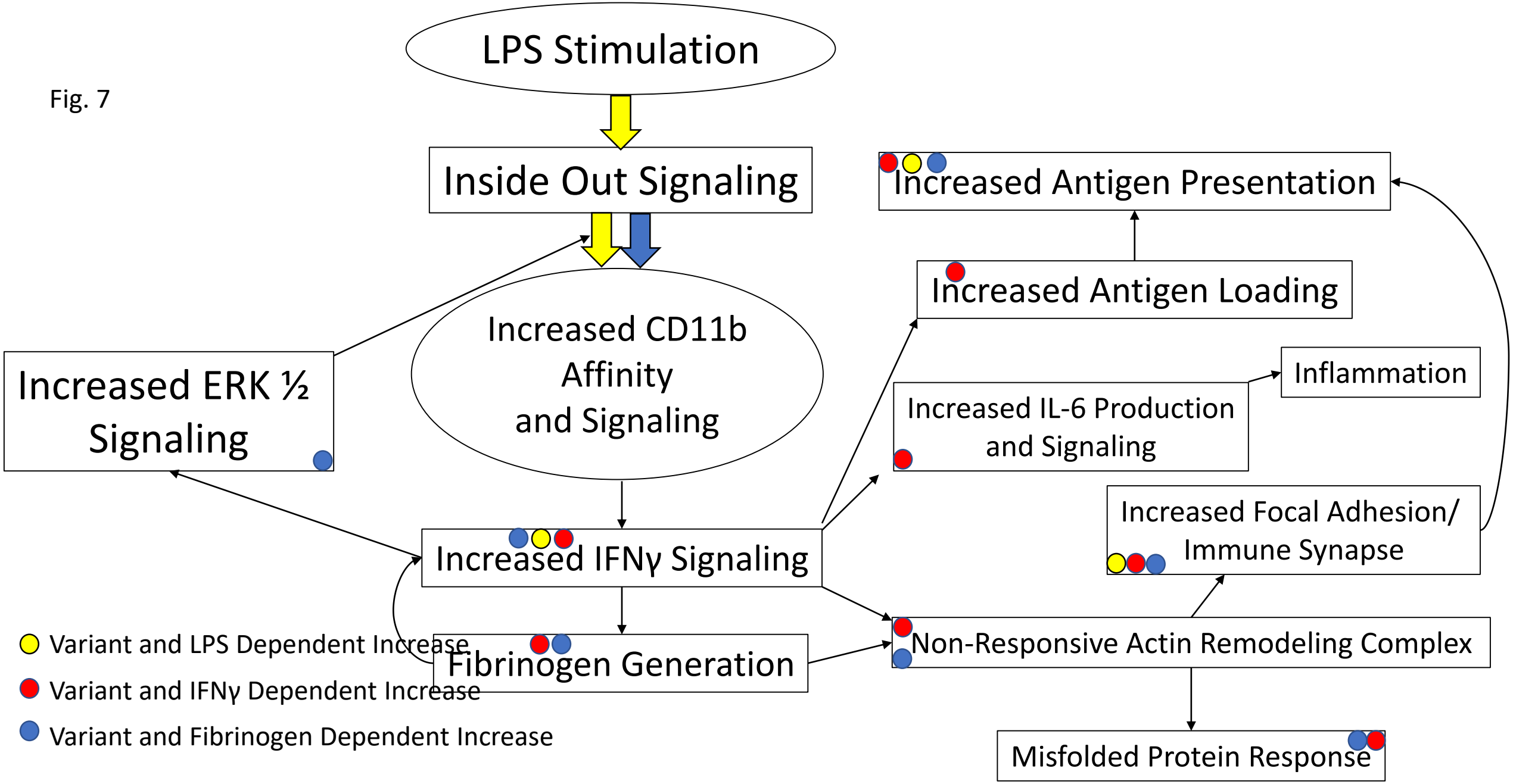
Percent Change in IFNγR2 (MFI)
Invariant
Variant
ITGAM Genotype

Fig. 7


LPS Stimulation
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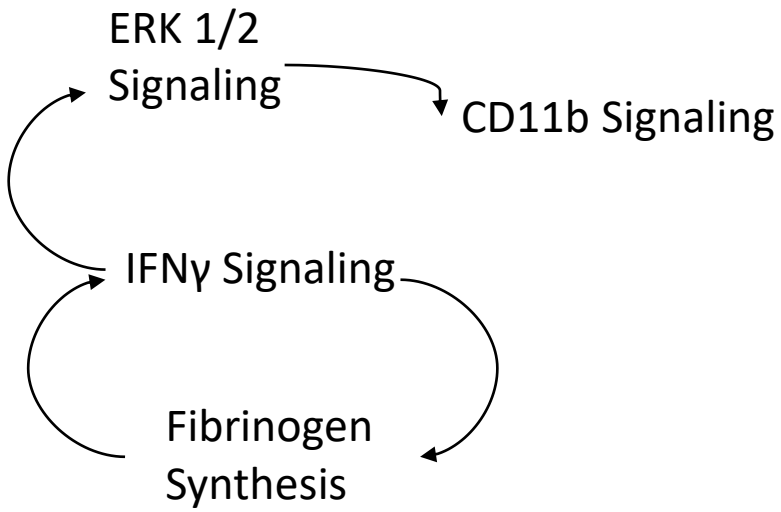
Increased CD11b Affinity and Signaling
Increased ERK ½ Signaling
Increased IFNγ Signaling
Fibrinogen Generation
Increased Antigen Presentation
Increased Antigen Loading
Increased IL-6 Production and Signaling
Inflammation
Increased Focal Adhesion/ Immune Synapse
Non-Responsive Actin Remodeling Complex
Misfolded Protein Response
Variant and LPS Dependent Increase
Variant and IFNγ Dependent Increase
Variant and Fibrinogen Dependent Increase

LPS Stimulation

Inside-Out
Signaling



Effects of *ITGAM* Genetic Variation on Mac-1-Mediated Functions of B Cells

Log # LR170037

Award Number W81XWH-18-1-0631

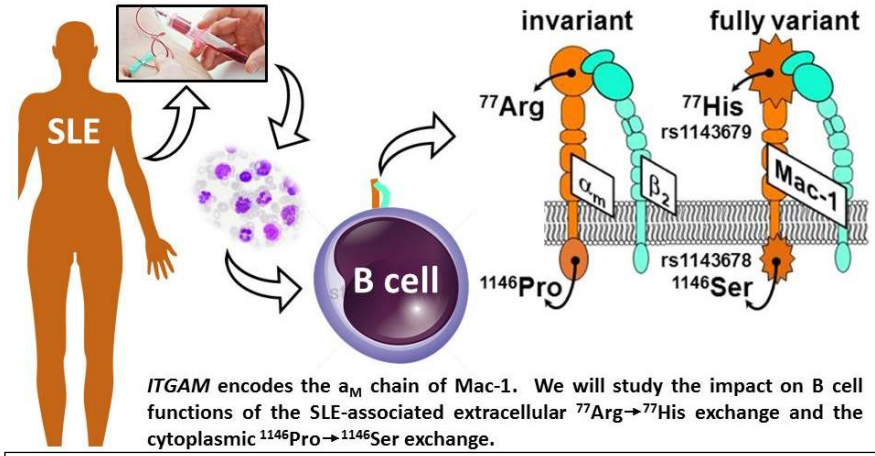
PI: Alexander J. Szalai **Org:** The University of Alabama at Birmingham **Award Amount:** $525,000

Study Aim(s)

- Define the impact of the *ITGAM* 77His and 1146Ser variants on CD11b dependent signaling in B cells.
- Assess the impact of 77His and 1146Ser on CD11b cytoskeletal associations, membrane mobility, and clustering on B cells.
- Show *ITGAM* variation impacts CD11b in B cells from SLE patients.

Approach

We have access to an already genotyped and already consented cohort of recallable healthy and SLE donors and a biobank of their Epstein - Barr virus (EBV) transformed B cells. EBV B cells from healthy donors will be used to assess the impact of *ITGAM* variation on CD11b phosphorylation and cell signaling and Mac-1 cytoskeletal association, mobility, and clustering. Clinical relevance will be validated using peripheral blood B cells freshly isolated from recalled healthy versus SLE affected donors. Approaches to be employed will include; tandem mass-spectrometry, single-cell network profiling, and multispectral imaging flow cytometry .



***ITGAM* encodes the a_M chain of Mac-1. We will study the impact on B cell functions of the SLE-associated extracellular ^{77}Arg→^{77}His exchange and the cytoplasmic ^{1146}Pro→^{1146}Ser exchange.**

We have provided the first evidence that the SLE-linked variant ^{1146}Ser residue in CD11b; (i) is a target for kinase-mediated phosphorylation of CD11b, (ii) associates with increased expression of CD11b by EBV-B cells, (iii) associates with an expansion of $CD19^+CD27^+CD24^+$ memory B cells, and (iv) intensifies an IFNγ→JAK/STAT→MHC pathway of antigen presentation resembling that seen in Wiskott-Aldrich Syndrome.

Timeline and Cost

Activities	Year 1	Year 2	Year 3
Define the impact of the *ITGAM* variants on CD11b dependent signaling in B cells.			
Assess the impact of 77His and 1146Ser on CD11b cytoskeletal associations, membrane mobility, and clustering on B cells.			
Show *ITGAM* variation impacts CD11b in B cells from SLE patients.			
Estimated Budget ($K)	**165**	**179**	**181**

Updated: January 5, 2023

Stated Goals/Milestones for Budget Year 2

■ accomplished ☐ not completed

- ■ local IRB and HRPO approved
- ■ EBV B cells from donors with different *ITGAM* genotypes identified
- ■ graduate student Joseph Blake recruited to work on this project
- ■ Western blots detected phosphorylation of ^{1142}Ser and ^{1146}Ser
- ■ mass spectrometry failed to detect phosphorylation of ^{1142}Ser and ^{1146}Ser
- ■ *in vitro* kinase assays detected phosphorylation of ^{1142}Ser and ^{1146}Ser
- ■ signaling network analysis
- ■ Preparation of manuscript
- ❑ Multispectral imaging flow cytometry studies
- ❑ Mac-1 cytoskeletal association/clustering studies

Comments/Challenges/Issues/Concerns

Covid-19 pandemic related issues (social distancing, restricted access to equipment, supply-chain disruptions, departure of personell) hindered our progress.

Budget Expenditure to Date

Projected: $525,000 Actual: $422,679.20